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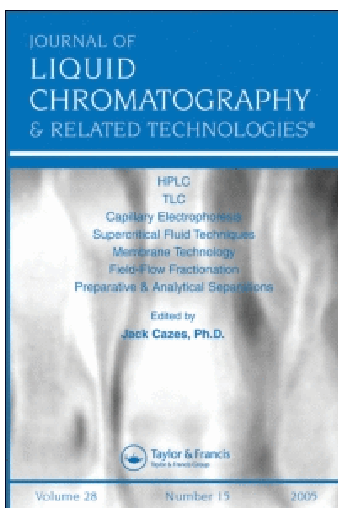

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SEPARATION OF TESTOSTERONE AND DIHYDROTESTOSTERONE IN SEMEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

We have established a method for the separation of testosterone and dihydrotestosterone in seminal plasma by high performance liquid chromatography. The separation column is a reversed phase column (ODS). The mobile phase is methanol-water (8:2) and the flowrate is 1.5 ml/min. It takes 12-14 min to separate a sample. The recoveries are 96.4% and 96.5% respectively by radioactivety labelled steroids. We have separated 600 seminal plasma samples with this method. The separated testosterone and dihydrotestosteroneare determined by radioimmunoassay. The result have shown that the method is satisfactory.

INTRODUCTION

Testosterone and Dihydrotestosterone are important reproductive hormones for male. They must be determined in Family Planning research for male. But their quanlity are very low and are almost same. Now

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they can be only determined by RIA indirectly. however,the cross reaction of T and DHT are produced by RIA. In order to avoid this reaction and obtain accurat measurenent of T and DHT respectively,T and DHT in semen must be separated. The separation of testosterone and dihydrotestosterone in semen was usually achieved by Celite column chromatogrophy[8-9]. This method , however, has many shortcomings. In recent years, more and more attention has been paid to high performance liquid chromatographic(HPLC) separation of the steroidal hormones and the separated are subsquently determined by radioimmunoassay(RIA) for a substance which is too low to be detected by UV-detector and Fluorometer of HPLC [1]. The HPLC technique has been employed to separate a dozen of steroidal hormones including T and DHT[1-7]. However, separation of T and DHT in human semen using this technique has so far not been reported. We have established a method for the separation of T and DHT in semen by HPLC and determined the separated substances by RIA. This method has been used to study two subjects in cooperation with WHO (Studies of normal semen values and blood hormone leveles of adult Chinese men,and Reproductive hormone profile of the serum and seminal plasma before and two years after vasectomy). The range of steroid concentration that can be determined by this method is from 10^{-10} to 10^{-12} g/ml.

Also, we found a substance X. (The nature is still unknwn to us. We only known it was produced by the rubber plugs during distillation under reduced pressure. So, it is present in all the seminal plasma samples.) Substance X shows HPLC behevior similar to DHT and not influencing RIA. We used X as an indicator for DHT.

MATERIALS AND METHOD

APPARTUE

High performance liquid chromatography (Gilson, France) equiped with a microcomputer (Apple 1le) and a date system (DATA MASTER), Ultraviolet detector (121), Fraction collector (201)(Gilson,France). Column: Partisil 5 ODS 3, 250x4.6 mm (Whtman, U.S.A).

The ^{3}H-labelled steroids were determined by 3801 liquid-scintillation spectroscope (Beckman, U.S.A).

CHEMICALS

Methanol: A.R, redistilled

Water: fresh and twice distilled

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The testosterone standard was purchased from the British Drug Houses. ^{3}H-T and its kits was supplied by the World Health Organization (WHO). ^{3}H-DHT and its kits was purchased from RADIM, S.P.A, Italy.

SEMEN AND ITS PREYREATMENT

In 1985 year, 600 semen samples were supplied by volunteers of China who were health and fertile. Their age were from 26 to 54 years. The semen samples was separated by centrifuging at 2500-3000 rpm and the seminal plasma was kept in a refrigerator at -70°C for later use. When the frozen seminal plasma thawed, 0.5 ml seminal plasma was extracted by 5 ml diethyl ether, followed by distillation under reduced pressure. The residue was dissolved in 400ul methanol solution (v:v=4:1). An aliquot of 250 ul was injected into the HPLC system.

SEPARATION OF SAMPLE

The separation was accomplished by using methanol-water mixture (V:V=4:1) as mobile phase at a flowrate of 1.5ml/min. A 250ul sampling loop was used to enable each sampling of 250ul. The temperature was controlled at 25±0.5°C. The retention times of T & DHT were 4.95± 0.15 min and 6.15±0.15 min respectively. A fluorescence detector was employed to ensure the retention time of 'DHT' and a fraction collector was used to collect T for 1.2 min (4.5-5.6 min)& DHT for 1.2 min(5.7-6.8) The collected samples were freeze dried with a freeze-drier and then determined by RIA.

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RESULTS

SEPARATION OF T & DHT

During the experiment we found that asubstance X exhibited HPLC behavior similar to DHT. This substance could be detected by both UV and fluorescence detector.They showed a single peak, fluorescence detection being much more sensitive. Then we used X to indicate the behavior of DHT by adding X to the prepared standard testosterone solution having aconcentration of 0.2 ug/100ul. A 250ul of the solution was injected. In order to obtain better separation in a short time, we finally decided to use the following conditions,the mobile phase: methanol water (V:V=4:1), the flowrate: 1.5ml/min, the room temperature:25±0.5°C. The chromatogram 1 is obtained according to the previous conditions.

The experiment on the separation of ^{3}H-T & ^{3}H-DHT according to the previous conditions shown in Fig.2.

The results as shown in Fig.1 & Fig.2 are basically the same.

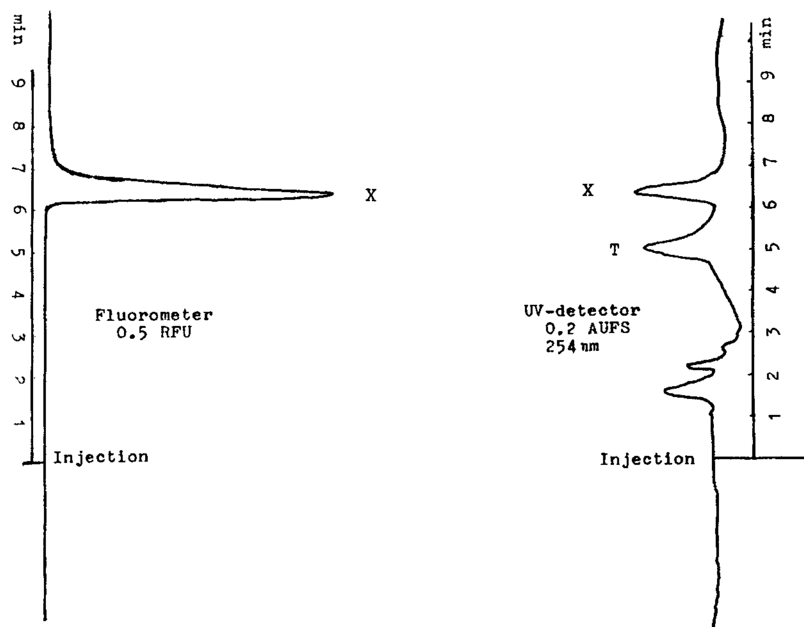

Figure 1. Separation of Testosterone and Dihydrotestosterone
Sampling loop: 250ul; sample size: 250ul; flowrate: 1.5ml/min; mobile phase: 80% methanol;chart speed; 10 mm/min.

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TABLE 1
Recoveries of T & DHT

NO	Addition(cpm)		Result(cpm)		Recovery(%)	
	T	DHT	T	DHT	T	DHT
1	13562	3122	13041	2929	96.2	94.1
2	13562	3112	13051	3073	96.2	97.5
3	13562	3112	13210	3175	97.4	102.0
4	13562	3112	12808	2900	94.4	93.2
5	13562	3112	13333	3060	98.3	98.3
6	13562	3112	12816	2898	94.5	93.1
7	13562	3112	13292	3028	98.0	97.3
Average					96.4	96.6
Standard Deviation					±1.57	±3.20

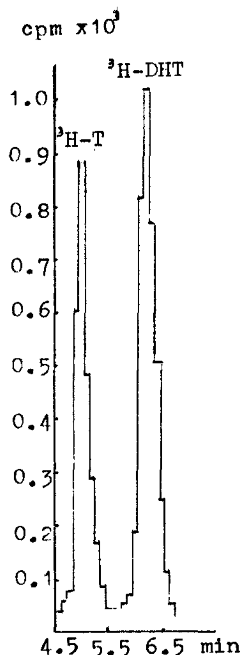

Figure 2. Chromatogram of ^{3}H-T and ^{3}H-DHT

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RECOVERY TESTS WITH ^{3}H-T & ^{3}H-DHT

The sample size ofthe mixture of ^{3}H-T & ^{3}-DHT was 10ul(a 20 sampling loop was used). They were collected into a scintillation bottle containing previously added scintillation liquid according to the scheduled collection time,and determined after standing overnight.(in order to get ^{3}H-lablled steroids from water phase into organic phase fully).

It is seen from table 1 that the results are satisfactrory.

RECOVERY TESTS BY ADDING ^{3}H-T & ^{3}H-DHT TO THE SEMEN SAMPLE

^{3}H-T & ^{3}H-DHT(3005 cpm & 1322cpm respectively) were added to asample of 0.5 ml semen. After extraction with diethyl ether,a similar experiment was performed according to the previous conditions.A portion of the separated substance was taken for radioactivity determination. The results X$\pm$ SD are 97.5$\pm$ 14.2% (n=47) for T & 91.5$\pm$12.7%(n=47) for DHT.

TABLE 2
Comparison of the RIA results by different separation methods

Liter-ature	T(nmol/l) Average	T(nmol/l) Range or ± SD	DHT(nmol/l) Average	DHT(nmol/l) Range or ± SD	Methods
a	0.77	0.71–0.83[b]	0.90	0.85–0.95[b]	HPLC
8	0.61	0.21–1.76	1.27	0.49–3.48	Celite
9	0.98	0.71–1.3	0.77	0.56–1.0	Celite
10	0.89	0.26–2.15	1.06	0.72–1.91	SEphadexLH-20 TLC
11	2.08	± 1.60	2.46	± 2.41	TLC
12	0.69	±0.059	1.47	±0.148	Oxidation

[a] The present method.

[b] 95% confidence interval.

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THE COMPARED WITH OTHER METHOD RESULTS

The previously established experimental conditions have been used to the separation of six hundred-plus samples of human semen for the subjects in cooperation with WHO. Radioimmunoassay has shown that the results are satisfactory. The results are presented in Table 2.

DISCUSSION

1. The contents of T & DHT in semen are low (10^{-10}-10^{-11}g/ml) and so their preparationcan only be achieved in the analytical mode. However,since the ethereal extract of seminal plasma can not be dissolved in small volume of methanol solution,finally we used 400ul 80%methanol for the solution.

2. Under the present experimental conditions,a retention time shifting tends to be serious if the partial sampling method is used. Therefore we utilized the full sampling method (i.e. to sample 250ul with a 250ul sampling loop) to ensure the basic constancy of the retention time.

3. Since in semen the T & DHT concentrations are very low, T is by no means detectable with an ultraviolet detector and DHT is not sensitive to either ultraviolet or fluorescence. Although the ^{3}H-labelled T & DHT can help define the separation conditions and can be used for the separation of practical sample, it is difficult to ensure the experimental conditions because of lack of direct detection; moreover,the number of samples is

very large and the experiments last very long. Fortunately we discoveried a substance X (the nature of which is still unkown to us), which shows HPLC behavior similarto DHT and which does not influence RIA. We used X as an indicator for DHT; and the fluorescence detector was employed because it is extremely sensitive to fluorescence. X had been used to check if the HPLC system was normal or not,prior to separation of practical samples. The X indicator is very necessary as is shown by the results of separation of the six hundred-plus seminal plasma samples. The tacer and recoveries of ^{3}H-T & ^{3}H-DHT and the practical samples have shown that this method is satisfactory.

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