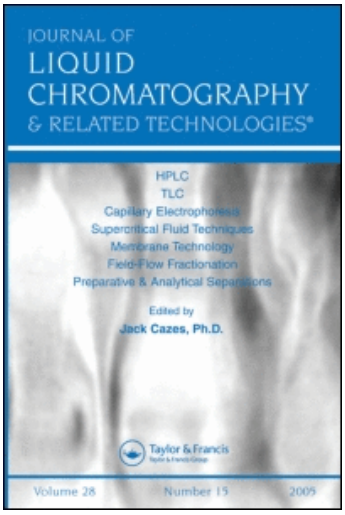

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Rapid High Performance Liquid Ch80Matography System for the Separation of Gonadal Steroids

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To cite this Article Keasler, Michael J.(1982) 'A Rapid High Performance Liquid Ch80Matography System for the Separation of Gonadal Steroids', Journal of Liquid Chromatography & Related Technologies, 5: 1, 125 — 139
To link to this Article: DOI: 10.1080/01483918208068825
URL: http://dx.doi.org/10.1080/01483918208068825

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(1), 125-139 (1982)

A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM FOR THE SEPARATION OF GONADAL STEROIDS

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SUMMARY

A radial compression reverse phase (C-18) column system in conjunction with a high performance liquid chromatograph has been developed which separates eighteen different gonadal steroids in a single run of forty minutes. In addition, three other gonadal steroids co-elute with two of the original eighteen but they could be separated by rechromatography on an alkyl (C-8) HPLC column. This combination of two columns allows the complete separation of twenty-one gonadal steroids or metabolites of the progesterone, pregnenolone, 5-α reductase, and aromatose pathways in less than one hour compared to a few weeks required when thin layer and paper chromatorgraphy are used to separate these steroid as in the present method employed.

INTRODUCTION

The concentration of various gonadal steroids in serum and/or tissue has been determined by extracting the steroid and quantifying them by radioimmunoassay. Despite the fact that highly specific antisera are used in the radioimmunoassay, cross reactions occur which interfere with the quantification of specific gonadal steroids, thus, a chromatographic system is required to separate the gonadal steroids (Figure 1) prior to radioimmunoassay. Numerous techniques for purification and separation of gonadal steroids (i.e. paper chromatography and thin layer chromatography [1-4], have been developed but all of these procedures are complicated and time consuming. Utilizing the techniques of high performance liquid chromatography (HPLC) in conjunction with the radial compression column system, it is possible to develop a rapid system for qualitation and quantitation by radioimmunoassay of collected fractions, or direct absorbance of gonadal steroids in serum or tissue. Also, the system can be used for quantification of radiolabeled metabolites formed in the incubation of

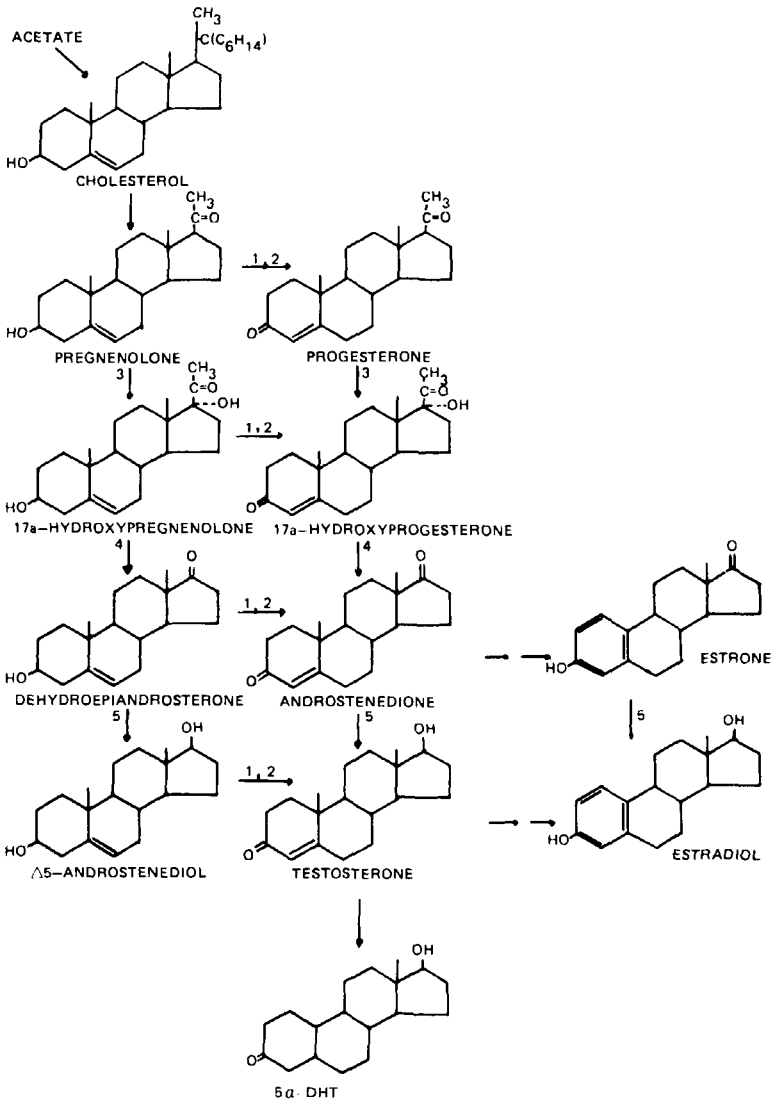

Figure 1: The various pathways (pregnenolone, progesterone, 5_{α}-reduced, and aromatase) for the conversion cholesterol to various gonadal steroids from different testicular tissues. The righthand column shows the Δ^4 and the left hand column the $\Delta 5$ pathway. Each conversion from left to right involves an isomerase and 3-β-hydroxysteroid dehydrogenase.

tissue with labeled precursors by counting an aliquot of each fraction collected from the HPLC eluent. Several researchers reported on the application of HPLC for separation of various gonadal steroids using isocratic systems [5,7], elution gradients with a non-polar reverse phase system [8], and DIOL columns [9]. These reported techniques do not allow adequate separation of gonadal steroids commonly found in serum or formed in tissue during metabolic studies (i.e. progesterone, pregnenolone, 5α reductase and aromatase pathway for gonadal steroids). This paper presents an HPLC separation system for the separation of twenty-one different gonadal steroids using the radial compression reverse phase (C-18 and C-8) column chromatographic system.

MATERIAL AND METHODS

The Waters (Milford, MA, U.S.A) HPLC system was employed for all of the studies. This system consists of a U6K injector, two 6000A solvent delivery systems, 450 variable-wavelength detector, 440 fixed wavelength (254nm) detector, 730 Data Module (for plotting and analysis of peak area and retention time) and the 720 Data Controller. A radial-Pak A column part of the radial compression system (reverse phase permanently bonded octadecylsilane (C-18) column -8 cm length - 10 μ particle size) or a reverse phase permanently bonded octasilane (C-8) column (8 cm length - 10 μ particle size). This radial compression separation system offers several advantages over the prepacked stainless steel columns: (1) enhanced reproducibility and reliability, (2) elimination of column channeling; and (3) consistent high efficiences. All experiments were performed at ambient temperature. The Waters HPLC was also equipped with an in-line precolumn filter. The Precision Sampling pressure lock syringe, 25μl, (Supelco, Bellfort, PA., U.S.A.) was used for sample injections.

Highly purified water was obtained by triple glass distillation of deionized water in our laboratory. The methanol, glass distilled-Omni Solv (MCB Manufacturing Chemists, Inc., Cincinnati, Ohio, U.S.A.) lot #10M/4 was used. The isopropanol, glass distilled-Omni-Solv was purchased from MCB lot #1N16. The acetonitrile was obtained from Waters Associate (Milford, MA, U.S.A.) number 84905. The steroids (nonradiolabeled) were obtained from Steraloids (Wilton, N.H. 03086) and the radioactive steroids were obtained from New England Nuclear

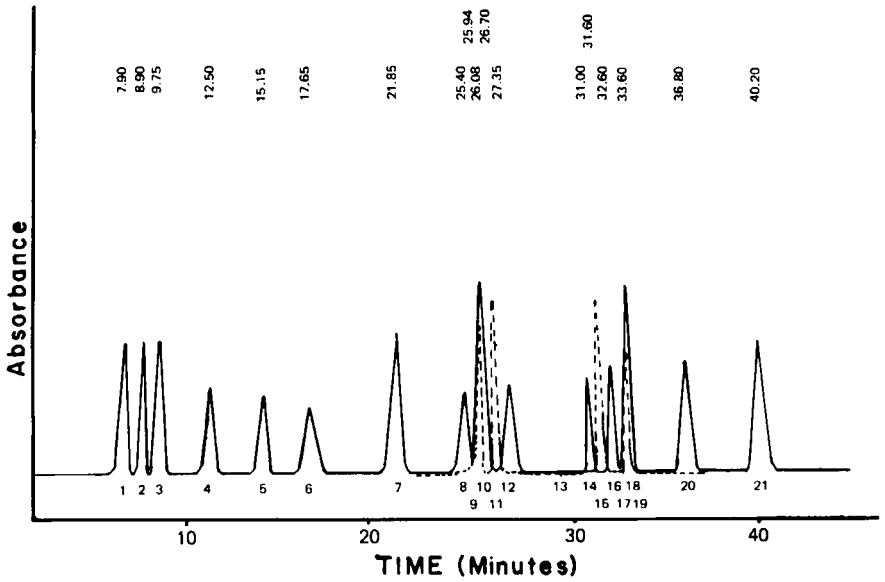

Figure 2: The HPLC separation of gonadal steroids using methanol-H_2O as the eluting solvent on a C-18 (10μ) reverse phase radial compression column. Solvent A = methanol/H_2O, (1/1,V/V). Solvent B = methanol. A linear gradient 2.0 ml/min. 0%B→30%B, 20 minutes followed by 30→100%B for the next 20 minutes. The Δ^4-pathways steroids and various other related steroids are shown in the solid lines. The dashed lines represent absorbance at 210 nm. The relative retention times are shown above each peak. The numbers below each peak refer to the names and numbers of the steroids found in abbreviation table.

(Boston, MA, U.S.A.). The buffers and solvents were filtered through 0.5 um Millipore to remove solid particles and degassed in order to avoid bubble formation.

RESULTS AND DISCUSSION

The pathway of gonadal steroid synthesis is similar to that found in all steroid forming tissues. Two major sources of gonadal steroids are: acetate, either formed from acetyl-coenzyme A during the metabolism of glucose or derived directly from blood; and cholesterol, formed elsewhere in the body and trans-

ported to the gonads. The relative importance of the two sources seems to depend on the species studied [10]. Cholesterol is different from the gonadal steroids in that it has a long side chain attached to the C-17 atom of the steroid nucleus. The side chain of cholesterol is split off at the first stage of this sterol to the first steroid Δ^5-pregnenolone [11].

Pregnenolone is converted to testosterone [12] along various pathways (Figure 1). The progesterone pathway (Δ^4) involves conversion of pregnenolone to progesterone, which can be converted to 17-hydroxyprogesterone (17 hydroxylase), then to androstenedione by a 17-20 lyase and finally to testosterone by a 17βhydroxysteroid dehydrogenase. The pregnenolone (Δ^5) pathway involves the conversion of pregnenolone to 17-hydroxypregnenolone, then to dehydroepiandrosterone, and finally to androstenediol by the same enzymes found in Δ^4 and Δ^5 pathway steroids, 5 α reductase pathway (DHT), hydroxylated testosterones, estrogens (aromatase pathway) in addition to testosterone esters (e.g. propionate and enanthate). These two latter steroids were added because they are used experimentally (testosterone propionate) for injection into rats, [16] and clinically (testosterone enanthate) for injection into humans to increase testosterone levels [17] but very little work has been done to quantitate these steroids after injection in the rat or human.

The HPLC was first set-up to find a column and solvent that would separate most of the steroids in a single run of 45 minutes or less. A C-18 radial compression column system was used with methanol as the solvent (Figure 2). This separation involves a gradient system (A = 50% methanol/50% H_2O, B = methanol) starting at 0%B and proceeding over 20 minutes to 30% B (70%A) then in 20 minutes to 100%B . This gradient elution system separates estrogens and the hydroxylated testosterone from the steroids of the Δ^4 and Δ^5 pathway and DHT from testosterone. The component steroids of the Δ^4 pathway (progesterone, 17-hydroxyprogesterone, androstenedione and testosterone) are well separated as well as are the steroids of the Δ^5 pathway, but several overlaps occur between the steroids of the Δ^4 and Δ^5 pathways. These include the coelution of pregnenolone and progesterone, testosterone and 17-hydroxypregnenolone and the close elution of androstenedione and dehydroepiandrosterone. This system re-

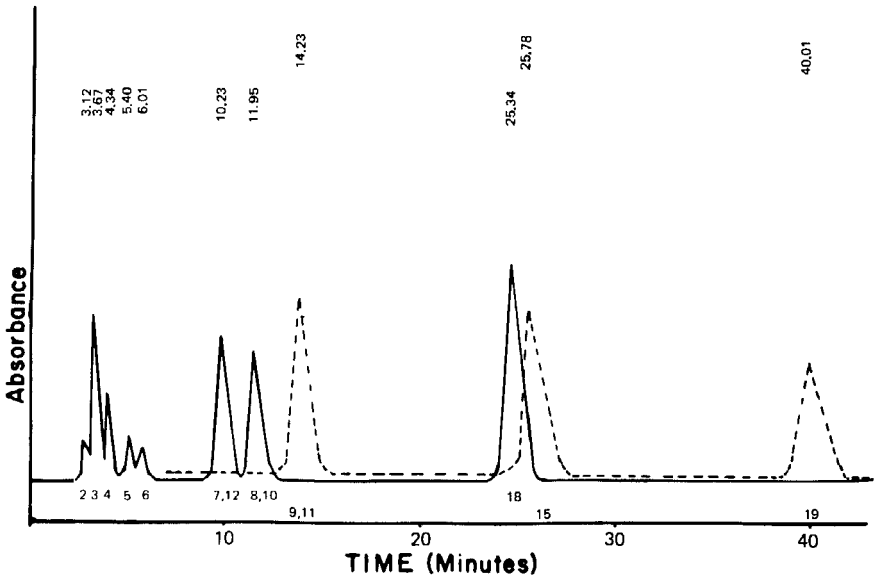

Figure 3: The HPLC separation of gonadal steroids using methanol-H_2O as the eluting solvent on a C-8 (10_{μ}) reverse phase compression column. Solvent A = methanol/H_2O (1/1,V/V). Solvent B = methanol. An isocratic elution using 85%A/15%B at a flow rate of 3.0 ml/min is employed. The Δ^4-pathway steroids and other related steroids are shown in the solid lines. The dashed lines represent absorbance at 210 nm. The relative retention times are shown above each peak. The numbers below each peak refer to the names of the steroid found in abbreviation table.

solved extremely well testosterone from testosterone propionate (10 minutes) and testosterone enanthate (15 minutes) so that studies of these derivatives in the presence of testosterone can easily be accomplished.

Therefore, eighteen of the steroids could be separated in a single run of forty minutes but a few overlaps occurred. Attempts to adjust the gradient using methanol failed to separate the overlapping peaks. Therefore, two methods were tried in order to resolve these overlapping peaks; first, change to a C-8

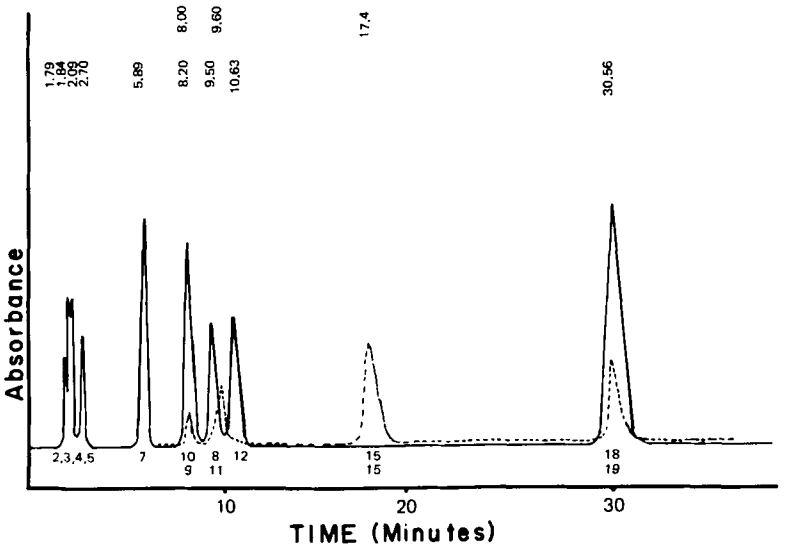

Figure 4: The HPLC separation of gonadal steroids using acetonitrile-H_2O as the eluting solvent on a C-18 (10μ) reverse phase radial compression column. Solvent A = acetonitrile/H_2O (1/9,V/V). Solvent B = acetonitrile. An isocratic elution using 65%A and 35%B at a flow rate of 3.0 ml/min is employed. The Δ^4-pathway and related steroids are shown by the solid lines. The dashed lines represent absorbance at 210 nm. The relative retention times are shown above each peak. The numbers below each peak refer to the names of the steroids as identified in abbreviation table.

type packing in the column, second, change to a different polarity solvent (acetonitrile or isopropanol).

The first approach was to change to a different column packing, namely, the C-8 radial compression column using the same solvent methanol. The optimum separation is shown in Figure 3. The column resolves the pregnenolone and progesterone by 15 minutes and separates the DHEA and 17-hydroxypregnenolone from the Δ^4 pathway steroids by 3-5 minutes with these steroids merging into a

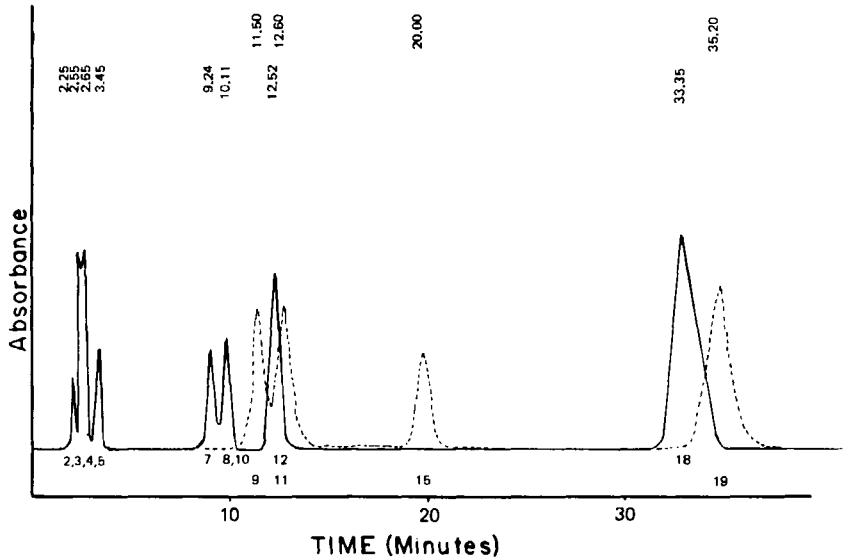

Figure 5: The HPLC separation of gonadal sterids using acetonitrile - H_2O as the eluting solvent on a C-8 (10μ) reverse phase radial compression column. Solvent A = acetonitrile/H_2O (1/9,V/V). Solvent B = acetonitrile. An isocratic elution using 70%A and 30%B at a flow rate of 3.0 ml/min is employed. The Δ^4-pathway and related steroids are shown by the solid lines. The dashed lines represent absorbance at 210 nm. The relative retention times are shown above each peak. The numbers below each peak refer to the names of the steroids as identified in abbreviation table.

single peak. Therefore, the Δ^4 and Δ^5-pathway steroids can be separated by using the C-8 column with methanol as solvent in an isocratic elution with methanol/water. This system also seems to compress the hydroxylated testosterone and merges into a single peak estradiol and androstenedione, as well as testosterone and 17-hydroxyprogesterone.

Next, changing the elution solvent from methanol to acetonitrile was tried on the original C-18 radial compression column (Figure 4) in order to find a single column for the elution of all twenty-one steroids. The results shown in Figure 4 indicate that the steroids of the Δ^4 and Δ^5-pathway are still

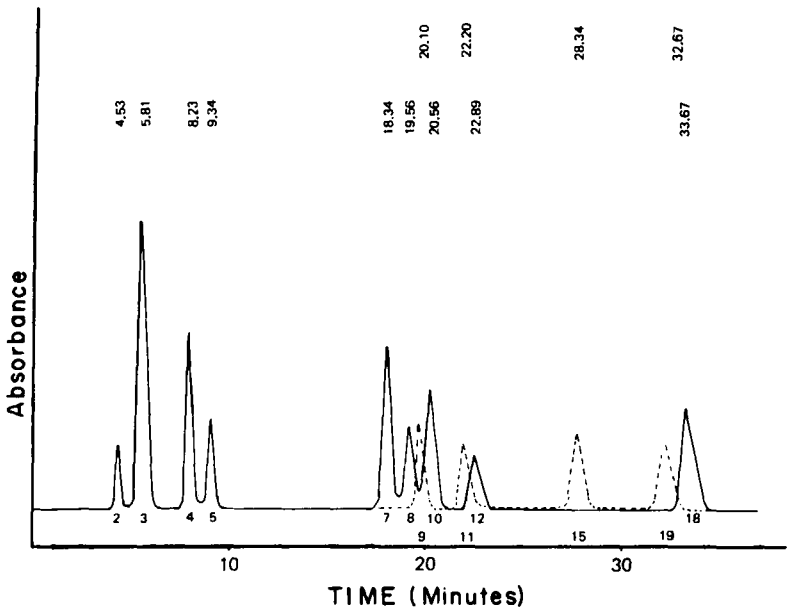

Figure 6: The HPLC separation of gonadal steroids using isopropanol-H_2O as the eluting solvent on a C-18 (10μ) reverse phase radial compression column. Solvent A = isoproponal/H_2O (1/9,V/V). Solvent B = isopropanol. A linear gradient elution starting at 85%A and 15%B and increasing to 60%A and 40%B over a forty minute period at a flow rate of 3.0 ml/min. The Δ^4-pathway and related steroids are shown by the solid lines. The dashed lines represent absorbance at 210 nm. The relative retention times are shown above each peak. The numbers below each peak refer to the names of the steroids as identified in abbreviation table.

separated but with some overlap particularly of pregnenolone and progesterone. In comparison with the elution using methanol the acetonitrile inverts the order of elution of 17-hydroxyprogesterone and testosterone. The elution distance between testosterone and progesterone has been increased from 7 minutes with methanol to 22 minutes with acetonitrile thus allowing better separation of steroids which elute in this area, namely, DHT, 5α-diol, 20β-hydroxylated progesterone. Therefore, this system offers an advantage if those particular

steroids are the ones of interest.

The acetonitrile was used to elute the C-8 radial compression with the results shown in Figure 5. Compared to the methanol, acetonitrile appears to not be as good an eluting solvent because the pregnenolone and progesterone are separated by on about three minutes. The DHEA and 17 hydroxypregnenolone are separated from all the Δ^4-pathway steroids except androstenedione.

Finally, isopropanol was used to elute the C-18 radial compression column. The results (Figure 6) are very similar to those found with methanol except the distance between the elution of testosterone and progesterone has been increased from 7 minutes to 13 minutes. It can also be seen that pregnenolone and progesterone are slightly separable by about one minute. Therefore, the use of isopropanol seems to offer only limited advantage over the use of methanol.

It is concluded that separation of the twenty-one gonadal steroids of the pregnenolone, progesterone, 5α reductase, and aromatase pathway required two columns. Elution from the C-18 column and rechromatography of certain areas on the C-8 column using methanol as solvent will result in the separation of the major gonadal steroids in 2-3 hours compared to the several weeks required with the present system of paper and thin-layer chromatography.

This system of separation of gonadal steroids has been applied to two different types of samples. First, serum and/or testicular tissue from rats injected with various amounts of testosterone propionate. The tissue and serum were individually extracted, and the resultant sample was injected in the HPLC using the conditions shown in Figure 2. Fractions were collected, dried and assayed for testosterone using a specific radioimmunoassay [18]. Three peaks of RIA reactive material are separable by HPLC (Figure 7). These peaks are tentatively identified as testosterone, 5α reduced androgens (DHT, 5αdiol), and possibly testosterone propionate (manuscript in preparation). Therefore, this HPLC separation system can be used to separate steroids prior to RIA in order to remove any contaminants from serum or tissue which might interfere in the RIA determination of steroids. Thus, the exact criteria for quantitation of steroids would be first elution at the proper position on the HPLC and RIA of

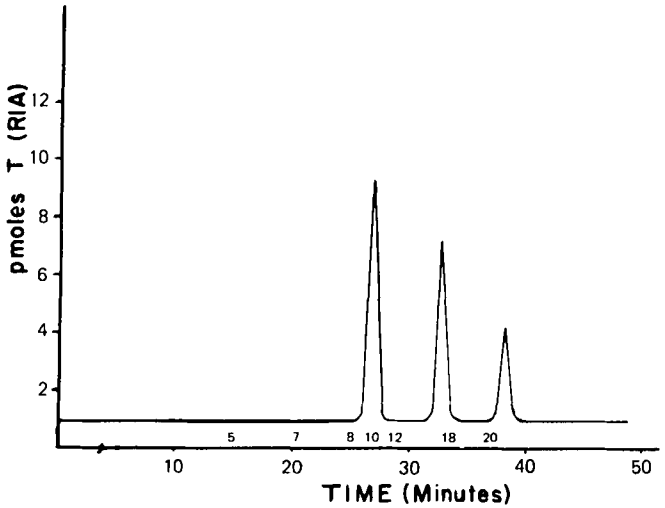

Figure 7: The HPLC separation of steroids found in the serum of T.P. injected rats. One milliliter of serum was solvent extracted and injected on a C-18 (10μ) reverse phase radial compression column. Solvent A = methanol/H_2O (1/1,V/V). Solvent B = methanol. A linear gradient 0→30%B for 20 minutes followed by 30→100%B for the next 20 minutes at a flow rate of 2.0 ml/min. was used to separate the steroids. Fractions (0.5 min) were collected in a LKB Redirac Fraction Collector. The fractions were dried and assayed for testosterone using the double-antibody radioimmunoassay (RIA).

only that peak position, since most contaminants would be removed. Second, samples obtained from metabolic studies where radioactive precursors were used to determine the steroids conversion in studying the metabolism of that steroid by a specific tissue or cell cultures. Follicles obtained from bovine ovaries were incubated for 3 hours with [4-^{3}H] progesterone. The tissue and media were extracted and injected into the HPLC, using the conditions shown in Figure 2. Fractions were collected at 0.5 minute intervals. The results are shown in

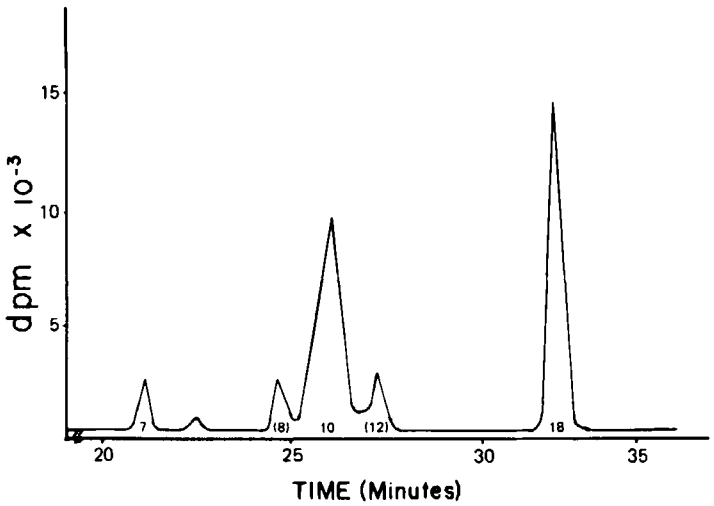

Figure 8: The HPLC separation of steroids resulting from the incubation of [4-^{3}H] progesterone with bovine follicles. The incubation media from the incubation of 500 mg of follicle with 3 ml of media containing [4-^{3}H]-progesterone. (95 Ci/mmole). The media is extracted and then injected onto a C-18 (10μ) reverse phase radial compression column. Solvent A = methanol/H_2O (1/1,V/V). Solvent B = methanol. A linear gradient 0→30%B for 20 minutes followed by 30→100%B for the next 20 minutes at a flow rate of 2.0 ml/min. is used to separate the steroids. Fractions (0.5min) were collected in by a LKB Redirac Fraction Collector. An aliquot (50μl) was removed from each fraction, air-dried, in a scintillation vial, and 15 ml of scintillation fluid (PPO, 5 g/ l of toluene) was added to each fraction before counting in a Packard Tricarb Liquid Scintillation Counter.

Figure 8 (also as shown by cold mass). The predominant products were testosterone and 17 hydroxyprogesterone with a small amount of estradiol also being formed. The exact details will be presented elsewhere (manuscript in preparation).

These two types of experiments demonstrate that HPLC provides a quantitative and rapid technique for the separation of gonadal steroids prior to RIA and that this technique can be used for the study of steroid metabolism in gonadal tissue, and specific gonadal cell cultures. Further studies of various gonadal tissue, serum samples, and gonadal cell culture are presently in progress and designed to take advantage of the rapid separation of the HPLC of gonadal steroids.

ACKNOWLEDGEMENTS

This research was in part supported by the Robert A. Welch Foundation Grant AU-829 and the University of Texas Biomedical Research Grant S-507-RR05745 from the Division of Research Resources of the National Institutes of Health. The assistance of Ralph Stevens for the serum samples and Ralf Zimmermann for the bovine follicle sample is gratefully acknowledged.

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ABBREVIATIONS

Trivial Name	Abbreviation	Peak Number in Figures	Chemical Name
Estriol	E_3	(1)	Estra-1,3,5(10)-triene-3, 16α, 17β-triol
7-α-Hydroxytestosterone	7 OH-T	(2)	4-androsten-7α, 17 -diol-3-dione
19-Hydroxytestosterone	19 OH-T	(3)	4-androsten-17β, 19-diol-3-one
11-α-Hydroxytestosterone	11α OH-T	(4)	4-androsten-11α, 17β-diol-3-one
11-β-Hydroxytestosterone	11β OH-T	(5)	4-androsten-11β, 17β-diol-3-one
16-β-Hydroxytestosterone	16β OH-T	(6)	4-androsten-16β, 17β-diol-3-one
Estradiol	E2	(7)	Estra, 1,3,5(10)triene-3, 17 -diol
17-Hydroxyprogesterone	17 OH-P 17 OH-P	(8)	17α-hydroxypregn-4-ene-3, 20-dione
17-Hydroxypregnenolone	17 OH Δ^5	(9)	17α, 3β-dihydroxypregn-5-en-20-one
Testosterone	T	(10)	17β-hydroxyandiost-4-eve-3-one
Dehydroepiandrosterone	DHEA	(11)	3β-hydroxyandrost-5-ene-17-one
Androstenedione	A	(12)	Androst-4-ene-3, 17-dione
Dihydrotestosterone	DHT	(13)	17β-hydroxy-5α-androstan-3-one
20-α-Hydroxyprogesterone	20αOH-P	(14)	4-Pregnen-20α-ol-3-one
Androsterone	AD	(15)	5α-Androsten-3α-ol-17-one
20β-Hydroxyprogesterone	20βOH-P	(16)	4-Pregn-20β-ol-3-one
5α-Androstandiol	5αdiol	(17)	5α-Androsten, 3α, 17β-diol
Progesterone	P	(18)	Pregn-4-ene-3, 20-dione
Pregnenolone	Δ^5	(19)	3β-hydroxypregn-5-en-20-ene
Testosterone propionate	TP	(20)	4-androsten-17β-ol-3-one propionate
Testosterone enanthate	TE	(21)	4-androsten-17β-ol-3-one enanthate