Combined Assay, Identification, and Foreign Related Steroids Test for Methandrostenolone by High-Speed Liquid Chromatography

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Abstract □ A high-speed liquid chromatographic system is described, which can be used for the simultaneous identification of the anabolic steroid methandrostenolone and its impurities and the quantitation of each of these compounds. Separation is effected by adsorption chromatography on a slurry-packed microparticulate silica gel column.

Keyphrases □ Methandrostenolone and related steroid impurities—simultaneous identification and quantitation by high-speed liquid chromatography □ High-speed liquid chromatography—simultaneous identification and quantitation of methandrostenolone and related steroid impurities

The synthetic steroid methandrostenolone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one) (I) is used as an anabolic agent, both medically and nonmedically (1, 2). It may be synthesized from methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) (II) either microbiologically (3) or chemically (4). Steroidal impurities which may be present (5) are II, 6α,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one (III), and 6β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one (IV). Of these, II has about twice the oral androgenic potency and about one-half of the anabolic activity of methandrostenolone (6). Therefore, its presence in any significant amount is undesirable and limited (5). The epimeric pair of 6-hydroxylated impurities would be expected to be less active than methandrostenolone, since, in general, substitution at the 6-position lowers androgenic activity (7). Nevertheless, the presence of such compounds as foreign related steroids should be controlled as much as possible within the limits of good manufacturing practice.

Present compendial assays of methandrostenolone (5, 8) are interfered with by the presence of impurities and are, therefore, less accurate then they might be. The BP (5) uses a simple UV measurement for the raw material and a condensation with dinitrophenylhydrazine, followed by colorimetric assay for the tablets, together with a TLC test for the foreign related steroids. The limits for the impurities are 0.5% for II and 2% each for III and IV. The NF (8) uses a straightforward UV measurement for both raw material and tablets and is only concerned with the presence of II as a foreign related steroid, setting a limit of 0.5% by TLC.

High-speed liquid chromatography (HSLC) is enjoying increasing usage for the analysis of steroids (9). The work described in this study is an assay method for I, together with a method for estimating the amount of each impurity. Since it is a chromatographic procedure, it is also an identification test. The method may be used for both composite and single-tablet analysis. It is fast, accurate, precise, and specific for each compound described.

EXPERIMENTAL

Reagents—Compound II[1], Compound III[2], Compound IV[2], *m*-dinitrobenzene[3], and all solvents were used as received. Compound I[4] was recrystallized from acetone and water and dried *in vacuo* before use.

HSLC Procedure—A high-speed liquid chromatograph[5] equipped with a fixed wavelength (254 nm) UV detector [attenuated 0.02 absorbance unit full-scale (aufs)], a septumless injector, and an electronic integrator[6] were used.

The silica gel[7] (4–9 μm) column (25 cm × 2.1 mm i.d. × 3.2 mm o.d.; 304 stainless steel) was prepared using a balanced-density slurry packing procedure similar to that described by Majors (10). A mobile phase of 3% (v/v) ethylene chloride and 15% (v/v) 2-propanol in *n*-hexane was used at a flow rate of 60 ml/hr (2600 psi) at ambient temperature.

Preparation of Standard Solutions—*II Standard*—Compound II, 12.5 mg, was accurately weighed into a 10-ml volumetric flask and dissolved in and diluted to volume with chloroform. Then 1.0 ml of this solution was pipetted into a 100-ml volumetric flask and diluted to volume with chloroform (Solution A, 12.5 μg/ml).

III and IV Standards—Approximately 15.6 mg each of III and IV was accurately weighed into separate 25-ml volumetric flasks and dissolved in and diluted to volume with chloroform. Then 1 ml of each solution was pipetted into separate 10-ml volumetric flasks and diluted to volume with chloroform (Solutions B and C, 62.5 μg/ml).

m-Dinitrobenzene (Internal Standard)—Approximately 9.5 mg of *m*-dinitrobenzene was accurately weighed into a 25-ml volumetric flask and dissolved in and diluted to volume with chloroform (Solution D, 380 μg/ml). Then 20 ml of Solution D was pipetted into a 100-ml volumetric flask and diluted to volume with chloroform (Solution E, 76 μg/ml).

Methandrostenolone Working Standard—Approximately 12.5 mg of recrystallized I was accurately weighed into a 5-ml volumetric flask. Aliquots (1 ml) of each of Solutions A, B, C, and D were pipetted into this flask, which was agitated gently to dissolve the

[1] National Formulary reference standard.
[2] British Pharmacopoeia, authentic substance.
[3] Fisher Scientific, Montreal, Quebec, Canada.
[4] Ciba/Geigy, Montreal, Quebec, Canada.
[5] Varian Aerograph, model 4100, Walnut Creek, Calif.
[6] Vidar Autolab, model 6300, Mountain View, Calif.
[7] LiChrosorb SI 60, Brinkmann Instruments (Canada) Ltd.; manufactured by E. Merck, Darmstadt, Germany.

Table I—Assay of Methandrostenolone[a] in Synthetic Mixtures

Sample	Amount, μg/ Injection	Recovery, %
1	1.240	99.6
2	2.480	99.8
3	2.610	101.9
4	2.750	99.6
	Mean recovery, %	= 100.2
	Coefficient of variation, %	= 1.1

[a] Containing 0.7% of III and 1.0% of IV.

methandrostenolone and diluted to volume with chloroform. This solution now contained I (2.5 mg/ml), II (2.5 μg/ml), III and IV (12.5 μg/ml), and *m*-dinitrobenzene (76 μg/ml) and corresponded to a solution of I containing 0.5% III and IV and 0.1% II.

Analysis of Methandrostenolone Tablets—*Tablet Extraction*—Single (5-mg) tablets were placed in 15 × 75-mm screw-capped test tubes and crushed with a glass rod to a fine powder. Two milliliters of Solution E was pipetted into each tube, which was closed and placed on a rotator[8] at 60 rpm for 20 min. The tube was centrifuged[9] at 3000 rpm for 5 min.

HSLC—Duplicate 1-μl aliquots[10] of the working standard solution of I were chromatographed, and the peaks corresponding to I and the internal standard were integrated. Duplicate 1-μl aliquots of each tablet extract were chromatographed, again integrating the peaks due to internal standard and I in each sample. The percent label claim of I was calculated from:

$$\% \text{ I} = \frac{N_M N_{\text{I.S.}}\ (\text{sample})}{N_M N_{\text{I.S.}}\ (\text{standard})} \times 100 \qquad \text{(Eq. 1)}$$

where N_M = integrator counts for peak from I, and $N_{\text{I.S.}}$ = integrator counts for internal standard peak.

Limit Test for II, III, and IV in Methandrostenolone Tablets—Duplicate (5-μl) aliquots[11] of the working standard solution of I were chromatographed, integrating the internal standard peak. Duplicate (5-μl) aliquots of the tablet extracts used for the assay were also chromatographed, integrating the internal standard peak. The ratio of impurities in the sample and standard was calculated from:

$$R_x = \frac{N_{\text{I.S.}} H_x\ (\text{sample})}{N_{\text{I.S.}} H_x\ (\text{standard})} \qquad \text{(Eq. 2)}$$

where R_x = ratio of compound, x, in the sample to that in the standard; $N_{\text{I.S.}}$ = integrator counts for the internal standard peak; and H_x = peak height for Compound x, where x can be II, III, or IV.

If R_x is greater than 1.0 for Compound x, the sample contains more than 0.1% (II) or 0.5% (III and IV) of that impurity.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of a synthetic mixture of I, II, III, IV, and the internal standard, *m*-dinitrobenzene. The impurities represent 1.0, 0.7, and 1.0%, respectively.

Figure 2 shows a plot of integrator count ratio for various amounts of I and a constant amount of internal standard (76 ng/injection) *versus* the weight of I chromatographed; the response was linear to more than 3 μg of I. Subsequent work was carried out with a nominal level of 2.5 μg of I/injection.

Response curves for II, III, and IV were prepared by calculating the ratios of the peak heights to internal standard counts and plotting these values against the amount of compound injected. Compound II was studied over the 1.25–12.5-ng/μl range, while III and IV were tested over the 2.5–50-ng/μl range; 5-μl injections were used in all cases. Relative to a 1-μl injection of I at the level of 2.5 μg/μl, these ranges represent 0.05–0.5% II and 0.10–2.0% III and IV.

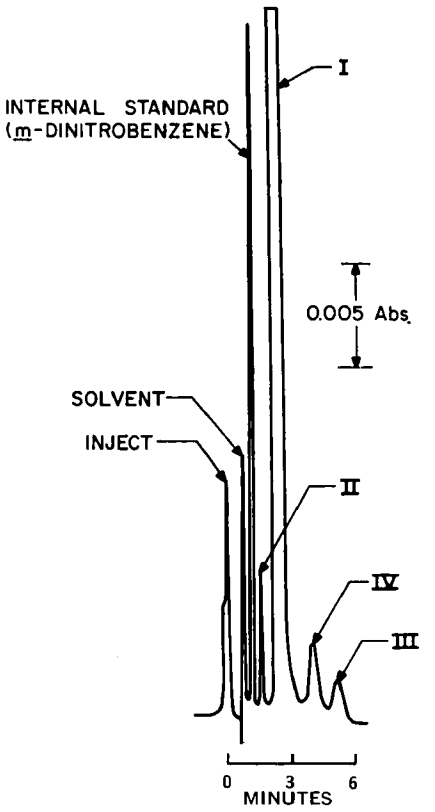

Figure 1—*High-speed liquid chromatogram of a synthetic mixture of methandrostenolone (I, 2.5 μg), methyltestosterone (II, 25 ng), 6α-hydroxymethandrostenolone (III, 17.5 ng), and 6β-hydroxymethandrostenolone (IV, 25 ng). Chromatographic conditions are given in the text.*

The calculated line of best fit in each case was a straight line through the origin ($y = mx$), with the relative standard deviations of the slopes being 20.3, 7.7, and 2.0% for II, III, and IV, respectively.

Methandrostenolone raw material and two separate reference standards (NF and BP) were chromatographed under the conditions described here and were shown to contain between 0.4 and 1.0% of each of the two impurities III and IV and negligible amounts of II. Recrystallization of the raw material from a water–acetone solution yielded I containing less than 0.03% of either impurity. This sample was used to prepare the working standard solution of I.

The chromatographic system was stable over the period required for the assay. Retention time variation on the peak from I was less than 2%. Compound I was quantitated by comparison of the ratios of integrator counts for peaks obtained for I and the internal standard when 1.0-μl aliquots of the tablet extract and working standard solution were chromatographed. Since the nominal concentration of these two compounds was the same in both solutions, direct comparison of peak height ratios multiplied by 100 gives the percent of label claim for I. Table I lists the assay results for synthetic solutions of I over a range of 1.2–2.75 μg/injection.

The limit test for the impurities in I could have been carried out with a single-injection technique, using the same chromatogram used for the assay. However, the results would have been less precise because of the small size of the impurity peaks when only 1.0 μl (2.5 μg) of solution was used (Fig. 1). For this reason, a 5-μl injection was used which gave peaks for the impurities that were large enough for quantitation but a nonlinear peak for I. Negligible interference of the parent peak with IV was observed under these conditions, and quantitation of the impurities was possible at levels as low as 0.05% for II and 0.1% for III and IV. Quantitation was effected by comparison of the ratios of impurity peak heights to in-

[8] Multi-Purpose Rotator, Scientific Industries, Springfield, Mass.
[9] HM-s centrifuge, International Equipment, Needham Heights, Mass.
[10] Hamilton 75N CH microliter syringe.
[11] Hamilton 701N CH microliter syringe.

Table II—Assay of Methandrostenolone and Impurities in Tablets

Lot		HSLC I, %	II, %	III, %	IV, %	Total, %	BP, %
A[a]	Mean =	100.0	0.06	0.35	0.76	101.2	98.8
	SD =	±2.6	±0.02	±0.04	±0.04	—	
A[b]		98.7	0.05	0.33	0.72	98.8	98.6
B[a]	Mean =	95.2	<0.05	0.51	0.67	96.4	97.7
	SD =	±3.7	—	±0.04	±0.04	—	
B[b]		96.6	<0.05	0.47	0.61	97.7	97.8

[a] Values are from analysis of 10 single tablets. [b] Values are from analysis of composite of 10 tablets, with both methods applied to same solution.

ternal standard counts from chromatograms of 5-μl aliquots of sample and working standard solutions. Peak height was used for the impurity peaks because the integrator was not always able to track peaks corresponding to small quantities of III and IV.

When six (5-μl) aliquots of the working standard solution of I were analyzed, coefficients of variation of 1.4, 21.4, 4.4, and 8.3% were obtained for I, II, IV, and III, respectively.

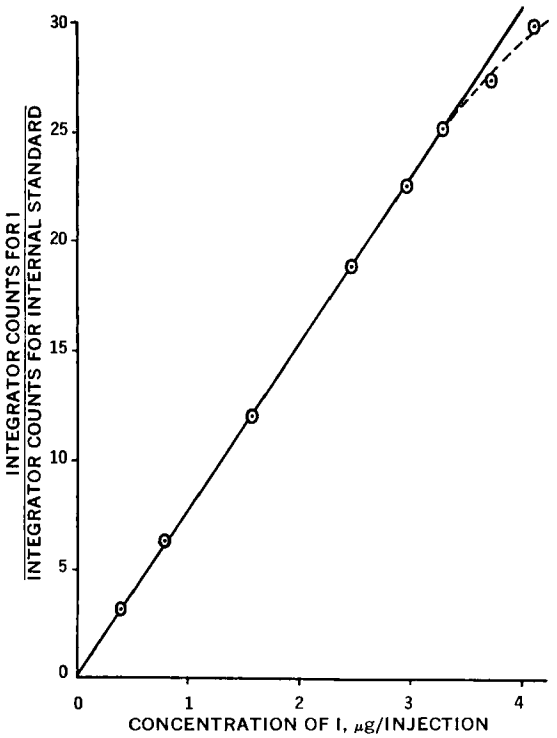

Figure 2—*Linearity curve for methandrostenolone from chromatograms of methandrostenolone standard solutions containing 76 ng/injection of* m-*dinitrobenzene as internal standard.*

Table II summarizes the results obtained when 10 single tablets (5 mg) of I, from each of two lots, were assayed by HSLC and by the BP method and when 10-tablet composites of each lot were assayed by both methods. The assay results are in excellent agreement when the total percent recovery by HSLC is compared to the BP method, especially in the case of the composite solutions assayed by both methods. The single-tablet assay results have different means, indicating a possible shortcoming in assaying only 10 tablets for content uniformity purposes. Alternatively, it may reflect real differences between two sets of tablets or differences in precision of the two methods at the single-tablet level. The coefficients of variation on the impurities were 33, 12, and 6% for II, III, and IV, respectively, at impurity levels of 0.05, 0.4, and 0.7%, respectively, and include about 3% tablet variation.

In conclusion, a precise, rapid, HSLC procedure was developed which allows either the assay of methandrostenolone and an estimate of its three impurities or a dual-injection assay of the parent compound and of these same impurities.

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