Journal of Chromatography, 338 (1985) 281—288
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2444

QUANTITATIVE DETERMINATION OF METHYLTESTOSTERONE AND METHYLTESTOSTERONE-d_3 IN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

Y. SHINOHARA*, S. BABA and Y. KASUYA

Tokyo College of Pharmacy, 1432-1, Horinouchi, Hachioji, Tokyo 192-03 (Japan)

(First received August 8th, 1984; revised manuscript received October 5th, 1984)

SUMMARY

A method for the quantitative estimation of methyltestosterone and methyltestosterone-d_3 in biological fluids has been developed using gas chromatography—mass spectrometry—selected-ion monitoring. Methyltestosterone-d_6 was used as an internal standard. Methyltestosterone and methyltestosterone-d_3 in serum were determined based on the peak height ratios of the molecular ions of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6. Sensitivity, specificity, precision, accuracy and reproducibility of the present method were demonstrated to be satisfactory for application to pharmacokinetic and bioavailability studies.

INTRODUCTION

Methyltestosterone (17β-hydroxy-17α-methyl-androst-4-en-3-one) is an orally effective synthetic androgen which has been used in the treatment of eunuchism, eunuchoidism, male impotence and female breast cancer [1—3].

The bioavailability/bioequivalency regulations of the United States Food and Drug Administration (FDA) which became effective on 7 July, 1977, listed 110 drugs and drug dosage forms which were known or suspected of having potential bioavailability/bioequivalency problems [4]. Although methyltestosterone is one of the drugs listed, there appears to be little information on its pharmacokinetic or bioavailability characteristics. Bioavailability and pharmacokinetic studies of methyltestosterone require sensitive, specific and reproducible analytical techniques. Alkalay and co-workers [5, 6] have employed spectrofluorometric determination to assess the bioavailability of methyltestosterone. The method, however, does not provide specificity and sufficient reproducibility.

0378-4347/85/$03.30

The use of gas chromatography—mass spectrometry (GC—MS) and stable-isotope-labelled drugs as diluents has found broad application in pharmacological studies [7—9]. In this technique, stable-isotope-labelled carriers serve as the ideal internal standard to correct for losses of a subtance under study in the initial isolation procedures. Pharmacokinetic and bioavailability studies represent one field in which the sensitivity and specificity of GC—MS offer an advantage. Recently, studies on the relative bioavailability of several different formulations of the same drug have been performed effectively by use of a stable-isotope-labelled variant as the reference with which the test unlabelled formulations are compared [10—12].

We have initiated studies to assess the relative bioavailability of methyltestosterone tablet formulations with coadministration of a stable-isotope-labelled methyltestosterone solution as internal biological standard. The present paper describes an analytical method for the simultaneous quantitative estimation of methyltestosterone and its 17α-methyl-d_3 analogue (methyltestosterone-d_3) in human serum. This method involves GC—MS with selected-ion monitoring (SIM) using methyltestosterone-d_6 as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

17α-Methyl-d_3-testosterone (methyltestosterone-d_3) and 17α-methyl-d_3-testosterone-19,19,19-d_3 (methyltestosterone-d_6) were synthesized in our laboratory as described elsewhere [13]. The isotopic compositions were 99.6 atom% deuterium (d_3, 98.64%; d_2, 1.36%; d_1, 0.00%) for methyltestosterone-d_3 and 99.3 atom% deuterium (d_6, 96.88%; d_5, 2.26%; d_4, 0.86%) for methyltestosterone-d_6. Non-labelled methyltestosterone was purchased from Tokyo Kasei Kogyo, Tokyo, Japan (reagent grade) and was recrystallized from *n*-hexane—ethyl acetate (5:1). All other chemicals and solvents were analytical grade and used without further purification.

Stock solutions

Stock solutions of methyltestosterone (2.494 mg per 250 ml), methyltestosterone-d_3 (2.509 mg per 250 ml) and methyltestosterone-d_6 (2.568 mg per 250 ml) were prepared in ethanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than six months. All analyses were performed by diluting the stock solutions with ethanol.

Gas chromatography—mass spectrometry—selected-ion monitoring

GC—MS—SIM measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with a Shimadzu high-speed multiple-ion detector—peak matcher 9060S. GC was performed on a glass column (1 m × 3 mm I.D.) packed with 1.5% SE-30 on Chromosorb W (80—100 mesh). The column temperature was 230°C and the temperature of both the injector and separator was 250°C. The temperature of the ion source was 270°C. Helium was used as the carrier gas at a flow-rate of about 25 ml/min. The electron energy was set at 20 eV and the trap current at 60 μA. The multiple-ion detector was focused on the ions at *m/z* 302, 305 and 308.

The recording was made on a Nippon Denshi Kagaku four-pen recorder U-626D5, the chart speed being 10 mm/min.

Sample preparation for GC—MS—SIM

Frozen serum samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100 × 16 mm) were added 1.0 ml of serum and 50 ng of methyltestosterone-d_6 dissolved in 20 μl of ethanol. The serum sample was allowed to stand for 30 min at room temperature. After adding 40 μl of 3 *M* sodium hydroxide solution, the serum sample was extracted with *n*-hexane (3 × 3 ml) by vortex-mixing for 10 sec. After centrifugation for 5 min at 1000 *g*, the organic layer was pipetted into a PTFE-lined screw-cap culture tube (100 × 16 mm) and washed with 1 ml of 5% acetic acid by vortex-mixing for 10 sec. After centrifugation for 5 min at 1000 *g*, the organic layer was pipetted into a PTFE-lined screw-cap conical centrifuge tube (100 × 16 mm). The solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 20 μl of ethanol by ultrasonication for 5 min. After centrifugation (1000 *g*, 1 min), a 2—4 μl aliquot of the solution was subjected to GC—MS.

Absolute recovery

To 1.0 ml of human blank serum were added 50 ng of methyltestosterone in 20 μl of ethanol. After the serum sample was allowed to stand for 30 min at room temperature, the same procedures of extraction, washing and evaporation as described under sample preparation for GC—MS—SIM were followed. To the residue thus obtained were added 50 ng of methyltestosterone-d_6 dissolved in 20 μl of ethanol. After ultrasonication and centrifugation, a 2—4 μl aliquot of the solution was subjected to GC—MS. The absolute recovery of methyltestosterone from human serum was estimated by comparing the peak heights of *m/z* 302 and *m/z* 308.

Calibration curves and quantitation

Calibration curves for methyltestosterone or methyltestosterone-d_3 were prepared by adding 0, 1, 2, 5, 20, 50 and 200 ng of methyltestosterone or methyltestosterone-d_3 to 1.0-ml portions of human blank serum. Each sample was prepared in triplicate. The samples were then carried through the entire procedure and the peak height ratios of *m/z* 302 versus *m/z* 308 (d_0/d_6) and *m/z* 305 versus *m/z* 308 (d_3/d_6) were determined in triplicate. The curves were obtained by an unweighted least-squares linear fitting of the peak height ratios versus the mixed molar ratios on each analysis of unknown samples. Serum concentrations were calculated by comparing the peak height ratios obtained from the unknown samples with those obtained from the standard mixtures.

Drug administration

Eight healthy adult male volunteers, ranging in age from 21 to 34 years and in weight from 61.2 to 88.8 kg, participated in the study. The bioavailability tests began at 6.30 a.m. After an overnight fast, they were administered orally either a 10-mg methyltestosterone tablet and a 10-mg methyltestosterone-d_3 solution (100 ml) or a 10-mg methyltestosterone solution (100 ml) and a 10-mg methyltestosterone-d_3 solution (100 ml). The tablet was administered

with 120 ml of water. The solution was followed by a 20-ml water rinse of the container. No food was permitted for 4 h after drug administration. Blood (17 ml) was drawn just before the oral dose and at 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h after dosing. The samples were allowed to clot and were then centrifuged to separate the serum. The serum samples were stored at −20°C until the time of assay.

On a separate occasion of one week after completion of the first study, the drug administration was repeated with the same subjects receiving the reverse treatment.

RESULTS AND DISCUSSION

The electron-impact mass spectra of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6 demonstrated that the respective relative

TABLE I

PERCENT CONTRIBUTION TO THE ION INTENSITIES OF VARIOUS SPECIES IN CHANNELS MONITORED

Compound	*m/z* 302	*m/z* 305	*m/z* 308
Methyltestosterone	100	0.51	0.29
Methyltestosterone-d_3	0.47	100	0.55
Methyltestosterone-d_6	0.37	0.51	100

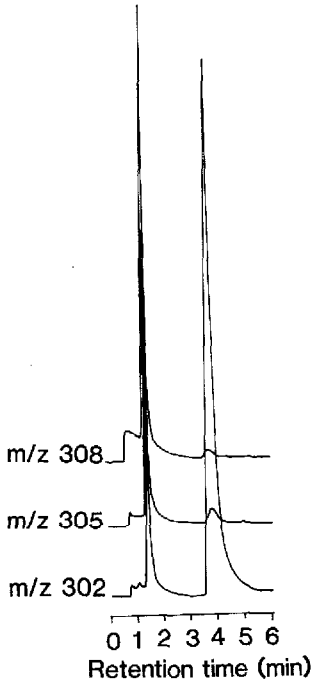

Fig. 1. Selected-ion monitoring of extracts from human serum sample spiked with methyl testosterone, methyltestosterone-d_3 and methyltestosterone-d_6.

abundances of the molecular ions at *m/z* 302, 305 and 308 were prominent. When the molecular ions were monitored, the sensitivity limit of the GC—MS—SIM assay was found to be 50 pg. A signal-to-noise ratio of $\geqslant 2.5$ was used as a criterion for a significant response for the injected methyltestosterone.

Because of the natural abundance of ^{2}H, ^{13}C and ^{18}O, a small peak at *m/z* 305 may appear in the mass spectrum of methyltestosterone. In addition, there is also the possibility that methyltestosterone-d_3 could contribute to the *m/z* 302 and methyltestosterone-d_6 to the *m/z* 302 and/or 305. Precise analysis of the GC—MS—SIM data from pure samples of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6 summarized in Table I indicated that no corrections for overlapping ions among the various isotopic compounds in question were necessary. The labelled compounds possessed sufficiently high isotopic purity and the contributions to the other ions were minor. Since the amount of methyltestosterone-d_6 in the analytical sample was 10- to 100-fold larger than that of methyltestosterone or methyltestosterone-d_3, the contribution of methyltestosterone-d_6 to the *m/z* 302 and 305 might be significant. However, this could be neglected because of the use of a constant amount of methyltestosterone-d_6 throughout all the assay samples.

In the GC—MS—SIM method used in the present study, an appropriate organic extraction solvent had to be chosen to enhance the sensitivity and to avoid the possibility of interferences. The extraction efficiency of methyltestosterone from serum samples was tested with *n*-hexane, diethyl ether, choroform and ethyl acetate. In spite of the high extraction efficiency obtained with diethyl ether, chloroform and ethyl acetate, the presence of numerous interfering peaks limited the use of these solvents. Fig. 1 shows the selected-ion monitoring after processing from a serum sample spiked with methyltestosterone (30 ng), methyltestosterone-d_3 (30 ng) and methyltestosterone-d_6 (50 ng) using *n*-hexane as extraction solvent. The presence of a large peak at 3.5—4.5 min, which corresponded to cholesterol, did not interfere with the analytes. The retention times of the analytes were the same (1.4 min). The total absolute recovery of methyltestosterone extracted from human serum with *n*-

TABLE II

ACCURACY OF SELECTED-ION MONITORING OF METHYLTESTOSTERONE IN SERUM

Added (ng/ml)	Found (ng/ml)					C.V. (%)	Relative error (%)
	Individual values*				Mean ± S.D.		
1.00	0.97	0.96	1.01	0.68	0.91 ± 0.15	16.5	—9.0
2.00	2.10	1.74	1.71	1.98	1.88 ± 0.19	10.0	—6.0
4.99	5.03	5.07	5.42	5.19	5.18 ± 0.18	3.4	3.8
19.95	20.61	19.51	19.79	19.76	19.92 ± 0.48	2.4	—0.2
49.87	50.87	51.60	50.26	49.45	50.55 ± 0.91	1.8	1.4
199.48	203.43	199.19	194.01	200.80	199.34 ± 3.97	2.0	—0.1

*Each individual value represents the mean of triplicate measurements.

TABLE III

ACCURACY OF SELECTED-ION MONITORING OF METHYLTESTOSTERONE-d_3 IN SERUM

Added (ng/ml)	Found (ng/ml)					C.V. (%)	Relative error (%)
	Individual values*				Mean ± S.D.		
1.00	1.10	1.09	0.79	1.07	1.01 ± 0.15	14.7	1.0
2.01	2.08	2.11	2.07	2.07	2.08 ± 0.02	0.9	3.6
5.02	4.83	4.87	4.84	4.89	4.86 ± 0.03	0.6	−3.2
20.07	19.96	20.55	20.53	19.36	20.10 ± 0.56	2.8	0.1
50.18	51.22	51.70	51.66	48.60	50.80 ± 1.48	2.9	1.2
200.72	207.39	203.54	195.43	195.81	200.54 ± 5.90	2.9	−0.1

*Each individual value represents the mean of triplicate measurements.

TABLE IV

DAY-TO-DAY PRECISION

n = 8 for each concentration.

Concentration (ng/ml)	Found (ng/ml)	C.V. (%)	Relative error (%)
Methyltestosterone			
4.99	4.98 ± 0.20	4.0	−0.2
19.95	20.14 ± 0.60	3.0	1.0
49.87	49.75 ± 0.38	0.8	−0.2
Methyltestosterone-d_3			
5.02	4.90 ± 0.09	1.8	−2.4
20.07	20.12 ± 0.33	1.6	0.2
50.18	50.24 ± 0.18	0.4	0.1

hexane was satisfactory, being about 70.4 ± 6.7% (n = 6). The present extraction procedures enabled sensitive quantitative analysis with a detection limit of 500 pg of methyltestosterone or methyltestosterone-d_3 per ml of serum.

Calibration curves were prepared by spiking 1.0 ml of blank human serum with various amounts (1—200 ng) of methyltestosterone or methyltestosterone-d_3 and a constant amount (50 ng) of methyltestosterone-d_6. Each sample was then analysed by monitoring the ions at m/z 302, 305 and 308. When the peak height ratios were plotted against the mixed molar ratios, a good correlation was found between the observed peak height ratios and the mixed molar ratios. Unweighted least-squares regression analysis gave a regression line of y = 1.0129x + 0.0042 (r = 1.0000) for methyltestosterone and y = 1.0317 x — 0.0023 (r = 1.0000) for methyltestosterone-d_3.

The accuracy of measurement was determined for methyltestosterone or methyltestosterone-d_3 added to 1.0-ml aliquots of blank human serum. The serum samples spiked with 1—200 ng of methyltestosterone or methyltestosterone-d_3 were analysed by the present method. The results presented in Tables II and III show that the estimated amounts of methyltestosterone or

methyltestosterone-d_3 were in good agreement with the actual amounts added.

Day-to-day precision of the assay was determined for a period of eight working days by performing triplicate analyses on serum samples containing 5, 20 and 50 ng/ml methyltestosterone or methyltestosterone-d_3. The results listed in Table IV demonstrate the excellent reproducibility.

The present method was applied for the quantitation of serum concentrations of methyltestosterone and methyltestosterone-d_3 after oral administration of a 10 mg + 10 mg mixture of methyltestosterone and methyltestosterone-d_3 solution to a healthy subject. There was no interference from metabolites of methyltestosterone and methyltestosterone-d_3 in the vicinity of the peaks of analytes in the mass fragmentograms. Serum concentrations of methyltestosterone and methyltestosterone-d_3 could be followed up to 8 h; the serum concentration—time curve is shown in Fig. 2. A bioavailability study of methyltestosterone is now in progress and will be described in detail elsewhere.

The present method provided a sensitive, simple and reliable technique for determining the serum levels of methyltestosterone and methyltestosterone-d_3 with good accuracy and precision. The method was confirmed to be applicable for assessing the relative bioavailability of methyltestosterone tablet formulations.

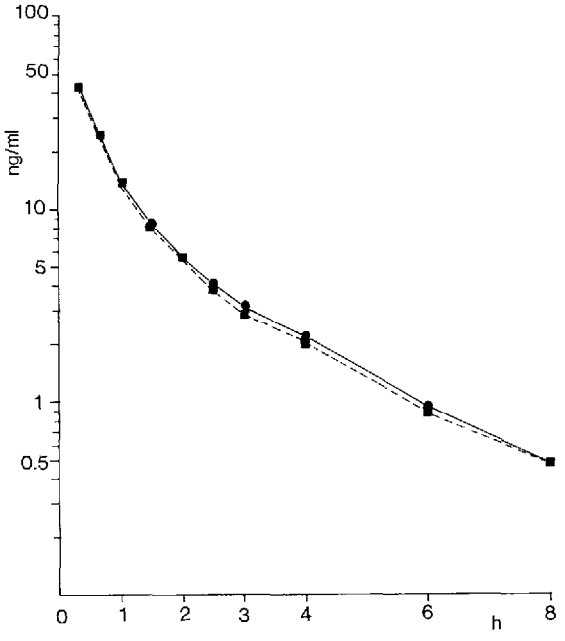

Fig. 2. Serum concentration—time curve for methyltestosterone (•) and methyltestosterone-d_3 (▪) after a single oral dose of a 10 mg + 10 mg mixture of methyltestosterone and methyltestosterone-d_3 solution to a healthy male volunteer.

REFERENCES

1 L. Ruzicka, M.W. Goldberg and H.R. Rosenberg, Helv. Chim. Acta, 18 (1935) 1487.
2 C.W. Emmens and A.S. Parkes, J. Endocrinol., 1 (1939) 323.
3 G.L. Foss, Brit. Med. J., ii (1939) 11.

4 Fed. Regist., 42 (1977) 1624.
5 D. Alkalay, L. Khemani and M.F. Bartlett, J. Pharm. Sci., 61 (1972) 1746.
6 D. Alkalay, L. Khemani, W.E. Wagner, Jr. and M.F. Bartlett, J. Clin. Pharmacol., 13 (1973) 142.
7 T.A. Baillie, Pharmacol. Rev., 33 (1981) 81.
8 W.A. Garland and M.L. Powell, J. Chromatogr. Sci., 19 (1981) 392.
9 M. Eichelbaum, G.E. von Unruh and A. Somogyi, Clin. Pharmacokinet., 7 (1982) 490.
10 H.A. Heck, S.E. Buttrill, Jr., N.W. Flynn, R.L. Dyer, M. Anbar, T. Cairns, S. Dighe and B.E. Cabana, J. Pharmacokinet. Biopharm., 7 (1979) 233.
11 D. Alkalay, W.E. Wagner, Jr., S. Carlsen, L. Khemani, J. Volk, M.F. Bartlett and A. LeSher, Clin. Pharmacol. Ther., 27 (1980) 697.
12 M. Eichelbaum, H.J. Dengler, A. Somogyi and G.E. von Unruh, Eur. J. Clin. Pharmacol., 19 (1981) 127.
13 Y. Shinohara, S. Baba and Y. Kasuya, International Symposium on Mass Spectrometry in the Health and Life Sciences, San Francisco, CA, September 9—13, 1984, p. 50 (Abstract).