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Optimization and validation of conventional and micellar LC methods for the analysis of methyltestosterone in sugar-coated pills

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Abstract

Two isocratic liquid chromatographic methods (conventional and micellar) for the determination of methyltestosterone in sugar-coated pills using fluoxymesterone as internal standard have been developed and validated. In conventional liquid chromatography a mobile phase 45% water:acetonitrile 55% (v:v), a flow-rate 1 ml min^{-1} and a C_{18} Hypersil ODS (250 × 4.6 mm, 5 μm) column (25 °C) were used. In micellar liquid chromatography the conditions were: mobile phase 40 mM sodium dodecyl sulfate: 10% propanol, flow-rate 0.5 ml min^{-1} and C_{18} Hypersil ODS (150 × 3.0 mm, 5 μm) column (60 °C). For both methods, UV absorbance detection at 245 nm was used and a separation up to base line was achieved. Prior to HPLC analysis a simple sample preparation was required.
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Keywords: Micellar and conventional LC; Methyltestosterone; Sugar-coated pills; validation

1. Introduction

Anabolic-androgenic steroids (AAS) are compounds derived from testosterone (T), having anabolic (tissue building) and androgenic (masculinizing) properties. Administration of AAS may be by oral or parenteral injection. Because unmodified T degrades rapidly upon entering the bloodstream, T molecules must first be chemically modified to retard absorption or degradation. T can usually be modified in three different ways (i) alkylation in 17α position (the majority are orally active), (ii) modification of the ring structure of the steroid to retard hepatic metabolism (some preparations use both types of modifications) and (iii) esterification of the 17β hydroxyl group (parenteral use) [1–3]. Although only a limited number of anabolic preparations are available for the veterinary practitioner, a wide range is available for abuse in the field of human athletics and thoroughbred horseracing [4]. The majority have a 17α-alkyl functional group (e.g. 17α-methyltestosterone (MT) protecting to the C17 hydroxy function from inactivation by first pass metabolism.

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MT is indicated for androgen replacement in impotence or for male climacteric symptoms [5] and also produces a sensation of well-being in middle-aged persons returning to normality within their working sense and even recovering the genetic activity [6]. As the other steroids, the use of MT as additive in livestock breeding has been banned in all EEC countries. In addition, MT has a growth-promoting effect in various animal species including fish and has been widely used to induce sex reversal in fish thereby producing monosex stocks [7,8].

Micellar liquid chromatography (MLC) is an alternative method to conventional liquid chromatography (CLC). The use of MLC for the separation of different samples is increasing due to some advantages with respect to CLC. For example, the low cost and toxicity of the mobile phases due to the few amount of solvent employed in the mobile phases, the enhanced selectivity and the simultaneous separations of hydrophobic and hydrophilic compounds [9,10]. The most important drawback of the MLC vs. CLC is the poor chromatographic efficiency due to the poor wetting of the stationary phase and low mass transfer of solutes between the mobile and stationary phases [11–14]. This lack in efficiency can be improved, however, by adding small amounts of organic modifiers, by keeping the same linear flow-rate (e.g. by using lower flow-rates and smaller column IDs than those used in CLC), or by increasing column temperature [15]. One of the main applications of MLC is the possibility of direct sample injection of biological material into the column due to the ability of micellar aggregates to dissolve sample proteins and other compounds [16,17].

MT is included in the International Olympic Committee doping list [18] and has also been determined in trout tissues by HPLC-UV [7] and in horse and human urine after oral administration in order to study its metabolism by GC-MS [4,19]. The optimization of the separation of complex mixtures of natural and synthetic AAS, including MT, by CLC and MLC have also been studied and applied to urine samples [20,21]. LC-MS is more selective than HPLC-DAD. However, it is very expensive and is not available in many analytical laboratories.

The US pharmacopoeia describes UV spectrometry for analysis of capsules and tablets containing MT, and thin-layer chromatography, infrared spectrometry (IR) or HPLC for pure MT powder [22]. MT and other steroids have also been examined in tablets and injectables by HPLC-IR [3]. However, it is worth mentioning the lack of official methods for assays in pharmaceuticals of active ingredients in reputed pharmacopoeias including MT. In other words, it is currently possible to find several methods based on RP-HPLC for MT determination which are not well-validated in pills. As an example Ghosh [23] has described 1.300 HPLC methods for hundreds of active ingredients. However, only a few of the proposed methods have been adequately validated [24–26].

In this paper, two simple, rapid, precise and sensitive CLC and MLC methods for MT determination in prepared samples from Longivol sugar-coated pills (containing 1 mg g^{-1} MT) using fluoxymesterone (FM) as internal standard, have been developed and validated. Validation process is mainly based on the ICH guideline [26].

2. Experimental

2.1. Chemicals and reagents

MT (17-hydroxy-17-methylandrost-4-en-3-one) and FM (9α-fluoro-11β, 17β-dihydroxy-17-methyl-4-androsten-3-one) were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) purum ($\geqslant 97\%$) was from Merck (Darmstadt, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH) and propanol (PrOH) were purchased from Promochem (Wesel, Germany). Millipore 0.45 μm nylon filters (Bedford, MA) was used. Water was purified with a Milli-Q system (Millipore, Molsheim, France). Other chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components, all from TSP (Riviera Beach, FL): a ConstaMetric 4100 solvent delivery system; a spectra Monitor 5000 photodiode-array

detector (DAD) covering the range 190–360 nm and interfaced to a computer for data acquisition and a recorder Model CI 4100 data module. A Rheodyne 20-μl loop injector (Cotati, CA), a Jones-Chromatography block heated series 7960 for thermostating columns (Seagate Technology, Scotts Valley, CA), a vacuum membrane degasser Model Gastor (SAS corporation, Tokyo, Japan), a bonded-silica Hypersil ODS (250 × 4.6 mm ID, 5 μm) column and a bonded-silica Hypersil ODS (150 × 3.0 mm ID, 5 μm) column from Phenomenex (Torrance, CA), were used.

2.3. Mobile phase and chromatographic analysis

The mobile phase were prepared daily by mixing Milli-Q water with acetonitrile (ACN) in CLC or aqueous solutions of SDS (prepared with Milli-Q water) with PrOH in MLC at the required volume ratio by programming the pump. All solvents and mobile phases were firstly filtered under vacuum through 0.45 μm nylon filters and degassed using a vacuum membrane degasser.

Once the column had been conditioned with the mobile phase, chromatograms were obtained at the programmed temperature (25 or 60 °C). For optimization purposes based on the use of different mobile phases, a methanolic solution containing MT (5 $\mu g\,ml^{-1}$) and FM (5 $\mu g\,ml^{-1}$) was injected (20 μl). The flow-rates in CLC and MLC were 1 and 0.5 $ml\,min^{-1}$, respectively, and UV-DAD detection in the range 190–360 nm was used. Peaks identification and purity were performed by comparison of they retention time and UV spectra with those of MT and FM previously registered by injection of each one individually. Analysis was carried out at 245 nm.

2.4. Sample preparation

Longivol sugar-coated pills (Medical, S.A., Cordoba, Spain) (mean weight 1 g) containing chicken living-embryo extract, E (50 mg), B_6 (50 mg), B_1 (20 mg), B_2 (3 mg), B_{12} (15 μg) vitamins, nicotinamide (15 mg), MT (1 mg), ethinyloestradriol (15 μg), methylteobromine (25 mg), magnesium and calcium inositol hexaphosphate (200 mg) and enteric coated excipient, were used.

Ten sugar-coated pills of product were adequately ground to a powder and homogenised. The amount corresponding to one sugar-coated pill (1 mg MT g^{-1}) was weighted, added with 1.0 mg FM (IS) and dissolved in MeOH (10 ml). The methanolic solution was shaken for 5 min, sonicated for 5 min to produce the complete dissolution of MT, and filtered through 0.45 μm nylon syringe filters. Then, 0.5 ml of the filtrate were completed to 10 ml using MeOH. The theoretical MT concentration was 5 $\mu g\,ml^{-1}$ (100% MT). Finally the mixture was injected into the HPLC system (20 μl).

Placebo samples were prepared by weighting, mixing and homogenising the ingredients of sugar-coated pills, and were processed in a similar way to the pharmaceuticals.

3. Results and discussion

3.1. Preliminary conditions

In previous papers, optimization studies for the separation of complex samples of AAS (natural and synthetic), including MT, have been studied in CLC using binary, ternary and quaternary mobile phases [20] and in MLC using SDS and different organic modifiers [21]. The optimum separations were achieved in CLC using 55% water:45% ACN (v:v), a Hypersil ODS (250 × 4.6 mm, 5 μm) (25 °C) and a flow-rate 1.0 $ml\,min^{-1}$, and in MLC using a mobile phase 40 mM SDS: 5% PrOH, a Hypersil ODS (150 × 3.0 mm, 5 μm) (60 °C) and a flow-rate 0.5 $ml\,min^{-1}$. In these conditions, a separation up to base line, including MT and FM, was achieved. On these grounds, ACN and SDS/PrOH were initially selected in CLC and MLC, respectively, to developed and validate an analytical method for MT in sugar-coated pills using FM as IS. In addition, the above packing characteristics (150 mm/3.0 mm ID/0.5 $ml\,min^{-1}$ in MLC and 250 mm/4.6 mm ID/1 $ml\,min^{-1}$ in CLC, respectively) were also selected. As consequence of column changes (temperature, flow-rate and ID), improvements in the mass transfer of solutes between chromatographic phases (column efficiency) and similar linear

velocities without loosing efficiency (similar retention), can be achieved, respectively, in MLC vs. CLC [15].

3.2. Chromatographic optimization

Taking into account the above results and with the aim to improve the analytical performances (e.g. reduce the run time analysis), the separation between MT and FM (IS) was reoptimized in CLC (range 45–60% ACN) and 55% ACN was finally selected. In MLC, 40 mM SDS was selected (this value assures a concentration over the critical micellar concentration (cmc = 8.1 mM) [27] and PrOH varied in the range 6–12% (10% PrOH was finally selected). In both conditions, separations up to base line were achieved. In summary, the optimal conditions for MT separation in CLC were a Hypersil ODS (250 × 4.6 mm, 5 μm) 25 °C column and a mobile phase 55% ACN:45% H_2O, and in MLC a Hypersil ODS (150 × 3.0 mm, 5 μm) 60 °C and 40 mM SDS:10% PrOH, respectively.

3.3. Separation performances

The separations obtained in CLC and MLC from a standard sample containing MT (5 μg ml^{-1}), and IS (5 μg ml^{-1}) are shown in Fig. 1. Estimates of the mean and RSD values ($n = 6$), using peak areas, are listed in Table 1. The RSDs

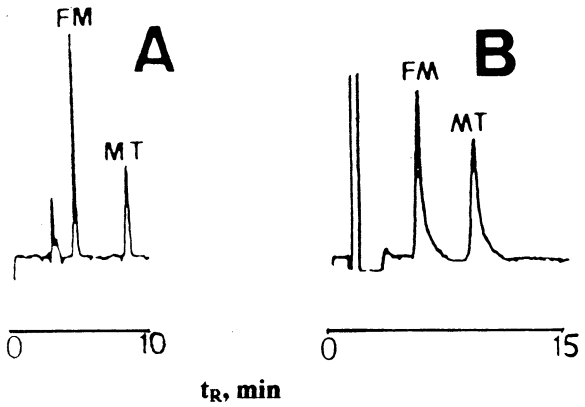

Fig. 1. Chromatograms in (A) CLC and (B) MLC for a standard mixture of MT and FM (5 μg ml^{-1}) obtained at 245 nm.

Table 1
Performances in CLC and MLC obtained from the separation of Fig. 1 involving MT and FM (IS)

	CLC		MLC	
	FM (IS)	MT	FM (IS)	MT
t_R, min	3.95	7.19	5.41	8.96
k	0.88	2.42	3.01	5.56
α	2.75		1.85	
ASF	1.02	1.00	1.33	1.35
RSD, %	1.64		1.96	
R_s	2.02		1.60	

Conditions as in Fig. 1, where k is the retention factor, ASF the asymmetry factor of the peaks, R_s the resolution between consecutive peaks, α is the separation factor and RSD the relative standard deviation of peak areas.

($n = 6$) of the retention factors, k, for MT in CLC and MLC were lower than 1%. Resolution, R_s, between consecutive peaks (MT vs. FM) were adequate. As can be observed, the data obtained from these compounds are adequate to develop an analytical method [28].

3.4. Calibration graphs, precision and detection limits

Standards containing mixtures of MT were prepared at eight different concentrations in the range 0.2–100 μg ml^{-1}, using FM as IS (5 μg ml^{-1}). These solutions were analyzed with the optimized conditions above described (Table 1). The results were analyzed by linear regression. The calibration equations, $Y = A + Bx$ (μg ml^{-1}), were obtained for MT by plotting peak area ratios of MT/IS (Y) versus the concentration (x). The parameters A (intercepts), B (slopes) and r (regression coefficients) were 0.049, 0.106 and 0.999 in CLC and 0.038, 0.115 and 0.999 in MLC, respectively.

Detection (LODs) and quantitation (LOQs) limits were calculated in CLC and MLC for a signal-to-noise (S/N) ratio of 3 and 10, respectively, from calibrations graphs. The values obtained in CLC were 4.0 and 10 ng g^{-1}, and in MLC were 0.6 and 2.0 ng g^{-1}, respectively.

4. Analysis of sugar-coated pills and validation methods

4.1. Linearity

Similar calibrations to those performed above were carried out in CLC and MLC for MT determination in Longivol samples. It was performed using placebo samples and seven different amounts of MT in the range of 50–150% around the theoretical value (range 2.5–7.5 μg ml^{-1}) and FM as IS). The calibration equations, $Y = A + Bx$ (μg ml^{-1}), were consistent with those obtained in Section 3.3. The correlation coefficients, r, found were 0.999 in each case.

4.2. Precision (repeatability and intermediate precision)

The precision was examined in CLC and MLC by analyzing six different sugar-coated pills ($n = 6$) by only one operator (No. 1), using calibration curves.

The repeatability was evaluated by only one operator within 1 day, whereas intermediate precision was evaluated for three different days. The mean and CV values obtained are shown in Table 2.

4.3. Accuracy

Placebo samples were spiked with different amounts of the active ingredient (MT) at 80, 100 and 120% (in triplicate for each one, $n = 9$) over the theoretical values. The mixtures obtained were processed according to sample preparation method (Section 2.4) and MT was determined using CLC and MLC. The mean values of the percent recoveries, % R, obtained are shown in Table 2. As expected, these values are consistent with the theoretical value for MT.

Table 2
Repeatability (RPT), intermediate precision (IP) and accuracy test for sugar-coated pills containing MT

		CLC	MLC
RPT	MT (mg g^{-1})	1.01 ± 0.02	1.02 ± 0.03
	CV (%)	2.3	2.9
IP	MT (mg g^{-1})	1.02 ± 0.03	1.03 ± 0.01
	CV (%)	2.9	3.3
R, %	80%	101 ± 1	102 ± 1
	100%	101 ± 2	102 ± 3
	120%	101 ± 2	101 ± 1
	Mean	101 ± 2	102 ± 2

4.4. Selectivity

Selectivity was assessed in CLC and MLC by a qualitative comparison of the chromatograms obtained from Longivol samples and the corresponding placebos. Fig. 2 shows the chromatograms obtained from placebo and from Longivol samples with and without adding MT. As it can be seen, possible interferences due to the substances present in samples were not observed. However, in CLC (Fig. 2B and C) FM elutes close behind a major peak. If chromatograms are not resolved up to base line, a minor modification of the separation conditions (e.g. mobile phase) or MLC (despite the larger asymmetry factor) is recommended. In addition, a detection and identification process based on retention times and a diode array detector (DAD) was carried out [29]. The CV ($n = 6$) of the retention factors for MT in CLC and MLC were lower than 1%. The UV spectrum of each peak in the chromatogram was stored and subsequently compared with standards (Fig. 1). The spectra were normalized and overlaid. Further, peak purity was investigated by displaying the spectra obtained at different points across the peak. From this study, the analyte chromatographic peak is only attributable to MT.

4.5. Robustness

In order to test the robustness of the CLC and MLC methods, Longivol samples containing 1 mg g^{-1} MT were analyzed by two operators ($n = 6$) (Nos 2 and 3) using standards prepared by themselves and under different chromatographic conditions than those used in the present methods (operator No. 1). The working conditions used and the results obtained in CLC and MLC

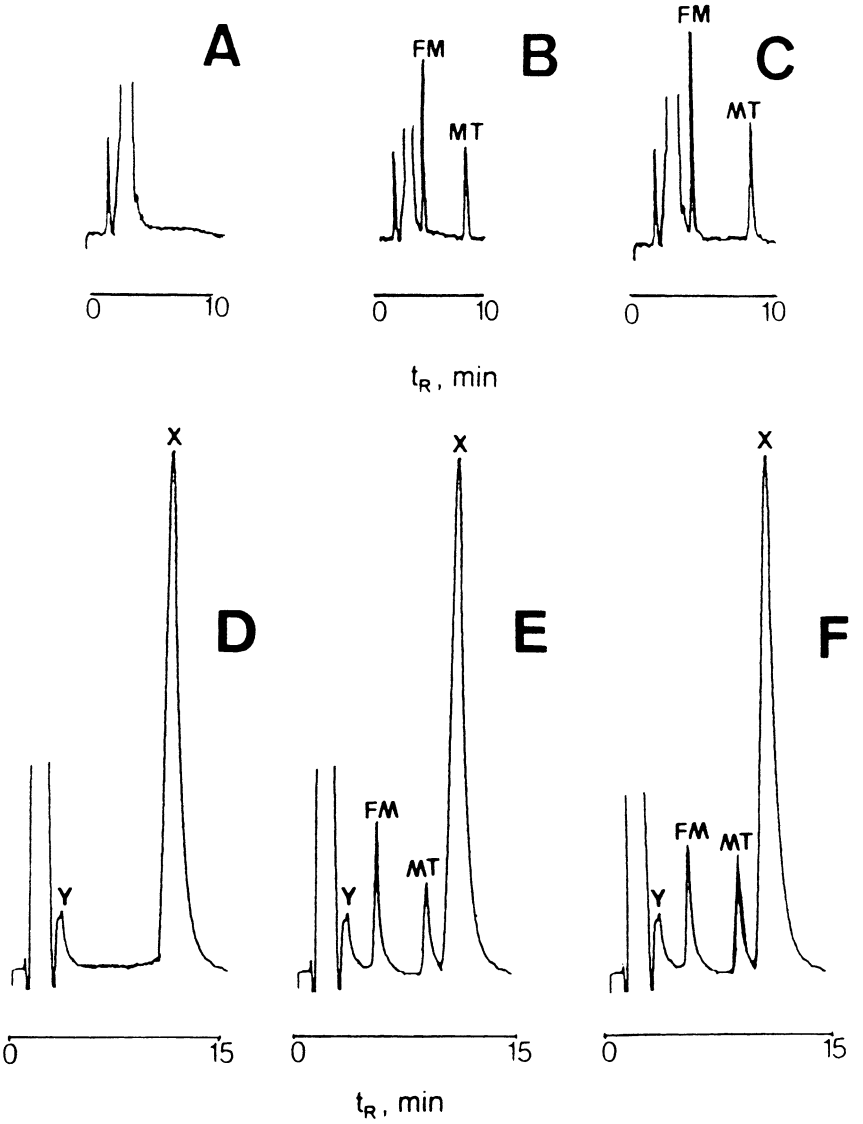

Fig. 2. Chromatograms obtained at 245 nm in CLC (A, B, C) and MLC (D, E, F) from placebos (A and D), Longivol samples containing MT 5 μg ml^{-1} and spiked with FM (5 μg ml^{-1}) (B and E), and Longivol samples containing MT 5 μg ml^{-1} and spiked with MT (5 μg ml^{-1}) and FM (5 μg ml^{-1}) (C y F). *X* and *Y* denotes unknown compounds.

for the operators are summarized in Tables 3 and 4.

4.6. CLC vs. MLC

A *t*-test was applied to MT results in CLC and MLC vs. the theoretical one (100% MT) (Table 2) showing no evidence of systematic error. An ANOVA test was also applied to the MT results obtained in CLC and MLC (Table 2). Since for MT the *P* values of the *F*-test were greater than 0.05 there are not statistically significant differences between CLC and MLC at the significance level of 5%.

CLC and MLC were compared using repeatability and intermediate precision MT data (Table

Table 3
Chromatographic conditions for robustness study in CLC and MLC, (SDS) = 40 mM

	CLC			MLC		
Conditions	Op. 1	Op. 2	Op. 3	Op. 1	Op. 2	Op. 3
Column	Hypersil ODS (250 × 4.6 mm, 5 μm)			Hypersil ODS (150 × 3.0 mm, 5 μm)		
Mobile phase	ACN:H_2O, v:v			%PrOH		
	55:45	50:50	52:48	10	8	9.5
F, ml min^{-1}	1	0.9	0.9	0.5	0.5	0.6
λ, nm	245	247	243	245	247	247
T, °C	25	25	25	60	55	57

Table 4
Robustness test for sugar-coated pills containing MT carried out by three operators ($n = 6$)

Operator	CLC		MLC	
	MT (mg g^{-1})	RSD (%)	MT (mg g^{-1})	RSD (%)
1	1.01 ± 0.02	2.3	1.02 ± 0.03	2.9
2	0.99 ± 0.03	3.2	1.00 ± 0.04	4.2
3	0.98 ± 0.03	2.8	0.98 ± 0.04	4.1
Mean	0.99 ± 0.15	2.8	1.00 ± 0.02	3.7

2). F-test was carried out showing no significant differences between CLC and MLC at the significance level of 5%. In summary, CLC and MLC methods can be considered as interchangeable.

5. Conclusions

Two simple, sensitive, accurate and reproducible HPLC methods (CLC and MLC) were developed for the analysis of MT in sugar-coated pills which required a simple sample preparation procedure prior to the HPLC analysis. Moreover, the robustness test indicates that different working conditions are possible because small variations in the main variables of the methods do not significantly affect the results.

These methods achieve the established pharmacopoeias requirements to be used as routine methods for the quality control and stability studies. Despite of CLC offers better chromatographic performances, MLC constitutes an alternative to CLC since presents several advantages such as a decrease of LOD and LOQ and the use of cheaper and less toxic mobile phases. In addition, MLC vs. CLC methods can be considered as interchangeable.

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