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Note

Reversed-phase high-performance liquid chromatography of C_{21} metabolites of progesterone

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Preliminary results of studies of the metabolism of progesterone in a perfused canine isolated gravid uterine preparation established that the preparation produced a number of metabolites that were poorly separated by the use of gas—liquid chromatography (GLC). The methods described here are the results of efforts to establish means for separating and identifying many of the C_{21} compounds that may be metabolites of progesterone. Lin et al. [1] have reported separation of the reduction products of progesterone by employing a combinaton of adsorption and reversed-phase high-performance liquid chromatography (HPLC). The methods described here are simple isocratic reversed-phase HPLC systems that separate C_{21} ketonic metabolites of progesterone, the stereoisomers of pregnanediol, and eight isomers of pregnane-3,6,20-triol. Separations have been achieved using a single commercially available column and three mobile phases.

EXPERIMENTAL

Materials

The solvents were HPLC-grade methanol and acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Distilled water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Solutions were filtered through 0.5-μm Millipore FHUP-047-00 filters and then sparged with helium for 30 min just before chromatography. Isomers of pregnane-3,6,20-triol were synthesized in our laboratory according to the method published by Allen

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and Knights [2]. The other steroids were obtained from commercial sources. Their purity was evaluated by paper chromatography, thin-layer chromatography, determination of melting point, and GLC [3], as well as by HPLC. Analysis by the latter two methods indicated trace impurities in some of the steroids.

Chromatography

Reversed-phase HPLC was performed using a DuPont system (DuPont, Wilmington, DE, U.S.A.). The system consisted of an 870 pump module equipped with a universal septumless injector with a 50-μl sample loop, an ultraviolet (UV) spectrophotometer detector with a flow cell with a pathlength of 10 mm and a capacity of 8 μl, and a refractive index (RI) detector. A dual-channel Liniar Model 285 recorder (Liniar Instruments, Irvine, CA, U.S.A.) was connected to the two detectors.

A 250 × 4.6 mm I.D. commercially packed column of Zorbax ODS (5—6 μm particle size) (DuPont) was employed for all separations. The mobile phases, flow-rates, and column pressures were as follows: acetonitrile—water (60:40, v/v), 1 ml/min, 44 bars for ketones, 48 bars for diols; methanol—water (70:30, v/v), 2 ml/min, 220 bars for ketones, 237 bars for diols; methanol—water (11:9, v/v), 2 ml/min, 252 bars for triols, 290 bars for 17-hydroxypregn-4-ene-3,20-dione. Chromatography was carried out at room temperature.

RESULTS AND DISCUSSION

The steroids chromatographed and their retention times (t_R) using methanol—water and acetonitrile—water as mobile phases are listed in Table I. The steroids within each one of the three groups of ketonic metabolites, diols, and triols are listed in order of increasing t_R in the system methanol—water (70:30) and have each been given an identification number.

An example of the separation of a mixture of some ketonic C_{21} steroids using the mobile phase methanol—water (70:30) is illustrated in Fig. 1 and the separation of the same mixture using acetonitrile—water (60:40) is shown in Fig. 2. From Fig. 1 it can be seen that 5α-pregnane-3,20-dione (9) and 3α-hydroxy-5β-pregnan-20-one (10) did not separate using methanol—water as the mobile phase. However, these steroids were well separated using acetonitrile—water (Fig. 2). In the chromatography employing acetonitrile—water as the mobile phase (Fig. 2), 3β-hydroxy-5α-pregnan-20-one (11) and 5β-pregnane-3,20-dione (6) did not separate and the separation of 20β-hydroxy-pregn-4-en-3-one (4) and progesterone (3) was not optimal. These two pairs of steroids were separated using the methanol—water system (Fig. 1). Mixtures of the four 3-hydroxypregnan-20-one epimers were separated using either mobile phase. Acetonitrile—water is the mobile phase of choice for the separation of the four 20-hydroxypregnan-3-ones, since 20β-hydroxy-5α-pregnan-3-one (13) and 20β-hydroxy-5β-pregnan-3-one (14) were not well separated using methanol—water. Other pairs of steroids not optimally resolved or unresolved using one mobile phase can be well separated in the alternative system, e.g., methanol—water is the better mobile phase system for the pairs progesterone (3) and 20α-hydroxy-5β-pregnan-3-one (7); 20α-hydroxy-5α-pregnan-3-one

TABLE I

RETENTION TIMES OF SOME POSSIBLE C_{21} METABOLITES OF PROGESTERONE

The experimental conditions employed are described in the section *Chromatography* in the text and in the legends of the figures. The retention times are those obtained by refractive index detection.

No.	Steroid	Retention time (min)		
		Acetonitrile—water (60:40, v/v)	Methanol—water	
			70:30, v/v	11:9, v/v
Ketones				
1	17-Hydroxypregn-4-ene-3,20-dione	9.6	7.2	30.2
2	20α-Hydroxypregn-4-en-3-one	16.0	14.2	
3	Pregn-4-ene-3,20-dione (progesterone)	22.1	15.4	
4	20β-Hydroxypregn-4-en-3-one	21.3	20.2	
5	20α-Hydroxy-5α-pregnan-3-one	25.2	21.8	
6	5β-Pregnane-3,20-dione	30.2	22.8	
7	20α-Hydroxy-5β-pregnan-3-one	22.9	24.3	
8	3β-Hydroxy-5β-pregnan-20-one	27.4	24.8	
9	5α-Pregnane-3,20-dione	35.8	25.8	
10	3α-Hydroxy-5β-pregnan-20-one	25.0	26.3	
11	3β-Hydroxy-5α-pregnan-20-one	29.9	27.7	
12	3α-Hydroxy-5α-pregnan-20-one	31.8	31.3	
13	20β-Hydroxy-5α-pregnan-3-one	33.8	32.8	
14	20β-Hydroxy-5β-pregnan-3-one	31.7	33.5	
Diols				
15	5β-Pregnane-3β,20α-diol	18.5	21.4	
16	5α-Pregnane-3β,20α-diol	20.8	23.1	
17	5β-Pregnane-3β,20β-diol	24.9	31.7	
18	5α-Pregnane-3β,20β-diol	25.9	32.8	
19	5β-Pregnane-3α,20α-diol	19.5	33.4	
20	5α-Pregnane-3α,20α-diol	23.4	35.9	
21	5β-Pregnane-3α,20β-diol	25.5	42.2	
22	5α-Pregnane-3α,20β-diol	29.9	45.6	
Triols				
23	5β-Pregnane-3α,6β,20β-triol		3.0	9.2
24	5β-Pregnane-3α,6β,20α-triol		4.0	15.4
25	5β-Pregnane-3α,6α,20β-triol		4.3	18.0
26	5α-Pregnane-3β,6β,20β-triol		5.0	21.4
27	5α-Pregnane-3β,6β,20α-triol		5.6	25.7
28	5α-Pregnane-3α,6α,20β-triol		5.7	28.1
29	5β-Pregnane-3α,6α,20α-triol		5.8	30.5
30	5α-Pregnane-3α,6α,20α-triol		7.9	44.7

(5) and 3α-hydroxy-5β-pregnan-20-one (10), and 3α-hydroxy-5α-pregnan-20-one (12) and 20β-hydroxy-5β-pregnan-3-one (14).

The results of chromatography of a mixture of the eight pregnanediol stereoisomers using the mobile phase methanol—water (70:30) are shown in Fig. 3A. The chromatogram of the same mixture obtained using acetonitrile—water (60:40) as the mobile phase is shown in Fig. 3B. Again, the problems

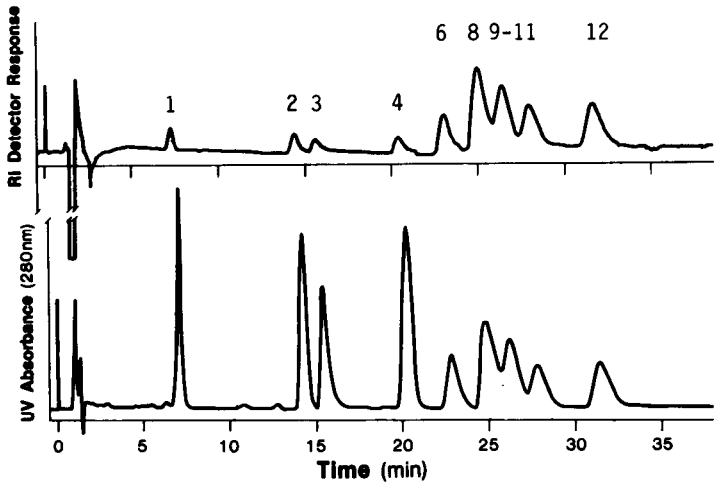

Fig. 1. Chromatogram of ketonic C_{21} steroids using the mobile phase methanol—water (70:30, v/v). A mixture of 10 μg each of 17-hydroxypregn-4-ene-3,20-dione, 20-hydroxypregn-4-en-3-ones, and progesterone, 40 μg each of pregnane-3,20-diones, and 75 μg each of pregnanolones dissolved in methanol was chromatographed on a Zorbax ODS column (250 mm × 4.6 mm I.D., 5—6 μm particle size). The flow-rate was 2 ml/min and the pressure was 220 bars. The effluent passed through two detectors in series, the first a UV spectrophotometer set at 280 nm, range 0.04, and the second a refractive index (RI) detector with the attenuator set at $0.05 \cdot 10^{-3}$ RI units. The dual-channel recorder was set at a span of 1 mV. The chart speed was 1 cm/min. For peak identification, see Table I.

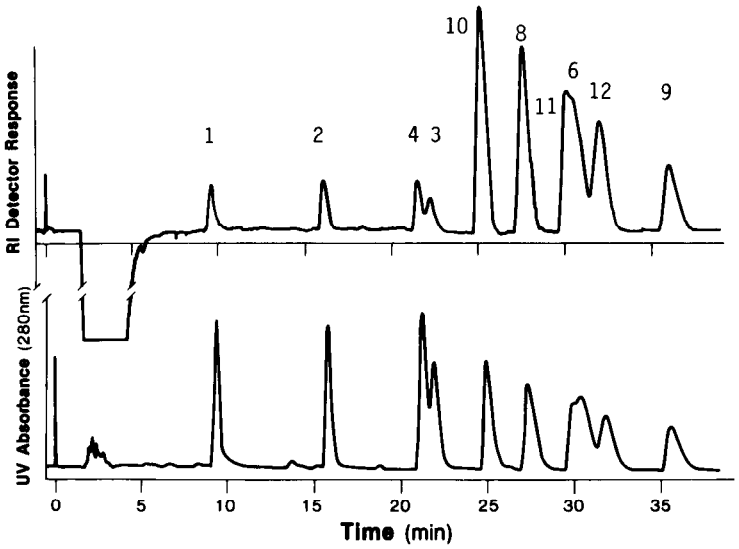

Fig. 2. Chromatogram of ketonic C_{21} steroids using the mobile phase acetonitrile—water (60:40, v/v). The flow-rate was 1 ml/min and the pressure was 44 bars. The UV spectrophotometer range was set at 0.08. The other parameters are as described in Fig. 1. For peak identification, see Table I.

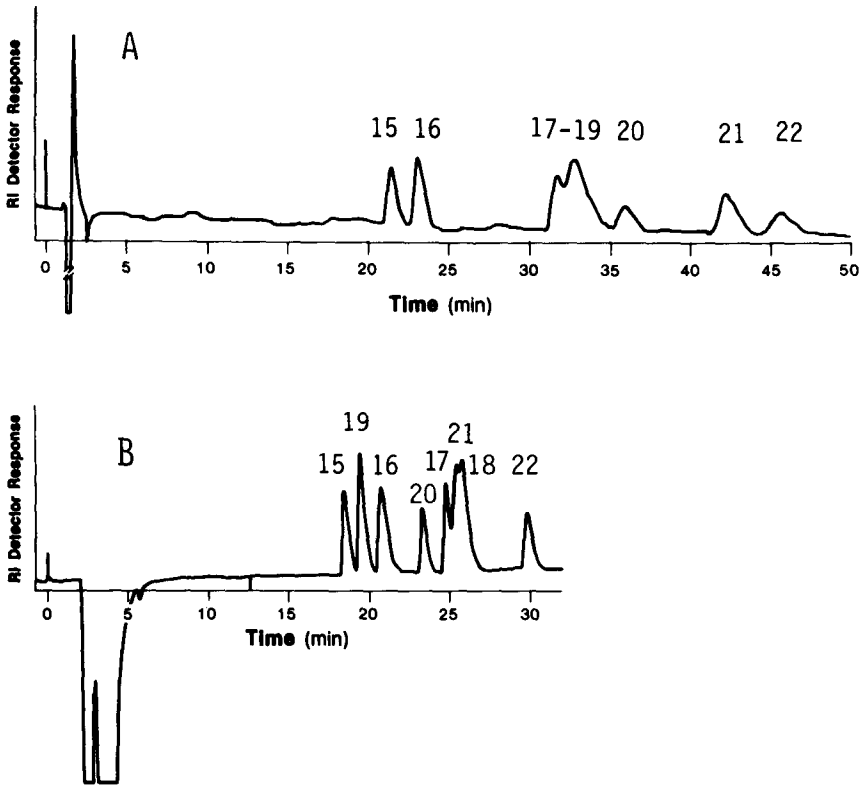

Fig. 3. Chromatograms of pregnanediols. A mixture of 70 μg of 5α-pregnane-3α,20β-diol and 85 μg each of the other pregnane-3,20-diols dissolved in methanol was chromatographed using (A) the mobile phase methanol—water (70:30, v/v), flow-rate 2 ml/min, pressure 237 bars, and refractive index detector attenuator at $0.05 \cdot 10^{-3}$ RI units, or (B) the mobile phase acetonitrile—water (60:40, v/v), flow-rate 1 ml/min, pressure 48 bars, and refractive index detector attenuator at $0.1 \cdot 10^{-3}$ RI units. The parameters for the column and recorder are as described in Fig. 1. For peak identification, see Table I.

encountered with one mobile phase system are solved by substituting the other system. Using the mobile phase methanol—water (Fig. 3A), 5α-pregnane-3β,20β-diol (18) and 5β-pregnane-3α,20α-diol (19) were not separated. 5α-Pregnane-3β,20β-diol (18) was only partially separated from 5β-pregnane-3β,20β-diol (17). However, a mixture of 5β-pregnane-3β,20β-diol (17) and 5β-pregnane-3α,20α-diol (19) will be resolved. The group of three steroids was separated from one another by employing acetonitrile—water as the mobile phase (Fig. 3B). Upon chromatography of the pregnanediols using this latter mobile phase, there was little separation of 5β-pregnane-3α,20β-diol (21) and 5α-pregnane-3β,20β-diol (18), and incomplete separation of 5β-pregnane-3α,20β-diol (21) and 5β-pregnane-3β,20β-diol (17). The two pairs of steroids have sufficiently different t_R values to allow separation using methanol—water as the mobile phase.

The components of a mixture of eight pregnane-3,6,20-triols were well separated using the Zorbax-ODS column and the mobile phase methanol—water

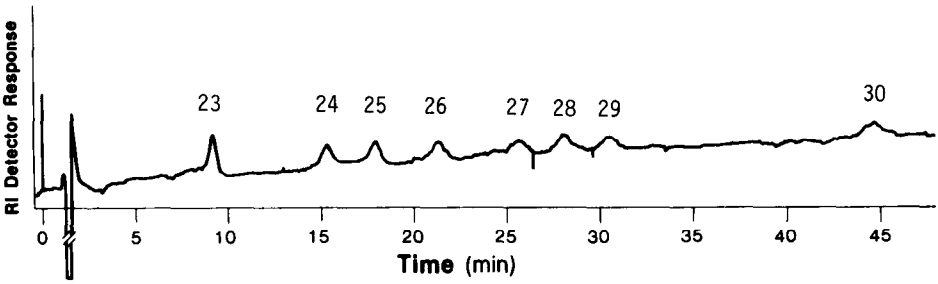

Fig. 4. Chromatogram of pregnane-3,6,20-triols. A mixture of between 37 and 45 μg of the pregnanetriols dissolved in methanol was chromatographed using the mobile phase methanol—water (11:9, v/v), flow-rate 2 ml/min, and pressure 252 bars. The parameters for the column, refractive index detector, and recorder are as described in Fig. 1. For peak identification, see Table I.

(11:9) (Fig. 4). This system was selected because of published results employing it for the HPLC of $C_{19}O_3$ steroids [4]. The attenuator setting on the refractive index detector was chosen as that optimal for securing both sufficient sensitivity with the limited amounts of these steroids available as reference standards and a satisfactory baseline. As can be seen from Table I, the employment of the mobile phase methanol—water (70:30) resulted in the separation of only certain of the pregnanetriols from one another. However, it would offer a further method of characterization by means of t_R for some of the previously separated pregnane-3,6,20-triols.

Lin et al. [1] have achieved separation of ketonic steroids and the pregnanediols using a combination of adsorption and reversed-phase partition HPLC. The adsorption systems consisted of two chromatographic tubes, each 300 × 2 mm I.D., packed in their laboratory with Partisil 5 (5 μm particle size, Whatman, Clifton, NJ, U.S.A.) connnected in series, and the mobile phases 0.25% ethanol in dichloromethane, hexane—isopropanol (97:3), and *n*-hexane—methanol—ethanol (96:3:1). The reversed-phase system consisted of two tubes, each 250 × 4 mm I.D., packed in their laboratory with Zorbax BP-ODS (7—8 μm particle size) and a mobile phase of 60% aqueous acetonitrile. The order of elution of the steroids obtained by us with the single-tube Zorbax-ODS column and acetonitrile—water (60:40) is similar to that obtained by Lin et al. [1] with their reversed-phase system with the exception that progesterone (3) preceeds 20α-hydroxy-5β-pregnan-3-one (7) in our system. Therefore, our results conform to their generalizations concerning the stereochemistry of isomers. For some ketonic steroids the use of the longer reversed-phase column appears to offer an advantage, since the greater differences in t_R between some of the pairs of steroids result in separations not achieved on the single-tube column with the mobile phase acetonitrile—water (60:40).

There are obvious benefits to be derived from prior fractionation of organic residues by Girard's *T* and digitonin partitions and various methods of chromatography [3]. However, the reversed-phase HPLC systems using a single column and easily exchangeable mobile phases employed in this investigation can be used in tandem with them or alone, depending on the complexity of the mixture and the purpose of the analysis, to achieve resolution of C_{21} metabolites of progesterone. The biological studies using the perfused canine

isolated gravid uterine preparation for which these methods were developed employed steroids labeled with ^{3}H and ^{14}C. Fractions of the elutriates from the HPLC column were collected and then assayed for radioactivity in a scintillation counter. Labeled metabolites of the progesterone injected were successfully separated and identified by these methods [5].

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