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Changes in Testosterone and Dihydrotestosterone Levels in Male Rat Accessory Sex Organs, Serum, and Seminal Fluid After Castration: Establishment of a New Highly Sensitive Simultaneous Androgen Measurement Method

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ABSTRACT: It is known that abnormal androgen dynamics in the tissues is a cause of androgen-dependent disorders. Investigation of tissue androgen levels could provide a clue to the elucidation of disorders. However, it is difficult to measure a trace amount of androgen in the tissues. We established a highly sensitive simultaneous quantification method of testosterone and dihydrotestosterone (DHT), which play the most important roles in the body among androgenic steroids in trace amounts, and investigated time course changes in testosterone and DHT levels in male accessory sex organs, serum, and seminal fluid after castration in rat models. In addition, changes in the testosterone/DHT ratio of male accessory sex organs and seminal fluid were observed. The simultaneous testosterone and DHT measurement method established by us was validated. Intra-assay variation and interassay precision and accuracy were all within ±20%, and the quantification limits of testosterone and DHT were both 15.6 pg/g. With the use of this method, the testosterone and DHT levels in the prostate, seminal vesicles, and serum immediately after castration were similar to those previously reported. The testosterone and DHT levels were 350 pg/g and 605 pg/g, respectively; which showed dominance of DHT in seminal fluid, although it was not as marked as that in the male accessory sex organs. Androgens decreased with time after castration in the accessory sex organs, serum, and seminal fluid. In the prostate and seminal vesicles, testosterone and DHT decreased to about 50% and about 2% of the normal levels, respectively, 72 hours after castration. The serum levels were under the quantification limits 6 hours after castration and thereafter. In seminal fluid, the testosterone and DHT levels decreased to 49% and 35% of normal levels, respectively, 72 hours after castration. The testosterone/DHT ratio in the male accessory sex organs was lower in the prostate (0.06) than in the seminal vesicles (0.13) immediately after castration. In the seminal fluid, changes in the ratio were small compared with those in the accessory sex organs and serum. These results showed that our method was capable of measuring testosterone and DHT in very small amounts of samples such as prostate biopsy specimens, and it might provide a clue to the elucidation of the pathology of androgen-dependent disorders.

Key words: LC-MS/MS, hormone, prostate, seminal vesicles.
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Androgen is an important male steroid hormone that regulates metabolism in many organs, and its effect is obvious in male sex organs. There are 4 androgens, and testosterone (T) synthesized by Leydig cells in the testis mainly presents in the circulation. Another dominant androgen is dihydrotestosterone (DHT) synthesized from T by 5-alpha reductase in the target organs. T and DHT play an important role in sexuality differentiation and development of secondary sex characteristics. In addition to T and DHT, androgens with very weak action, dehydroepiandrosterone (DHEA) and androstenedione (4-dione), are secreted by the adrenal gland and testis (Partin et al, 1998). Clinically, the incidences of benign prostatic hypertrophy (BPH) and prostatic cancer are high in men past middle age, and the occurrence of these diseases is correlated with the dynamics of androgen (Gray et al, 1991; Marcelli and Cunningham, 1999; Taplin and Ho, 2001). Moreover, abnormal androgen dynamics is reported to be a cause of osteoporosis, sterility, and erection disorder in men (Blaquier et al, 1972; Mulligan and Schmitt, 1993; Weinbauer et al, 1997; Orwoll, 1998; Vanderschueren et al, 2000). Study of androgen dynamics is important in investigating the causes of these disorders. There have been several reports of androgen content in serum and tissues (Hirosumi et al, 1995; Nakayama et al, 1997; Gottreich et al, 2000; Dohle et al, 2003; Torres et al, 2003). However, accurate measurement of tissue androgen content is difficult because of abundant impurities. Thus, we established a highly sensitive simultaneous T and DHT measurement method. With this method, we measured

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time course T and DHT levels in the prostate, seminal vesicles, serum, and seminal fluid after castration and compared the results with those previously reported. At the same time, the time course changes in the T/DHT ratio in the organs after castration were investigated.

Materials and Methods

Animals

Mature male Wistar rats aged 10 weeks were purchased from SLC Japan Inc (Shizuoka, Japan). The rats were maintained in an air-conditioned room under a lighting cycle (light on from 0700 to 1900 hours) and given pellets and water for 1 week before the experiment. Teikoku Hormone Mfg Co, Ltd, performed the experiment after approval by the Animal Protection Committee.

Experimental Method

The rats were weighed and divided into 7 groups with sampling time points of 0, 3, 6, 12, 24, 48, and 72 hours after castration (5 animals per group). Bilateral orchiectomy was performed under pentobarbital anesthesia (50 mg/kg, intraperitoneal injection), followed by excision of the prostate (abdominal lobe) and seminal vesicles at the specified time point. The prostate and seminal vesicles were weighed, frozen in liquid nitrogen, and stored at −70°C until analysis. Blood was collected in a blood sampling tube, clotted, and centrifuged (738 × *g* for 10 minutes). The separated serum was stored at −70°C. Seminal fluid (0.5 mL) in the seminal vesicles was collected in a test tube, and 9.5 mL of physiological saline was added. It was then centrifuged (266 × *g* for 2 minutes, twice), and the supernatant was stored at −70°C.

Chemical Products

Standard T and DHT were purchased from Sigma Chemical Co (St Louis, Mo). Tri-deuterium–labeled T (T-d3) and tri-deuterium–labeled DHT (DHT-d3) were provided by Teikoku Hormone Mfg Co, Ltd (Kawasaki, Japan). All solvents used were of analytical grade.

Instruments

For liquid chromatography–tandem mass spectrometric analysis (LC-MS/MS), Micromass Quattro II and HP1100 liquid chromatograph (Hewlett-Packard, Corvallis, Ore) were connected, and a hypersil octadecylsilane (ODS) column (internal diameter 125 × 3 mm, membrane thickness 3 μm, Hewlett-Packard) was used.

Analytical Methods of DHT and T

The prostate and seminal vesicles were completely powdered with liquid nitrogen and transferred to 30-mL centrifuge tubes. Serum and seminal fluid were transferred to 10-mL tubes. Internal standards of T and DHT, 1 ng each, were added to the tubes. Steroids in the prostate and seminal vesicles were extracted with 20 mL of 40% hexane/ethyl acetate. The organic layer was washed sequentially with 5% sodium bicarbonate and water and dried with sodium sulfate. Steroids were then extracted with 70% acetonitrile/water on a C18 column (washed with 6 mL of methanol and water beforehand), and the obtained steroids were dried in a centrifugal evaporator. To increase the analytical sensitivity, the dried steroids were reacted with 200 μL of 2% fluoro-1-methylpyridinium-*p*-toluenesulfonate (Tokyo Kasei Kogyo, Tokyo, Japan). After the reaction, the pyridinium derivatives produced were dried under nitrogen gas and washed with 50 μL of methanol, 1 mL of H_2O, and 20 μL of 1 N HCl, and the steroid derivatives were re-extracted on a C18 column. The steroid extract was measured by LC-MS/MS. The HPLC conditions were: solvent, methanol/0.1% CH_3COOH-H_2O 65:35; temperature 40°C; and flow rate 0.2 mL/min. For T, *m*/*z* 380.3 was activated as a precursor ion and decomposed. Among the product ions, *m*/*z* 253 was monitored. For DHT, *m*/*z* 382.3 was activated as a precursor ion, and the produced *m*/*z* 255 ions were monitored. As for the product ions of the internal standards (T-d3 and DHT-d3), *m*/*z* 256 and *m*/*z* 258 ions were monitored.

Preparation of Calibration Curves and Validation

Validation was performed with the vehicle containing no androgen or serum from castrated rats (Table). Standard T (0, 15.6, 20, 62.5, 125, 500, and 1000 pg) and DHT (0. 15.6, 20, 62.5, 125, 500, and 1000 pg) were added to 7 tubes, and 1 ng of T-d3 (T-19-C^2H_3) and 1 ng DHT-d3 ([17,16,16-$^2H_3$]-DHT) were added as the internal standards. T and DHT were measured as described above, and the accuracy and precision were analyzed. The accuracy was calculated by dividing the difference between the mean analytical value and the initial amount of the standard by the initial amount of the standard. The precision was calculated by dividing the standard deviation by the mean analytical value. Accuracy and precision are presented as percentages.

Statistical Analysis

All data were analyzed with Microsoft Excel software (Microsoft Corp, Redmond, Wash). Values were expressed as mean ± SE. Statistical analyses for comparisons among multiple treatment groups were based on the method of 2-way analysis of variance (ANOVA) with multiple comparisons. The results of all statistical tests were considered significant at $P < .05$.

Results

Establishment of a Highly Sensitive Quantification Method

The typical patterns of selective ion monitors (SIM) of T and DHT are shown in Figure 1.

The calibration curves of T and DHT were linear, and the correlation coefficients were .999972 and .999767, respectively. In the validation results, quantification limits of T and DHT were both 15.6 pg/g, and the accuracy and precision were both within ± 20% (Table).

Accuracy and precision of our method: quantification of androgens

	Testosterone				Dihydrotestosterone			
Standard, pg	Analysis, pg	SD	Accuracy, %	Precision, %	Analysis, pg	SD	Accuracy, %	Precision, %
Tissue Interassay								
15.6	15.7	2.3	0.7	14.8	17.4	2.2	11.8	12.5
125	125.8	3.0	0.7	2.4	125.7	2.4	0.5	1.9
1000	1026.9	21.6	2.7	2.1	998.4	13.6	−0.2	1.4
Tissue Intra-assay								
15.6	15.8	0.8	1.6	5.0	14.0	1.9	−10.2	13.6
20	19.0	1.5	−5.2	7.8	16.3	1.1	−18.4	6.8
62.5	66.3	3.0	6.1	4.5	55.5	0.8	−11.2	1.5
500	564.8	5.7	13.0	1.0	459.8	9.2	−8.0	2.0
Plasma Interassay								
15.6	16.4	1.4	5.4	8.2	16.8	1.6	7.9	9.5
125	124.8	4.2	−0.2	3.4	122.7	5.0	−1.8	4.1
1000	1001.7	9.6	0.2	1.0	995.3	22.5	−0.5	2.3
Plasma Intra-assay								
15.6	14.3	0.3	−8.3	2.2	16.9	1.1	8.2	6.7
125	122.1	7.5	−2.3	6.2	126.1	4.1	0.9	3.3
1000	936.1	20.9	−6.4	2.2	960.5	11.1	−3.9	1.2

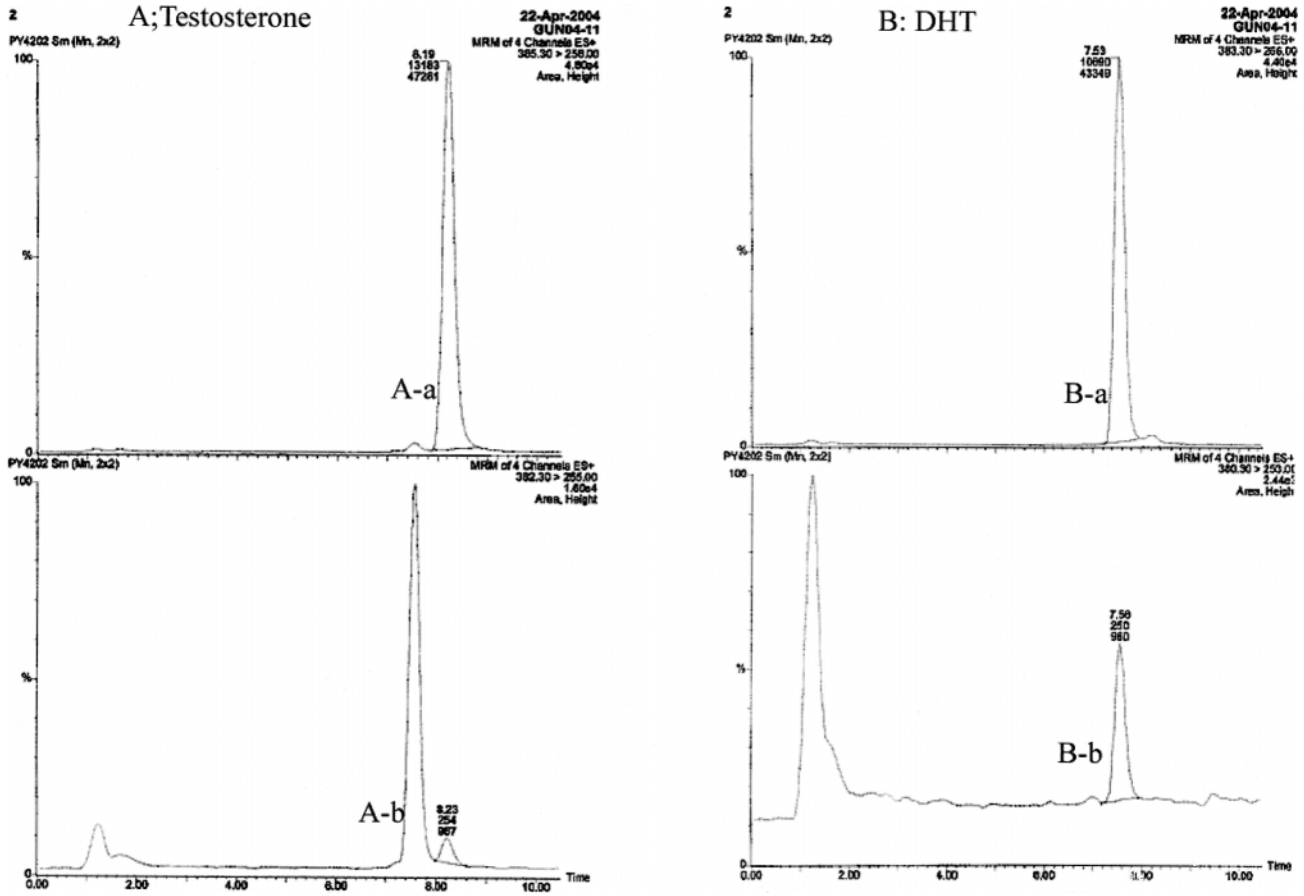

Figure 1. Typical selective ion monitor (SIM) patterns for testosterone and testosterone-d3 **(A)**, and dihydrotestosterone (DHT) and DHT-d3 **(B)**. A-a: Peak for testosterone. A-b: Peak for testosterone-d3. B-a: Peak for DHT. B-b: Peak for DHT-d3.

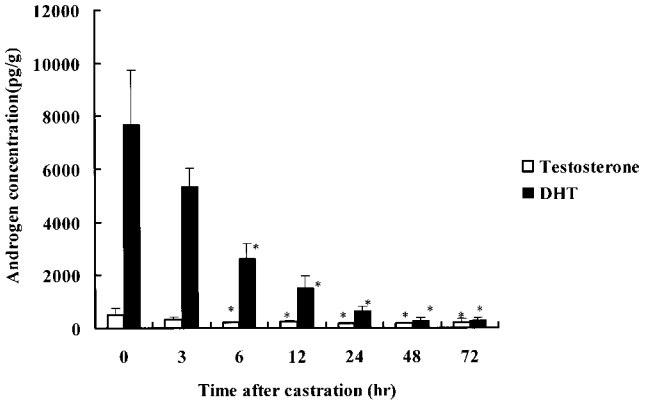

Figure 2. Changes in testosterone and dihydrotestosterone (DHT) concentrations in rat prostate. Data are presented as mean ± SE, n = 5. * Significantly different from normal controls ($P < .05$).

Time Course Changes in Androgen Concentration in Rat Prostate, Seminal Vesicles, Serum, and Seminal Fluid After Castration

T and DHT decreased with time after castration in the prostate, seminal vesicles, serum, and seminal fluid (Figures 2 through 5). Seventy-two hours after castration, T and DHT decreased to 42% and 3% of the normal levels, respectively, in the prostate and 52% and 2%, respectively, in the seminal vesicles. In serum, androgen concentrations were under the quantification limits 6 hours after castration and thereafter. Three hours after castration, T and DHT decreased to 4% and 50% of the normal levels, respectively. In seminal fluid, T and DHT decreased to 49% and 35% of the normal levels, respectively, 72 hours after castration.

Time Course Changes in T/DHT Ratio in Rat Prostate, Seminal Vesicles, Serum, and Seminal Fluid After Castration

In the male accessory sex organs, the ratio of T in the prostate (0.06) was lower than that in the seminal vesicles

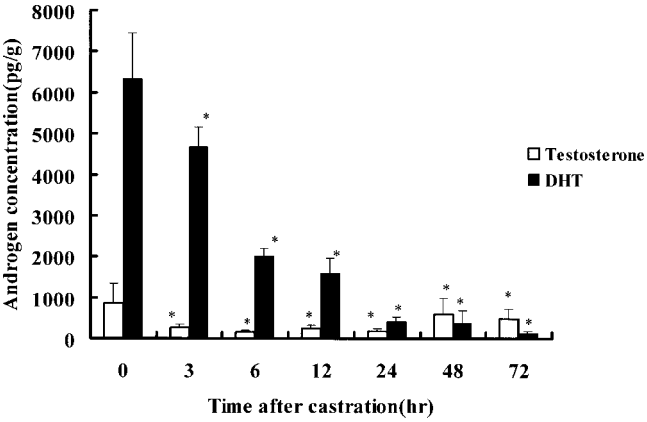

Figure 3. Changes in testosterone and dihydrotestosterone (DHT) concentrations in rat seminal vesicle. Data are presented as mean ± SE, n = 5. * Significantly different from normal controls ($P < .05$).

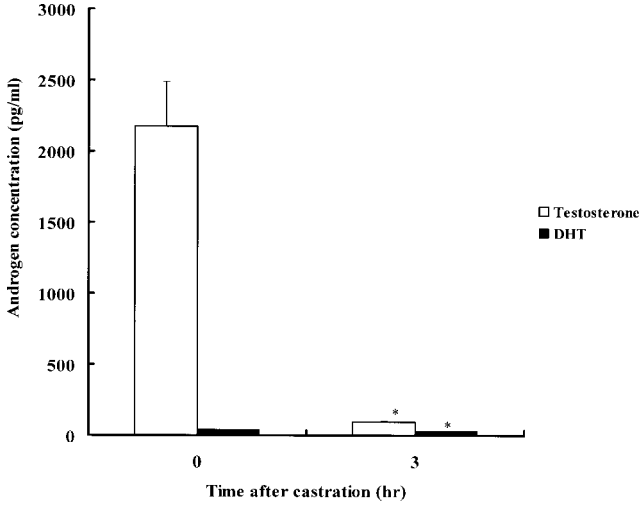

Figure 4. Changes in the testosterone and dihydrotestosterone (DHT) concentrations in rat serum. Data are presented as mean ± SE, n = 5. * Significantly different from normal controls ($P < .05$).

(0.13) immediately after castration (0 hours). The T/DHT ratio increased with time after castration (prostate: 3 hours, 0.06; 6 hours, 0.07; 12 hours, 0.15; 24 hours, 0.29; 48 hours, 0.65; 72 hours, 0.71; seminal vesicles: 3 hours, 0.15; 6 hours, 0.17; 12 hours, 0.2; 24 hours, 0.41; 48 hours, 1.05; 72 hours, 3.35). In serum, the T/DHT ratio was high at 0 hours but rapidly decreased within 3 hours after castration (0 hours, 53.7; 3 hours, 4.13). In seminal fluid, no rapid change was observed in the T/DHT ratio until 72 hours after castration and remained at about 0.5–0.8.

Discussion

Although accurate quantification of androgen in the tissues is important for elucidating the causes of disorders derived from abnormal androgen dynamics, it is difficult because the tissue content is low and abundant impurities

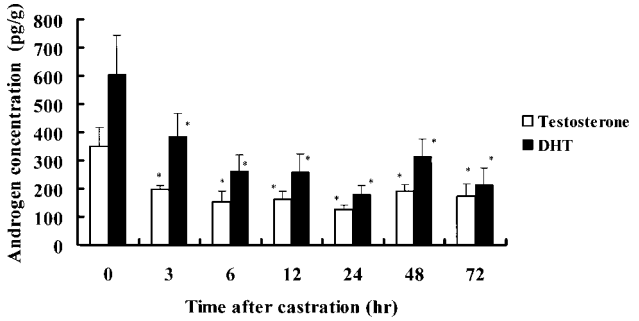

Figure 5. Changes in the testosterone **(A)** and dihydrotestosterone (DHT) **(B)** concentrations in rat seminal fluid. Data are presented as mean ± SE, n = 5. * Significantly different from normal controls ($P < .05$).

are present. The results of androgen measurements by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) have been reported (Morioka et al, 1982; Rassaie et al, 1992). Stable measurement of samples such as serum that contain fewer impurities can be performed by these methods, but sensitivity is not sufficient for measuring androgens in small samples of the prostate and seminal vesicles because of cross-reactions between the impurities in the tissue and the specific antibody. Thus, we investigated mass spectrometry as a measurement method using no specific antibody. Reliability of this method was high compared with RIA and ELISA because this method measured ion masses. We thought that measurement by tandem mass spectrometry might further increase the measurement specificity (Ishigai et al, 1997). We had previously investigated gas chromatography–mass spectroscopy (GC-MS) for determination of prostatic T, but the quantification limit was 156 pg, which was less sensitive than LC-MS/MS. Furthermore, LC-MS/MS is a more suitable technique for the simultaneous determination of androgens than GC-MS because of the thermoinstability and low volatility of steroids. Thus we selected the LC-MS/MS method. We had established and reported a highly sensitive simultaneous measurement method of T and DHT in human prostate tissues, in which T and DHT were measured by LC-MS/MS after pretreatment with carboxymethoxylamine hemihydrochloride (CMA; Shibata et al, 2000). However, sensitivity was not high enough to measure androgen in a specimen of about 10 mg, which was the maximum sample amount in a prostate biopsy. To increase the sensitivity and specificity, the formation of pyridinium derivatives with 2% fluore-1-methylpyridinium-*p*-toluenesulfonate was introduced before LC-MS/MS, and the highly sensitive simultaneous T and DHT measurement method was established (Mukaiyama et al, 1975; Martin and Quirke, 1994). On the basis of the validation, this method is capable of simultaneously measuring T and DHT with about 10 mg of tissue.

The T and DHT levels in the male accessory organs, prostate, and seminal vesicles were compared immediately after castration. DHT was dominant in 2 organs as previously reported, and the T and DHT levels were similar to those in previous reports (Damassa and Gustafson, 1988; Hirosumi et al, 1995; Nakayama et al, 1997). The T/DHT ratio in the prostate was lower than in the seminal vesicles. Seventy-two hours after castration, T in the prostate and seminal vesicle decreased to 42% and 52% of the normal levels, respectively. These mild decreases resulted from minute amounts of T that originally existed in those tissues. However, 72 hours after castration, DHT in the prostate and seminal vesicle rapidly decreased to 3% and 2% of normal levels, respectively. Accordingly, the T/DHT ratio increased to 0.71 in the prostate 72 hours after castration and exceeded 1 in the seminal vesicles 48 hours after castration. These findings suggested that the activity of 5-alpha reductase in the prostate was higher than in the seminal vesicles (George, 1997); therefore, the T/DHT ratio of prostate was lower than that of seminal vesicles.

As for the serum T and DHT levels, T was dominant, as previously reported (Gottreich et al, 2000, Torres et al, 2003). T decreased rapidly to 4% and DHT to 50% of the normal level within 3 hours after castration. The androgen levels were under the quantification limit thereafter; thus, it was not possible to perform the measurement. These findings suggested that T in the circulation did not accumulate and was supplied by production in the testis. As for seminal fluid, the relationship between fructose in seminal fluid and serum T in castrated patients has been reported (Gonzales, 1994, 2001). There are some reports concerning T and DHT levels in human seminal fluid (Paulson et al, 1986; Zalata et al, 1995; Anderson et al, 1997). The results of our study suggested that DHT was dominant in the normal state. The T/DHT ratio in seminal fluid was higher than in the male accessory sex organs. Time course changes in the androgen levels after castration in seminal fluid were slow compared with those in the male accessory sex organs, and the T and DHT levels were still maintained at 49% and 35% of the normal levels, respectively, 72 hours after castration. In this experiment, we collected seminal fluid in the seminal vesicles. We did not measure quantity of androgen in ejaculated seminal fluid. The androgen levels in seminal fluid after castration might be affected by androgen in the seminal vesicle.

This study indicated that the highly sensitive simultaneous T and DHT measurement method established by us was capable of measuring the tissue androgen levels with the use of small amounts of specimens. The androgen levels in the male accessory sex organs and serum immediately after castration measured by our method were similar to those of current methods reported, and our method was more accurate. Our method allowed the measurement of androgen levels in the prostate during hormone therapy and in tissues with age-related decreases, which might lead to the elucidation of the causes of androgen-dependent disorders.

References

Anderson RA, Kelly RW, Wu FCW. Comparison between testosterone enanthate–induced azoospermia and oligozoospermia in a male contraceptive study. V. Localization of higher 5α-reductase activity to the reproductive tract in oligozoosermic men administered supraphysiological doses of testosterone. *J Androl.* 1997;18:366–371.

Blaquier JA, Cameo MS, Burgos MH. The role of androgens in the maturation of epididymal spermatozoa in the guinea pig. *Endocrinology.* 1972;3:839–842.

Damassa DA, Gustafson AW. Effects of chronic infusions of sex steroid–binding protein on the testosterone-mediated inhibition of gonadotropin secretion and maintenance of sex accessory glands in male rats. *Endocrinology.* 1988;4:1885–1892.

Dohle GR, Smit M, Weber RF. Androgens and male fertility. *World J Urol.* 2003;5:341–345.

George FW. Androgen metabolism in the prostate of the finasteride treated, adult rat: a possible explanation for the differential action of testosterone and 5α-dihydrotestosterone during development of the male urogenital tract. *Endcrinology.* 1997;3:871–877.

Gonzales GF. Test for androgen activity at the male reproductive tract in infertile men. *Arch Androl.* 1994;3:235–242.

Gonzales GF. Function of seminal vesicles and their role on male fertility. *Asian J Androl.* 2001;4:251–258.

Gottreich A, Zuri I, Barel S, Hammer I, Terkel J. Urinary testosterone levels in the male blind mole rat (*Spalax ehrenbergi*) affect female preference. *Physiol Behav.* 2000;3:309–315.

Gray A, Feldman HA, McKinlay JB, Longcope C. Age, disease, and changing sex hormone levels in middle-aged men: results of the Massachusetts Male Aging Study. *J Clin Endocrinol Metab.* 1991;5:1016–1025.

Hirosumi J, Nakayama O, Chida N, Inami M, Fagan T, Sawada K, Shigematsu S, Kojo H, Notsu Y, Okuhara M. FK143, a novel nonsteroidal inhibitor of steroid 5 alpha-reductase: (2) in vivo effects on rat and dog prostates. *J Steroid Biochem Mol Biol.* 1995;4:365–373.

Ishigai M, Ishitani Y, Kumaki K. Characteristics of mass spectrometric analyses coupled to gas chromatography and liquid chromatography for 22-oxacalcitriol, a vitamin D3 analog, and related compounds. *J Chromatogr B Biomed Sci Appl.* 1997;1–2:11–17.

Marcelli M, Cunningham GR. Hormonal signaling in prostatic hyperplasia and neoplasia. *J Clin Endocrinol Metab.* 1999;10:3463–3468.

Martin J, Quirke E. Chemical derivatization for electrospray ionization mass spectrometry. 1. Alkyl halides, alcohols, phenols, thiils, and amines. *Anal Chem.* 1994;8:1302–1315.

Morioka M, Takeda K, Mitsuhata N, Ohashi T, Ohmori H. Endogenous androgen levels of human prostatic tissues. *Nippon Naibunpi Gakkai Zasshi.* 1982;58:876–885.

Mukaiyama T, Ikeda S, Kobayashi S. A novel method for the preparation of various 2-pyridyl sulfides from alcohols. *Chem Lett.* 1975;1159–1162.

Mulligan T, Schmitt B. Testosterone for erectile failure. *J Gen Intern Med.* 1993;9:517–521.

Nakayama O, Hirosumi J, Chida N, Takahashi S, Sawada K, Kojo H, Notsu Y. FR146687, a novel steroid 5 alpha-reductase inhibitor: in vitro and in vivo effects on prostates. *Prostate.* 1997;4:241–249.

Orwoll ES. Osteoporosis in men. *Endocrinol Metab Clin North Am.* 1998;2:349–367.

Partin AW, Rodriguez R. The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In: Walsh PC, Retik AB, Vaughan ED, Wein AJ, eds. *Campbell's Urology.* 8th ed. Philadelphia, Pa: Saunders; 1998.

Paulson RJ, Bernstein GS, Marrs RP, Lobo RA. Idiopathic oligospermia and peripheral androgen metabolism. *Fertil Steril.* 1986;46:480–483.

Rassaie MJ, Kumari GL, Rao PN, Shrivastav TG, Pandey HP. Influence of different combinations of antibodies and penicillinase-labeled testosterone derivatives on sensitivity and specificity of immunoassays. *Steroids.* 1992;3:112–118.

Shibata Y, Ito K, Suzuki K, Nakano K, Fukabori Y, Suzuki R, Kawabe Y, Honma S, Yamanaka H. Changes in the endocrine environment of the human prostate transition zone with aging: simultaneous quantitative analysis of prostatic sex steroids and comparison with human prostatic histological composition. *Prostate.* 2000;1:45–55.

Taplin ME, Ho SM. Clinical review 134: the endocrinology of prostate cancer. *J Clin Endocrinol Metab.* 2001;8:3467–3477.

Torres JM, Ruiz E, Ortega E. Development of a quantitative RT-PCR method to study 5alpha-reductase mRNA isozymes in rat prostate in different androgen status. *Prostate.* 2003;1:74–79.

Vanderschueren D, Boonen S, Bouillon R. Osteoporosis and osteoporotic fractures in men: a clinical perspective. *Baillieres Best Pract Res Clin Endocrinol Metab.* 2000;2:299–315.

Weinbauer G, Nieschlag E. Endocrine control of germ cell proliferation in the primate testis. What do we really know? *Adv Exp Med Biol.* 1997;424:51–58.

Zalata A, Hafez T, Verdonck L, Vermeulen L, Comhaire F. Androgens in seminal plasma: markers of the surface epithelium of the male reproductive tract. *Int J Androl.* 1995;18:271–277.