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Conventional and micellar liquid chromatography method development for danazol and validation in capsules

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Abstract

Two isocratic liquid chromatographic methods (conventional and micellar) for the determination of danazol (DZ) in capsules using canrenone (CAN) as internal standard have been developed and validated. In conventional liquid chromatography a mobile phase 35% water:acetonitrile 65%, 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 (MLC) the conditions were: mobile phase 40 mM sodium dodecyl sulfate:2% pentanol, 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 280 nm was used and a separation up to base line was achieved. Prior to HPLC analysis a simple sample preparation was required. The recoveries found in the accuracy test were 99 ± 10 and 101 ± 8%, in conventional liquid chromatography (CLC) and MLC, respectively. Repeatability and intermediate precision expressed as R.S.D. were lower than 5% for both methods. Detection limits obtained were 2.4 and 3.0 ng g^{-1} in CLC and CLM, respectively.

Keywords: Micellar and conventional LC; Danazol; Capsules; Validation

1. Introduction

Available anabolic steroid products are subclassified as ester derivatives and nonesters. Danazol (DZ) [17α-pregna-2,4-dien-20-ynol(2,3-D)-isoxazol-17-ol] is a nonester synthetic hormone with weak androgenic effects and structurally related to testosterone and ethisterone. DZ, with an ethynyl moiety at C17 and isoxazole ring fused to positions 2 and 3 of the A ring is considerably more lipophilic than the other 17-OH steroids (e.g. testosterone, boldenone, nandrolone). The additional chromophoric species in DZ structure (Fig. 1 shows the presence of a conjugated triene chromophore) produces a significantly different UV absorption spectrum: a maximum at 280 nm (higher than typical anabolic steroids) allows its identification [1].

Clinically, DZ is a gonadotropin inhibitor indicated for the treatment of endometriosis, hereditary angioedema and fibrocystic breast disease. Although its mechanism of action has not

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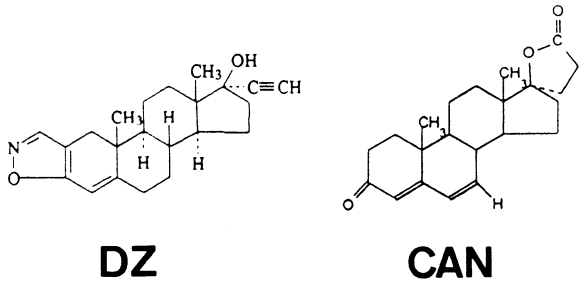

Fig. 1. Structures of DZ and CAN, internal standard.

been fully elucidated, it does cause reduction in plasma levels of luteinizing hormone and follicle-stimulating hormone [2]. DZ is also used in doping field as other anabolic steroids to increase muscle development and strength, decrease healing time following injury, diminishing fatigue, and increase aggressiveness [3–6]. The adverse effects associated with anabolic use of DZ are dependent on dose and duration of use. These effects have been described with more frequent incidence in females (amenorrhea, breakthrough bleeding or spotting, decreased breast size, irregular menstrual periods, weight gain) and with less one in both females and males (muscle spasms, unusual weakness and virilism [1,7,8].

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. The most important drawback of the MLC versus 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. 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. 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 [9,10].

In the literature there are few information on DZ, especially in MLC. The analysis of DZ has been described in the USP employing UV measurements at 286 nm [11], by quantitative TLC for the validation of the purity analysis of DZ [12] and in capsules by CLC with UV detection at 260 nm using methanol–chloroform–water as mobile phase [13]. DZ has also been determined by HPLC in human plasma and serum [2,14,15] and for photodegradation studies [16]. In this study it has been reported the photosensitivity of DZ in solution to UV light and the stability (at least 6 months) to daylight. In addition, complex samples of natural and synthetic androgenic anabolic steroids (AAS), including DZ, have also been studied in CLC [17] and MLC [18]. Currently, it is possible to find a plethora of methods based on RP–HPLC for the determination of active ingredients in pharmaceuticals. Ghosh [19] has described 1300 HPLC methods for hundreds of them. However, only a few of the proposed methods have been adequately validated [20–22].

In this paper, two simple, rapid, sensitive, accurate, precise, reproducible and robust CLC and MLC methods for DZ determination in prepared samples from Danatrol® capsules using UV absorbance detection at 280 nm, have been developed and validated. These methods can be considered as an alternative to those methods reported by the most important pharmacopoeias for quality control purposes.

2. Experimental

2.1. Chemicals and reagents

DZ [17α-pregna-2,4-dien-20-ynol(2,3-D)-isoxazol-17-ol] and canrenone (CAN) (17α-17-hydroxy-3-oxopregna-4,6-diene-21-carboxylic acid γ-lactone) were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) purum ($\geq$97%) was from Merck (Darmstadt, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH) and pentanol (PeOH) were purchased

from Promochem (Wesel, Germany).) Millipore 0.45 μm 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 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, 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), a bonded-silica Hypersil ODS (250 mm × 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, USA), 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 or PeOH 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 DZ (5 μg ml^{-1}) and CAN (5 μg 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 their retention time and UV spectra with those of DZ and CAN previously registered by injection of each one individually. Analysis was carried out at 280 nm.

2.4. Sample preparation

Danatrol® capsules (Sanofi Winthrop, S.A., Barcelona, Spain) containing 100 mg DZ per sampling unit (SU) of mean weight = 230 mg, lactose, starch, talc and magnesic stearate as excipients, were used.

Ten capsules of product were adequately ground to a powder and homogenized. The amount of the powder corresponding to one capsule was weighted and dissolved in MeOH (50 ml). The methanolic solution was shaken for 5 min, sonicated for 5 min to produce the complete dissolution of DZ, and filtered through 0.45 μm nylon syringe filters. Then, 25 μl of the filtrate were added with 0.5 ml 100 μg ml^{-1} CAN (IS) and completed to 10 ml using MeOH. The theoretical DZ concentration after dilution was 5 μg ml^{-1} (100% DZ). Finally, the mixture was injected into the HPLC system (20 μl).

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

3. Results and discussion

3.1. Preliminary conditions

The chromatographic properties of DZ and other 17-hydroxy steroids has been examined and compared using UV detection in CLC. This study concludes that the isoxazole derivative DZ has the highest retention factor than enones and dienones [1]. These results have been corroborated in CLC and MLC optimization studies for the separation of complex samples of natural and synthetic AAS (DZ was always more hydrophobic than the other steroids studied since the highest retention factors were obtained). CLC and MLC used binary, ternary and quaternary mobile phases [17], and SDS and different organic modifiers (ACN, THF, PrOH, BuOH and PeOH), respectively [18]. 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}. These optimized CLC and MLC separations allowed the separation of all AAS. On these grounds, ACN and SDS/PrOH were initially selected in CLC and MLC, respectively, to develope and validate an analytical method for DZ in capsules using CAN 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 versus CLC [10].

3.2. Chromatographic optimization

Taking into account the above results and with the aim to improve the analytical performances (e.g. reduce the strong retention of DZ exhibited in CLC and MLC, run time analysis, selectivity), the separation between DZ and CAN (IS) was studied in CLC (range 55–70% ACN) and 65% 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) [23] and PrOH varied in the range 6–12%. However, when Danatrol samples were checked using PrOH as modifier, interferences from excipients and IS (CAN) overlapped. To solve this impediment, other IS (e.g. dexamethasone, bolasterone) were checked with identical results. Using the preliminary information in MLC above mentioned [17], PeOH was tested in the range and 0.2–2.5%, with satisfactory results (2% PeOH was finally selected). In summary, the optimum conditions for DZ separation in CLC were an Hypersil ODS (250 × 4.6 mm, 5 μm) column at a temperature of 25 °C and a mobile phase of 65% ACN:35% H_2O; and in MLC an Hypersil ODS (150 × 3.0 mm, 5 μm) column at a temperature of 60 °C and a mobile phase of 40 mM SDS:2%PeOH.

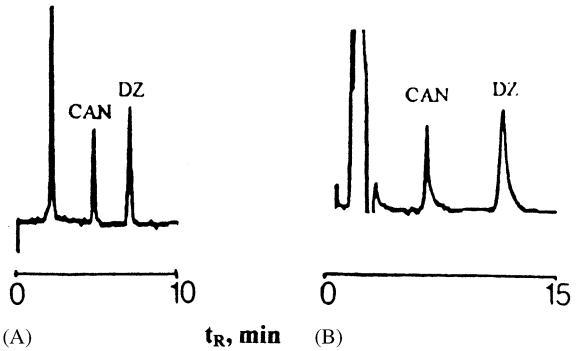

Fig. 2. Chromatograms obtained at 280 nm in CLC (A) and MLC (B) for a standard mixture of DZ and CAN (5 μg ml^{-1}).

Table 1
Performances in CLC and MLC obtained from the separation of Fig. 2 involving DZ and CAN (IS)

	CLC		MLC	
	CAN (IS)	DZ	CAN (IS)	DZ
t_R (min)	5.08	7.46	6.13	11.24
k	1.81	2.62	3.98	8.14
α	1.44		2.05	
ASF	1.00	1.03	1.63	1.50
R.S.D. (%)		1.35		2.44
R_s	3.22		3.00	

Conditions as in Fig. 2, where k is the retention factor, ASF the asymmetry factor of the peaks, R_s the resolution between the peaks, α is the separation factor and R.S.D. the relative standard deviation of peak areas.

3.3. Separation performances

The separations obtained in CLC and MLC from a standard sample containing DZ (5 μg ml^{-1}) and IS (5 μg ml^{-1}) are shown in Fig. 2. As can be observed, CLC and MLC separations up to base line were achieved. In CLC the run time analysis and asymmetry factor are lower than in MLC. However, in MLC the separation factor, α, overcomes to that reported in CLC. Estimates of the mean and R.S.D. values ($n = 6$) using peak areas, are listed in Table 1. The R.S.D. ($n = 6$) of the retention factors, k, for DZ in CLC and MLC

were lower than 1%. As can be observed, the data obtained from these compounds are adequate to develop an analytical method [24].

3.4. Calibration graphs and detection limits

Standards containing mixtures of DZ were prepared at eight concentration levels in the range 0.2–100 μg ml^{-1}, using CAN 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 DZ by plotting peak area ratios of DZ/IS (Y) versus the concentration (x). The parameters A (intercepts), B (slopes) and r (regression coefficients) were 0.105, 0.311 and 0.999 in CLC and 0.100, 0.192 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 of LODs and LOQs in CLC were 2.4 and 7.7 ng g^{-1}, respectively, and in MLC were 3.0 and 9.6 ng g^{-1}, respectively.

4. Analysis of capsules and validation methods

4.1. Linearity

Similar calibrations to those performed above was carried out in CLC and MLC for DZ determination in Danatrol® samples. It was performed using placebo samples and seven different amounts of DZ in the range of 50–150% around the theoretical value (range 2.5–7.5 μg ml^{-1}) and CAN 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 capsules ($n = 6$) by only one operator (No 1), using calibration curves. The repeatability (within-run precision) 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.

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

		CLC	MLC
RPT	DZ (mg g^{-1})	101±5	104±6
	R.S.D. (%)	2.9	3.3
IP	DZ (mg g^{-1})	99±3	103±1
	R.S.D. (%)	4.4	4.9
R (%±R.S.D.)	80%	98±3	100±7
	100%	101±5	99±5
	120%	99±6	103±4
	Mean	99±10	101±8

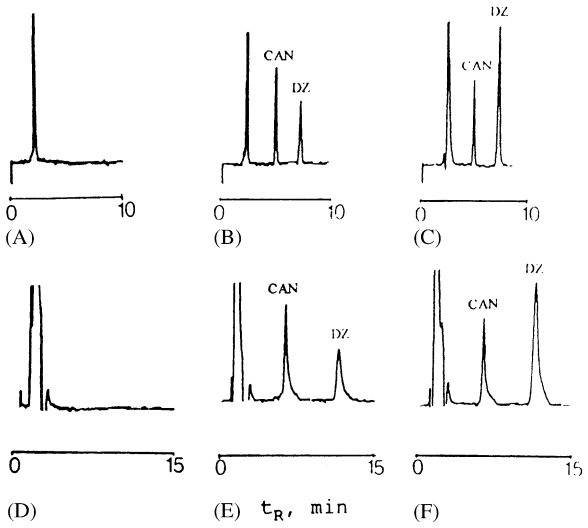

Fig. 3. Chromatograms obtained at 280 nm in CLC (A, B, C) and MLC (D, E, F) from placebos (A and D), Danatrol samples containing DZ 5 μg ml^{-1} and spiked with CAN (5 μg ml^{-1})(B and E), and Danatrol samples containing DZ 5 μg ml^{-1} and spiked with DZ (5 μg ml^{-1}) and CAN (5 μg ml^{-1}) (C and F).

4.3. Accuracy

Placebo samples were spiked with different amounts of the active ingredient (DZ) at 80, 100 and 120% (in triplicate for each one, $n = 9$) over

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			%PeOH		
	65:35	68:32	70:30	2	1.8	1.6
F (ml min^{-1})	1	0.9	0.9	0.5	0.4	0.4
λ (nm)	280	286	284	280	286	284
T (°C)	25	25	25	60	55	57

Table 4
Robustness test for capsules containing DZ carried out by three operators ($n = 6$)

Operator	CLC		MLC	
	DZ (mg per SU)	R.S.D. (%)	DZ (mg per SU)	R.S.D. (%)
1	101 ± 5	5.4	104 ± 6	5.1
2	102 ± 5	4.9	105 ± 5	4.7
3	97 ± 4	4.3	97 ± 8	4.1
Mean	100 ± 3	2.7	102 ± 5	4.6

SU, sampling unit.

the theoretical values (100 mg DZ/SU). The mixtures obtained were processed according to sample preparation method (see Section 2.4) and DZ was determined using CLC and MLC. The mean values of the percent recoveries obtained are shown in Table 2. As expected, these values are consistent with the theoretical value for DZ.

4.4. Selectivity

Selectivity was assessed in CLC and MLC by a qualitative comparison of the chromatograms obtained from Danatrol® samples and the corresponding placebos. In Fig. 3 are shown the chromatograms obtained from placebo samples and from Danatrol® samples with and without adding DZ. As can be observed, possible interferences due to the substances present in samples were not observed. In addition, a detection and identification process based on retention times and a diode array detector (DAD) was carried out [25]. The R.S.D. ($n = 6$) of the retention factors for DZ 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. 2). The spectra were normalized and overlaid. Impurities were investigated further by displaying the spectra obtained at different points across the peak with negative result.

4.5. Robustness

In order to test the robustness of the CLC and MLC methods, six samples were analyzed by two operators (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 in CLC and MLC for the operators are summarized in Table 3, and in Table 4 the results obtained in each case.

4.6. CLC versus MLC

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

2). *F*-test was carried out showing no significant differences between CLC and MLC at the significance level of 5%.

An ANOVA test was also applied to the DZ results (Table 2). Since for DZ 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%. In other words, CLC and MLC methods from the point of view of precision and accuracy can be considered as interchangeables.

5. Conclusions

Two simple, sensitive, accurate and reproducible HPLC methods (CLC and MLC) were developed for the analysis of DZ in capsules 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 of DZ in capsules. In general CLC offers better chromatographic performances and lower retention than MLC. However, MLC constitutes an alternative to CLC since presents several advantages such as the selectivity factor and the use of cheaper and less toxic mobile phases. Additionally, MLC versus CLC methods can be considered as interchangeables.

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References

[1] F. Taylor, Jr, C. Randall, J. DeRuiter, J. Chromatogr. Sci. 28 (1990) 162.
[2] W.D. Hooper, G.R. Cannel, R.G. Dickinson, J. Chromatogr. 416 (1987) 347.
[3] S. Helms, J.T. Pento, Anabolic steroid use by athletes, US Pharmacist (January 1985) 49–52.
[4] H.A. Haulpt, G.D. Rovere, Am. J. Sports Med. 12 (1984) 469.
[5] C. Birdsong, Am. Pharm. 26 (1986) 43.
[6] M.B. Mellion, Am. Fam. Phys. 30 (1984) 113.
[7] J.E. Wright, Exerc. Sport Sci. 8 (1980) 149.
[8] The United States Pharmacopeia, 24th ed., DI, The United States Pharmacopoeial Convection, Rockville, MD, 2000, pp. 1099–1101.
[9] A. Berthod, C. Garcia-Alvarez-Coque, Micellar Liquid Chromatography, Marcel Dekker, New York, 2000.
[10] A. Santos-Montes, R. Izquierdo-Hornillos, J. Chromatogr. B 724 (1999) 53.
[11] The United States Pharmacopeia, 24th edn., The United States Pharmacopoeial Convection, Rockville, MD, 2000, p. 496.
[12] K. Ferenczi-Fodor, Z. Vegh, Z. Pap-Sziklay, J. Planar Chromatogr. 6 (1993) 198.
[13] R.T. Sane, M. Chakraborty, V.K. Nayak, B.L. Chauhan, J. Chromatogr. 358 (1986) 448.
[14] A. Rahman, N.E. Hoffman, Anal. Lett. 22 (1989) 377.
[15] K. Selinger, H.M. Hill, J.A. Anslow, D. Gash, J. Pharm. Biomed. Anal. 8 (1990) 79.
[16] E.A. Gad Kariem, M.A. Abounassif, M.E. Hagga, H.A. Al-Khamees, J. Pharm. Biomed. Anal. 23 (2000) 413.
[17] R. Gonzalo-Lumbreras, R. Izquierdo-Hornillos, J. Chromatogr. B 742 (2000) 47.
[18] R. Gonzalo-Lumbreras, Ph.D. Thesis, Universidad Complutense, Madrid, 2001.
[19] M.K. Ghosh, HPLC Methods for Drug Analysis, Springer, Berlin, 1992.
[20] J.M. Green, Anal. Chem. 68 (1996) 305–309.
[21] The United States Pharmacopoeia, 23th edn., The United States Pharmacopoeial Convection, Rockville, MD, 1995, p. 1982.
[22] International Conference on Harmonization, Draft Guideline on Validation of Analytical Procedures, Federal Register, 60 (1995) 11260.
[23] C. Tandford, Hydrophobic Effect. Formation of Micelles and Biological Membranes, second ed, Wiley, New York, 1980.
[24] U.D. Neue, D.J. Phillips, T.H. Walter, M. Capparella, B. Alden, R.P. Alden, R.P. Fisk, LC–GC Int. 8 (1995) 26.
[25] B.K. Logan, Anal. Chim. Acta 288 (1994) 111.