Determination of 4-Pregnene-3-ones in Thymus Tissue Samples by High Performance Liquid Chromatography*

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Z. Naturforsch. **39c**, 1012–1014 (1984);
received April 1984

High Performance Liquid Chromatography (HPLC), 4-Pregnene-3-ones, Thymus Gland, Disposable Extraction Columns

A high performance liquid chromatography method for determining the levels of 4-pregnene-3-ones in tissue containing fat, *e.g.* in thymus is reported. Following the extraction with chloroform/methanol the fat has been separated from the steroidal-fraction by using disposable extraction-columns. The steroidal-fraction has been analysed in two separation systems, on a RP_{18}-column with a methanol/water gradient and on an amino column with a isopropanol/hexane gradient.

The applicability of the described method is demonstrated with calf thymus samples.

Introduction

Recently some steroids were isolated from the thymus by liquid-gel chromatography and identified with GC-MS [1, 2].

According to these results there seems to be some relations between the age of the thymus donor and the steroid type and concentration in the organ investigated [3]. This prompted us to analyse the kind and the amount of the steroids in thymus and their dependence on different physiological and pathological parameters.

The previously advocated method [1, 2] to be used for routine steroid analysis for thymus tissue samples seems less suitable, whereas an HPLC analysis is obviously more advantageous.

Suitability and limits of the HPLC for this problem were investigated with model analyses. The mixtures were containing steroids previously isolated from thymus [1, 2] and some biogenetically related ones. Conditions which gave the best separations are described in Results and Discussions.

* Natural Product Chemistry 92, for part 91 see J. Reisch, R. A. Hussain and I. Mester, Phytochemistry in press.
** A part of the Ph. D. dissertation.

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0341-0382/84/0900-1012 $ 01.30/0

At first, the investigation was limited to 4-pregnene-3-one derivatives due to their assumed biological importance. Furthermore, they could be easily determined by means of a fixed-wavelength detector at 254 nm.

Experimental

Reagents

The standard substances: progesterone, cortisol, 11-deoxycortisol, and 17α-hydroxyprogesterone were obtained from Merck (D-6100 Darmstadt, F.R.G.) cortisone, corticosterone from Fluka (CH-9470 Buchs, Switzerland), 21-deoxycortisol, 11β-hydroxyprogesterone, 20α-dihydroprogesterone from Sigma (D-8000 Munich, F.R.G.), and 11-deoxycortisol from Ega (D-7924 Steinheim, F. R. G.).

The disposable-extraction-columns were purchased from Baker (D-6080 Groß-Gerau, F.R.G.). HPLC-grade methanol, isopropanol and hexane were supplied by Merck or Baker. The water was demineralised and bidestilled.

Sample preparation

The thymus tissue samples with the twentyfold volume of chloroform/methanol 2:1 were mixed in a "Commercial blendor" with a high-grade steel beaker (Waring, New Hartford, Conn. USA). For further details see Fig. 1.

Chromatography

The liquid chromatography system consisted of a Varian Model 5000 pump with a pair of microprocessor-controlled proportioning valves for binary low pressure gradient formation, equipped with a 254 nm UV-detector (Varian, Palo Alto, Calf. USA). Hyperchrome® columns, 250 × 4.6 mm (Bischoff Analytical Technic, D-7250 Leonberg, F.R.G.) packed with Lichrosorb® 5 µm RP_{18}- or amino-particles were employed. The elutions were carried out at room temperature with a flow rate of 1 ml/min. The linear gradient in the case of RP_{18} column was water/methanol 35:65 to 15:85 in 13 min (system A) and isopropanol/hexane 17:83 to 57:43 in 10 min in the case of amino phase (system B). The signals were identified by comparison with those of standard substance (see Reagents).

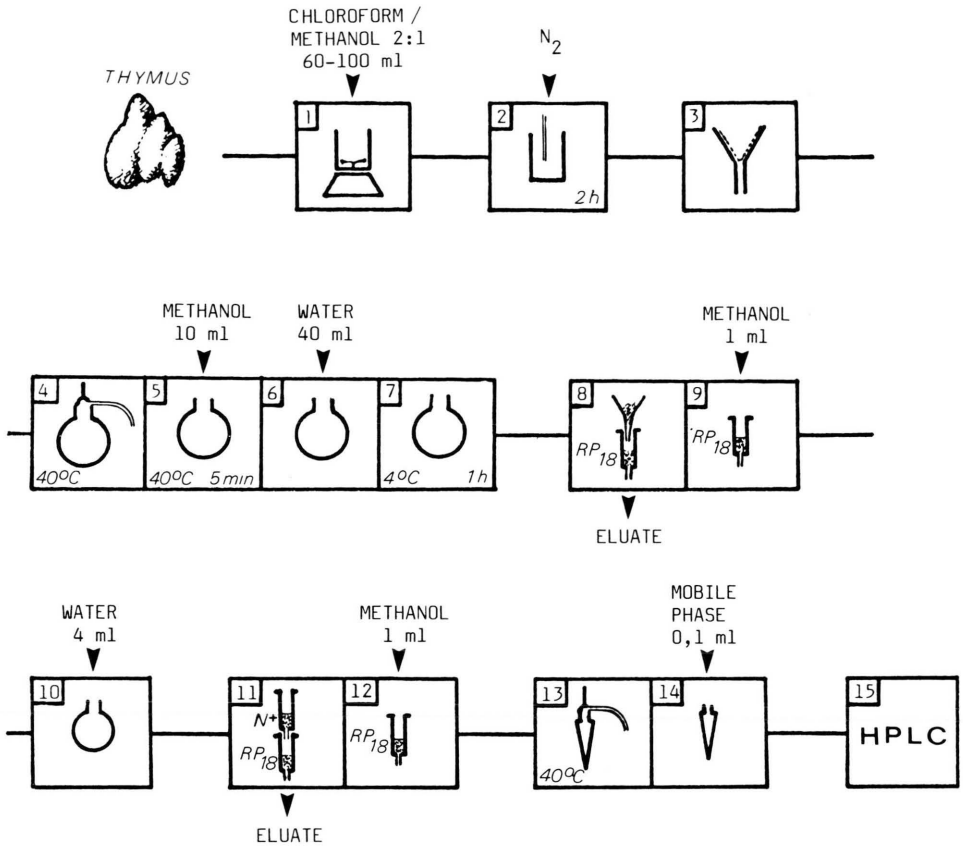

Fig. 1. [1] twentyfold volume of chloroform/methanol 2:1 to the tissue sample is added, [2] under N_2 atmosphere for 2 h, [3] filtration, [4] evaporation of the solvent, [5] addition of 10 ml methanol to the residue, [6] addition of 40 ml water, [7] 1 h cooling in a refrigerator, [8] filtering through glaswool and a Baker disposable extraction column RP_{18}, [9] elution with 1 ml methanol, [10] 4 ml water are added, [11] passing through a combination of a quaternary amine and a RP_{18} Baker disposable extraction column, [12] elution with 1 ml methanol, [13] evaporation of the methanol, [14] 0.1 ml from the mobile phase used in the HPLC separation system is added.

Results and Discussion

According to the age of the donor, the thymus contains more or less fat, while the steroids are only present in relatively low concentrations. Therefore special attention should be paid to the preparation method, whereby the procedure sketched in Fig. 1 seems to be the most suitable. In this method the fat is eliminated by addition of water to the methanolic solution and filtration through glaswool. The steroids in the mother liquor were concentrated on a RP_{18} column.

In model analyses of fat and steroid mixtures the recoveries are 80–90% according to the fat content.

But there were difficulties with structurely related steroids (structure isomers) *e.g.* 11-deoxycortisol and corticosterone. Therefore not all the 4-pregnene-3-ones were precisely detected in one system. But since these compounds could be analyzed simultaneously by the other system, a combination of the two chromatogrammes allows a definite assertion. For steroids which could be identified in each system a double proof detection is achieved. However, to which level this method is applicable depends on the natural variable proportions of fat, gland tissue and blood in samples investigated (Fig. 2). Some results gained from calf thymi are shown in Table I. Remarkable is the presence of the

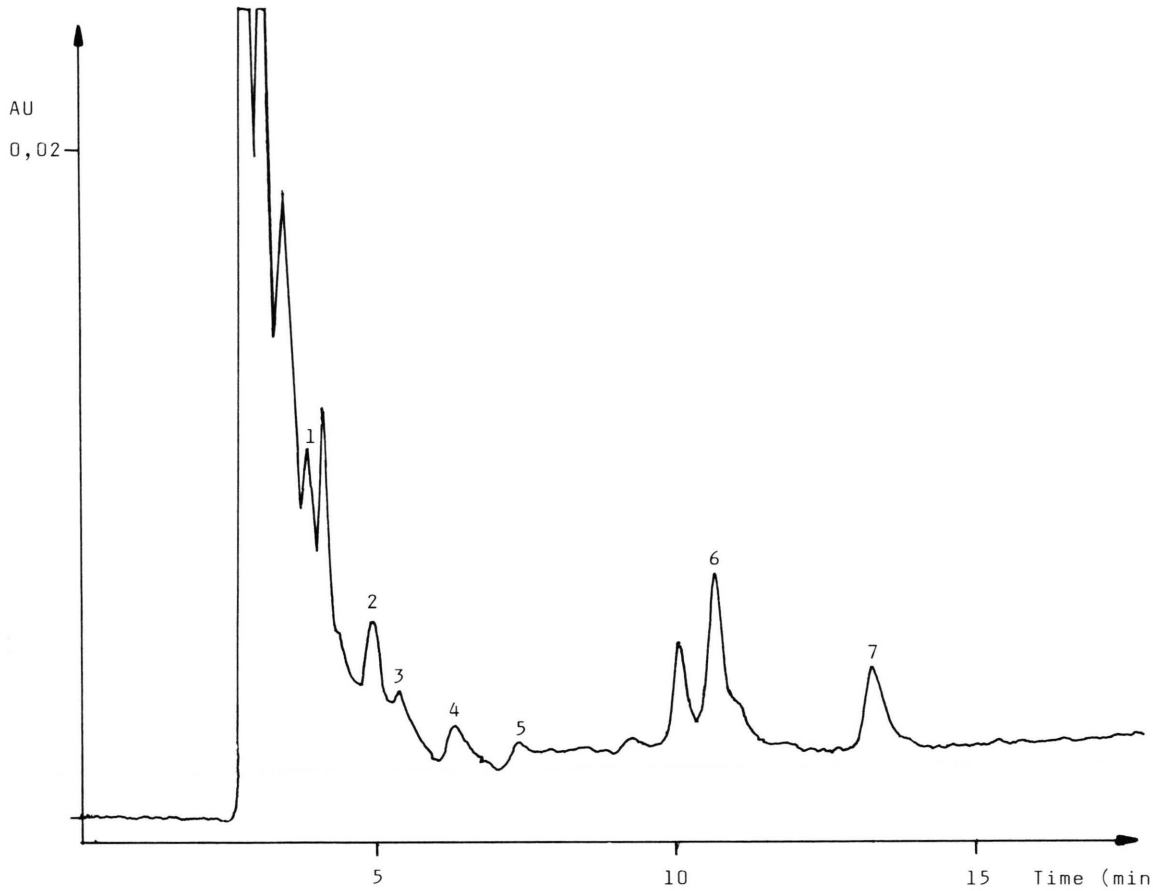

Fig. 2. Representative chromatogramme from an extract of a calf thymus, separated in system B. Peaks: 1 = progesterone, 2 = 20α-dihydroprogesterone, 3 = 17α-hydroxyprogesterone, 4 = 11β-hydroxyprogesterone, 5 = 11-deoxycorticosterone, 6 = corticosterone, 7 = cortisol.

Table I. Results of the investigation of some calf thymus glands.

Steroids	C_A	C_B	C_C	C_D
Cortisone	+	+		++
Cortisol	++	+++	+++	+++
Corticosterone	+++	+++	+++	+++
11-Deoxycortisol	+++			
21-Deoxycortisol	++			
11-Deoxycorticosterone			+	+
11β-Hydroxyprogesterone		+	+	+
17α-Hydroxyprogesterone	++	+	+	+
20α-Dihydroprogesterone	+	+	++	++
Progesterone	+	+	++	++

+++: Present in relatively large amounts (> 200 ng/g fresh tissue).
++: Present in small amounts (100–200 ng/g fresh tissue).
+: Present in detectable traces (< 50 ng/g fresh tissue).
Calf ($C_A - C_D$): sex; age; reason for the forced slaughter. C_A: ♀; 3 months; navel inflammation. – C_B: ♀; 2 months; pneumonia. – C_C: ♀; 7 weeks; intestine invagination. – C_D: ♂; 5 weeks; ?

20α-dihydroprogesterone (20αOHP), since the 20α-hydroxy steroid dehydrogenase – which reduces progesterone to 20αOHP – seems to be a marker for certain differentiation stages of T-lymphocytes [4].

Acknowledgement

The authors would like to thank Dr. med. vet. A. Rübekeil (D-2908 Friesoythe, F.R.G.) for providing the calf thymus samples and useful information.

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