Journal of Chromatography, 423 (1987) 285–291
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3909

Note

Combined use of rapid-flow fractionation and high-performance liquid chromatography for the determination of serum testosterone and application to study of stress response to physical exercise

KITARO OKA* and TOSHIHIKO HIRANO

Division of Clinical Pharmacology, Tokyo College of Pharmacy, Tokyo 192-03 (Japan)

and

MAKOTO NOGUCHI

Division of Pharmacy, Kawasaki City Ida Hospital, Kanagawa 211 (Japan)

(First received April 29th, 1987; revised manuscript received July 21st, 1987)

Recent developments in the high-performance liquid chromatography (HPLC) of serum 3-oxo-4-ene steroids, such as corticosteroids, demonstrate the possibility of determining their concentrations of the order of 1 ng/ml of serum or plasma using a UV absorbance detector and a silica gel column [1–3]. The clean-up procedure for such highly sensitive HPLC should be possible by rapid-flow fractionation (RFF), which allows optimization of the lowest solvent polarity required for extracting target molecules using a minimal solvent volume [2, 4]. Thus RFF and HPLC used in conjunction were found to provide chromatograms without interference. In the present study, this technique was used to determine the concentrations of male testosterone in serum.

Four different methods for the assay of testosterone have been reported: radioimmunoassay [5–7], enzyme immunoassay [8, 9], competitive protein binding technique [10, 11] and the recently developed gas chromatography–mass fragmentometry method [12–14]. The use of HPLC for this purpose has not been reported so far. Such an application of HPLC should be examined in consideration of the fact that the physiological concentration of testosterone in male serum or plasma is 3.6–11.7 ng/ml, a level within the detectable range of a UV absorbance detector. The concentration of testosterone in plasma has been reported to show diurnal variation [13]. The extent of the variation is not as significant as that for corticosteroids. Also, testosterone concentration shows episodic rhythm

0378-4347/87/$03.50

throughout the day, possibly due in some cases to motional stress [15]. These physiological phenomena appear to warrant detailed study using an improved assay technique such as HPLC. Thus, the variation of serum testosterone concentrations before and after physical exercise was studied using an HPLC method.

EXPERIMENTAL

RFF apparatus

The apparatus and operational procedure have been described in our previous paper [2]. In brief, the system comprised two glass columns, one for testosterone extraction and another for washing the extract to eliminate the acidic constituents. The support material in each column was diatomaceous earth granules of particle size larger than 50 μm and prepared from Celite powder (No. 545, Johns Manville, U.S.A.) by precipitation in distilled water. Inner sizes of the extraction and washing columns were 3.5 and 0.8 ml, respectively. The void volume of the system was ca. 4.0 ml. The loading capacity was 0.5 ml of serum or plasma and the flow-rate was 0.7–0.9 ml/s under a nitrogen pressure of 2–3 kg/cm^2 for routine operation. The flow-rate used in the optimization of solvent polarity for this purpose was 9 ml/min using a KPW-10 (Kusano, Tokyo, Japan) solvent-delivery system for adjustment. A Uvilog-III (Kusano) UV absorbance detector and a syringe-loading sample injector Model 7125 (Rheodyne, Cotati, CA, U.S.A.) were also used.

Reagents, solvents and reference compounds

Inorganic chemicals were purchased from Cica-Merck (Tokyo, Japan). The solvents for RFF and HPLC were of reagent grade and obtained from Wako (Osaka, Japan). Testosterone and 19-nortestosterone were purchased from Sigma (St. Louis, MO, U.S.A.).

HPLC apparatus

A continuous-flow BIP-I (Jasco, Tokyo, Japan) solvent-delivery system, a variable-wavelength Uvidec-100-V (Jasco) UV detector set at 245 nm and an RC-150 (Jasco) pen recorder were used. The flow-rate was 2 ml/min. A syringe-loading sample injector Model 7125 (Rheodyne) was installed. A conventional packed column (LiChrosorb Si-60, particle size 5 μm, 250 mm $\times$ 4 mm I.D., Cica-Merck) was pre-washed with 200 μl of 1% sulphuric acid and then with an appropriate amount of distilled water before use. The sensitivity of the detection system was 0.002 absorbance units full scale (a.u.f.s.).

Optimization of extraction

The following preliminary experiment was carried out using distilled water instead of serum. The whole RFF column system comprising extraction and washing columns was conditioned with 7 ml of methanol and then with 7 ml of diethyl ether. After 0.5 ml of water had been injected through the plug, the extraction column was connected to the solvent-delivery pump and the washing column to the UV detector. An appropriate mixture of diethyl ether and *n*-hexane

pre-saturated with water was introduced as the mobile phase, and the system was pre-conditioned at a flow-rate of 1 ml/min until a flat baseline was obtained at a sensitivity of 0.32 a.u.f.s. Dichloromethane solution (10 μl) spiked with 10 μg of testosterone or 19-nortestosterone was injected. The solvents used were 2, 5, 10, 20, 30, 40 and 50% diethyl ether in *n*-hexane (v/v), and diethyl ether. The most appropriate solvent composition was determined from the extraction chromatograms obtained.

A preliminary experiment was then carried out using a serum sample. A 0.5-ml portion of the serum spiked with 10 μg of testosterone or 19-nortestosterone was introduced into the extraction column, followed by *n*-hexane containing 30 or 40% diethyl ether at a flow-rate of 9 ml/min. The solvent polarity thus determined was 30% diethyl ether in *n*-hexane.

Routine operation with RFF

The general procedure is described in our pevious paper [2]. The acidic contaminants were eliminated from the washing column with 70 μl of 5% sodium hydroxide. After the columns had been connected, 0.5 ml of serum specimen spiked with 10 ng of 19-nortestosterone dissolved in 10 μl of ethanol was introduced into the extraction column through the plug. A reservoir column with an inner volume of 7 ml was attached to the extraction column and filled with 7 ml of 30% diethyl ether in *n*-hexane. This column was then connected to the nitrogen cylinder with its gauge set at 2–3 kg/cm^2 to flush the solvent out of the columns within 10–12 s. The eluent was collected in a glass tube and the solvent was evaporated to dryness. The resulting residue was redissolved in 20 μl of 1% methanol in dichloromethane, to be injected later into the HPLC system. The columns were cleaned for subsequent use as described in our previous paper [2].

Determination of testosterone concentration

The mobile phase for HPLC was 5.3% ethanol in *n*-hexane containing 0.1% distilled water, and the flow-rate was 2 ml/min. The sensitivity of the system was 0.002 a.u.f.s. A calibration curve was obtained from the peak-height ratios of testosterone and 19-nortestosterone, using female serum obtained from a volunteer whose menstrual cycle was in follicular phase.

Recovery of testosterone

The testosterone recovery rate was determined as follows. Testosterone in amounts of 1, 5 and 10 ng was added to 0.5 ml of pooled female serum sample containing less than 200 pg of testosterone. The internal standard was added to the extract after the RFF operation. The recovery was calculated from peak-height ratios deducted by an endogenous peak ratio of the two steroids.

RESULTS

The results of testosterone extraction–monitoring by RFF are shown in Fig. 1 and were essentially the same as those obtained for 19-nortestosterone. A logarithmic relationship between capacity ratios of the extraction peaks and solvent

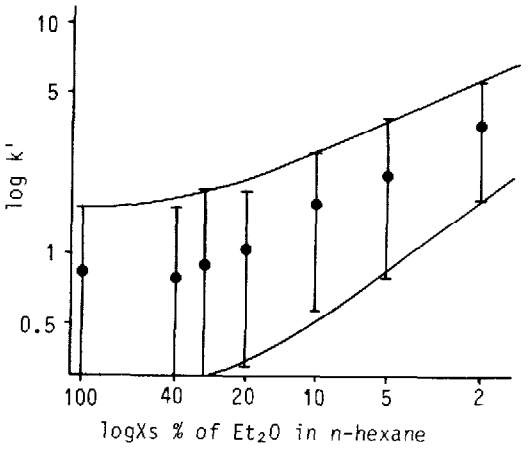

Fig. 1. Optimization of extraction solvent polarity for serum testosterone using an RFF apparatus equipped with a solvent-delivery pump and UV detector. Horizontal axis shows the logarithm of the stronger solvent (diethyl ether) fraction in each solvent mixture. Vertical axis shows the logarithm of capacity ratio, $k' = V_R/V_0 - 1$, where V_R is the chromatographic retention volume and V_0 is the void volume of the column. Each vertical line indicates an extraction peak width, where the peak position is indicated by a black circle. It appears reasonable that the lowest polarity sufficient for extracting serum testosterone with minimal solvent volume is that of 30–40% diethyl ether in *n*-hexane. One unit of k' corresponds to a void volume of 4.0 ml as described in the text. Since k' at 30% diethyl ether was 1.6, the optimized solvent volume was 6.4 ml. In routine RFF operation, 7.0 ml of solvent were used.

compositions indicated that both testosterone and 19-nortestosterone could be recovered from distilled water using diethyl ether–*n*-hexane. For the clean-up of the steroids by frontal extraction, solvent polarities higher than that of 40% diethyl ether are required. The minimum volume of the extraction solvent was ca. 6.4 ml, as evident from the fact that the peak widths of steroids in the solvent mixtures containing more than 40% diethyl ether spanned equally from 0 to 1.6 in k' units, one k' unit corresponding to 4.0 ml of the solvent. This volume was the same as the void volume of the present RFF system. It was thus necessary to select a solvent mixture of less than 40% diethyl ether so as to eliminate serum components with polarities exceeding those of the steroids.

To ensure optimal solvent polarity, the RFF technique was tested using a serum specimen and mixtures with 30 and 40% diethyl ether at higher flow-rates. Essentially, the same results for the amount of solvent volume required were obtained with both solvent compositions. Two steroids were recovered from the serum, each at a volume less than 7 ml. Thus, the 30% solvent composition was chosen for routine RFF operations. The chromatograms in Fig. 2 show that this technique is free from interference.

Steroid recovery was quantitative with the average value being $100.0 \pm 3.3\%$ at concentrations of 1, 5 and 10 ng/ml. The intra- and inter-assay coefficients of variation were less than 4%. The equation of the calibration curve obtained with the pooled female serum was $y = 0.1188x - 0.0015$, where x = testosterone concentration (ng/ml) and y = the peak-height ratio of testosterone against 19-nortestosterone. The correlation coefficient (r) was 0.9970.

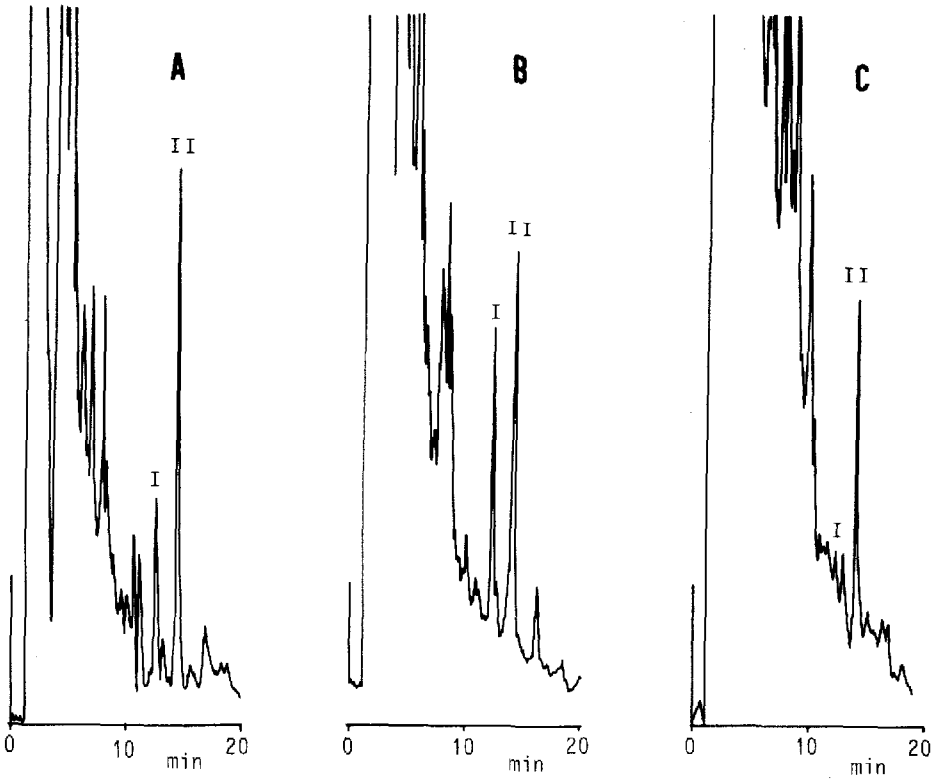

Fig. 2. Typical chromatograms of the serum testosterone of a healthy male volunteer before (A) and after (B) 10-km running: 2.7 and 6.1 ng/ml, respectively. Chromatogram C was obtained from a female volunteer in the luteal phase of her menstrual cycle. Peaks: I = testosterone; II = 19-nortestosterone.

TABLE I

INCREASE IN SERUM TESTOSTERONE LEVELS AFTER 10-km RUNNING

Statistical difference before and after: $p < 0.001$.

No. of volunteer	Serum testosterone (ng/ml)		Factor of increase
	Before	After	
1	4.60	8.28	1.80
2	5.18	13.53	2.61
3	1.59	8.74	5.50
4	2.71	9.55	3.52
5	2.56	12.38	4.84
6	1.35	6.13	4.54
Mean ± S.D.	3.00 ± 1.57	9.76 ± 2.74	3.80 ± 1.42

DISCUSSION

These results demonstrate that the combined use of RFF and HPLC provides a sensitive, selective and simple chromatographic method for the determination of testosterone concentration in male serum or plasma. The plasma sample was subjected to the same experimental procedure. The technique was carried out on samples from six healthy male volunteers aged from 19 to 24 years. The average concentration of testosterone in the serum at rest was 3.00 ± 1.57 ng/ml. This value is basically in agreement with those reported for Japanese men [12]. The present data confirm that our method is sufficiently accurate for application to biological studies. The entire assay can be performed within a period of only 20 min.

The present technique was also used in the study of the response serum testosterone to physical stress, and the results obtained are shown in Table I. Several researchers have reported the effects of prolonged stress on the concentration of this hormone in the serum, indicating it to decrease markedly possibly as a result of its decreased production rather than its increased consumption [15]. The administration of adrenocorticotropic hormone for four days was also noted to suppress its secretion [16]. Our results on six volunteers before and after 10-km running for 30–40 min clearly show concentrations after running to be 3.80 ± 1.42 times those before running. The variance in results may possibly arise from differences in the periods of time of physical exercise. A short period of physical stress such as that required for 10-km running may increase testicular and adrenal function via the hypothalamic–pituitary axis, whereas a long period may suppress these functions by exhausting the production of this hormone in the testes. These particular biological phenomena of testosterone observed in the present study do not appear to effect those of well-known glucocorticoids that are secreted only from the adrenal cortex.

ACKNOWLEDGEMENTS

We are indebted to Professor Hisao Iwane of the Tokyo Medical College for his assistance in conducting the running test, and to Mrs. Sachiko O'hara for her secretarial assistance.

REFERENCES

1 K. Oka, K. Minagawa, S. Hara, M. Noguchi, Y. Matsuoka, M. Kono and S. Irimajiri, Anal. Chem., 56 (1984) 24.
2 K. Oka, N. Ohki, M. Noguchi, Y. Matsuoka, S. Irimajiri, M. Abe and T. Takizawa, Anal. Chem., 56 (1984) 2614.
3 K. Oka, M. Noguchi, T. Kitamura and S. Shima, Clin. Chem., 33 (1987) 1639.
4 K. Oka, T. Ijitsu, K. Minagawa, S. Hara and M. Noguchi, J. Chromatogr., 339 (1985) 253.
5 D.S. Skelley, L.P. Brown and P.K. Besch, Clin. Chem., 19 (1973) 146.
6 S. Furuyama, D.M. Mayes and C.A. Nugent, Steroids, 16 (1970) 415.
7 J.M. Kjeld, C.M. Puah and G.F. Joplin, Clin. Chim. Acta, 80 (1977) 271.
8 G.B. Wisdom, Clin. Chem., 22 (1976) 1243.
9 A.M.G. Bosch, W.H.J.M. Stevens, C.J. Wijngaarden and A.H.W.M. Schuurs, Fresenius' Z. Anal. Chem., 290 (1978) 98.
10 R. Horton, T. Kato and R. Sherins, Steroids, 10 (1967) 245.

11 J.A. Demetriou and F.G. Austin, Clin. Chem., 16 (1970) 111.
12 S. Baba, Y. Shinohara and Y. Kasuya, J. Chromatogr., 162 (1979) 529.
13 S. Baba, Y. Shinohara and Y. Kasuya, J. Clin. Endocrinol. Metab., 50 (1980) 889.
14 Y. Shinohara, S. Baba and Y. Kasuya, J. Clin. Endocrinol. Metab., 51 (1980) 1459.
15 A. Aakuaag, O. Bentdal, K. Quigstad, P. Walstad, H. Roenningen and F.Fonnum, Int. J. Androl., 1 (1978) 22.
16 I.Z. Beitins, F. Bayard, A. Kowarski and C.J. Migeon, Steroids, 21 (1973) 553.