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Short communication

HPLC method development for testosterone propionate and cipionate in oil-based injectables

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Abstract

Two isocratic liquid chromatographic methods for the determination of testosterone propionate (TP) and cipionate (TC) in oil-based injectables using methyltestosterone and bolasterone as internal standards, respectively, have been developed and validated. Mobile phases 57% water:acetonitrile 43% (v:v) and 54% water:acetonitrile 46% (v:v) were used for TP and TC, respectively. For both methods, a bonded-silica Luna CN (250 mm × 4.6 mm i.d., 5 μm) (25 °C) column, a flow-rate 1 ml min^{-1} and UV absorbance detection at 245 nm were used and two separations up to base line were achieved. Prior to HPLC analysis, sample preparation was required, including extraction of TP and TC from oil-based injectables using the surfactant sodium dodecyl sulphate.

Keywords: HPLC; Validation; Testosterone propionate and cipionate; Oil-based injectables

1. Introduction

Testosterone (T) is the most representative natural androgenic-anabolic steroid (AAS) belonging to the C19 steroid group characterized by a Δ^4-3 ketonic group in the A ring and a hydroxylic group in the 17 site [1]. Other AAS are synthesized from T in order to produce AAS with strong anabolic properties and low androgenicity. Alkylation in the 17α position results in derivatives that are orally actives (e.g. methyltestosterone). Esterification in 17β position with organic acids results in derivatives, such as testosterone propionate (TP) or cipionate (TC), with improved oil solubility, employed for intramusculary administration in injectable forms. For these compounds, the ratio of solubility between oil and water gives good correlated predictions of the ratios of solubility between blood and target organs [2]. T esters are readily hydrolyzed in tissues by esterases. This reaction cannot take place while the esterified steroid is dissolved in fat, and thus, the duration of its action is extended [3]. Shorter chain esters must be injected more frequently than longer ones if consistent blood levels are desired. These levels probably lead to the greatest efficiency of the drug and the highest anabolic/androgenic ratio [4].

The determination of T misuse (usually administered in esterified form) is currently based on the urinary T/epitestosterone ratio, although in some rare instances physiological or pathological conditions could compromise the application of this general criterion.

Some authors have proposed GC/MS for the determination of T esters in plasma and hair as a definitive proof of the administration of exogenous T [5–8]. Analysis of T esters in chemicals, bulk materials and pharmaceutical preparations, has been investigated by gas chromatography/combustion/isotope ratio (GC/C/IRMS), based on the $^{13}C/^{12}C$ ratio [9]. An alternative MS technique is HPLC/electrospray MS, which has been used for characterizing steroid esterified with long-chain fatty acids [10]. A simple one-step extraction from oils or tablets was found to be an adequate sample preparation procedure for screening, tentative identification and quantitation of T esters by HPLC-UV [11]. A general screening method by HPLC with UV–vis-particle beam mass spectrometry for the determination of anabolic steroids in oil-based injectables, water suspensions,

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dietary supplements, and herbal drugs marketed in the form of capsules or tablets has also been described [12]. Moreover, residues of AAS in misplaced injection sites and anabolic preparations are monitored by HPLC-UV-DAD [13]. In addition, the analysis of testosterone esters in oil-based injectables has been described in the USP employing TLC and UV spectrometry for testosterone propionate and enanthate, and TLC and GC-FID for testosterone cipionate [14]. Currently, it is possible to find a plethora of methods based on RP-HPLC for the determination of active ingredients in pharmaceuticals. Ghosh [15] has described 1300 HPLC methods for hundreds of them. However, only a few of the proposed methods have been adequately validated [14,16,17].

In this paper, two simple, rapid, sensitive, accurate, precise, reproducible and robust HPLC methods for TP or TC determination in oil-based injectables using a Luna CN column and UV absorbance at 245 nm, have been developed and validated. A simple one-step extraction using the surfactant sodium dodecyl sulphate (SDS) was found to be adequate for these analyses. These methods can be considered as an alternative to those reported by the most important pharmacopoeias for the quantitation of major components (TP or TC) in oil-based injectables.

2. Experimental

2.1. Chemicals and reagents

Testosterone (T) (17β-hydroxy-4-androsten-3-one), TP (4-androsten-17β-(1-oxopropoxy)-3-one), TC (4-androsten-17β-(3-ciclopentyl-1-oxopropoxy)-3-one), methyltestosterone (MT) (17α-methyl-4-androsten-17β-ol-3-one) and bolasterone (BLS) (7,17-dimethylandrost-4-en-17β-ol-3-one), were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) purum (≥97%) was from Merck (Darmstadt, Germany). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Scharlab (Barcelona, Spain). Millipore 0.45 mm nylon filters (Bedford, MA, USA) 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, USA): 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 PU-1580 solvent delivery system from Jasco Corporation (Tokyo, Japan), a Rheodyne 20 μl loop injector (Cotati, CA, USA), a Jones-Chromatography block heated series 7960 for thermostating columns (Seagate Technology, Scotts Valley, CA, USA), a vacuum membrane degasser Model Gastor (SAS Corporation, Tokyo, Japan), two bonded-silica columns: Hypersil ODS (250 mm × 4.6 mm i.d., 5 μm) and Luna CN (250 mm × 4.6 mm i.d., 5 μm) from Phenomenex (Torrance, CA, USA), were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) was also used.

2.3. Mobile phase and chromatographic analysis

The mobile phases were prepared daily by mixing Milli-Q water with acetonitrile (ACN) 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 (30 °C). For optimization purposes based on the use of different mobile phases, a methanolic solution containing TP or TC (5 μg ml^{-1}) and MT or BLS (5 μg ml^{-1}), respectively, was injected (20 μl). The flow-rate was 1 ml min^{-1}, and UV-DAD detection in the range 190–360 nm was used. Peaks identification and purity were performed by comparison of their retention time and UV spectra with those of TP or TC, previously registered by injection of each one individually. Analysis was carried out at 245 nm.

2.4. Sample preparation

Testex Leo ampoules (1 ml) (Nycomed Leo, S.A., Madrid, Spain) containing 25 mg ml^{-1} TP and olive oil and ethyl oleate as excipients, or Testex Leo 250 Prolongatum (Nycomed Leo, S.A., Madrid, Spain) ampoules (2 ml) containing 125 mg ml^{-1} TC and chlorobutanol, olive oil and ethyl oleate as excipients, were used.

Ten ampoules of each product containing TP or TC were mixed, and 200 μl of them were added with 0.1 M SDS to 10 ml (TP) or 25 ml (TC). These mixtures contained 500 μg ml^{-1} (TP) or 1000 μg ml^{-1} (TC). They were vigorously shaken for 5 min, sonicated for 15 min and centrifuged for 5 min to separate the oil from the aqueous phase. The aqueous phase was removed (without disturbing the oil) and used to prepare 5 μg ml^{-1} TP or TC solutions using 0.1 M SDS (100% TP or TC). The recoveries found for TP and TC were close to 100%, respectively (see Section 4.1). After extraction and for quantitation purposes, samples were added with MT(IS) for TP or BLS(IS) for TC to obtain 5 μg ml^{-1} of IS. Finally, these solutions were injected in the HPLC system (20 μl).

Placebo samples were prepared by mixing and homogenizing the excipients of injectables, and processed in a similar way to the pharmaceuticals.

3. Results and discussion

3.1. Column selection

In previous papers, the optimization of the HPLC separation of complex mixtures (including T) containing urinary

anabolics and corticoids [18], and natural and synthetic anabolic steroids [19], involving several mobile and stationary phases, was carried out. From these studies, an Hypersil ODS column (250 mm × 4.6 mm i.d., 5 μm) (30 °C) and H_2O:ACN (60:40, v:v) were selected to obtain preliminary information about the retention of TP and TC versus T. Using this column, TP and TC exhibited a strong retention (hydrophobicity) versus T. *k*-values for these compounds follow the sequence TC > TP > T requiring, then, higher concentrations of ACN for adequate time analysis.

Available columns with embedded polar groups such as CN columns versus C18 give somewhat different selectivity effects [20,21]. The interaction mechanism of CN columns with neutral solutes such as TC or TP are mainly based on the polarity of the nitrile group. In addition, CN columns have much smaller hydrophobicity, presumably due to the polarity of the nitrile group, as compared with C18 columns. The steric resistances are also smaller, which may result from the dipole repulsion of aligned CN groups, resulting in a stationary phase that is more ordered and accessible. The hydrogen-bond acidity is also smaller, perhaps due to an interaction of CN groups with non-ionized silanols. However, the column cation exchange activity is much larger, due to ionized silanols [22]. For these reasons, a Luna CN column (250 mm × 4.6 mm i.d., 5 μm) (30 °C) has been tested to improve the separation of TC, TP and T described previously, providing the separation up to base line of these compounds in an analysis time of about 20 min (lower than using C18 columns). Thus, this column has been used in further experiments.

3.2. Chromatographic optimization and separation performance

Taking into account the above results, an optimization of the mobile phase was carried out with the aim to obtain two different separations for TP and TC, using an adequate IS, and to improve the analytical performances obtained above (e.g. reduce the retention of TP and TC, run time analysis, selectivity).

Mobile phases containing ACN in the range 40–50% were studied. The IS selection was based on the above and previous information about the hydrophobicity of 20 steroids studied [18,19]. MT and BLS were selected for the analysis of TP and TC, respectively. Taking into account α, R_S and run time analysis, mobile phases containing 43% or 46% ACN provided two optimal separations up to base line (Fig. 1) for TP and MT (separation 1) in about 7 min, and for TC and BLS (separation 2) in about 9 min, respectively. The performances in HPLC involving these compounds are summarized in Table 1. Estimates of the mean and R.S.D. values ($n = 6$) using peak areas are also listed in Table 1. The R.S.D. ($n = 6$) of the retention factors, *k*, for TP and TC were lower than 1%. As can be observed, the data obtained from these compounds are adequate to develop an analytical method [23].

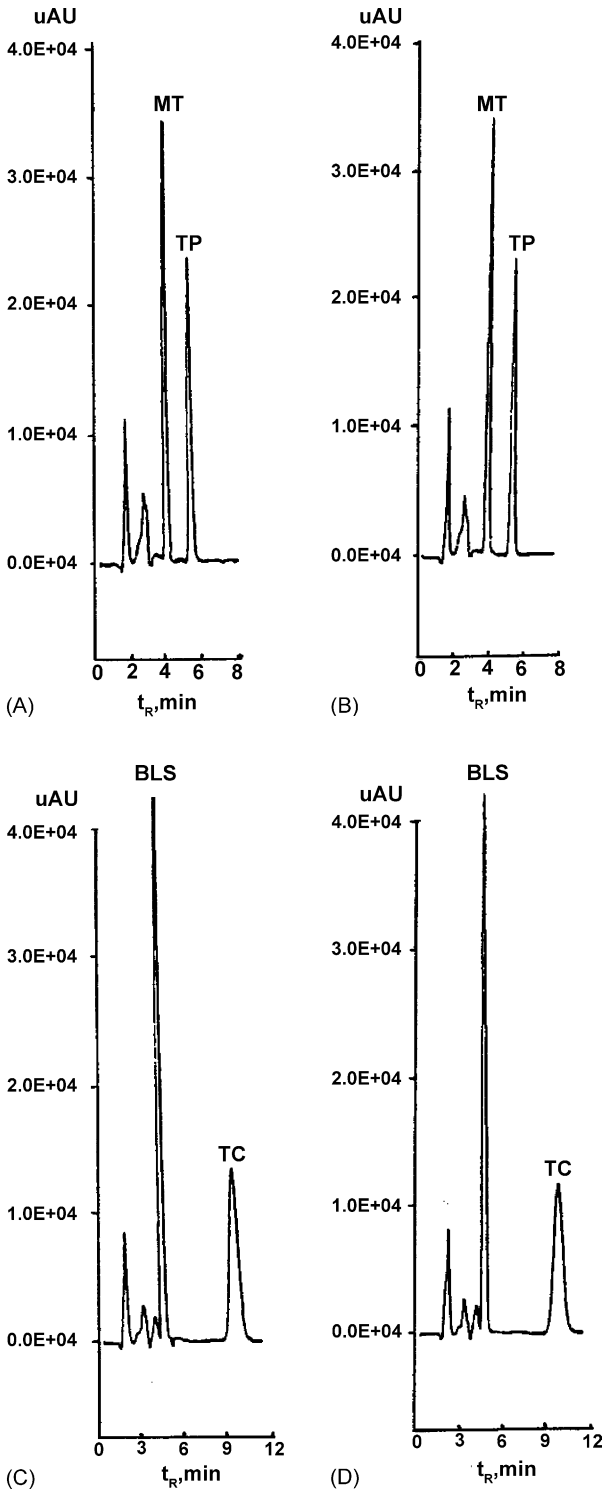

Fig. 1. Chromatograms obtained at 245 nm from standard samples containing TP and MT(IS) (A) and TC and BLS(IS) (C); from Testex Leo ampoules containing 5 μg ml^{-1} TP and spiked with 5 μg ml^{-1} MT (B), and Testex Leo 250 Prolongatum samples containing 5 μg ml^{-1} TC and spiked with 5 μg ml^{-1} BLS (D). Conditions: Luna CN column and mobile phases containing 43% ACN (A and B) and 46% ACN (C and D).

Table 1
Performances obtained from the separations of Fig.1 involving TP and MT(IS), and TC and BLS(IS)

	Separation 1		Separation 2	
	MT(IS)	TP	BLS(IS)	TC
t_R (min)	4.58	6.24	4.43	9.48
k	0.79	1.44	0.73	2.70
α	1.82		3.70	
ASF	1.0	1.0	1.0	1.2
R.S.D. (%)	2.3		2.5	
R_s	18.4		22.4	

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 the separation factor and R.S.D. the relative standard deviation of peak areas.

3.3. Calibration graphs, and detection and quantitation limits

Standards containing mixtures of TP or TC in 0.1 M SDS were prepared at 15 concentrations levels in the range of 0.2–100 $\mu g\,ml^{-1}$, using MT or BLS as IS (5 $\mu g\,ml^{-1}$), respectively. These solutions were analyzed with the optimized conditions described above (Fig. 1). The results were analyzed by linear regression. The calibration equations, $Y = A + Bx$ ($\mu g\,ml^{-1}$), were obtained for TP and TC by plotting peak area ratios of TP/MT or TC/BLS (Y) versus concentration (x). The parameters A (intercepts), B (slopes) and r (regression coefficients) were 0.027, 0.194 and 0.999 for TP and 0.034, 0.138 and 0.999 for TC, respectively.

Detection (LODs) and quantitation (LOQs) limits were calculated for a signal to noise (S/N) ratio of 3 and 10, respectively, from calibration graphs. The values obtained of LODs were 13 and 5 $ng\,ml^{-1}$ and LOQs were 43 and 17 $ng\,ml^{-1}$ for TP and TC, respectively.

4. Analysis of oil-based injectables and validation methods

4.1. Extraction of active ingredients from oil-based injectables

Oil-based injectables required sample pretreatment. An approach is to make a solution by increasing the solubility of these drugs in water through addition of co-solvents, cyclodextrins, detergents and mixed micelles [24–26]. A simple one-step extraction using MeOH was initially tested with unsatisfactory results, probably due to the hydrophobic character of TP and TC. However, when samples were processed using 0.1 M SDS (see Section 2.4), the recoveries ($n = 6$) obtained by means of the calibration curves were 99 ± 3% and 99 ± 4% for TP and TC, respectively. Under these conditions SDS does not modify the separation.

4.2. Linearity

Similar calibrations to those performed above were carried out for TP or TC determination in Testex Leo and Testex Leo 250 Prolongatum samples, respectively. It was performed using placebo samples and seven different amounts of TP or TC in the range 50–150% around the theoretical value (range 2.5–7.5 $\mu g\,ml^{-1}$) and MT or BLS as IS, respectively. The calibration equations were consistent with those obtained in Section 3.3. The correlation coefficients, r, found were 0.999 and 0.997 for TP and TC, respectively.

4.3. Precision (repeatability and intermediate precision)

The precision was examined by analyzing six different injectables ($n = 6$) 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 R.S.D. values obtained are shown in Table 2.

4.4. Accuracy

Placebo samples were spiked with different amounts of the active ingredients (TP or TC) at 80%, 100% and 120% (in triplicate for each one, $n = 9$) over the theoretical values (25 or 125 $mg\,ml^{-1}$ TP or TC, respectively). The mixtures obtained were processed according to sample preparation method (see Section 2.4) and TP or TC were determined. The mean values of the percent recoveries obtained are shown in Table 2. As expected, these values are consistent with those obtained in Section 4.1 and with the theoretical value for TP or TC.

4.5. Selectivity

Selectivity was assessed by a qualitative comparison of the chromatograms obtained from Testex Leo and Testex Leo Prolongatum samples and the corresponding placebos. Fig. 1 shows the chromatograms obtained from placebo samples and from samples with and without addition of TP or TC. As can be observed, possible interferences due to substances present in the samples were not observed. In addition, a detection and identification process based on retention times and a diode array detector (DAD) was carried out [27]. The R.S.D. ($n = 6$) of the retention factors for TP or TC were lower than 1%. The UV spectrum of each peak in the chromatogram was stored and subsequently compared with standards (Fig. 1).

Table 2
Repeatability (RPT), intermediate precision (IP) and accuracy, R, test for oil-based injectables containing TP or TC

		TP	TC
RPT	$mg\,ml^{-1}$	25.2	126
	R.S.D. (%)	1.3	0.7
IP	$mg\,ml^{-1}$	25.1	127
	R.S.D. (%)	1.7	0.9
R (% ± R.S.D.)	80%	100 ± 2	100 ± 1
	100%	101 ± 1	102 ± 1
	120%	100 ± 2	99 ± 1
	Mean	101 ± 1	101 ± 1

Table 3
Chromatographic conditions for robustness study

Conditions	TP			TC		
	Operator no. 1	Operator no. 2	Operator no. 3	Operator no. 1	Operator no. 2	Operator no. 3
Column	Luna CN (250 mm × 4.6 mm i.d., 5 μm)			Luna CN (250 mm × 4.6 mm i.d., 5 μm)		
Mobile phase	H_2O:ACN (v:v)			H_2O:ACN (v:v)		
	57:43	59:41	55:45	54:46	56:44	53:47
F (ml min^{-1})	1.1	1.0	0.9	1.1	1.0	0.9
λ (nm)	247	245	243	247	245	243
T (°C)	27	30	33	27	30	33

Table 4
Robustness test for oil-based injectables containing TP or TC carried out by three operators ($n = 6$)

Operator	TP		TC	
	mg ml^{-1}	R.S.D. (%)	mg ml^{-1}	R.S.D. (%)
1	24.7 ± 0.3	1.3	127 ± 0.9	0.7
2	25.2 ± 0.3	1.3	128 ± 0.8	0.6
3	24.9 ± 0.4	2.0	128 ± 0.8	0.6
Mean	25.0 ± 0.2	1.7	128 ± 0.8	0.7

The spectra were normalized and overlaid. Impurities were investigated further by displaying the spectra obtained at different points across the peak with negative result.

4.6. Robustness

In order to test the robustness of the methods, six samples 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 for the operators are summarized in Table 3, and Table 4 shows the results obtained in each case.

5. Conclusions

The chromatographic behavior of TP and TC versus testosterone was evaluated by a separation previously described which used a Hypersil C18 column and water–ACN as mobile phase. These compounds exhibited a strong retention (hydrophobicity) versus testosterone. This separation was improved using the same mobile phase and a semi-polar Luna CN column. After optimization of the mobile phase and selection of internal standards, two separations up to base-line in few minutes, were achieved. From these separations, two simple, sensitive, accurate and reproducible HPLC methods were developed and validated for the analysis of TP or TC in oil-based injectables. Moreover, the robustness test indicates that different working conditions are possible because small variations in the main variables of both methods do not significantly affect the results. Both methods required sample pre-treatment that includes extraction in SDS prior to the HPLC analysis (recoveries were close to 100%). This sample pre-treatment was simpler than the USP ones [14] and/or more efficient than those using MeOH [12] or mixtures MeOH–water 90:10 [12,14] (further re-extraction with 100% MeOH for TC was required [14]). On the other hand, CN versus conventional C18 columns [11,12], require lower analysis time and organic modifier. Moreover, these methods could be an alternative when LC–MS or GC–MS is not available.

These methods achieve the established pharmacopoeias requirements to be used as routine methods for the quantitation of major components (TP or TC) in oil-based injectables.

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