Assessment of Urinary Total Testosterone Production by a Highly Sensitive Time-Resolved Fluorescence Immunoassay

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Radioimmunoassay (RIA) that involves purification of the analyte by organic solvent extraction is widely used. Although the extraction RIA is reliable when properly validated, it is time consuming and radioactive, we measure urinary total testosterone with a highly sensitive rapid and accurate time-resolved fluorescence immunoassay (TRFIA) method. High affinity antitestosterone antibody and Eu³⁺ labeled Donkey antisheep IgG as tracers were used. The assay was evaluated for specificity, sensitivity, analytical recovery, precision and dilution linearity by the TRFIA method on urine samples. A satisfactory standard curve for testosterone TRFIA has been developed with good sensitivity (5.1 pmol/L). The validity of the assay for

urinary total testosterone was confirmed by the good correlation between the results obtained by TRFIA (X) and those RIA (Y) ($Y = 0.075 + 0.971X$, $R = 0.992$). Specificity, analytical recovery, precision and dilution linearity studies were determined and all found to be satisfactory. Male urinary total testosterone excretion ranged from 64.00 to 374.11 nmol/24 hr, which was about four times more than the range for women urinary testosterone excretion (14.16–100.65 nmol/24 hr), which suggests that a direct, reliable, easy to automate, highly sensitive and specific TRFIA type assay for the measurement of total testosterone in urine samples has been developed. *J. Clin. Lab. Anal.* 22:403–408, 2008. © 2008 Wiley-Liss, Inc.

Key words: testosterone; urine; time-resolved fluorescence immunoassay; assessment

INTRODUCTION

Although testosterone are quantified primarily in serum or plasma, they can also be measured in urine. Much research has been done to emphasize the importance of testosterone analysis in urine on androgen-related disorders in men and women (1–7). Unlike serum or plasma testosterone that can be misleading owing to the circadian rhythm variation (8), total urinary testosterone levels are mainly independent of short-term changes in blood, and therefore, they can be used as an index of androgen production (2,9). Testosterone are mainly excreted in human urine as glucuronic or sulphate conjugates (mostly testosterone glucuronide), and only 1% is excreted as free testosterone. For this reason, in most cases an enzymatic hydrolysis with β -glucuronidase is required to obtain the corresponding steroid. The most widely used assay

to measure urinary testosterone is radioimmunoassay (RIA) methodology that involves purification of the analyte by organic solvent extraction and column

Abbreviations: RIA, radioimmunoassay; TRFIA, time-resolved fluorescence immunoassay; LH, luteinizing hormone; SD, standard deviation; UV, ultraviolet; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay.

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Grant sponsor: National Natural Science Foundation of China; Grant number: 30470497.

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Received 17 December 2007; Accepted 10 September 2008

DOI 10.1002/jcla.20283

Published online in Wiley InterScience (www.interscience.wiley.com).

chromatography (10–12). Although the extraction/chromatographic RIA method is highly reliable when properly validated, it has some disadvantages. The method is cumbersome, time consuming, and costly. In recent years there has been increased use of mass spectrometry assay methods to quantify steroid hormones (13,14). These methods are touted to become the gold standard for all steroid hormone measurements, because multistep procedures are required for the measurement, which is not practical for diagnostic testing. However, direct immunoassay methods for measuring urinary total testosterone, which do not incorporate a purification step prior to quantification of the analyte, are not available.

The purpose of our study was to develop a direct and convenient fluorometric method based on time-resolved fluorescence immunoassay (TRFIA) of an Eu^{3+} chelate to measure urinary total testosterone excretion without hydrolysis and solvent extraction. We compared the results of this assay with those of RIA.

MATERIALS AND METHODS

Chemicals

Antitestosterone antibodies raised in sheep (Product code: S013) and donkey antisheep IgG antibody (Product code: SA026) were purchased from Micropharm, London, UK. Testosterone standard (purity, 86500) was purchased from Sigma-Aldrich Co., St. Louis, MO. The preparation was reconstituted according to the manufacturer's recommendation and diluted in 10 mmol/L PBS (pH 7.4), containing 2.0% BSA and 0.04% NaN_3 , to give the desired standard concentrations of 0, 1.0, 3.0, 10.0, 30.0, 100.0 nmol/L. All other steroid hormones used for cross-reactivity studies (see Table 1) were obtained from Sigma-Aldrich, Co., St. Louis, MO.

Testosterone-3-CMO-BSA, Tween 20, and bovine serum albumin were all from Sigma-Aldrich Co., St. Louis, MO. DTTA- Eu^{3+} (N1-[*p*-isothiocyanato-benzyl]-diethylene-triamine-N1; N2; N3; N4-tetraacetate- Eu^{3+}) was from Tianjin Radio-Medical Institute (Tianjin, China). Other chemicals used were of analytical grade. Buffers were as described previously (15).

Urine Samples

Urine samples were collected in large containers without preservatives from normal healthy volunteers (137 men and 123 women) aged between 21 and 45 years after signing the college consent form that was approved by Shanghai JiaoTong University School of Medicine Ethics Committee. Total daily urinary excretion of

TABLE 1. Specificity of Testosterone TRFIA

Steroid hormone	% Cross reactivity
Testosterone	100
Testosterone-3-glucuronide	61.1
Dihydrotestosterone	20.4
Androstenedione	0.5
DHEA	0.03
DHEA-3-glucuronide	0.07
Danazol	0.09
Triamcinolone	0.2
Estradiol	0.02
Progesterone	0.02
Corticosterone	0.13
Prednisolone	0.29
Cortisol	0.002
17-OH-progesterone	0.01
Cholesterol	0.02
Pregnenolone	0.06

testosterone was determined by weight in grams then aliquots were transferred into universal tubes providing four separate samples. All urine samples were then stored at -20°C in a freezer until required for assaying.

Kits for Correlation Studies

Testosterone RIA kits (Coat-A-Count[®] Total Testosterone) were purchased from DPC Diagnostic Products Corporation, Los Angeles, CA.

Purification and Labeling of Donkey Antisheep IgG Antibody

Before Europium labeling of donkey antisheep IgG antibody, prepacked columns of Sephadex G-25M were used for desalting and buffer exchange. About 1 mg of purified donkey antisheep IgG antibody was dissolved in 50 mmol/L sodium carbonate buffer (pH 9.8), containing 0.9% NaCl. This solution was added to a glass vial containing 0.8 mg of DTTA- Eu^{3+} and then incubated overnight at 4°C . The labeled donkey antisheep IgG antibody was separated from excess free label by gel filtration using a Sepharose CL-6B column (1.5×50 cm) and the elution buffer. The elution was monitored at 280 nm with a Model EM-1 Econo UV monitor, and a total of 8.8 mL of labeled donkey antisheep IgG antibody was collected. The concentration of donkey antisheep IgG antibody in the purified conjugate solution was determined with the Beer-Lambert method (the UV absorbed by the DTTA- Eu^{3+} was subtracted); Eu^{3+} -DTTA labeled donkey antisheep IgG antibody gave an absorbance of 1.29 at 280 nm. The concentration of IgG was about $4.71 \mu\text{mol/L}$,

which was calculated from the formula: $\text{IgG}(\mu\text{mol/L}) = (\text{A280} \times 0.008 \times \text{Eu}^{3+} \mu\text{mol/L}) \times 1,000,000 / (1.34 \times 160,000)$. The Eu^{3+} concentration was measured against Eu^{3+} calibrators in the 1235 fluorometer after fluorescence enhancement. The Eu^{3+} concentration was about $32.97 \mu\text{mol/L}$. The average number of Eu^{3+} coupled to one donkey anti-sheep IgG antibody was 7.0, calculated from the molar concentrations of Eu^{3+} and Donkey anti-sheep IgG antibody [$\text{Eu}^{3+}(\mu\text{mol/L}) / \text{IgG}(\mu\text{mol/L})$]. The labeled donkey anti-sheep IgG antibody could be stored at 37°C for at least 4 weeks without any loss of immunoreactivity.

Coating of Polystyrene Microtiter Strips

Coating of polystyrene microtiter strips (Nunc, Roskilde, Denmark) was performed by a modification of our previously described methods (15). Concentrated testosterone-3-CMO-BSA (1.0 mg/mL) was diluted to $5 \mu\text{g/mL}$ in coating buffer. The coated strips were stored at $4-8^\circ\text{C}$ in a dry sealed bag. No loss of immunoreactivity was observed during storage for 4 weeks at 37°C .

Time-Resolved Immunofluorometric Assay for Total Urinary Testosterone

Urine samples can be assayed by our TRFIA directly without any extraction. Two-step assay procedures were used. In brief, $50 \mu\text{L}$ of standards or urine samples were incubated at room temperature for 30 min in testosterone-3-CMO-BSA-coated wells with $100 \mu\text{L}$ of assay buffer containing the diluted antitestosterone antibody (1:1000). Subsequently the assay wells were aspirated and washed six times with the washing buffer. We added $200 \mu\text{L}$ of Eu^{3+} -labeled donkey anti-sheep IgG antibody to every well and then gently mixed them for 30 min at RT. The bound Eu^{3+} -label donkey anti-sheep IgG antibody was then dissociated from the surface with $200 \mu\text{L}$ of enhancement solution and the resulting fluorescent chelate solution was subjected to single photon counting with a time-resolved fluorometer (Arcus 1235, Wallac, Turku, Finland).

DPC RIA for Urinary Total Testosterone

A modification of the procedures described in the instructions enclosed in the kits was used to extract urine samples, separate testosterone from other steroids by hydrolysis and assay by RIA. Briefly, 1 mL of urine sample was hydrolyzed by $200 \mu\text{L}$ HCl (12 N). After incubating in a boiling water-bath for 15 min, each hydrolyzed sample was diluted 1-in-25 with the zero calibrator (for example add $10 \mu\text{L}$ of hydrolyzed sample to $240 \mu\text{L}$ of zero calibrator). Fifty micro liters of the

standards, controls or hydrolyzates were dispensed into the corresponding groups of tubes. We added 1.0 mL of ^{125}I -labeled total testosterone to every tube and then gently mixed them with a vortex-type mixer. After a 3-hr incubation at 37°C with continuous shaking at 400 rpm, the tubes were washed twice. The remaining bound radioactivity was measured with the γ scintillation counter.

Statistical Analysis of Testosterone Concentrations

We determined the distributions of urinary total testosterone in healthy volunteers. The Student–Newman–Kewls test was used for statistical analysis. A significant difference was defined as comparison resulting in $P < 0.05$.

RESULTS

Optimization of Testosterone Standard Curve

Typical standard curve data (log-log plot) for testosterone TRFIA are shown in Figure 1. The precision profile of the assay was determined from 15 replicates, the coefficients of variation at the concentration standards of 0, 1.0, 3.0, 10.0, 30.0, 100.0 nmol/L were all $< 4.0\%$. Sensitivity (minimum detection limit) for the testosterone TRFIA standard curve was determined according to the method of Abraham (16) in which it represents the amount of testosterone that produced a statistically significant drop in the signal compared with the signal at the zero standard and found to be 5.1 pmol/L . When the limit of detection was determined according to The International Union of Pure and Applied Chemistry as the mass of analyte

Fig. 1. Typical standard curve ($\square$) and precision profile ($\bullet$) for TRFIA of testosterone. Each point on the standard curve is the mean of five duplicate measurements. The zero standard resulted in a mean 736,268 counts per second (cps).

required to give a decrease in the signal of zero standard of 2.5 SD, the sensitivity was 25.41 pmol/L.

Specificity of Antitestosterone Antibody

Cross reactivities of various steroid hormones with the testosterone antibody using TRFIA standard curve is shown in Table 1. Testosterone TRFIA shows high specificity for testosterone and testosterone-3-glucuronide. The following compounds were spiked into a steroid-free diluent and the following results were obtained. Percent change is calculated as $\% \text{change} = \frac{(\text{concentration of spiked sample})}{\text{amount of compound spiked}} \times 100$.

Analytical Recovery

We assessed the analytical recovery of TRFIA by adding three amounts of testosterone to give final exogenous concentrations of 5.0, 20.0, and 40.0 nmol/L to five urine samples. The endogenous testosterone concentrations were 0.34, 5.05, 12.62, 22.84, and 40.26 nmol/L. Fifty micro liters of each of these exogenous testosterone was spiked into 950 μ L of urine samples, for a spiking ratio of 1 to 19, leaving the urine matrix of the spiked sample relatively intact. To calculate expected values, 95% of the unspiked value was added to 5% of the spiking solution concentration. We calculated the percentage recovery after we subtracted the concentrations of endogenous testosterone from the experimentally determined amounts. Recoveries ranged from 87 to 102% (Table 2).

TABLE 2. Analytical Recovery of Testosterone Added to Urine Sample

Testosterone, nmol/L			
Added	Expected	Observed	Recovery ^a , %
5.0	0.37	0.38	101
	4.85	4.61	95
	12.04	11.56	96
	21.75	21.53	99
	38.3	33.32	87
20.0	0.82	0.82	100
	5.30	5.40	102
	12.49	12.11	97
	22.20	21.31	96
	38.75	39.13	101
40.0	5.32	4.95	93
	9.80	9.80	100
	16.99	16.82	99
	26.70	26.70	100
	43.25	40.22	93

^aMean = 97.27%; SD = 4.04; *n* = 15.

Precision

Within-run precision studies were done by assaying urine samples in three concentrations 18 times each. We assessed between-run precision by analyzing control samples in ten successive runs and evaluated day-to-day precision by testing the control samples on ten consecutive days. All coefficient of variations were well below 8.6% (Table 3).

Dilution

Table 4 shows the results of our evaluation of the dilution linearity of TRFIA when we used samples serially diluted with assay buffer. Expected values were derived from initial concentrations of total testosterone in the undiluted urine samples. Correlating the results obtained from TRFIA with the expected concentrations, we found that the dilution curves were linear over the whole range of concentrations. Expected and measured values were well correlated.

Correlation Studies of Testosterone TRFIA

To further verify the results of our TRFIA, all urine samples were also assayed by TRFIA and RIA. We observed linear relativity between TRFIA and RIA results when the assays were performed in duplicate at the same time (Fig. 2). The linear regression equation was as follows: RIA result = 0.075 + 0.971 (TRFIA result). The correlation coefficient *r* was 0.992.

Urinary Testosterone Ranges

Urinary total testosterone levels were detectable in normal healthy volunteers with TRFIA. The data were tested and were found to be normally distributed. In female volunteers (*n* = 123), urinary total testosterone was from 14.16 to 100.65 with a mean of 48.27 $\pm$ 18.00 (SD) nmol/24 hr. In males (*n* = 137), urinary total testosterone was from 64.00 to 374.11 with a mean of

TABLE 3. Precision of TRFIA for Urinary Total Testosterone

Testosterone level, mean $\pm$ SD (nmol/L)	Coefficient of variation (%)
Within run (<i>n</i> = 18)	
6.43 $\pm$ 0.35	5.4
54.14 $\pm$ 3.62	6.6
95.77 $\pm$ 4.80	5.0
Between run (<i>n</i> = 10)	
6.48 $\pm$ 0.56	8.6
55.28 $\pm$ 3.51	6.3
93.96 $\pm$ 2.00	2.1
Day to day (<i>n</i> = 10)	
6.12 $\pm$ 0.51	8.3
55.04 $\pm$ 2.81	5.1
92.48 $\pm$ 6.33	6.8

TABLE 4. Dilution Linearity of Samples With High Testosterone Concentrations (nmol/L)^a

Dilution factor	Sample 1		Sample 2		Sample 3	
	Expected	Observed	Expected	Observed	Expected	Observed
None	NA	10.23	NA	45.56	NA	65.49
× 2	5.12	5.03	22.78	20.33	32.75	30.43
× 4	2.56	3.39	11.39	10.76	16.37	17.04
× 8	1.28	1.4	5.70	4.95	8.19	7.45
× 16	0.64	0.65	2.85	2.86	4.09	4
× 32	0.32	0.23	1.42	1.33	2.05	2.11

NA, not applicable.

^aWe conducted a regression analysis of expected (x) vs. measured (y) values (in units per milliliter). Respective slopes, intercepts and *r* values were 1.0163, 0.1238, and 0.9817 for sample 1; 0.8911, 0.1793 and 0.9993 for sample 2; and 0.9349, 0.3427 and 0.9977 for sample 3.

Fig. 2. Correlation between total testosterone concentrations in urine samples as determined with TRFIA and RIA ($r = 0.992$, $n = 260$).

214.86 ± 60.39 (SD) nmol/L. Urinary total testosterone levels in male are significant higher than those in female ($P < 0.01$). Urinary total testosterone levels in patients with chronic renal failure were significantly higher than those in health volunteers ($P = 0.001$). There was significant difference between males and females regarding urinary total testosterone levels ($2019.72 \text{ nmol/24 hr} \pm 130.04$ vs. $476.21 \text{ nmol/24 hr} \pm 92.11$, respectively; $P = 0.0001$). Urinary total testosterone levels in patients with chronic renal failure were significantly lower than those in healthy volunteers ($P = 0.001$). There was significant difference between males and females regarding urinary total testosterone levels ($98.72 \text{ nmol/24 hr} \pm 51.04$ vs. $20.21 \text{ nmol/24 hr} \pm 12.11$, respectively; $P = 0.0001$).

DISCUSSION

Acid hydrolysis at elevated temperatures, which is a more general method that can hydrolyze both glucuronide and sulphate conjugates, has been used for many years to cleave the steroid from its conjugate (17). However, it has two disadvantages: it may alter the

structure or the constitution of the steroid and the pigment formed can be taken up in the extract (18). We have developed a direct and convenient TRFIA method for the measurement of total testosterone in urine samples, and this, to our knowledge, has not been published elsewhere in the literature. The antibody we used in the study shows a high specificity for testosterone (100%) and testosterone-3-glucuronide (61.1%), which can account for the feasibility of our direct immunoassay method. The novel feature of the current assay is that it does not require an initial extraction step. Assay time is shortened by 1.0 hr (3.25 hr for DPC RIA). The assay reagents were used in an instrument so that the assay could be performed on an automated platform.

After europium chelates were developed as labels in the 1980s (19,20), TRFIA has been reported as an ideal immunoassay technique. Sensitivity is higher with this technique than with methods based on tritiated tracers or enzyme-conjugated tracers. In addition, TRFIA offers an alternative challenging immunoassays based on ^{125}I labels with its advantage of a stable, non-radioactive tracer. Fluorescence of the Eu^{3+} tracer is long lived and allows for the differentiation of the short-lived background fluorescences of biological material, plastics and optics (21). The highly specific activity of the label increases the sensitivity of immunoassays while minimizing nonspecific binding of the labeled bioaffinity molecule (22,23). TRFIA are now widely used for routine estimation of steroid hormones (24–26).

The validity of the direct urine assay for total testosterone was confirmed by the good correlation between the results obtained by TRFIA and those by RIA. The performance characteristics of TRFIA are in many respects equivalent to or better than those reported for RIA (10–12). Its analytical sensitivity was about 5.1 pmol/L, compared with about 0.21 nmol/L for the currently available DPC RIA. The high sensitivity of our TRFIA method allows the measurement of testosterone concentrations in urine samples after 10- to 40-fold dilution. This is very beneficial to eliminate

interference in the direct assay and will be advantageous for the development of TRFIA methods to estimate the low testosterone levels in saliva samples from men and women. Analytical recovery studies, parallelism (dilution of urine samples) and precision data were determined for the TRFIA found to be satisfactory. Urinary total testosterone excretion in male healthy volunteers ranged from 64.0 to 374.11 nmol/24 hr, which was about four times more than the range for women total urinary testosterone excretion (14.16–100.65 nmol/24 hr). Our data are generally in agreement with those results obtained by testosterone RIA kits (DPC Diagnostic Products Corporation, Los Angeles, CA). It is important to realize that reference values for total urinary testosterone may vary widely from one laboratory to another when different assay methods are being used. They also may vary to some extent when the same assay method is used by different laboratories. The variations in reference values will be especially evident as laboratories switch from immunoassay methods to GC-MS or LC-MS assays.

Overall, the novel TRFIA described here provides a rapid and sensitive method for the measurement of urinary total testosterone. Compared with previous methods, it is simple to automate and can reduce reagent and labor costs. The biggest advantages of this method are its ease and the reduced analysis time, which enable high throughput of samples. The reagents are stable over long periods of time, and no harmful radioactive material is needed. The data obtained for validation of testosterone TRFIA revealed a reliable performance of the assay for routine use.

ACKNOWLEDGMENTS

This work was partly funded by the National Natural Science Foundation of China (project 30470497).

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