Journal of Chromatography, 190 (1980) 169–174

CHROM. 12,556

Note

High-performance liquid chromatography of the reduction products of progesterone

JIANN-TSYH LIN, ERICH HEFTMANN* and IRVING R. HUNTER

Plant Biochemistry Research Unit, Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, Calif. 94710 (U.S.A.)

(Received November 19th, 1979)

In connection with our work on steroid metabolism in pea plants[1], we needed a chromatographic method for the separation of progesterone reduction products. Available thin-layer chromatographic techniques[2] failed to resolve the complex mixture of radioactive progesterone metabolites. High-performance liquid chromatography (HPLC) is highly efficient, but the reduction products of progesterone have not been investigated systematically so far[3]. The oxidation products containing a conjugated carbonyl group, such as the adrenocortical hormones, absorb UV radiation at 254 nm and are therefore easily detected in HPLC by fixed-wavelength detectors. The reduction products with isolated double bonds or oxo groups, on the other hand, require a variable UV detector, and for the saturated pregnane derivatives only the refractive index (RI) detector is suitable.

In the present study we have compared the performance of adsorption with that of reversed-phase partition chromatography in order to arrive at some generalizations concerning their relative merits in the separation of stereoisomers[4]. We have tested a total of 25 pregnane derivatives, of which 4 were diones, 11 monohydroxy-monoketones and 10 diols.

EXPERIMENTAL**

The HPLC apparatus was assembled from commercially available components. The pump was of the dual-piston reciprocating type, Constametric II (Laboratory Data Control, Division of Milton-Roy, Riviera Beach, Fla., U.S.A.), and the sample injection valve was an Altex Model 905-23 (Altex, Berkeley, Calif., U.S.A.), with a loop volume of 275 μl.

The adsorption column consisted of two stainless-steel chromatographic tubes (Alltech, Arlington Heights, Ill., U.S.A.), each 300 × 2 mm I.D., packed with Partisil 5 (5 μm; Whatman, Clifton, N.J., U.S.A.) connected in series. The column was packed in our laboratory from a balanced-density slurry of the silica gel in a

* To whom correspondence should be addressed.

** Reference to a company and/or product named by the Department is only for purpose of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

mixture of tetrabromoethane and tetrachloroethane in a HPLC slurry packing unit (Model 29426; Haskel, Burbank, Calif., U.S.A.) and was washed with hexane. The reversed-phase column consisted of two tubes, each 250 × 4 mm I.D. (Alltech), packed with Zorbax BP-ODS (7–8 μm; DuPont, Wilmington, Del., U.S.A.). Zorbax BP-ODS in a mixture of chloroform and bromoform was also packed and washed with methanol in the HPLC slurry packing unit.

The variable-wavelength detector was an Altex Model 155 with a flow cell having a 10-mm pathlength and a 20-μl volume, set at 280 nm. The RI detector was a Waters Model R-401 (Waters Assoc., Milford, Mass., U.S.A.). A single-channel recorder, Linear Model 355 (Linear, Irvine, Calif., U.S.A.) was attached to the output of either detector.

HPLC was performed at room temperature (23°). All solvents were "HPLC grade" quality (Fisher Scientific, Fair Lawn, N.J., U.S.A.) and the eluents were sonicated for about 15 min before use.

TABLE I

RETENTION TIMES OF PREGNANE DERIVATIVES

Conditions: 1: See Fig. 1; 2: same, except: eluent, hexane–isopropanol (97:3), pressure, 1700 p.s.i.; RI detector; 3: see Fig. 4.

Compounds	*Retention time (min)*		
	1	*2*	*3*
Diones			
4-Pregnene-3,20-dione (progesterone)	44	—	40
5-Pregnene-3,20-dione	51	—	52
5α-Pregnane-3,20-dione	18	—	62
5β-Pregnane-3,20-dione	22	—	51
Monohydroxymonoketones			
3β-Hydroxy-5-pregnen-20-one (pregnenolone)	37	—	36
20α-Hydroxy-4-pregnen-3-one	—	18	25
20β-Hydroxy-4-pregnen-3-one	—	17	35
3α-Hydroxy-5α-pregnan-20-one	32	—	51
3α-Hydroxy-5β-pregnan-20-one	61	—	41
3β-Hydroxy-5α-pregnan-20-one	39	—	50
3β-Hydroxy-5β-pregnan-20-one	27	—	45
20α-Hydroxy-5α-pregnan-3-one	53	—	43
20α-Hydroxy-5β-pregnan-3-one	100	—	38
20β-Hydroxy-5α-pregnan-3-one	53	—	59
20β-Hydroxy-5β-pregnan-3-one	95	—	56
Diols			
5-Pregnene-3β,20α-diol	—	15	21
5-Pregnene-3β,20β-diol	—	15	27.5
5α-Pregnane-3α,20α-diol	—	15	35
5α-Pregnane-3α,20β-diol	—	16	44
5α-Pregnane-3β,20α-diol	—	15	29
5α-Pregnane-3β,20β-diol	—	13	38
5β-Pregnane-3α,20α-diol	—	26	29
5β-Pregnane-3α,20β-diol	—	22	38
5β-Pregnane-3β,20α-diol	—	14.5	27
5β-Pregnane-3β,20β-diol	—	14.5	38

RESULTS AND DISCUSSION

Our results are summarized in Table I. The chromatographic conditions are shown in the figure legends. Adsorption chromatography (Fig. 1) separated 5α-pregnane-3,20-dione from the more polar 5β-epimer and progesterone from the more polar Δ^4-analog (Table I). In the reversed-phase partition system the saturated diones were eluted not only later than the unsaturated diones but also in reverse order. Thus, the four diones were completely separated by adsorption but not by partition chromatography.

Among the monohydroxymonoketones, the 20-epimers are not separable by adsorption under the conditions given in Table I, but the adsorption chromatogram shown in Fig. 2 gave a useful separation, the 20α-epimer being more polar than the

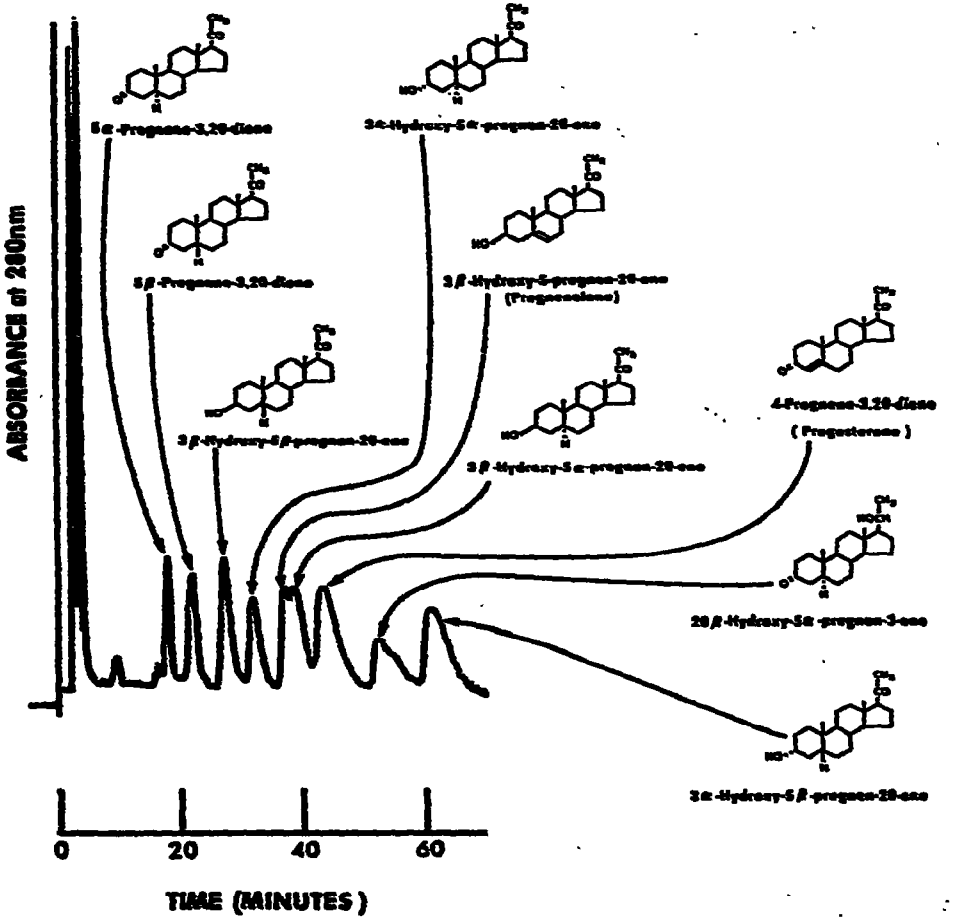

Fig. 1. Elution curve for 3 diones and 6 monohydroxymonoketones of the pregnane series. Between 17 μg (5α-pregnane-3,20-dione) and 135 μg (3α-hydroxy-5β-pregnan-20-one) of the steroids, dissolved in dichloromethane, were chromatographed on a column of Partisil 5, 600 × 2 mm I.D. Eluent, 0.25% ethanol in dichloromethane; flow-rate, 1 ml/min; pressure, 3200 p.s.i. Detector at 280 nm; range, 0.05. Recorder speed, 6 cm/h; span, 10 mV.

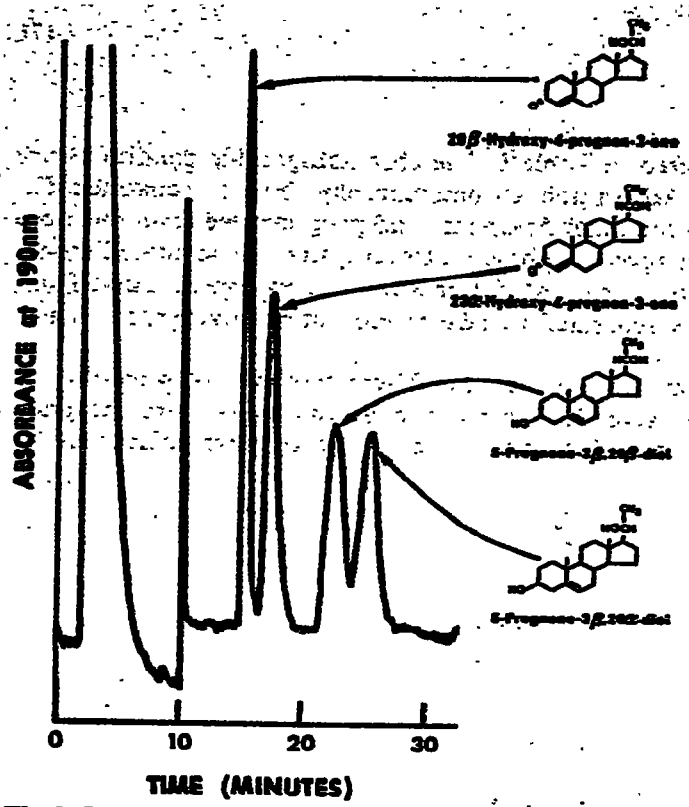

Fig. 2. Separation of 2 pairs of pregnane derivatives, epimeric at C-20. Two μg of each, 20β- and 20α-hydroxy-4-pregnen-3-one, and 90 μg of each, 5-pregnene-3β, 20β-diol and 5-pregnene-3β,20α-diol, dissolved in dichloromethane, were chromatographed on a column of Partisil 5, 600 × 2 mm I.D. Eluent, *n*-hexane–methanol–ethanol (96:3:1); flow-rate, 1 ml/min; pressure, 3500 p.s.i. Detector at 190 mm; range, 0.2. Recorder speed, 12 cm/h; span, 10 mV.

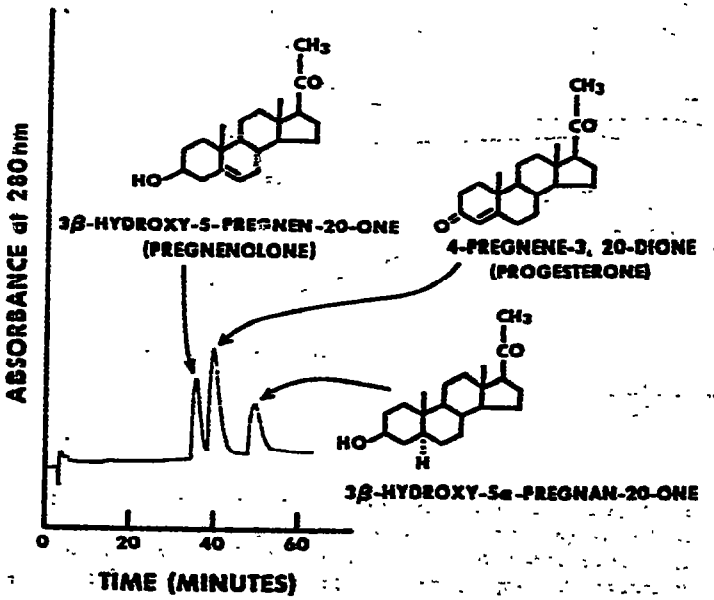

Fig. 3. Separation of Δ^5-, Δ^4- and 5α-steroids of the pregnane series by reversed-phase HPLC. 35 μg of pregnenolone, 35 μg progesterone and 70 μg 3β-hydroxy-5α-pregnan-20-one, dissolved in the mobile phase of 60% aqueous acetonitrile, were chromatographed on a column of Zorbax BP-ODS, 500 × 4 mm I.D. Flow-rate, 1 ml/min; pressure, 1450 p.s.i. For detector and recorder, see Fig. 1.

20β-epimer in each case. However, the best way to resolve such epimers is by reversed-phase partition (Table I and Fig. 4). In that system the 20α-epimers are consistently more polar than the corresponding 20β-epimers.

Comparing steroids epimeric at C-3, we find that the equatorial hydroxyl group (3β in the A/B-*trans* series and 3α in the A/B-*cis* series) is invariably more strongly adsorbed (Fig. 1). The differentiation between progesterone, pregnenolone and 3β-hydroxy-5α-pregnan-20-one is biochemically important, but their chromatographic resolution by adsorption is difficult (see Fig. 1), because they all have a similar conformation. However, complete separation can be achieved by partition chromatography (Fig. 3). Reversed-phase partition chromatography was less useful for separation of the 3α- from the 3β-epimers among the monohydroxymonoketones.

For the separation of the most polar reduction products of progesterone, the

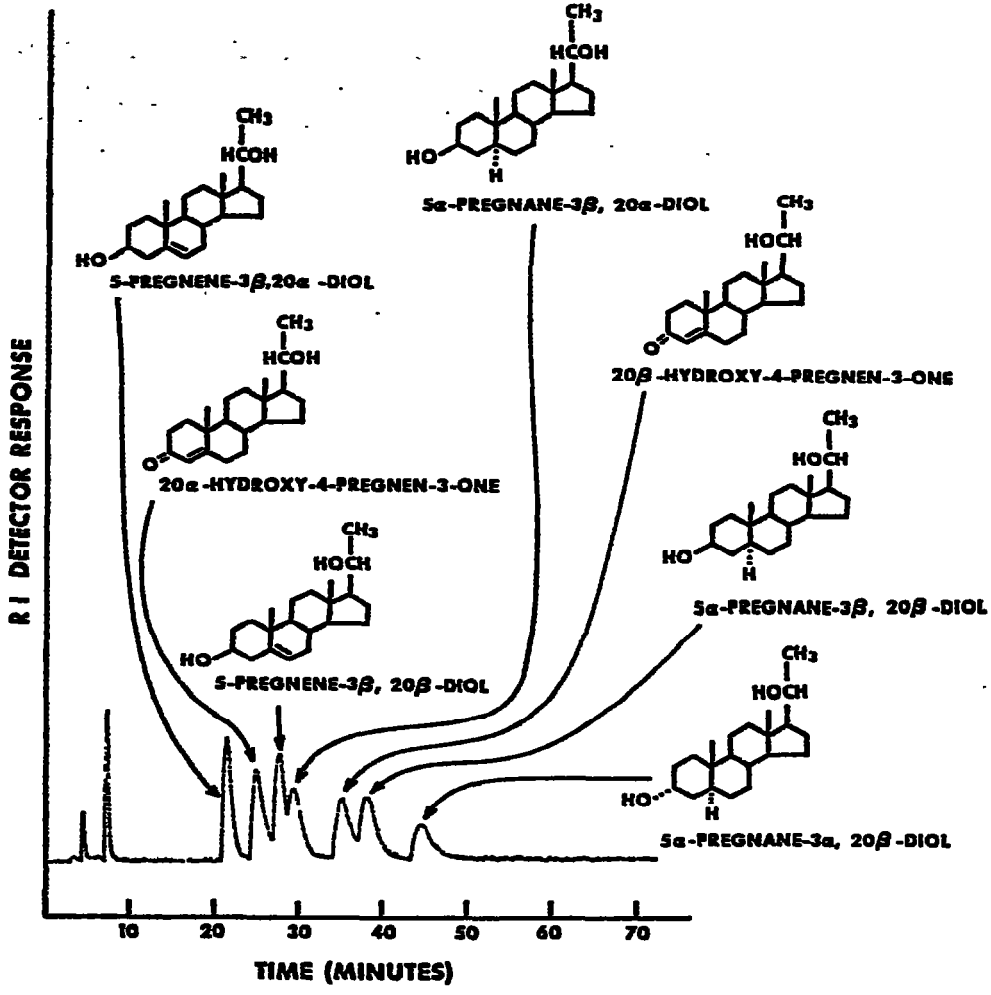

Fig. 4. Elution curve for 2 monohydroxymonoketones and 5 diols of the pregnane series. About 85 μg of each steroid was chromatographed in the reversed-phase system specified in Fig. 3, except the pressure was 1390 p.s.i. RI detector; sensitivity, 8×. Recorder speed, 12 cm/h; span, 10 mV.

diols, adsorption chromatography was useful where partition chromatography failed and *vice versa* (Table I and Fig. 4). Thus, the 20-epimers were generally better resolved by partition than by adsorption, the α-epimer always being eluted ahead of the β-epimer. In the A/B-*trans* series the 3-epimers were better resolved by reversed-phase partition than by adsorption, whereas in the A/B-*cis* series adsorption chromatography was superior. In both systems the 3β-steroids were eluted ahead of the corresponding 3α-steroids. Δ^5-Steroids cannot be separated from their 5α-analogs in the adsorption systems (Table I and Fig. 2), but they can be separated by the reversed-phase partition system and the Δ^5-steroids were eluted ahead of the 5α-steroids. Thus, a combination of adsorption and reversed-phase partition chromatography could potentially resolve even the complex mixtures encountered in biological samples.

ACKNOWLEDGEMENTS

Gifts of reference compounds from the Medical Research Council (D. N. Kirk, Westfield College, Hampstead, London, Great Britain) and the National Institutes of Health (D. F. Johnson, Laboratory of Chemistry, NIAMDD, Bethesda, Md., U.S.A.) are gratefully acknowledged.

REFERENCES

1 J. T. Lin, W. M. Proebsting and E. Heftmann, *Phytochemistry*, 18 (1979) 1667.
2 E. Heftmann, *Chromatography of Steroids*, Elsevier, Amsterdam, 1976, p. 93.
3 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
4 E. Heftmann, *J. Liquid Chromatogr.*, 2 (1979) 1137.