Journal of Chromatography, 277 (1983) 79—85
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1771

QUANTITATION OF TESTOLACTONE AND 4,5-DIHYDROTESTOLACTONE IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

V.L. PASCUCCI and R.L. YEAGER

Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD (U.S.A.)

R.J. SHERINS and R.V. CLARK

Developmental Endocrinology Branch, NICHHD, N.I.H., Bethesda, MD (U.S.A.)

and

J.F. GALLELLI and D.C. CHATTERJI*

Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, N.I.H., Bethesda, MD 20205 (U.S.A.)

(First received January 18th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A rapid, sensitive, and selective assay is described for the quantitation of both testolactone and its recently identified metabolite, 4,5-dihydrotestolactone, in plasma and urine using high-performance liquid chromatography. The procedure includes a methylene chloride extraction prior to chromatography and quantitation using peak height ratios (ultraviolet absorbance detection, 242 nm) of testolactone and 4,5-dihydrotestolactone to the internal standard, testosterone. A sensitivity of 20 ng/ml for both testolactone and 4,5-dihydrotestolactone is easily achieved using only 0.5 ml of sample. Mean recoveries for testolactone and its metabolite are 95.0% and 81.8%, respectively, and the mean coefficient of variation of the procedure is 3.5% for the drug and 7.1% for the metabolite. This method is currently being used to study the pharmacokinetics of testolactone and 4,5-dihydrotestolactone in male patients. A steady-state plasma concentration versus time profile from a representative patient is included.

0378-4347/83/$03.00 © 1983 Elsevier Science Publishers B.V.

INTRODUCTION

Testolactone (Fig. 1A), is an aromatase inhibitor which effectively blocks the peripheral aromatization of androgens into estrogens. The drug has been used to treat women with breast cancer who are post-menopausal or who have had an oophorectomy [1, 2], and more recently to study sex steroid regulation of pituitary gonadotropin secretion [3, 4]. Testolactone is currently under investigation for treatment of certain disorders in men in which estrogen overproduction is suspected, such as idiopathic oligospermic infertility [5, 6] and pubertal gynecomastia.

Although testolactone has been used therapeutically for about 20 years, to our knowledge there is no published procedure for quantitating testolactone in biological fluids. In this publication we describe a rapid, sensitive, and selective high-performance liquid chromatographic (HPLC) procedure for the quantitation of testolactone in both plasma and urine. The assay method also permits the concurrent detection and quantitation of 4,5-dihydrotestolactone (Fig. 1B), a metabolite of testolactone which has been recently isolated and identified in our laboratory [7]. This procedure is currently being used to study the pharmacokinetics of testolactone and its metabolite in male patients.

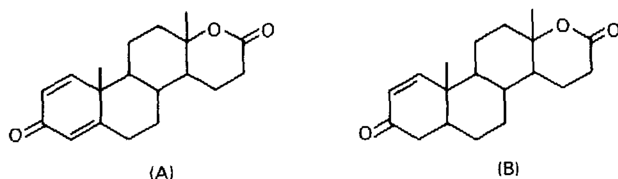

Fig. 1. Chemical structures of (A) testolactone (13-hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid δ-lactone) and (B) 4,5-dihydrotestolactone (13-hydroxy-3-oxo-13,17-secoandrosta-1-en-17-oic acid δ-lactone).

EXPERIMENTAL

Materials and reagents

U.S.P. Reference Standards of testolactone and testosterone were used to prepare plasma and urine standards and internal standard solution, respectively. A metabolite of testolactone, 4,5-dihydrotestolactone, was isolated and purified according to ref. 7. Briefly, urine collected from a testolactone patient was extracted with methylene chloride (glass-distilled, HPLC grade from Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Following separation and concentration, the organic layer was injected onto the HPLC system (conditions described below). A preparative HPLC column (Zorbax® C_8 reversed phase, 25 cm × 9.4 mm I.D., from Dupont, Wilmington, DE, U.S.A.) was used to separate and collect the suspected metabolite in the eluent. The eluent was then extracted with methylene chloride, which was subsequently evaporated to dryness. The isolated metabolite was used in the preparation of plasma and urine standards.

Acetonitrile, methanol, and methylene chloride, glass-distilled and certified

HPLC grade, were obtained from Burdick and Jackson Labs. Water was double-distilled in glass. All other chemicals used were reagent grade. All HPLC solvents were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under reduced pressure prior to use.

Chromatographic system and conditions

A Spectra-Physics liquid chromatograph, Model 3500B (Spectra-Physics, Santa Clara, CA, U.S.A.) was equipped with a Kratos variable-wavelength ultraviolet spectrophotometric detector, Spectroflow 773 (Kratos, Westwood, NJ, U.S.A.) set at 242 nm. The system included a Zorbax C_8 reversed-phase analytical column (25 cm × 4.6 mm I.D., 5-μm particle size) from Dupont. A 7 cm × 2 mm I.D. guard column packed with Co:Pell ODS (30—38 μm) particles (Whatman, Clifton, NJ, U.S.A.) preceded the analytical column. The mobile phase consisted of 40% (v/v) acetonitrile and 0.1% glacial acetic acid in water. The flow-rate was maintained at 1.2 ml/min. The pressure was 110.3 bars and the absorbance units full scale (a.u.f.s.) varied between 0.005 and 0.08.

Procedures

To 0.5 ml of plasma or urine sample or standard was added 0.25 μg testosterone (25 μl of 10 μg/ml in methanol) to serve as internal standard. To each sample were added 4.0 ml of methylene chloride. The samples were mixed for 15 min using an automatic shaking apparatus (Eberbach, Ann Arbor, MI, U.S.A.). They were then centrifuged at 2000 *g* for 5 min. The top (aqueous) layer was removed and discarded, and the bottom (organic) layer was transferred to a clean vial using a pasteur pipet. The methylene chloride layer was then evaporated to dryness using a gentle air stream at 40°C. The residue was reconstituted with 150--300 μl (plasma samples) or 300—500 μl (urine samples) mobile phase and mixed by vortexing. A 100-μl aliquot was injected onto the HPLC system.

Plasma and urine standards were prepared by adding known amounts of testolactone and 4,5-dihydrotestolactone stock solutions (10 or 100 μg/ml in methanol) to human plasma and urine to yield final concentrations ranging from 20 ng/ml to 10 μg/ml. Testolactone and 4,5-dihydrotestolactone were quantitated by comparison of the peak height ratio of drug or metabolite to internal standard using a calibration curve. The peak height ratios were plotted against concentrations of testolactone or 4,5-dihydrotestolactone and analyzed by weighted least squares linear regression [8] to generate daily calibration curves. Weighted regression analysis was necessary to accurately quantitate concentrations of samples which varied over the two orders of magnitude represented by the standard curve. A weighting factor equal to the square of the reciprocal measured concentration was used. Interday variability was determined by the reproducibility of the daily standard curves ($n \geqslant 5$) with respect to both their slopes and the calculated values for testolactone and its metabolite at six different concentrations ranging between 0.019 and 2.9 μg/ml. Intraday variability was assessed from the results of replicate analyses ($n \geqslant 5$) of plasma standards containing 0.025, 0.25 and 2.5 μg/ml of both drug and metabolite. These concentrations were representative of the low, medium

and high range of concentrations used in our standard calibration curves. Aqueous solutions containing known amounts of testolactone, 4,5-dihydrotestolactone, and testosterone were compared with the plasma or urine standards undergoing analysis to calculate the percent recovery at each of the six concentrations of the standard curve. Statistical analyses to determine means, standard deviations, standard errors, and coefficients of variation were performed using the programs available for the Hewlett-Packard 85 computer (Hewlett-Packard, Corvallis, OR, U.S.A.)

RESULTS AND DISCUSSION

HPLC procedure for plasma

Representative chromatograms from assayed samples of control human plasma, a prepared plasma standard, and plasma from a male patient treated with testolactone to which testosterone has been added are shown in Fig. 2. Retention times for testolactone, 4,5-dihydrotestolactone, and testosterone are 9.2, 11.6, and 13.6 min, respectively. No interfering peaks are observed in plasma from untreated patients. There is no interference from endogenous testosterone since, even in male plasma, endogenous testosterone levels (3—12 ng/ml) are only about 1—2% of the exogenously added internal standard testosterone (500 ng/ml).

The calibration curves constructed from daily runs of plasma standards were linear and highly reproducible. Plasma samples up to 10 μg/ml have been assayed using this procedure, and their results indicate that the linearity of response extends at least to this concentration. The mean slope (peak height ratio versus concentration in μg/ml) of six calibration curves for testolactone obtained during a 1-month period was 2.161 ± 0.046 (S.D.), C.V. = 2.1%. For 4,5-dihydrotestolactone, the mean slope was 0.742 ± 0.022 (S.D.), C.V. = 3.0%. The mean coefficient of variation of the procedure for the range of concentrations included in the standard curves is 3.5% for testolactone and 7.1% for 4,5-dihydrotestolactone (Table I). These data indicate the assay procedure is highly reliable and reproducible.

The intraday variability of the method was determined at three different concentrations spanning the range of the standard curve. At 0.025 μg/ml (n = 5), 0.25 μg/ml (n = 6), and 2.5 μg/ml (n = 6) of testolactone, the coefficients of variation of testolactone content were found to be 10.7%, 5.2%, and 3.5%, respectively. For 4,5-dihydrotestolactone content, the coefficients of variation at the low, medium, and high concentrations listed above were, respectively, 6.6% (n = 5), 1.0% (n = 6), and 3.8% (n = 6).

The recoveries of testolactone and 4,5-dihydrotestolactone at all six concentrations used in standard curve calibration averaged 95.0 ± 6.5% (S.D.), C.V. = 6.8% and 81.8 ± 1.5% (S.D.), C.V. = 1.8%, respectively. The recovery of the internal standard, testosterone, was 91.5 ± 2.9% (S.D.), C.V. = 3.2%.

Using the procedure described, 20 ng/ml testolactone and 20 ng/ml 4,5-dihydrotestolactone can easily be detected while maintaining a signal-to-noise ratio of at least 3 (a.u.f.s. = 0.005).

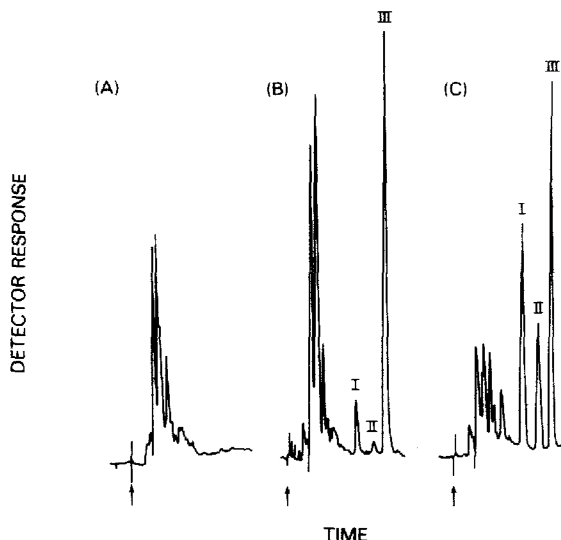

Fig. 2. Representative chromatograms of human plasma samples following sample preparation described in text. (A) Blank human plasma. (B) Human plasma to which was added testolactone (I) and 4,5-dihydrotestolactone (II), 0.05 μg/ml of each, and testosterone (III). (C) Plasma sample from male patient treated with 2 g/day of testolactone with testosterone added (testolactone and metabolite concentrations found to be 0.25 and 0.41 μg/ml, respectively). a.u.f.s. = 0.01.

TABLE I

INTERDAY VARIABILITY OF ASSAY PROCEDURE

Concentration (μg/ml)	*n*	Mean observed concentration (μg/ml)	S.D.	C.V. (%)
Testolactone				
0.019	5	0.019	0.0005	2.4
0.048	5	0.049	0.003	6.1
0.144	6	0.145	0.007	5.1
0.393	6	0.370	0.011	3.0
1.206	6	1.193	0.035	2.9
2.929	6	3.107	0.049	1.6
			Mean	3.5
4,5-Dihydrotestolactone				
0.019	6	0.018	0.002	11.2
0.046	6	0.055	0.009	15.8
0.147	6	0.142	0.010	7.4
0.416	6	0.390	0.008	2.0
1.249	6	1.241	0.044	3.5
2.868	6	3.059	0.092	3.0
			Mean	7.1

HPLC procedure for urine

The assay method described can also be used for the quantitation of testolactone and 4,5-dihydrotestolactone in urine. No interfering peaks were observed in urine samples from untreated patients, and the sensitivity, recovery, and reproducibility are similar for both plasma and urine. In general, higher concentrations of both testolactone and metabolite are found in patient urine than in plasma.

To facilitate quantitation of these higher urine concentrations, it is sometimes helpful to double the amount of internal standard added per sample (0.05 μg testosterone) and increase the reconstitution volume of the extracted urine samples (300—500 μl). With the exception of these minor changes, the sample preparation of urine and plasma samples is identical.

Analysis of plasma from testolactone-treated patients

Sequential blood samples were obtained from male patients receiving 500 mg testolactone or a placebo four times daily for at least 3 months. Samples were drawn at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 9.0, and 12.0 h after a morning dose of testolactone. The next dose, normally administered 6 h after the morning dose, was not given. An aliquot of plasma from each sample was analyzed for both testolactone and 4,5-dihydrotestolactone as described. A concentration versus time profile of the results obtained from one patient is shown in Fig. 3. The sensitivity of the assay procedure described was adequate for the quantitation of both testolactone and its metabolite. In no patient sample did drug or metabolite levels fall below 20 ng/ml during the 12-h period monitored, and no detectable levels of testolactone or its metabolite were observed in plasma samples from patients who received placebo.

The assay procedure described for testolactone and 4,5-dihydrotestolactone offers easy and rapid sample preparation, selectivity for both drug and metabolite, and the sensitivity required for pharmacokinetic studies of both agents in humans.

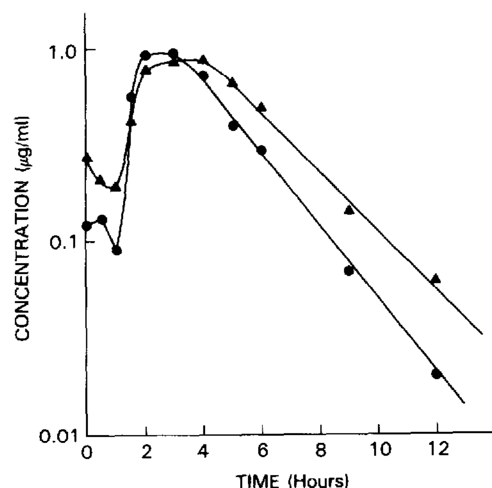

Fig. 3. Plasma concentration versus time profile of testolactone (●) and 4,5-dihydrotestolactone (▲) in a male patient as quantitated by the assay procedure described.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Alice Rogot for technical assistance and Mrs. Audrey Pellegrino for preparation of the manuscript.

REFERENCES

1 A. Segaloff, K.K. Meyer, J.B. Weeth, E.L. Rongone and M. Cunningham, Cander, 15 (1962) 987–991.
2 R.M. Barone, I.M. Shamonki, P.K. Sitteri and H.L. Judd, J. Clin. Endocrinol. Metab., 49 (1979) 672–676.
3 S.P. Marynick, D.L. Loriaux, R.J. Sherins, J.C. Pita and M.B. Lipsett, J. Clin. Endocrinol. Metab., 49 (1979) 396–398.
4 S.J. Winters, R.J. Sherins and D.L. Loriaux, J. Clin. Endocrinol. Metab., 48 (1979) 553–558.
5 R.A. Vigersky and A.R. Glass, J. Clin. Endocrinol. Metab., 52 (1980) 897–902.
6 R.V. Clark and R.J. Sherins, Clinical Trial of Testolactone for Treatment of Idiopathic Male Infertility, Abstract presented at the 8th Annu. Meet. Am. Soc. Andrology, Philadelphia, PA, March 22, 1983.
7 R.L. Yeager, V.L. Pascucci, B.A. Mico, R.J. Sherins and D.C. Chatterji, Drug Metab. Dispos., submitted for publication.
8 A.I. Nichols and C.C. Peck, General Weighted Least Squares Nonlinear Regression Program, Version 1, Users Manual, Uniformed Services University for The Health Sciences Technical Report No. 50, Bethesda, MD, May 1981.