International Journal of Pharmaceutics, 13 (1983) 333–343
Elsevier Biomedical Press

High-performance liquid chromatographic (HPLC) analysis of methenolone esters in pharmaceutical formulations *

V. Cavrini, A.M. Di Pietra and M.A. Raggi

Istituto di Chimica Farmaceutica e Tossicologica, via Belmeloro 6, 40126 Bologna (Italy)

(Received May 26th, 1982)
(Accepted August 2nd, 1982)

Summary

A specific high-performance liquid chromatographic (HPLC) procedure suitable as a stability-indicating assay for the methenolone esters (the acetate and enanthate) analysis in pharmaceutical formulations was investigated. The analysis was carried out under isocratic and reversed-phase conditions using a UV detector (240 nm). A RP8-HPLC system was of choice for the methenolone enanthate analysis.

Introduction

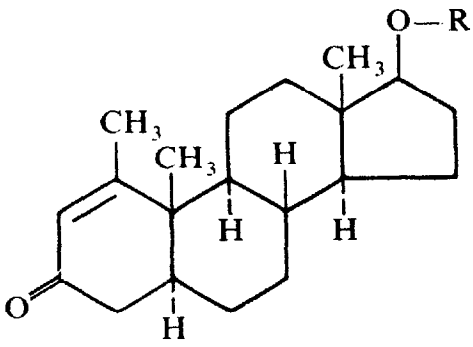
Methenolone, 1-methyl-17β-hydroxy-5α-androst-1-en-3-one (I), is a synthetic steroid with a potent anabolic action essentially devoid of androgenic activity in therapeutic doses (Counsell and Brueggemeier, 1979). The acetate (II) and enanthate (III) esters are used for improving the general conditions and for speeding the recovery in convalescent states. The enanthate has also been used in the treatment of advanced neoplasms of the breast (Wade, 1977).

The restricted studies pertaining to the methenolone esters analysis include TLC (Sonanini and Anker, 1967) and gas chromatographic (Guenter, 1978) methods.

Gas chromatography–mass spectrometry has been used in doping control for the methenolone analysis in urine samples (Rumboldt and Barbir, 1980; Clausnitzer et al., 1980). The official methods of assay for oily injections of anabolic steroids structurally related to methenolone, such as nandrolone esters (UPS XX, 1980; BP,

* Dedicated to Prof. Michele Amorosa on the occasion of his 70th birthday.

0378-5173/83/0000-0000/$03.00



(I) R=H
(II) R=—$COCH_3$
(III) R=—$CO(CH_2)_5CH_3$

1980), are based on the reaction between the drug and isoniazid to form a colored hydrazone that is determined spectrophotometrically. These methods are of limited specificity due to an inability to distinguish between the steroid ester and the parent steroid alcohol, which represents both a potential process impurity and a potential hydrolytic degradation product. The influence of the acyl moiety on the potency and duration of action as well as on the anabolic-to-androgenic ratio should call for a specific assay method based on the intact molecule.

Recently, specific high-performance liquid chromatographic methods for the quantitative steroid esters determination in pharmaceutical formulations have been developed (Li Wan Po et al., 1979; Ast and Abdou, 1979; Smith, 1979; Van Dame, 1980; Carignan et al., 1980; Munson and Wilson, 1981), but the application of this technique to methenolone esters analysis was not reported. The present study, therefore, was undertaken to provide a rapid reversed-phase HPLC procedure suitable as a stability-indicating assay for the methenolone esters in pharmaceutical formulations.

Materials and methods

Materials

Prednisolone was USP grade (E. Merck, Darmstadt). Methenolone acetate (Schering AG) was used as received. Methenolone enanthate was obtained from commercial formulations by the following procedure. The contents of 3 containers were mixed and shaken with 50 ml of dilute methanol (90%) and then centrifuged. The process was repeated twice and the combined methanolic extracts were evaporated at reduced pressure. The oily residue was purified by column chromatography on silica gel (eluent: *n*-hexane–ethylacetate 6 : 4) and by subsequent crystallization from methanol. M.P. 69–70°C (Kaspar et al., 1959: 70–70.5°C). IR (nujol): 1732 (CO ester), 1665 (CO ketone), 1525, 1235, 850 cm^{-1}. Elemental analysis for $C_{27}H_{42}O_3$—found C 77.92, H 10.21; calcd. C 78.26, H 10.25.

Methenolone was derived from the enanthate ester by alkaline hydrolysis in methanolic solution. M.P. 149–150°C (MeOH–H_2O) (Wiechert and Kaspar, 1960: 149.5–152°C). IR (nujol): 3420 (OH), 1655 (CO ester), 1600, 1290, 1210, 1125, 1075 cm^{-1}. Elemental analysis for $C_{20}H_{30}O_2$—found C 79.35, H 10.26; calcd. C 79.42, H 10.0.

The acetonitrile and methanol used for chromatography were HPLC grade from C. Erba, and the water was doubly glass-distilled.

Thin-layer chromatography

The isolation and the purification of (I) and (III) were monitored by thin-layer chromatography. The following chromatographic systems were used.

Adsorption TLC Flexible TLC sheets (Baker-flex silica gel IB2-F) with fluorescent indicator were used. Mobile phase: *n*-hexane–ethylacetate (6 : 4). Detection: (a) UV lamp (254 nm); (b) 2,4-dinitrophenylhydrazine reagent. The R_f values were: 0.35 (I); 0.70 (II); 0.81 (III).

Reversed-phase TLC. Silica gel 60 F_{254} silanized precoated plates (E. Merck, Darmstadt) were used. Mobile phase: acetone–water (3 : 1). Detection as above. R_f: 0.62 (I); 0.51 (II); 0.39 (III).

High-performance liquid chromatography

Instruments. A Varian liquid-chromatograph model 5020, equipped with a Valco (Field Instruments, Richmond, U.K.) high-pressure injection valve fitted with a 10-μl sample loop was used. All measurements were made at ambient temperature using a variable wavelength detector UV-50 (Varian), connected with y/t recorder with integrator (Bryans Southern Instruments). The detector wavelength was adjusted to 240 nm with a sensitivity of 0.05 aufs.

Chromatographic conditions. (A) RP 18-HPLC. The chromatography was carried out isocratically using a reversed-phase MicroPack MCH-10 (monomeric C_{18} bonded onto 10 μm silica gel) column. 30 cm × 4 mm i.d. The mobile phases were: (a) 90% acetonitrile in water at a flow-rate of 1.5 ml/min; and (b) 92% acetonitrile in water at a flow-rate of 2 ml/min. (B) RP 8-HPLC. The chromatography was performed on a 25 cm × 4 mm i.d. column of LiChrosorb RP8 (7 μm). Various mixtures of methanol–water were tried as mobile phase. For the assay procedure the methanol–water (90 : 10) solvent system was chosen and the flow-rate was kept constant at 1 ml/min.

Calibration curves

Stock solutions of methenolone acetate, methenolone enanthate and prednisolone (the internal standard) were prepared by dissolving 20 mg of each drug in 100 ml of acetonitrile or methanol. For methenolone acetate analysis standard solutions in acetonitrile containing 40–100 μg/ml of methonolone acetate and 100 μg/ml of prednisolone (the internal standard) were prepared. For methenolone enanthate analysis the standard solutions contained 40–120 μg/ml of the drug and 40 μg/ml of methenolone acetate (the internal standard) in acetonitrile (RP18-HPLC) or methanol (RP8-HPLC). Duplicate preparations were made for each standard solution. A 10-μl injection volume was used and all injections were carried out in triplicate. Calibration curves were constructed by plotting the peak height ratios of the drug to internal standard versus weight ratios of the drug to internal standard.

Sample preparation

Tablets. Ten tablets were weighed and tritured to a fine powder. An accurately

weighed sample of powder, equivalent to about 5 mg of methenolone acetate, was extracted 3 times with 7-ml aliquots of acetonitrile in a 50-ml screw-capped centrifuge tube by agitation for about 10 min. The extracts were then filtered, combined in a 25-ml volumetric flask and diluted with acetonitrile to volume. A 3-ml aliquot of the resulting solution were transferred into a 10-ml volumetric flask containing exactly 5 ml of the internal standard (prednisolone) solution. It then was diluted to volume with acetonitrile and shaken.

Injectables. The contents of several containers were well mixed, and the sample was worked up in two different ways.

(a) An accurately measured volume of sample, equivalent to about 100 mg of methenolone enanthate, was diluted with acetonitrile to provide a solution containing approximately 200 μg/ml of methenolone enanthate. A 3-ml aliquot of this solution was transferred into 10-ml volumetric flask containing 2 ml of internal standard (methenolone acetate) solution. It then was diluted to volume with acetonitrile and shaken.

(b) A volume of injection, equivalent to about 100 mg of the drug, was accurately transferred into a 50-ml screw-capped centrifuge tube and 25 ml of dilute methanol (90%) were added. The mixture was shaken for about 15 min, centrifuged at 10°C for 10 min and the resulting clear methanolic layer was transferred into a 100-ml volumetric flask. The extraction process was repeated twice and the combined methanolic extracts were brought to volume with methanol. The resulting solution was diluted further, 1: 5 with methanol. Using methanol instead of acetonitrile, the procedure described in (a) was then followed, beginning with 'a 3-ml aliquot of this solution was transferred...'.

Synthetic suspension. Aqueous suspension containing, per ml, methenolone acetate (1 mg), polyethyleneglycol 4000 (30 mg), polysorbate (3.0 mg), methylparaben (2.0 mg), propylparaben (0.2 mg) and sodium chloride (10 mg) was maintained at room temperature over one-month period. A 3-ml aliquot of well-mixed suspension was transferred accurately into a separation funnel and extracted 3 times with 7-ml portions of chloroform. The extracts were combined into 25-ml volumetric flask and then brought to volume with chloroform. This solution was diluted 1 : 2 with internal standard (prednisolone) solution and then analyzed.

Assay procedure

Prior to injection into the chromatograph the analytical solutions were filtered through a 0.45-μm teflon membrane filter (Gelman Sciences). All formulations were first chromatographed without internal standard to ensure that interfering peaks were not present. A 10-μl aliquot of the analytical solutions was injected and triplicate injections were made for each solution. The standard solutions were chromatographed both at the beginning and at the end of a day's run and these results were averaged. The peak height ratio of the drug to internal standard was used for further calculations.

Results and discussion

A high-performance liquid chromatographic (HPLC) method capable of separating such closely related compounds as methenolone and its esters (the acetate and enanthate) was investigated. To this end, a reversed-phase mode of chromatography was chosen because the polar free steroid elutes before the corresponding esters, thus allowing ready quantitation of the active ingredient (the steroid esters) in the dosage forms. Initially, the chromatography was performed on a reversed-phase C_{18}-column. Using the acetonitrile–water (90 : 10) mixture as mobile phase an adequate separation of methenolone, methenolone acetate and prednisolone (the internal

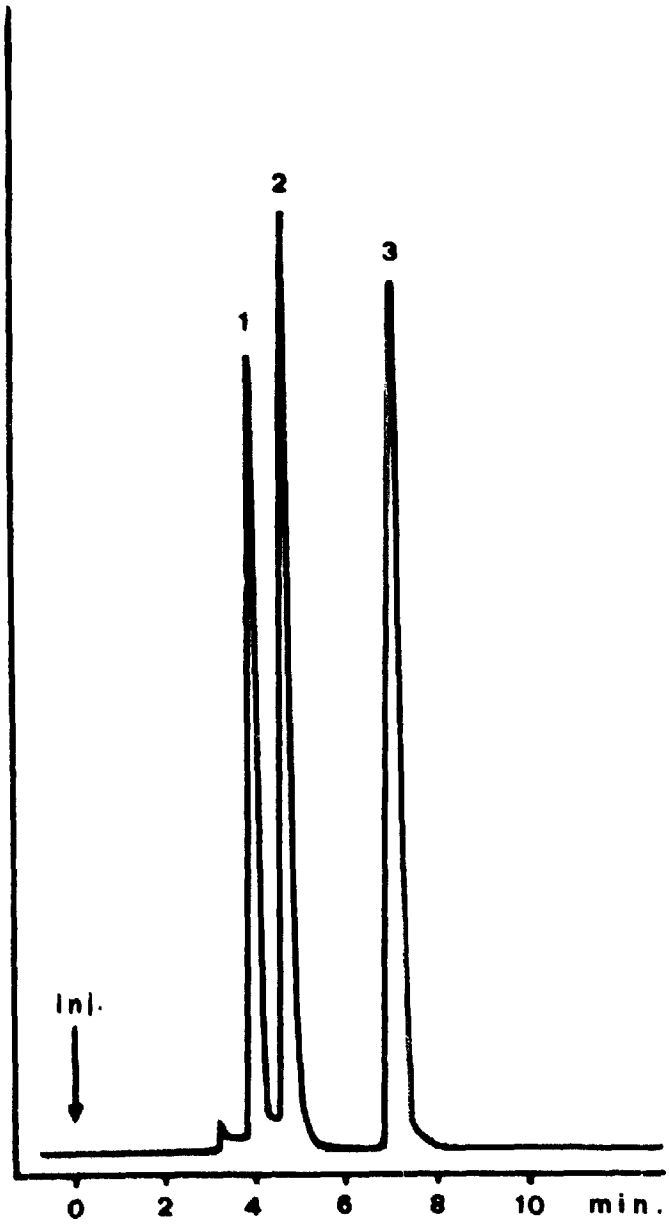


Fig. 1. RP-18-HPLC separation of prednisolone (1), methenolone (2) and methenolone acetate (3). Column: MicroPack MCH-10; mobile phase: methanol–water (90 : 10) at a flow-rate of 1.5 ml/min.

TABLE I

DATA FOR THE CALIBRATION CURVES AND THE SYSTEM SUITABILITY

Drug	Internal standard	column	Linear regression parameters [a]			System suitability	
			Slope	Intercept	Correlation coefficient	RSD% [b]	Resolution [c]
Methenolone acetate	Prednisolone	RP 18	0.761	−0.005	0.9992	0.45	4.8
Methenolone enanthate	Methenolone acetate	RP 18	0.336	0.010	0.9984	0.52	6.2
	Methenolone acetate	RP 8	0.560	0.030	0.9980	0.40	3.6

[a] Peak height ratio of drug to internal standard on the y-axis versus weight ratio of drug to internal standard on the x-axis.

[b] From 6 replicate injections of a single standard solution.

[c] Resolution factor between the peaks from drug and internal standard.

TABLE 2

ASSAY RESULTS FOR METHENOLONE ESTERS IN PHARMACEUTICAL DOSAGE FORMS

Drug	Dosage forms	Column	Found [a] % of claim	RSD%
Methenolone acetate	Tablets	RP 18	100.21	1.40
	Suspension	RP 18	100.80	1.25
Methenolone enanthate	Injection	RP 18	101.0	0.71
		RP 8	99.23	0.65

[a] Average of 5 determinations.

standard) was achieved (Fig. 1). Under these chromatographic conditions a specific quantitative determination of methenolone acetate in aged tablets and in a synthetic suspension was carried out. A linear calibration curve was found (Table 1) in the range 0.4–1.0 μg of amount injected. The assay results (Table 2) demonstrate that methenolone acetate in aged tablets as well as in a synthetic suspension have not undergone appreciable hydrolytic degradation to methenolone. The inactive ingredients (parabens) and chloroform, the extracting solvent in the suspension analysis, eluted early and did not interfere.

The RP 18-HPLC method was also used for the methenolone enanthate analysis in oily injections. Methenolone acetate was the internal standard. Composition and flow-rate of the mobile phase were varied (92% in acetonitrile and 2 ml/min, respectively) to achieve a convenient analysis-time. The elution order was: methenolone ($t_r = 2.9$ min), methenolone acetate ($t_r = 3.5$ min) and methenolone enanthate ($t_r = 9.1$ min). An acetonitrile concentration as high as 95% failed to separate methenolone and its acetyl ester. For the quantitative methenolone enanthate determination a linear calibration curve was found (Table 1) in the concentration range studied. The assay results (Table 2) demonstrate the applicability of this procedure, but the requirement of a high percentage of organic modifier, due to high lipophilicity of methenolone enanthate, makes the method expensive. In order to provide a more inexpensive chromatographic system, the RP 18-column was abandoned in favor of a RP 8-column. Methanol–water mixtures of varying composition were tried to achieve optimum chromatographic conditions. The effect of the methanol concentration in the mobile phase on the capacity factors of methenolone, methenolone acetate and methenolone enanthate is shown in Fig. 2. Plotting the data as log K′ versus methanol concentration in the mobile phase (Fig. 3), a linear correlation ($r = 0.996$) was found for methenolone enanthate, according to partitioning mechanism between the stationary and mobile phases (Munson and Wilson, 1981), whereas non-linear behavior was observed for methenolone and its acetyl ester. These data suggest that the retention of the more polar compounds (I and II) may not be due entirely to partitioning mechanism and that adsorption phenomena may play a role. At the high organic modifier concentrations, required for elute methenolone enanthate within a reasonable time, hydrogen bonding of the I and II

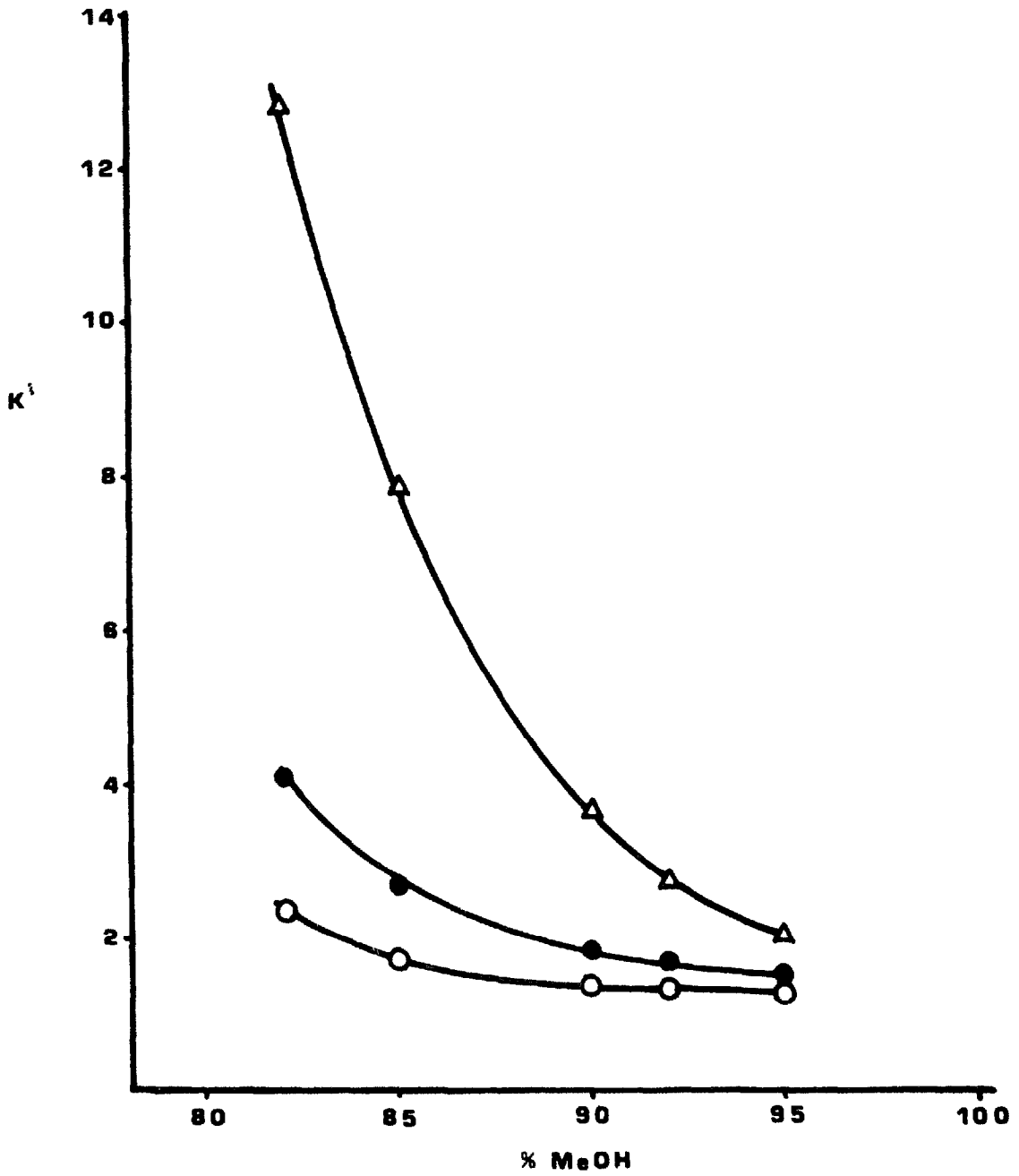


Fig. 2. Effect of methanol concentration in mobile phase on the capacity factor (K') for methenolone (○), methenolone acetate (●) and methenolone enanthate (△). Column: Licrosorb RP 8 (7 μm); flow-rate: 1 ml/min.

molecules with the free silanol groups will not be overcome by the interaction of the mobile phase with these hydroxyl groups. To develop an analytical method for III, a mobile phase of 90% (v/v) methanol in water was chosen, enabling adequate resolution of the compound under examination, internal standard and potential impurities (methenolone) with a short analysis-time (Fig. 4). In the concentration range studied a linear calibration plot was obtained (Table 1). Commercial samples

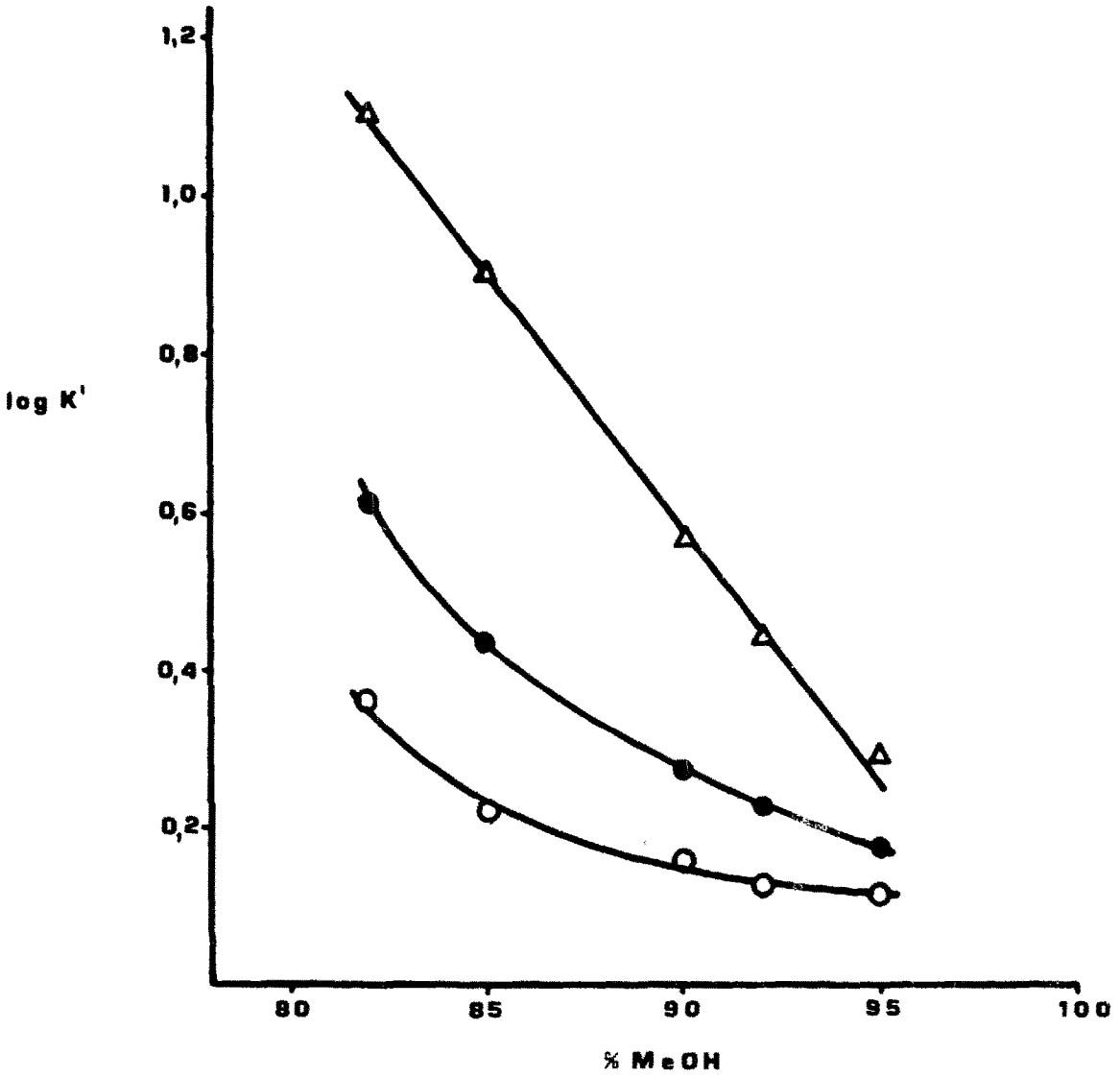


Fig. 3. Relationship of log K' and methanol concentration in mobile phase for I (○), II (●) and III (△). Chromatographic conditions as in Fig. 2.

were analyzed and were found to contain methenolone enanthate levels in good agreement with the nominal content (Table 2). Methenolone ester concentration as low as 0.1 μg/ml could be detected; this sensitivity makes the proposed RP 8-HPLC method suitable also for a rapid control for use in suspected doping cases.

The analysis of III in oily injections was carried out by immediate chromatography of the sample diluted to approximately 60 μg/ml (Method A). This simple procedure, however, involving injections onto the reversed-phase column of oil-containing solutions required a frequent column washing with methylene chloride to maintain column efficiency and selectivity (Rabel, 1981). Differently approached, the extraction of methenolone enanthate with the methanol–water (90 : 10) solvent system, followed by centrifugation at low temperature (10°C) (Method B), enables

the removal of the bulk of its oil vehicle; under these experimental conditions column cleaning was not required.

In summary, these results correspond with recent publications recommending

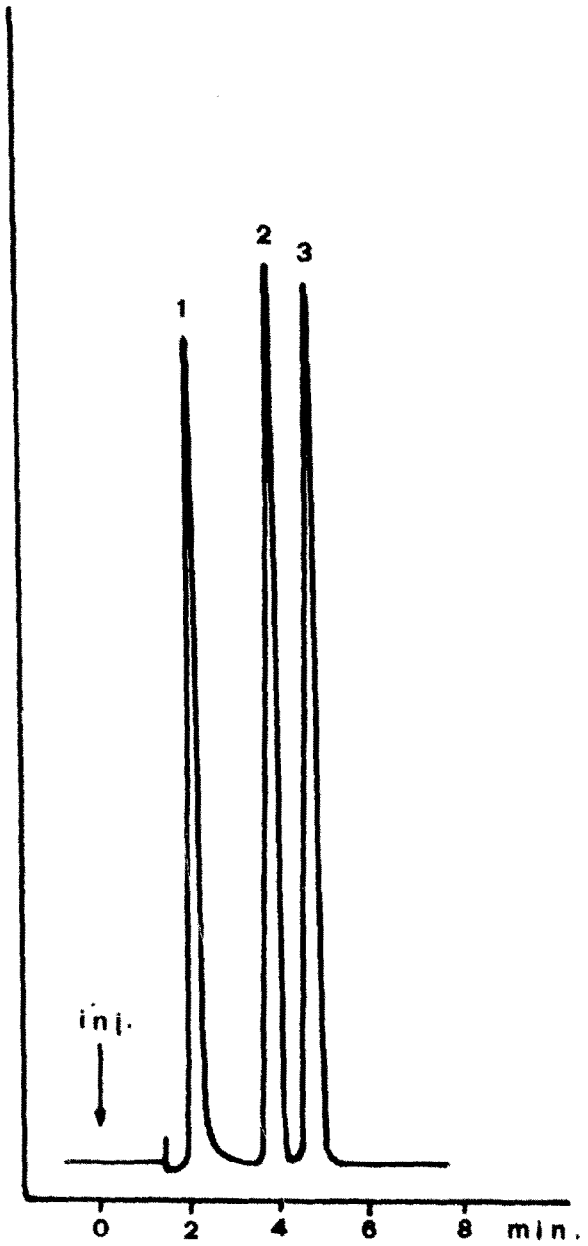


Fig. 4. RP 8-HPLC separation of methenolone (1), methenolone acetate (2), and enanthate (3). Column: LiChrosorb RP 8 (7 μm); mobile phase: methanol–water (90:10) at a flow-rate of 1 ml/min.

reversed-phase HPLC procedures as a suitable stability-indicating assay for steroid esters in pharmaceutical formulations and suggest the preferential use of a RP 8-column over a RP 18-column for analyzing such a highly lipophilic steroid as methenolone enanthate.

References

Ast, T.M. and Abdou, H.M., Analysis of fludrocortisone acetate and its solid dosage forms by high-performance liquid chromatography. J. Pharm. Sci., 68 (1979) 421–423.

Carignan, G., Lodge, B.A. and Skakun, W., High-performance liquid chromatographic analysis of testosterone esters in oily solutions. J. Pharm. Sci., 69 (1980) 1214–1216.

Clausnitzer, C., Behrendt, D. and Storch, A., Selected ion monitoring—eine moderne methode zum screening und zur identifizierung von anabolen steroiden. Med. Sport (Berlin), 20 (1980) 180–184.

Counsell, R.E. and Brueggemeier, R., The male sex hormones and analogs. In Wolff, M.E. (Ed.), Burger's Medicinal Chemistry, 4th Edn., John Wiley, New York, pp. 873–916.

Guenter, H.O., Detection of growth promoting products in foods. Fresenius' Z. Anal. Chem., 290 (1978) 389–416; Chem. Abstr. 89 (1978) 127804h.

Kaspar, E., Wiechert, R., Schenck, M. and Popper, A., Ger. 1, 117, 113 (1959 to Schering A-G); Chem. Abstr. 56 (1962) 11665e.

Li Wan Po, A., Irwin, W.J. and Yip, Y.W., High-performance liquid chromatographic assay of betamethasone 17-valerate and its degradation products. J. Chromatogr., 176 (1979) 399–405.

Munson, J.W. and Wilson, T.D., High-performance liquid chromatographic determination of hydrocortisone cypionate: method development and characterisation of chromatographic behavior. J. Pharm. Sci., 70 (1981) 177–181.

Rabel, F.M., Care of reverse phase high performance liquid chromatographic columns when injecting extracts of fat- or oil-based preparations. J. Ass. Off. Anal. Chem., 64 (1981) 1258.

Rumboldt, Z. and Barbir, Z., Doping control at the Eighth Mediterranean Games. Lijec. Vjesn, 102 (1980) 261–8; Chem. Abstr. 94 (1981) 115313n.

Smith, E., Liquid chromatographic determination of desoxycorticosterone acetate in oil injections. J. Ass. Off. Anal. Chem., 62 (1979) 812–817.

Sonanini, D. and Anker, L., Nachweis vonsteroiden mittels verteilungsdünnschichtchromatographie (Androgene, Oestrogene, Gestagene, Anabolica). Pharm. Acta Helv., 42 (1967) 54–64.

The British Pharmacopoeia, Pharmaceutical Press, London, 1980, pp. 641–642.

United States Pharmacopeia XX, National Formulary XV, United States Pharmacopeial Convention, Rockville, 1980, pp. 541–543.

Van Dame, H.C., Quantitative determination of steroid acetates in pharmaceutical preparations by high performance liquid chromatography. J. Ass. Off. Anal. Chem., 63 (1980) 1184–1188.

Wade, A., In Martindale (Ed.), The Extra Pharmacopoeia, 27th Edn., The Pharmaceutical Press, London, 1977, pp. 1407–1408.

Wiechert, R. and Kaspar, E., Über Steroidpyrazoline und ihre spaltung. Chem. Ber., 93 (1960) 1710–1715.