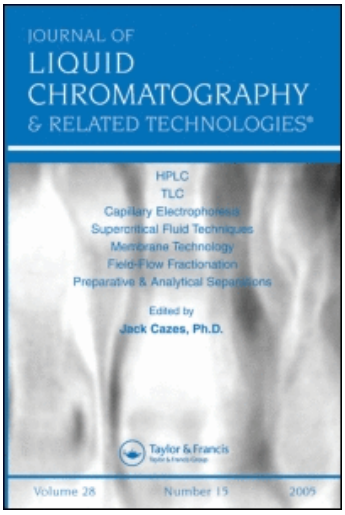

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A Rapid Method of Quantitating Steroids Resulting from the Incubation of Gonadal Tissues with Radioactive Precursors

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A RAPID METHOD OF QUANTITATING STEROIDS RESULTING FROM THE INCUBATION OF GONADAL TISSUES WITH RADIOACTIVE PRECURSORS

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ABSTRACT

A rapid method has been developed for the quantitation of steroid metabolites resulting from the incubation of specific gonadal cell types or gonadal tissue with radioactive precursors. The method involves the use of high performance liquid chromatography (HPLC) for separating the steroids and a flow-through radioactive detector (Flo-One HP) for quantitating the radioactive ^{3}H precursor and metabolites in the presence or absence of ^{14}C-steroid recovery tracers. A comparison is made between the results obtained directly by the Flo-One HP radioactivity detector and the fraction collection method, (counting aliquots from individual fractions in the liquid scintillation counter). In addition, the results using an electronic stream splitter in the analysis of a percentage of the effluent directly by Flo-One HP are evaluated. The remaining percentage is collected in a fraction collector and is used for further analysis (e.g. recrystallization, RIA, further purification and characterization).

The separation and quantitation of steroids from various tissues has been routinely done by one of several techniques including direct colorimetric analysis (1), radioimmunoassay (2), gas chromatography (3), and gas chromatography-mass spectrometry (4). These techniques are very sensitive and accurate for tissue studies involving determination of the exact amount of various steroids present, but for metabolic studies using radioactive precursors, these techniques are not effective for quantitation. Previously, for the quantitation and separation of radioactive gonadal steroids resulting from the incubation of specific gonadal cell cultures or tissues with radioactive precursors, the major procedure used was a combination of paper and thin layer

chromatography. This involved the initial extraction of the tissue; and, the extract was initially chromatographed on paper, followed by radioactivity scanning to locate the radioactive zones. The zones containing the radioactivity (5,6) were then eluted. Each of the eluted zones was separated further by thin layer chromatography. The developed thin-layer plates were then scanned for radioactivity, the silica gel removed from the plate and eluted with solvent. The solvent was filtered through a fritted glass filter to remove silica gel before quantitation by recrystallization (until a constant $^{14}C/^{3}H$ ratio was established). This procedure was difficult and time consuming, and required from one to four weeks for completion (7). More recently, with the development of HPLC, the separation of radiolabeled steroids (8-12) has become much more efficient but the quantitation still required the fraction collecting of the HPLC eluent into individual tubes, removing an aliquot from each tube to a scintillation vial, and then counting directly in the scintillation counter (fraction collection method).

This paper describes the use of an HPLC system (8) for the separation of gonadal steroids found in gonadal tissue or specific cell cultures. This HPLC system is interfaced with one of three separate options for the quantitation of the radioactive steroids. First, the eluent from the HPLC can be collected in tubes using a fraction collector with a rapid tube change (fraction collection method). Second, the eluent from the HPLC can be quantitated directly with a flow through radioactive detector (Flo-One HP). This detector permits the direct quantitation of both ^{14}C and ^{3}H labeled steroids used in metabolic studies of gonadal tissues. Third, the eluent from the HPLC is split using an electronic stream splitter with a certain percentage being directed to the flow radioactive detector and the remaining collected in tubes for further analysis (i.e. purification, recrystallization, NMR, GC-mass spectrometry, etc.). The latter two detection system in conjunction with the HPLC permit the separation and quantitation of gonadal steroid (radiolabeled) in less than one hour.

MATERIAL AND METHODS

The Waters (Milford, MA,) HPLC system was employed for all of the studies. This system consists of a U6K injector, one 6000A and one M45 pump, a 450 variable-wavelength detector, a 440 fixed-wavelength (254nm) detector, a 730 Data Module (for plotting and analysis of peak area and retention time) and a 720 Data Controller. A radial-Pak A (reversed-phase permanently bonded 10μm octadecylsilane C_{18} column, 8 cm in length) was used as part of a radial compression system.

A 3μm reversed-phase C_{18} (12% ODS) column, 4.6 mm ID x 15 cm (Custom LC, Houston, Texas) was employed for high-resolution HPLC. The Waters HPLC was equipped with an in-line precolumn filter. A 25 μl Precision Sampling pressure lock syringe, (Supelco, Bellfort, PA.) was used for sample injections.

Highly purified water was obtained by triple glass distillation of de-ionized water in our laboratory. Methanol (glass distilled-Omni Solv, MCB Manufacturing Chemists, Inc., Cincinnati, Ohio, Lot #10M/4) was used. The steroids were from Steraloids (Wilton, N.H.) and the radioactive steroids were obtained from New England Nuclear (Boston, MA.). The buffers and solvents were filtered through a 0.45μm Millipore filter and then degassed.

For the detection of radioactive metabolites one of the following methods was employed. First, the Water's HPLC system was interfaced with a RediRac Fraction Collector, Model 2112 (LKB, Rockville, MD.), with fractions being collected at 0.5 min intervals. A 50 μl aliquot was removed from each fraction, dried in a scintillation vial, 10 ml of scintillation fluid (PPO, 5 gm/liter of toluene) was added. Each sample fraction was counted for 5.0 min in a Packard Liquid Scintillation Counter. Second, the Waters HPLC system was interfaced directly with the Flo-One HP radioactivity detector (0.500 ml flow cell), using Flo Scint II as the nongelling scintillation fluid (Flo Scint II: effluent, 3:1, v/v). The No. 2 pen of the Waters Data Module was connected directly to the output of the Flo-One HP, resulting in a plot of dis-

integrations per 6 sec (either ^{14}C or ^{3}H). The ^{3}H and ^{14}C counts were determined simultaneously with the Flo-One HP which automatically corrects for the ^{14}C crossover and prints out the corrected number of dpm of ^{3}H and ^{14}C. Third, the calculation and the data obtained from aliquots counted directly in the Packard Liquid Scintillation Counter were compared to the data from the Flo-One HP. This was accomplished by splitting the column effluent after passing through the Fixed Wavelength 440 detector (Water Associates, Milford, Mass.) with the Electronic Variable Ratio Splitter, Model ES (Radiomatic Instruments and Chemical Co., Tampa, Florida) 50% at 2-sec intervals.

Human testicular biopsy tissues were incubated *in vitro* with ^{3}H pregnenolone by a previously described technique (13). The involves incubation of teased testicular tissue (20 mg) with a saturating concentration of ^{3}H-pregnenolone in 3 ml of incubation medium in an atmosphere of O_2: CO_2 (95:5) at 37°C under constant shaking for 3 hr. At the end of the incubation, ^{14}C-labeled recovery tracers and nonlabeled carrier steroids were added. The metabolites formed and remaining substrate were extracted, dried and injected into the Water HPLC system using the 3 μm C_{18} (12% ODS column) described earlier. The procedure for incubating the interstitial cell cultures with radioactive precursors has been described elsewhere (14).

RESULTS AND DISCUSSION

Previously, the separation and quantitation of gonadal steroids involved several steps (thin layer and paper chromatography) or derivatization (gas chromatography, colorimetric, gas chromatography-mass spectrometer) of the steroids was necessary. These techniques were excellent for quantitation of large amounts of steroids but for radiolabeled steroids used in metabolic studies these techniques could not be used, because these techniques were not sensitive for the quantitation of radioactivity. More recently with the development of the HPLC for the separation of gonadal steroids (8,9,10), radiolabeled steroids could be quantitated by collection of the eluent from the HPLC with fraction collector method. This involved fraction collecting of

the HPLC effluent. Each of the individual tubes could then be quantitated in a liquid scintillation counter. These methods proved excellent for quantitation, but required a large number of scintillation vials, scintillation fluid, time to aliquot the samples, and liquid scintillation counting time. This paper presents the comparison of the fraction collector method with two other direct method for the direct quantitation of radiolabeled steroids separated by the HPLC.

COMPARISON OF FRACTION COLLECTION AND DIRECT RADIOACTIVE DETECTION METHODS

A simple method for the quantitation of radioactive steroids after HPLC is to collect fractions at short time intervals, and to count aliquots in scintillation vials, in a liquid scintillation counter. The procedure requires sufficiently rapid tube changes so that no more than one drop is lost between fractions. The results of fraction collecting an HPLC analysis of an extract of an incubation of interstitial cells with ^{3}H-testosterone is shown in Figure 1A. Individual fractions were collected every 0.5 min. Two major peaks and one minor peak are seen. Allowing 5 minutes for counting each fraction, it took 8-10 hours to count 80 fraction obtained from each sample.

A comparison of the fraction collection method with the direct analysis by interfacing the eluent from the HPLC with a flow-through radioactivity detector (Flo-One HP) is shown in Figure 1 A + B. In Figure 1B, (radioactive detector) two major and four or five minor peaks are detected and found to be well resolved from each other. This greater resolution is due to the fact that the Flo-One HP gives an updated signal to the recorder every 6 seconds. This data is then plotted on Pen 2 of the Waters Data Module. To obtain similar results by the fraction collection method, fractions would have to be collected every 0.1 minute over the 40 min collection period, i.e. 400 tubes. Thus, the flow-through detector is more rapid and yields greater resolution than the fraction collector method. The time and cost of sample analysis is also lower for the flow-through radioactive detector.

The quantitative analysis by the fraction collection method is compared with the Flo-One HP method in Table I for two similar samples from study of

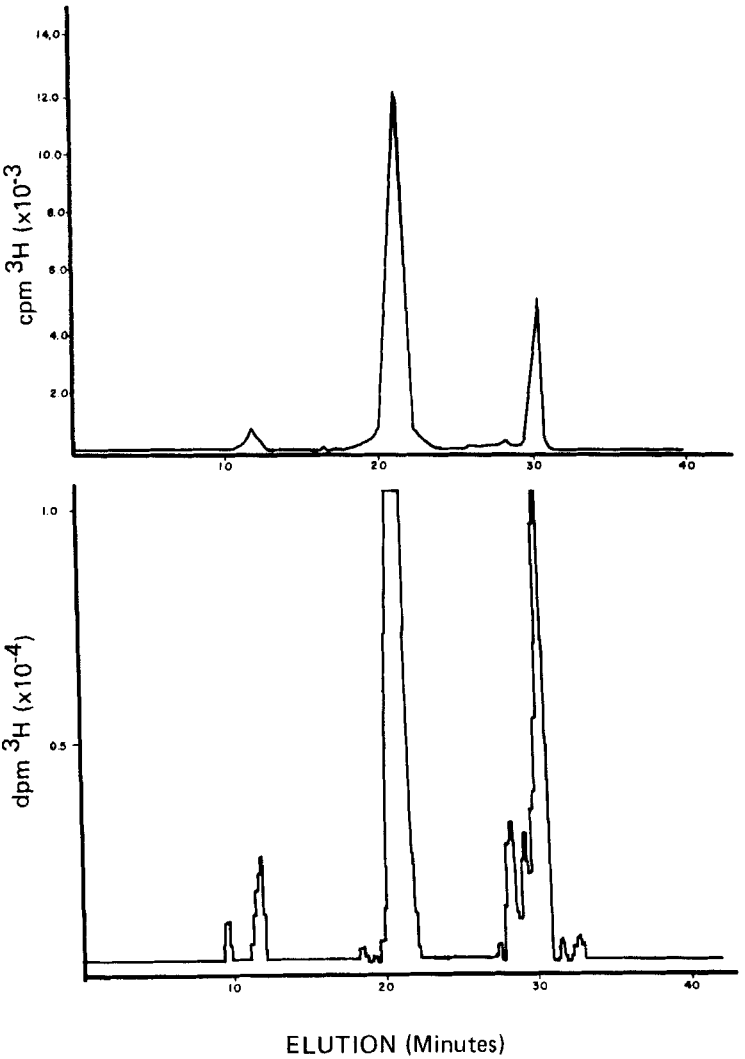

Figure 1: HPLC analysis of an incubate of isolated testicular interstitial cells with ^{3}H-testosterone using a 10µm C_{18} radial compression column eluting with a methanol/water gradient. Conditions: Solvent A = methanol/H_2O, (1/1,V/V) and Solvent B = methanol. A linear gradient 2.0 ml/min. 0%B→30%B, 20 minutes followed by 30→100%B for the next 20 minutes. The upper panel is a plot of cpm/aliquot of fractions from the Redi Rac 2112 fraction collector, fractions are collected every 0.5 min (1.0 ml each) and count for 5.0 min each in liquid scintillation counter. The lower panel is the actual plot the Flo-One HP radioactivity detector signal for ^{3}H dpm printed out by Pen 2 of the Waters Data Module.

Table I: Comparison of Counts Obtained With Flo-One HP Flow Radioactive Detector and Fraction Collection Followed by Liquid Scintillation Counting

Sample #	Peak*#	Flo-One/HP (dpm)	F.C. (dpm)	Flo-One dpm/F.C. dpm
84 F	1	2,860	3,636	0.78
	2	65,400	79,070	0.82
	3	9,040	12,325	0.73
	4	48,628	58,387	0.83
			153,418 dpm	
			(99% recovery)	
			S.D. = 0.045	S.E. = 0.023
84 B	1	2,770	3,614	0.76
	2	39,304	52,727	0.75
	3	18,810	22,872	0.82
			79,213 dpm	
			(91% recovery)	
			S.D. = 0.039	S.E. = 0.021
		TOTAL:	S.E. = 0.039	S.E. = 0.015

* Peak # is the order of elution of the major radioactive peaks shown in Figure 1.

the metabolism of radioactive steriods by interstitial cell cultures. The overall recoveries from HPLC were 91-99%, and the mean ratio dpm Flo-One/dpm scintillation counter was 0.784 for the seven peaks (Figure 1) shown on Table I. Thus, the overall efficiency of the flow-through detector for ^{3}H was 20% (0.784 x 25% efficiency set on the Flo-One HP) or about 1/2 that of the liquid scintillation counter. In addition, the standard deviation and error are very small, indicating that the efficiency for the flow through detector is maintained for low counting (2860 dpm) as well as high counting (48,628 dpm) samples. The Flo-One HP radioactivity detector can thus quantitate the radioactive steroids in the eluate from HPLC in less than one hour per sample compared to 5-10 hrs for the fraction collection method counting 5 minutes/tube.

ELECTRONIC SPLITTER - ANALYSIS OF A CERTAIN PERCENTAGE OF THE SAMPLE

The major drawback of using only the Flo-One HP and mixing the HPLC effluent with a nongelling scintillation is that the entire sample must be utilized, thus, the sample cannot be used for further analysis. Instead of the use of the liquid scintillation mixing, a solid scintillator could be used. This would allow complete recovery of the sample, but for detectable counts above background 10-20,000 dpm of ^{3}H and 250-400 dpm of ^{14}C would be required. The solid scintillation also has the disadvantage that the radioactive sample may interact with the solid support (usually silica) and slowly bleed from the solid scintillator giving high background levels, or actual bind to the solid scintillation.

Ideally, the system of choice should have high sensitivity (800 dpm for ^{3}H) and allow at least partial recovery of the sample. The following method was used to accomplish this objective. The eluent from HPLC was diverted to an electronic splitter (ES) using microbore tubing. Fifty percent of the eluate was diverted to the fraction collector and the remaining 50% to the Flo-One HP radioactive detector. This method was employed to analyze human testicular biopsy specimens, which were incubated with ^{3}H-pregnenolone for 3 hours. At the end of the incubation, before the samples were extracted, ^{14}C-recovery tracers were added. The samples were extracted and the extract analyzed by HPLC or paper and thin-layer chromatography. The results obtained by each technique were very similar (manuscript in preparation), but HPLC using the conditions shown in Figure 2 was much more rapid. After elution from HPLC, the sample was divided by the ES (50% to fraction collector, 3A and 50% to Flo-One HP, 3B). The results obtained by the fraction collection method, Figure 3 A, were corrected to the entire sample (multiplied times two), with the Flo-One HP graph not corrected to the entire sample with the dpm for only 1/2 of the sample. The plot, Figure 3C, showed the actual histogram for ^{3}H as plotted by the Water Data Module (Pen 2). As can be seen from Figure 3A, B, C, the profiles for the two methods are almost identifical for both ^{14}C and ^{3}H. The Flo-One HP corrected the ^{3}H channel for the ^{14}C crossover

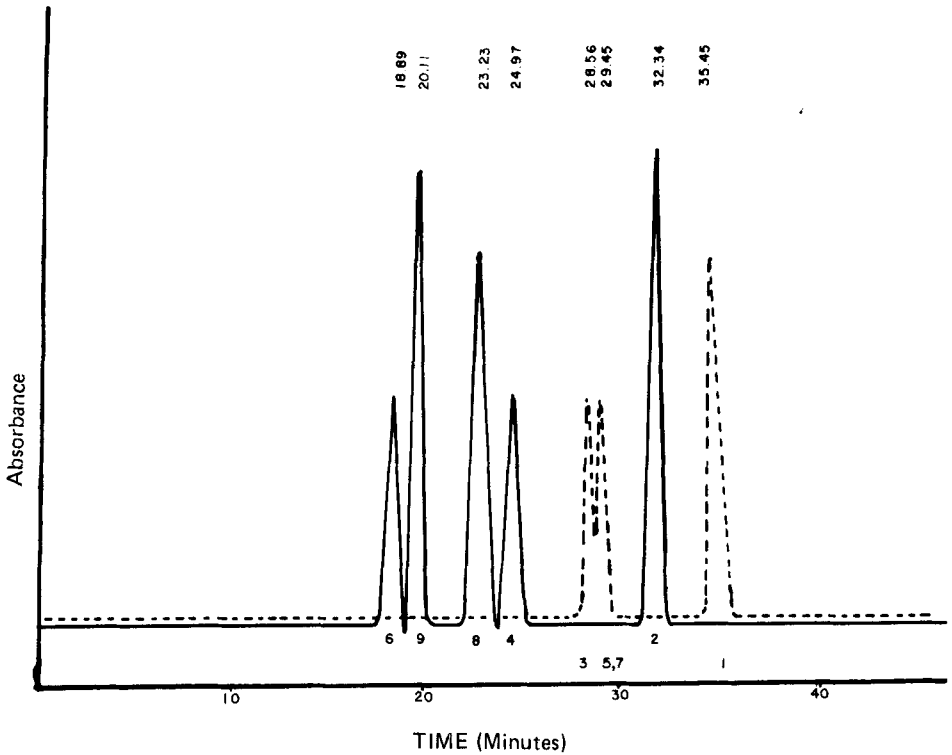

Figure 2: HPLC separation of gonadal steroids obtained by using methanol-/water as the eluent on a 3 μm C_{18} reversed-phase stainless steel column. Solvent A = methanol/water (1/1,v/v) and solvent B = methanol are used. A gradient elution at a flow rate of 1.0 ml/min from 0→10% B over 20 minutes with Waters Curve 6 (linear), followed by Waters Curve 2 to 50% B in the next 10 minutes, and then a linear increase to 100% B in the next 15 minutes is used. Steroid absorbance of the progesterone pathway steroids is are plotted by a solid line; the dashed line represents the pregnenolone pathway steroids. The relative retention times are shown above each peak. The number below each peak refers to the identity of each peak: (1)pregnenolone, (2) progesterone, (3) dehydroepiandrosterone, (4) 17-hydroxyprogesterone, (5) 17-hydroxy-pregnenolone, (6) androstenedione, (7) Δ^5-androstenediol, (8) testosterone, (9) estradiol.

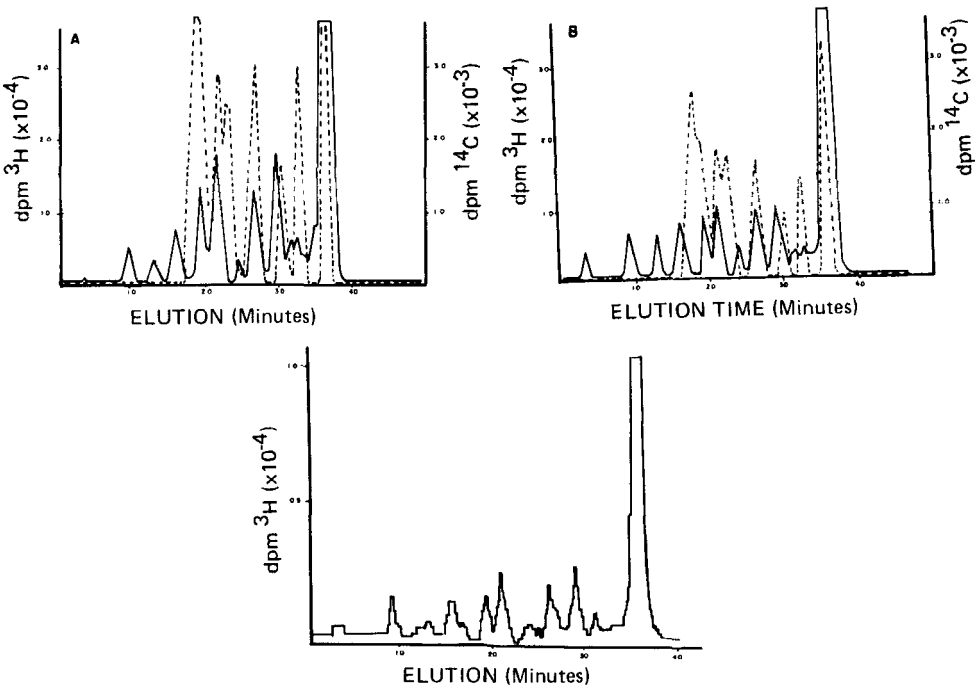

Figure 3: HPLC of a human testicular biopsy specimen incubated with ^{3}H-pregnenolone. At the end of the incubation ^{14}C recovery tracers were added the sample extract with organic solvent, and the steroids were analyzed on the HPLC using condition shown in Figure 2. The eluent from HPLC was split with an electronic splitter, 50% was diverted to fraction collector and 50% was analyzed directly by the flow-through radioactivity detector. Panel A is a plot of the fraction collector number (0.5 minute/fraction) vs. dpm, corrected for the entire sample (multiplied by 2). Panel B shows the direct analysis of the sample by the Flo-One HP radioactive detector, analysis for ^{14}C and ^{3}H being printed every 0.5 minute. The ^{14}C and ^{3}H were then plotted on the same time scale as the fraction collected samples. The dashed line represent ^{14}C and the solid line ^{3}H. The lower panel is the actual plot of the Water Data Module Pen 2 (10,000 dpm full scale). The time scale is different from panel A and B, since it is a direct reproduction of actual plot (0.50 cm/minute) for ^{3}H dpms.

(number of ^{14}C counts in the ^{3}H channel) and automatically calculated the number of dpm for both ^{14}C and ^{3}H. This feature was advantagous, because a ^{3}H precursor and ^{14}C recovery tracers are used in most gonadal steroid metabolism studies because of the loss of various steroids in the extraction and subsequent purification steps. The overall recoveries for the two method are about the same for ^{3}H and ^{14}C, as shown in Table II with a several different radioactive steroid peaks being analyzed (dpm/peak).

The results in Table II show that the data obtained by using the fraction collection method, compare very well with those obtained with the radioactivity flow detector when the eluate is split in half for each of the two modes of detection. The overall ratio of dpm for the scintillation counting to the radioactivity detector is about one, the efficiency for ^{14}C is 52.42% in the ^{14}C over ^{3}H channel. With the 25% crossover of ^{14}C into the ^{3}H channel the overall efficiency for the complete ^{14}C (52.42 + 25.0%) is 77.42% compared with 90-95% for the scintillation counter. The overall ^{3}H efficiency for the radioactivity flow detector is about 37.19% with a standard deviation of 3.56 and a standard error of 1.26, compared to the 50-60% efficiency for the liquid scintillation counter. Therefore, the radioactive flow detector can be used to quantitate rapidly ^{14}C and ^{3}H simultaneously in a sample and using the electronic splitter a portion (1-99%) of the eluate can collected in fraction collector tubes for further analysis. Eight human testicular biopsy specimens have been analyzed by this procedure. The HPLC and Flo-One HP separation and quantitation method represents a rapid method for studying steroidogenesis in human testes.

In conclusion, radiolabeled steroids from metabolic studies can be separated by HPLC and quantitated directly by the Flo-One HP. In addition, with the use of an electronic stream splitter a certain percentage of the eluate from the HPLC can be diverted to the radioactivity flow detector with the remaining percentage of the sample used for further analyses (recrystallization, derivatization, or further purification by HPLC). These methods result in almost identical quantitative data compared to the previous used frac-

Table II: Ratio of dpm scintillation counter to dpm on Flo-One HP radioactive flow detector and % efficiency for several peaks of testicular biopsy sample (50% split)

A. Peak*#	^{14}C	dpm scintillation/dpm Flo-One HP[1]	% eff. ^{14}C[2]
	1	1.05	52.5
	2	1.27	63.5
	3	0.93	46.5
	4	0.93	41.5
	5	1.15	57.5
	6	0.96	48
		MEAN = 1.048	52.42
		S.D. = 0.138	6.90
		S.E. = 0.056	2.81
B. Peak*# -	^{3}H	dpm scintillation/dpm Flo-One HP[3]	% eff. ^{3}H
	1A	2.20	44.0
	2A	1.89	37.8
	3A	1.68	33.6
	4A	1.80	35.0
	5A	2.20	44.0
	6A	1.89	37.8
	7A	1.68	33.6
	8A	1.80	36.0
		MEAN = 1.89	37.19
		S.D. = 0.205	3.56
		S.E. = 0.072	1.26

1. Ratio of ^{14}C dpm for $^{14}C/^{3}H$ channel.
2. % efficiency for $^{14}C/^{3}H$ channel with 25% ^{14}C in ^{3}H channel.
3. The % efficiency for the radioactivity detector was set at 20%; thus, final % efficiency is 20% times the dpm scintillation/dpm Flo-One HP

* The peak in order of elution from the HPLC and relative to the standards shown in Figure 2.

tion collection method, with only a decrease of 30-40% in counting efficiency for ^{3}H. Finally, by employing these methods the quantitation is obtained immediately compared to the lengthy steps involved in the fraction collection method.

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