大 韓 不 妊 學 會 雜 誌 : 第14卷 第2號 1987
Kor. J. Fertil. Steril., Vol. 14, No. 2, 1987, 12.

Luminescence Immunoassays and Their Applications for Dihydrotestosterone and Testosterone(I) : Establishment of LIA.

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=국문초록=

Dihydrotestosterone과 Testosterone의 섬광면역 측정법과 응용(I) : 측정법의 정립

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5α-dihydrotestosterone(DHT)과 testosterone(T)은 남성 생식기관의 주 생성 호르몬들로 그 구조가 매우 비슷하여 이들 각 개를 특이하게 측정(specific determination)하는 방법이 개발되지 않고 있다. 본 연구는 고속액체 크로마토그라피(HPLC)를 이용하여 이들을 분리한 후 섬광면역측정법(Luminescence immunoassay, LIA)으로 정량하는 방법을 개발하여 이들의 응용 가능성을 검토하고져 하였다.

DHT와 T의 retention time은 각각 10.3min, 17.6min이었다. DHT-LIA와 T-LIA에서 다른 스테로이드들과의 교차반응도는 방사면역측정법(RIA)과 대동소이하였다. 정도관리(quality control) 시료의 intra-assay variation은 DHT-LIA가 8.7%, T-LIA가 6.0%의 변이계수를 나타내었고, inter-assay variation의 변이계수는 각각 12.0%및 15.3%이었다. 실측치(y)와 기대치(x)간의 관계를 보면, DHT-LIA경우는 $Y=0.94X+0.9$($r=0.989$), T-LIA는 $Y=1.01X+0.06$($r=0.988$)로 두 측정치 사이에는 통계적으로 유의한 차이가 없었다.

위의 측정방법을 이용하여 DHT-enanthate와 T-enanthate 처리후 혈청내 DHT 및 T의 농도변화를 조사한 실험결과 LIA와 RIA의 값사이에 유의한 차이가 없었다.

위의 결과로 보아 본 실험에서 개발된 DHT와 T의 섬광면역측정법은 정립되었다고 사려된다.

Key Words : LIA, Testosterone, Dihydrotestosterone, Dihydrotestosterone enanthate, Testosterone enanthate, LIA.

INTRODUCTION

5-α-dihydrotestosterone(DHT) and testosterone(T) are structually very similar and clinically important steroids for the diagnoses and managements of a large number of endocrine diseases, including hypogonadism, Klinfelter's syndrome and testicular feminization in man and testosterone producing tumors in women.

During the last two decades, many procedures for the determinations of DHT and T in biological samples have been developed and simplified with a large number of different techniques such as gas or liquid chromatography(Harper et al., 1976 ; Gaskell et al., 1980), high-performance liquid chromatography(HPLC ; Kemp et al., 1981), gas-chroma-

*This work was partly surported by KOSEF, Korea Science and Engineering Foundation.

tography-mass-spectrometry(GCMS, Gaskell et al., 1980 ; Gould et al., 1986), radioimmunoassays(RIA, Morena et al., 1980 ; Baxendale and James, 1983), enzymeimmunoassay(EIA ; Turkes et al., 1979, 1980 ; Elder and Lewis, 1985 ; Marcus and Durnford, 1985). On the other hand, luminescence immunoassay(LIA) for T has been reported(Pazzagli et al., 1982 ; Kreysing et al., 1987).

Despite of these developments and advancements, the accuracy of T determination is still compromized by the cross activity of DHT. Recently, to improve the assay quality and to overcome this difficulty, polyclonal antibodies were raised to T-haptens conjugated to proteins through the 15 beta position. These antisera showed low cross reactivity and prevented accurate estimation of T especially in female plasma samples because of high cross reactions with progesterone and other C-21 steroids (Rao and Moore, 1976 ; Miyake et al., 1982). On the other hand, many investigators tried to produce the specific monoclonal antibodies for T (Kohen et al., 1982 ; Fantle and Wang, 1984 ; White et al., 1985). However, these monoclonal antibodies showed also higher cross reactivity with other steroid hormones especially in female samples. Until now, the antibody with satisfactory specificity for accurate measurement of DHT and T in biological samples without chromatographic purification of samples has not been generated.

Therefore, luminescence immunoassays for DHT and T, combined with HPLC in order to study the pharmacokinetics of DHT and T derivatives were established.

MATERIALS AND METHODS

Animals

Eight cynomolgus monkeys (Macaca fascicularis) orchidectomized more than 2 years age weighing(2.8~4.6kg), were used in this study. They were fed a pelleted diet supplemented with fresh fruits and had access to tap water and libitium.

Four monkeys(3.5±0.8kg) received a single i.m. injection of 1ml sesame oil suspension of DHT-enanthate(DHTE, 32.7mg/ml, containing 23.6mg DHT). Another 4 animals(3.3±1.1kg) were injected i.m. with 32.8mg of testosterone enanthate (TE, 23.6mg pure testosterone) in sesame oil.

Blood was withdrawn under ketamine anaesthesia(8~12mg/kg, Ketavet, Marke-Davis, Munich, FRG) before injection and on six consecutive days afterwards and then at 3~4 days interval for 1 month. The blood samples were stored overnight at 4℃ and then centrifuged at 3,000 rpm for 20 min and the serum was stored frozen at −20℃ until used.

Reagent

All steroids, testosterone-3-carboxymethyloxime(T-3-CMO), microperoxidase(POX, E.C. MP-11), N,N, dicyclohexylcarbodiimide, N, hydroxysuccinimide, bovine serum albumin (BSA, fraction V powder) and 30% hydrogen peroxide solution were obtained from Sigma Chem. GmbH. Sodium hydroxide, special grade solvents and Silica gel 60 thin layer chromatography (TLC) plates were purchased from Merck, FRG. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl(EDAC, MW=191.7), lyopilized immunobead and immunobead covalently bound rabbit gamma globulin were purchased from Bio-Rad Laboratories GmbH, FRG. Tritium labelled dihydrotestosterone, testosterone were obtained from Amersham International Ltd., Amersham, Bucks, UK. A diluent prepared by dissolving 1g of bovine immunoglobulin G(bIgG) in 0.154mol/1 sodium chloride was used as an assay buffer.

Microperxidase was dissolved in Tris-HCl buffer(0.01mol/1, pH 7.4) as a stock solution (2g/1) and then diluted into 10μmol/1 in 0.1 mol/1 borate buffer(pH 8.6) containing 9g of NaCl and 100mg of BSA.

Chromatography

The samples were extracted with 5ml of diethyl ether after adding ca. 1,000 cpm of tritiated tracers. The aqueous phase was frozen in methanol bath(−40℃). The organic phase was evaporated at 37℃ in water bath. The dried residue was reconstituted with 1 ml of methanol for conventional RIA or dissolved in 220μl of n-hexane and transfered into minivials for HPLC separation. Two hormones, DHT or T were separated prior to immunological quantitation through Diol columns(Bischoff, 10μm, 18×4.6mm i.d., and for precolumn, 5μm, 250×4.6mm i.d) in this HP LC system(Waters Associates, Konigstein, FRG). This system was set up with two pummps(M 6,000 and M 45), an autosampler(Wisp 710B), gradient system(M27), UV detectors (M441 and Pye-Unicum PU 4020) and a data module(M730). The fractions for DHT and T were collected with a time regulated collector (LKB Ultro-Rack type 2070). The following conditions were used : flow rate, 1.4ml/min ; column pressure, 620psi ; isocratic separation (pump A) of the steroids with n-hexane/2-propanol(96 : 4, v/v) ; elution of the more polar steroids with pump B (n-hexane/2-propanol, 75 : 25, v/v) ; UV detection at 254 nm and 206nm for DHT ; running time was 30min.

The retention time of the steroids was determined in a run with standards and this profile was checked routinely every weeks. Those for DHT and T were 10.3(10.2~11.2) and 17.6(17.3~19.1).

The recovery rates for DHT and T using internal tritiated steroids were 63.6±4.2 and 65.9±7.9.

Preparation of luminescent marker conjugates

6-(N-4-aminobutyl)-N-ethylamino-2, 3-dihydrophthalazine-1, 4-dione (aminobutylethyl isoluminol ; ABEI) was synthesized according to the procedure developed by Schroeder et al.,(1978) and were characterized by FD-mass spectroscopy. The synthesized ABEl was coupled to DHT-3-CMO and to T-3-CMO according to the mixed anhydride method of Erlanger et al.,(1957).

Antibodies to T-3-CMO-BSA were raised in rabbits and their Ig G fractions were purified through proteinA-SepharoseCL-4B affinity chromatography column. The amino groups of Ig G fractions were coupled to carboxyl groups of immunobeads(Bio-Rad) by a carbodiimide method.

Procedures for luminescence immunoassays

One hundred microlitre of serum extract or 100μl of standard solution(range 3.81 to 2,000pg/100μl of diluent) was added in duplicate to the tubes(12×55, Sarstadt). Subsequently, antibody-coupled immunobeads(10μg /tube) were added and then mixed with 100 μl of DHT-3-CMO-ABEI(ca. 60,000cps). The mixtures were incubated overnight at 4℃. The bound forms were separated by washing twice with 1ml Tween 20(0.02% in physiological saline) and then incubated at 60℃ for 1hour with 250μl of NaOH(2mole/1 in distilled water). The luminecence reation was started by adding 100μl of POX(10μmol/1) and 100μl of hydrogen peroxide(final dilution, 0.15%). The light emission was measured by Berthold LB 950 luminometer.

The concentrations of DHT or T in serum were defined by the arithmatic means($\bar{X}$)± standard deviations(SD). Data from the phamacokinetic study of DHTE or TE were analyzed using the Student's t-test.

RESULT

High pressure liquid chromatography

The present method provides a good separation of DHT and T because these are clearly separated within 30min. The mean retention times and overall recoveries after extraction and chromatography are summarized in Table 1.

The fractions between the steroids were collected and the radioactivities were mea-

Table 1. Retention time and recovery percents after separations of DHT and T by HPLC

	DHT	T
Retention time(min, range)	10.3 (10.2~11.2)	17. 6 (17.3~18.1)
Recovery rate(%)		
Intratesticular		
Sample of rat(n=48, range)	24.31±5.54(14.0~36.6)	70.10±4.95(52.1~78.0)
Monkey sera(n=120, range)	70.79±4. 7(56.6~80.5)	65. 9±7. 9(37.6~75.1)
Human sera(n=54, range)	63. 6±4. 2(36.8~66.3)	65. 9±7. 9(45.3~76.8)

sured for the calculations of recovery. The contamination of the column between two different samples as measured by radioactivity in the individual fractions was less than 0.5%. The retention times for DHT and T fractions were 10.3 and 17.6 respectively. The overall recoveries of DHT and T in monkey or human sera except the recovery of DHT from intratesticular samples of rat were more than 50%.

Reliability of LIA and RIA

Dose response curves : Fig. 1A. and Fig. 1B. show a representative dose response curve for DHT and for T respectively. Table 2 summarizes the mean values for the slope

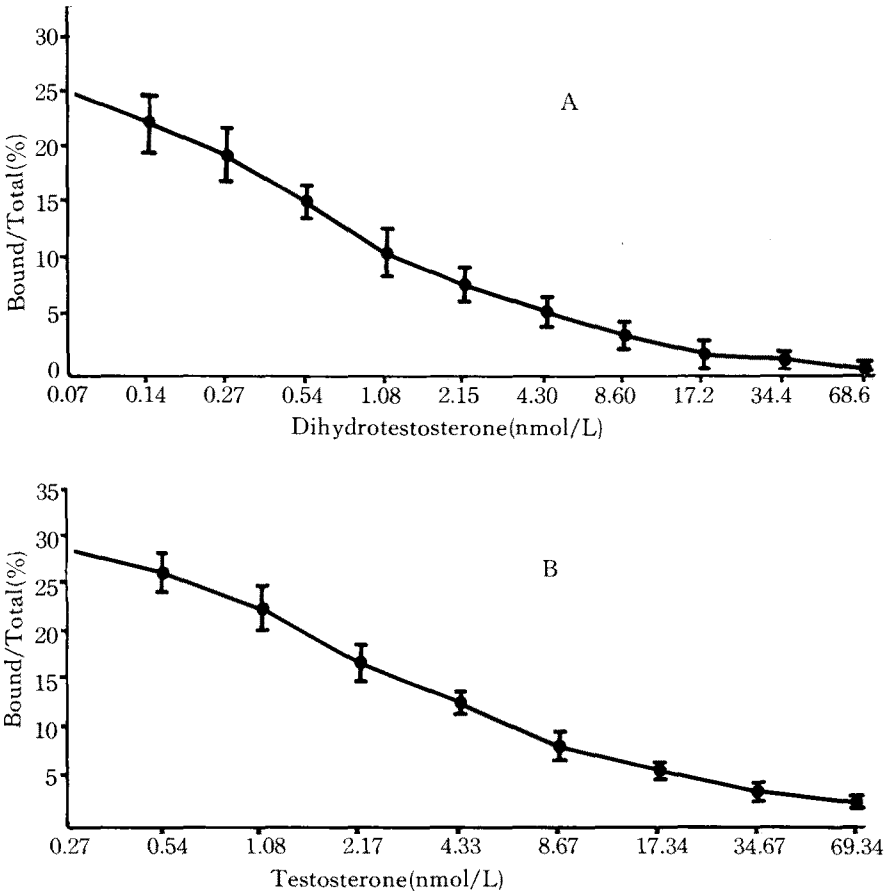

Fig. 1. Dose response curves of LIA for DHT(upper panel) and for T(lower panel). The bars represent arithmatic mean ± standard deviations from 8 consecutive assays.

Table 2. Values (mean±SD) for slope factor, intercept and sensitivity of the performed assays

	Dihydrotestosterone		Testosterone	
	LIA	RIA	LIA	RIA
N	109	165	217	223
Slope(b)	1.11(0.14)	2.14(0.28)	2.34(0.92)	2.28(0.07)
Correlation(r)	0.96(0.003)	0.99(0.008)	0.97(0.007)	0.99(0.005)
Intercept(Y) (nmol/l)	5.26(0.78)	5.25(1.33)	5.46(0.93)	5.33(1.21)
Sensitivity(pg/tube)	1.7 (0.9)	4.3 (0.7)	2.1 (0.7)	5 (0.3)

The numerals in the parentheses represent 1 standard deviations.

Table 3. Cross reactivity values using the same antibodies coupled to immunobead matrix as determined by DHT-or T-LIA

Steroids	Cross reactivity(%)			
	DHT-LIA	DHT-RIA	T-LIA	T-RIA
Dihydrotestosterone	100	100	95.5	95
Testosterone	82.3	76.3	100	100
Androstenediol	2.7	2.1	2.7	2.1
Androstenedione	4.6	3.2	1.5	1.1
Androsterone	2.1	1.8	1.6	0.9
Others	1.1	0.1	0.1	0.1

Other steroids are cortisol(<0.001), 17 β-estradiol, dihydroepiandrosterone and progesterone.

factors, intercept and sensitivity for DHT or T.

Antiserum dilution : The titre was defined as the antiserum dilution at which 50% of a given amount of labelled steroid was bound. The titres of this antiserum in LIA were 1/300 of the antibody coupled immunobead and those in RIA were 1/56,000 The minimal detectable doses(pg/tube) in eight consecutive experiments were 1.7 for DHT and 2.1 for T. The results of sensitivity shows that the properties of the present LIA were very similar to those of the conventional RIA.

Specificity : The cross reaction was calculated from the ratio of mass(X) of immunogenic steroid required to displace 50% of the radiolabelled steroid to the mass(Y) of the cross reacting steroid required to displace the same fraction of the labelled steroid : cross reaction = X/Y × 100%. The present result(Table 3) shows that the cross reactivities in the LIA were very similar but were slightly higher than those in the RIA. Thistable also shows that both assays require the separation step by extraction-chromatography in order to remove the interferences of both hormones.

Accuracy : This was determined by performing recovery experiments whereby increasing amounts of the steroids were added to the charcoal-stripped steroid free serum and the level of steroid present was assayed(Fig. 2). Linear regression analyes of the measured DHT and T concentrations without any chromatography were Y(measured value) = 0.94 (expected value) + 0.9 for DHT and Y = 1.01 + 0.06 for T.

Precision profile : Male and female serum samples were analyzed with a high degree of precision in each of 8 consecutive assays. The results were analyzed and summarized in Table 4. The intra- and inter-assay variabilities were closely comparable for the two assay systems.

Correlations of LIA and RIA results : Figure 3 and 4 show the correlations between the results obtained using LIA and RIA using same antiserum.

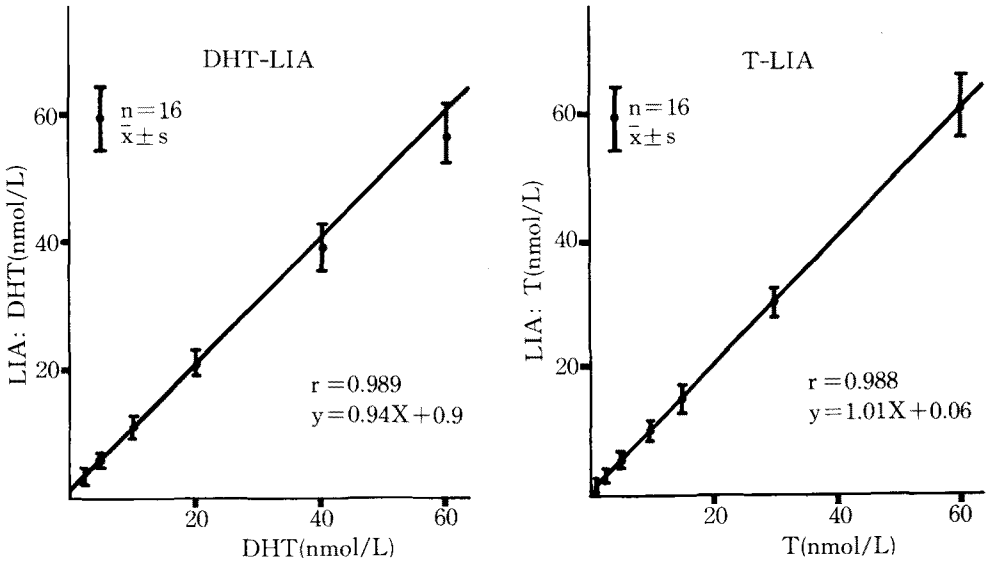

Fig. 2. Linear regression between DHT and T estimates obtained and those expected. The bars represent arithmatic mean ± standard deviations. The observed levels were determined by performing recovery experiments whereby increasing amounts or the steroids were added to the charchoal-stripped steroid free medium. Y axis represents observed value and X asis shows expected values.

Table 4. Performance data of the developed LIA for DHT and T

	DHT-LIA	T-LIA
Correlations between expected values(r)	0.989	0.988
Within-assay variation(nmol/1)	2.3±0.3(CV= 8.7%)	21.7±1.3(CV= 6.0%)
Between-assay variation	2.5±0.3(CV=12.0%)	22.2±3.4(CV=15.3%)

These results were summarized from more than 20 assays.

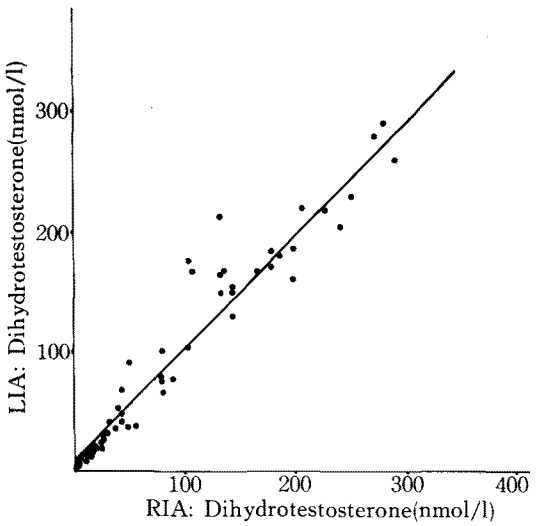

Fig. 3. Comparisions of serum DHT concentrations determined by LIA and RIA methods. Y(LIA values)=1.02×X(RIA values) +4.42, r=0.977, n=87.

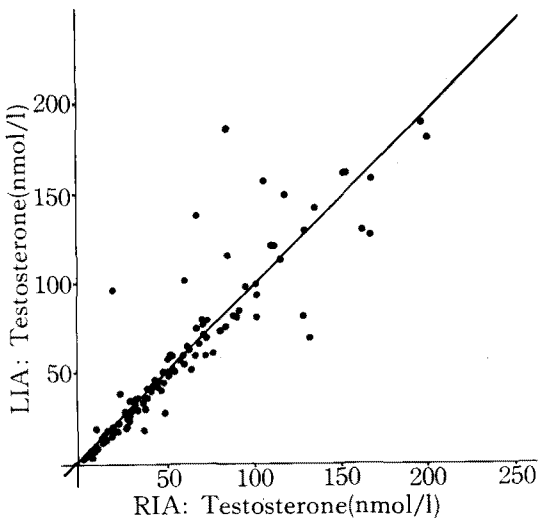

Fig. 4. Comparisions of serum T concentrations determined by LIA and RIA methods. Y(LIA values)=0.97×X(RIA values)+ 2.50 r=0.93, n=182.

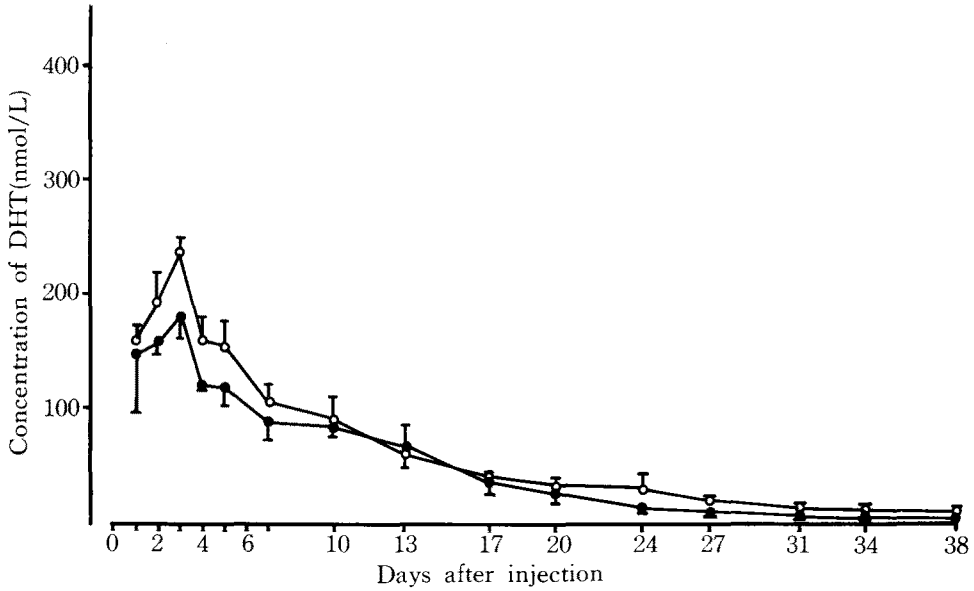

Fig. 5. Serum concentrations of DHT following a single i.m. injection of DHTE. Values are means ± standard deviations of four monkeys. Where no SDs are shown, the value lies within the symbol. The thick solid line with closed circles represents the values measured by LIA and thin solid line with open circle that by RIA.

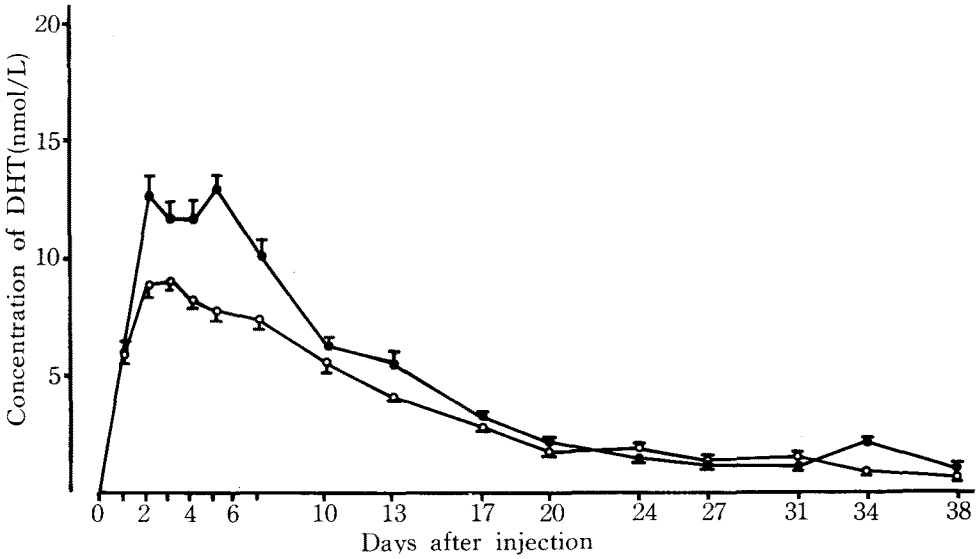

Fig. 6. Serum concentrations of DHT following a single i.m. injection of TE. Legends are same as in Figure 5.

The present results show that the correlations were very satisfactory.

Application to pharmacokinetic study of DHTE and TE in monkey : The pharmacokinetic serum pattern of DHT and T measured by LIA were extremely similar to those by RIA(Fig. 5). After the injections of DHTE, serum DHT level was elevated from less than 1nmol/1 to 147.1 plus/minus 41.7nmol/1 on the third day. Thereafter this level was declined rapidly to around 14nmol/1 on 27th day and then 1.03 plus/minus 0.5nmol/1 at 38

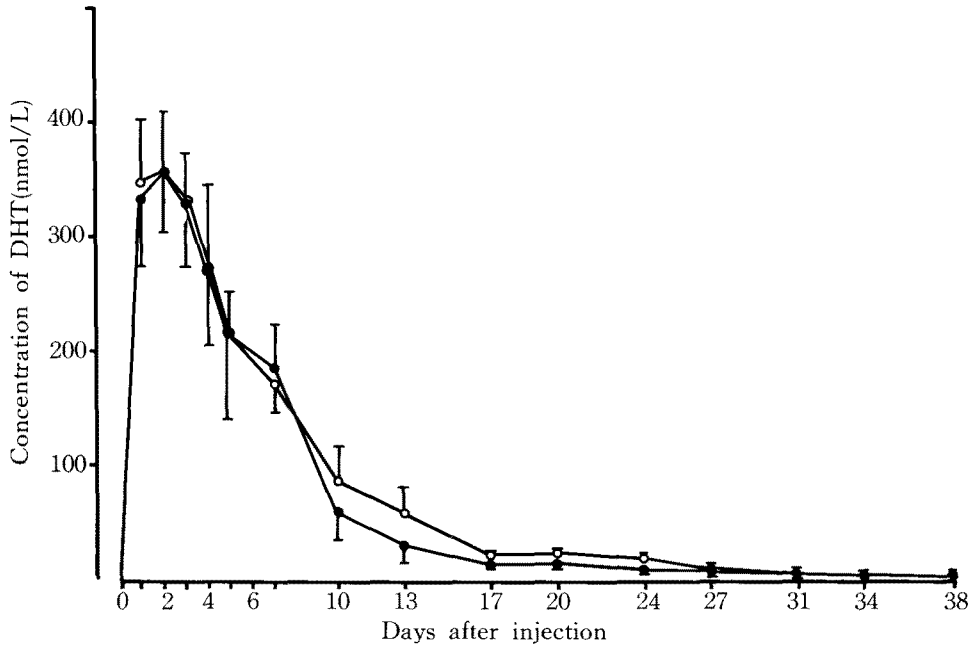

Fig. 7. Serum concentrations of T following a single i.m. injection of TE. Legends are same as in Figure 5.

th day.

When the monkeys were injected with TE (Fig. 6), the serum DHT levels rose from less than 1 nmol/1 to 13.0 plus/minus 3.9nmol/1 up to 6th day after injection and then the values declined gradually to normal level(0.8 plus/minus 0.1nmol/1) until day 38. On the other hand, the serum testosterone levels rose from less than 1 nmol/1 to 338.9 plus/minus 111.9nmol/1 for the first day and remained in this range for 2~3 days(Fig. 7). Thereafter this level was decreased rapidly so that the upper physiological limit was reached between days 17 to 24.

DISCUSSION

The present study describes a new chemiluminescence immunoassay for DHT, utilizing the labelled tracer of DHT-3-CMO-ABEI. On the other hand, the LIA for T which had been already established by Kreysing et al., (1987, personal communication) had been re-evaluated again. Both assay systems have been validated using the usual criteria of sensitivity, precision, spcificity, parallelism etc, comparing with the conventional RIA systems. The overall performances are very similar to those of RIA and show that the present LIA systems appear to be suitable for use in clinical assays for males and females and also for the biological studies.

The main problems in developing highly sensitive direct LIA methods for use with crude biological samples probably arises from interference with light-producing reaction (Kohen et al., 1980 ; Pazzagli et al., 1982). In addition to this, the cross activity by the similar substances compromises the accuracy of steroids determination in both systems like LIA and RIA in the biological samples. Because the specific antisera only for DHT or T are not available and satisfactory until now, the direct LIA is not established. The present report shows the rapid and simple separation step using HPLC system for both hormone.

The steroid esters have long been used to

prolong their activities(Junkmann, 1957 ; Nieschlag et al., 1976 ; Schulte-Beerbuehl and Nieschlag, 1980 ; Sokol et al., 1982 ; Schruemeyer and Nieschlag, 1984 ; Weinbauer et al., 1986 ; Fujioka et al., 1986). The pattern of testosterone secretion after injection of TE in sesame oil was very similar to the previous study of Weinbauer et al.,(1986) but the absolute values of T and DHT in serum after injection was two or three times higher than those of Weinbauer et al.,(1986).

They reported that the secretion and duraton of the this T ester depend on the vehicle used for injection and the rate of enzymatic hydrolysis. The present study suggests that there was also a large individual variation of absorption and secretion of injected esters. The present study also confirmed that this ester required injecinjections at two to three weeks interval in amount 30-to 40mg in order to provide serum testosterone level above the normal limit.

When DHTE injected, serum T level was less than 2nmol/1 in all cases. However the DHT level after a single intramuscular injection to castrated monkey showed a smilar pattern of TE treatment, that is, the DHT level in serum was elevated to supraphysiological levels and declined rapidly on the first week and then gradually decreased to 14.3 nmol/1 at third week. This result demonstrated that DHTE could be used to maintain the DHT levels during three weeks. On the other hand, the physiological significances of the DHT on the testicular fuucton should be studied. The present study also shows that the DHTE does not increase the T level in surum.

When the DHT or T concentrations in serum after the injection of these androgen esters were measured, the secretion pattern and the absolute amount of DHT and T measured by LIA were extremely similar to those by RIA. Therefore, the present study shows that the established methods are quitely useful for the use in the clinical study.

Acknowledgement

We grately thank to Dr. Hyun K. Kim of NICHHD Bethesda, for the gift of the DHT- and T-esters and the expert techinical assistances for monkey handling to Mr. M. Heuermann. Our thanks are extended to Ms. Karin Brunswicker and R. Sandhowe for T RIA and also to Dr. G.F. Weinbauer and Professor E. Nieschlag of Max-planck Gesellschaft, Muenster, FRG.

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