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Analysis of nandrolone esters in oily injections by reversed-phase high-performance liquid chromatography*

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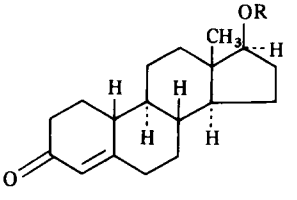
Abstract: A specific liquid chromatographic (HPLC) procedure is presented for the analysis of nandrolone esters (phenylpropionate, decanoate and undecanoate) in commercial oily injections. The analysis was carried out under isocratic, reversed-phase (RP8 column) conditions using a UV detector (240 nm). The system discriminates between nandrolone alcohol, a potential impurity, and its esters and permits the quantitation of trace benzaldehyde derived from the oxidative degradation of benzyl alcohol. The proposed HPLC method was found to be more specific and accurate than the pharmacopoeial spectrophotometric assay procedure (isoniazid reagent). The interference of benzaldehyde with the isoniazid method was also investigated.

Keywords: *Reversed-phase high-performance liquid chromatography; nandrolone esters; anabolic steroids; benzaldehyde interferences; benzaldehyde determination.*

Introduction

Nandrolone, 17β-hydroxyestr-4-en-3-one or 19-nortestosterone (Scheme 1), is an anabolic steroid with reduced androgenic effects. In the form of a variety of esters (phenylpropionate, decanoate and undecanoate) nandrolone is used in a wide



I : R = H
Ia : R = CO $(CH_2)_2$ Ph
Ib : R = CO $(CH_2)_8CH_3$
Ic : R = CO $(CH_2)_9CH_3$

Scheme 1
Structure of nandrolone (**I**) and its phenylpropionate (**Ia**), decanoate (**Ib**) and undecanoate (**Ic**) esters.

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assortment of conditions, ranging from post-operative recovery to chronic debilitating diseases. The duration of action of the drug and the anabolic-to-androgenic ratio has been found to be affected by the nature of the acyl moiety [1, 2]. A specific assay method able to distinguish the various steroid esters and the steroid alcohol, a potential impurity from synthesis and/or hydrolytic degradation, is therefore required. The pharmacopoeial methods [3–5] for the estimation of nandrolone esters in pharmaceutical dosage forms are based on a colorimetric method [6], which involves a reaction between the steroid and isoniazid (isonicotinic acid hydrazide) followed by spectrophotometric determination of the resulting coloured hydrazone. This procedure lacks specificity as it depends on the reaction of the 4-ene-3-one conjugate system, present in nandrolone and its esters as well as in a variety of Δ^4-3-ketosteroids [7, 8]. When applied in conjunction with a preliminary paper [9], thin-layer [10] and column [11] chromatographic separation, the isoniazid method was specific for the intact molecule of steroid hormones, but the procedures were laborious and time-consuming. These considerations and the lack of information concerning the analysis of nandrolone esters [12–14] led us to develop a rapid, specific and stability-indicating method suitable for determining the drug in commercial dosage forms (oily injections), based on reversed-phase high-performance liquid chromatography. In the present paper the proposed HPLC procedure is described and the assay results are compared with those from the pharmacopoeial method (isoniazid assay).

Experimental

Materials and reagents

Nandrolone (Menarini, Italy), nandrolone phenylpropionate and decanoate (Organon, Netherlands) and nandrolone undecanoate (Crinos, Italy) were all kindly supplied by their manufacturers and used as received. Reagent grade benzaldehyde, acetophenone, benzyl alcohol and isoniazid (isonicotinic acid hydrazide) were obtained from Carlo Erba, Milan, Italy; reagent grade diphenyl from Janssen Chimica, Belgium. These chemicals were all used without further purification.

Benzaldehyde isonicotinyl hydrazone was prepared by heating under reflux for 1 h an 80% ethanol solution containing equimolar quantities of benzaldehyde and isoniazid. The crude product was purified by recrystallization from ethanol. Its physical properties were: melting point: 199–200°C (lit. [15]: 198°C); IR spectrum (nujol mull): 3180 cm^{-1} ($\nu N - H$), 1685 cm^{-1} ($\nu C = 0$), 1560 cm^{-1}, 1280 cm^{-1}, 1150 cm^{-1}, 845 cm^{-1}, 760 cm^{-1}, 700 cm^{-1}. Methanol (C. Erba, Italy) and tetrahydrofuran (Merck, F.R.G.) used for chromatography were HPLC grade; water was deionized double distilled.

The isoniazid reagent was prepared according to the USP method [3, 4].

Apparatus

HPLC analyses were performed on a Varian 5020 liquid chromatograph equipped with a Valco injection valve fitted with a 10 µl sample loop. All measurements were made at ambient temperature using a variable wavelength detector (Varian UV-50) and a chart recorder (Bryans Southern Instrument). The detector wavelength was adjusted to 240 nm (sensitivity: 0.2–0.05 a.u.f.s.) and peak height was used for quantitation.

Spectrophotometric analyses were performed using a Varian DMS 90 UV-visible double-beam spectrophotometer and a Jasco Uvidec-4 digital single-beam spectrophotometer with 1 cm cells.

Chromatographic conditions

Separations were performed isocratically on a 250 × 4 mm i.d. column containing 7-μm Lichrosorb RP-8. The mobile phases used were: (phase A) methanol–tetrahydrofuran–water (82:3:15 v/v/v) for nandrolone phenylpropionate analysis, and (phase B) methanol–tetrahydrofuran–water (90:2:8 v/v/v) for nandrolone decanoate and undecanoate analysis. The separation between benzaldehyde and benzyl alcohol was performed using a mobile phase consisting of methanol–water (52:48 v/v). The flow rate was kept constant at 1.0 ml min^{-1}.

Stock solutions

Stock solutions for the HPLC procedure were all prepared in methanol at the indicated concentrations: nandrolone esters and diphenyl (0.2 mg ml^{-1}); benzaldehyde (20 μg ml^{-1}), benzyl alcohol (2 mg ml^{-1}) and acetophenone (80 μg ml^{-1}).

Stock solutions for the isoniazid method were prepared in chloroform: nandrolone phenylpropionate, decanoate and undecanoate (25 μg ml^{-1}), benzaldehyde (0.5 mg ml^{-1}).

Calibration curves

Standard solutions containing 40–80 μg ml^{-1} of nandrolone phenylpropionate with 20 μg ml^{-1} of diphenyl (internal standard) were prepared in methanol. Similarly standard solutions were prepared for nandrolone decanoate and undecanoate containing 20–60 μg ml^{-1} of the analyte with 20 μg ml^{-1} of nandrolone phenylpropionate (internal standard). Standard solutions containing 2–8 μg ml^{-1} of benzaldehyde with 8 μg ml^{-1} of acetophenone (internal standard) and 1 mg ml^{-1} of benzyl alcohol were prepared in methanol.

A 10 μl volume of each standard solution was injected in triplicate. The ratios of drug peak height to that of internal standard were plotted against their respective concentration ratios to obtain the calibration curves.

Nandrolone esters analysis

Commercially available single component dosage forms (oily injections) of nandrolone esters were analysed. The nandrolone phenylpropionate and decanoate injections were solutions of the drug in peanut oil containing 0.1 g ml^{-1} of benzyl alcohol; the nandrolone undecanoate injection was a solution in olive oil.

Sample preparation

HPLC method. A 1.0 ml quantity of the nandrolone ester injection was accurately transferred into a 50 ml screw-capped centrifuge tube and 20 ml of 85% ethanol were added. The mixture was then shaken for 15 min at room temperature, centrifuged for 10 min and the clear alcoholic layer was transferred into a 100 ml volumetric flask. The extraction process was repeated three times and the combined ethanolic extracts were brought to volume with 85% ethanol. An aliquot of the resulting solution (equivalent to about 0.5, 0.25 and 0.32 mg of nandrolone phenylpropionate, decanoate and undecanoate, respectively) was accurately transferred into 10 ml volumetric flask containing 1.0 ml of the appropriate internal standard solution (Table 1). It then was diluted to volume with 85% ethanol and shaken.

Table 1
Data for the calibration curves and the system suitability

Nandrolone ester	Internal standard	Linear regression parameters*				System suitability			
		Slope	Intercept	Correlation coefficient	Working range ($\mu g\ ml^{-1}$)	R.S.D.(%)†	Resol.‡	Detection limit§ ($\mu g\ ml^{-1}$)	(a.u.f.s.)
Phenylpropionate	Diphenyl	0.338	−0.0150	0.9994	40–80	0.34	4.5	0.60	(0.2)
Decanoate	Nandrolone phenylpropionate	0.681	−0.0387	0.9997	20–60	0.28	9.6	0.35	(0.1)
Undecanoate	Nandrolone phenylpropionate	0.584	−0.0228	0.9999	20–60	0.46	11.2	0.40	(0.1)

*Peak height ratio of drug to internal standard on the *y*-axis versus weight ratio of drug to internal standard on the *x*-axis.
†From six replicate injections of a single standard solution.
‡Resolution factor between the peaks from drug and internal standard.
§Based as a signal-to-noise ratio of 5:1.

USP method. A 1.0 ml quantity of the injection was diluted with chloroform to provide a solution containing approximately 25 μg of nandrolone ester per millilitre. This solution was then analysed by the USP method.

Assay procedure

HPLC. All formulations were first chromatographed without internal standard in order to verify the absence of interfering peaks at the retention position for the internal standard. A 10 μl aliquot of each analytical solution was injected into the liquid chromatograph in triplicate. The unknown samples were run concurrently with the appropriate standard solutions. The peak height ratio of drug to internal standard was determined and used for calculating the amount of nandrolone ester in each of the samples analysed.

USP. A 5-ml aliquot of both the sample and standard chloroform solutions was subjected to spectrophotometric determination (isoniazid method), as directed by the USP procedure.

Benzaldehyde determination

Sample preparation. A 1.0 ml aliquot of the nandrolone ester injection was extracted three times with 15-ml portions of 85% ethanol, following the same procedure used for the steroid extraction. The alcoholic extracts were combined and diluted to 50 ml with 85% ethanol.

Assay procedure. Method A. A 5-ml aliquot of the sample solution was transferred to a 10 ml volumetric flask, the internal standard (acetophenone) solution (1.0 ml) was added and the volume was adjusted with methanol. Aliquots (10 μl) of the sample and standard solutions were successively injected in triplicate into the chromatograph. The ratios of peak heights (benzaldehyde to acetophenone) were determined and the amount of benzaldehyde in each of the analysed samples was then calculated by interpolating the calibration curve.

Method B. A 5-ml aliquot of the sample solution was spiked with varying amounts (1–3 ml) of benzaldehyde stock solution, a fixed amount (1.0 ml) of internal standard (acetophenone) solution was added and the volume was adjusted to 10.0 ml with methanol. A 10-μl volume of each solution was injected in triplicate.

The regression line of peak height ratio of benzaldehyde/acetophenone versus the amount of benzaldehyde added to sample solution was then constructed. The x-intercept of the regression line was used for calculating the amount of benzaldehyde in the product analysed.

Results and Discussion

Chromatography

According to recent publications [16–21] and previous experience [22] concerning HPLC steroid ester analysis, a reversed-phase mode of chromatography (RP8 column) was chosen for the specific determination of nandrolone esters in pharmaceutical formulations. Preliminary studies were conducted to select the appropriate mobile phase. After several trials with mixtures of acetonitrile–water or methanol–water of

varying compositions, it was found convenient to use mixtures of methanol–water containing a small quantity of tetrahydrofuran (THF). Thus, the methanol–THF–water (82:3:15 v/v/v) solvent system was used for nandrolone phenylpropionate analysis (diphenyl was the internal standard), while methanol–THF–water (90:2:8 v/v/v) was the mobile phase for nandrolone decanoate and undecanoate analysis (the phenylpropionate as internal standard). Chromatograms representative of the separations obtained are reported in Figs 1 and 2, respectively. As can be seen, in each case an adequate separation between nandrolone alcohol (a potential impurity), internal standard and the analysed ester (the active ingredient) was achieved in a reasonable analysis time.

Nandrolone esters analysis

Under the described chromatographic conditions, some commercial oily injections containing a single nandrolone ester were analysed. For each drug a linear calibration curve was found over the studied concentration range. In Table 1 are summarized the data for the calibration curves and the system suitability.

Recovery studies were performed analysing synthetic oily solutions containing known amounts of the nandrolone esters **Ia**, **Ib** and **Ic**. With 85% ethanol as the extracting solvent [23], a quantitative recovery was achieved for all three esters (Table 2); using 90% methanol the recovery of the decanoate and undecanoate esters was reduced to

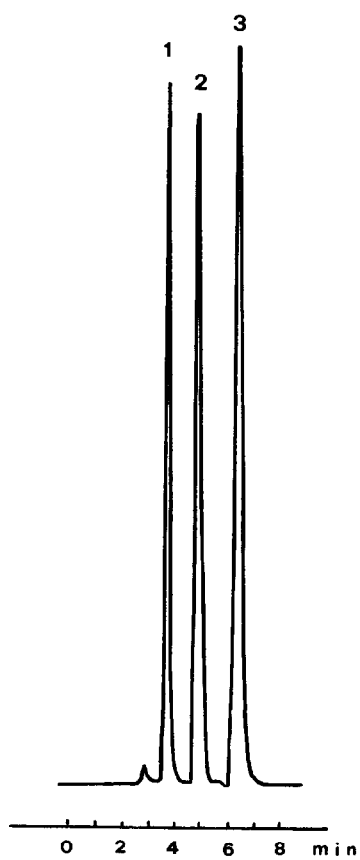

Figure 1
Separation of nandrolone (1), diphenyl (internal standard) (2), and nandrolone phenylpropionate (3). Column: LiChrosorb RP-8 (7 μm); mobile phase: methanol–THF–water (82:3:15 v/v) at a flow rate of 1 ml min^{-1}. Detection: 240 nm, 0.1 a.u.f.s.

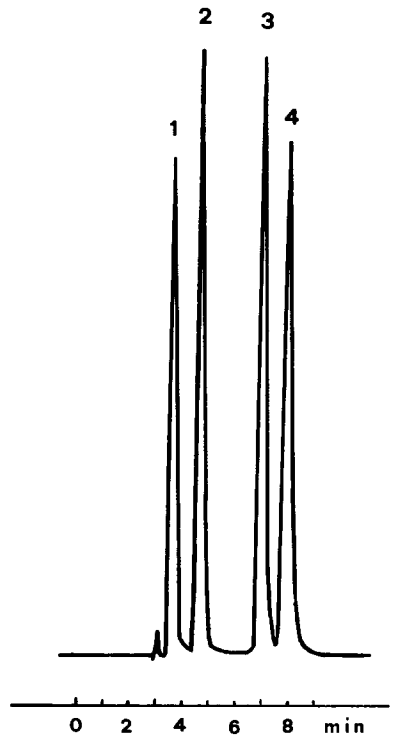

Figure 2
Separation of nandrolone (1) and its phenylpropionate (2), decanoate (3) and undecanoate (4) esters. Column as in Fig. 1; mobile phase: methanol–THF–water (90:2:8 v/v) at a flow rate of 1 ml min^{-1}. Detection: 240 nm, 0.1 a.u.f.s.

Table 2
Precision and accuracy of the assay for nandrolone esters **Ia**, **Ib** and **Ic** in synthetic oily solutions by HPLC

Steroid ester	Steroid ester content (mg ml^{-1})	Found* (mg ml^{-1})	Recovery (%)	R.S.D. (%)
Ia	25	24.85	99.40	0.57
	10	9.95	99.50	0.61
Ib	25	25.10	100.40	0.70
Ic	80	79.80	99.75	0.60

* Average of five determinations.

≈87%. Commercially available oily injections were then analysed by the proposed HPLC procedure and the assay results are reported in Table 3. As can be seen, the data obtained are in good agreement with the label claim and demonstrate that the nandrolone esters in current as well as in aged injections have not undergone appreciable degradation to nandrolone. Benzyl alcohol, used as an antimicrobial agent in some of the formulations analysed, eluted early and did not interfere with the analysis. A typical chromatogram of the extract from a commercial nandrolone decanoate injection is shown in Fig. 3. For comparison purposes, all commercial formulations were also analysed by the spectrophotometric (isoniazid reagent) USP method. The results obtained (Table 3), when compared with those from the HPLC procedure and the

Table 3
Assay results for the analysis of nandrolone esters in commercial oily injections by HPLC and USP methods

Drug product	Nandrolone ester	Label claim (mg ml^{-1})	HPLC*		USP*	
			Found†	R.S.D. (%)‡	Found†	R.S.D. (%)‡
A (aged)	Phenylpropionate	25	98.80	0.40	107.60	1.80
B	Phenylpropionate	25	98.85	0.45	101.60	2.20
C	Phenylpropionate	10	98.60	1.02	113.80	2.10
D	Decanoate	5	99.40	0.50	110.90	0.90
E	Decanoate	25	100.80	0.65	100.65	0.95
F	Undecanoate	80.5	98.90	0.42	99.50	1.03

*Average of five determination.
†Expressed as a percentage of label claim.
‡R.S.D. = relative standard deviation (%).

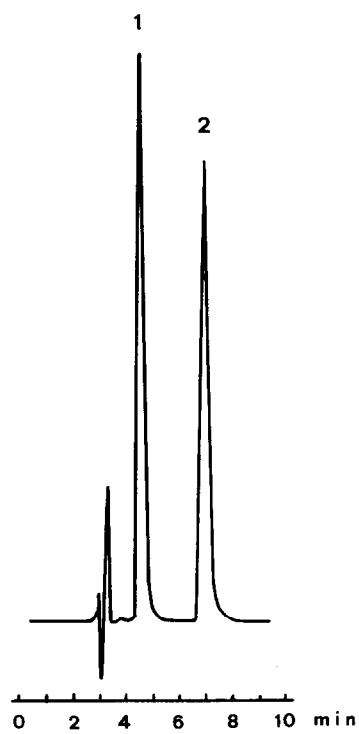

Figure 3
Typical chromatogram obtained for an extract from nandrolone decanoate injection (Product E); 1, nandrolone phenylpropionate (internal standard); 2, nandrolone decanoate. Chromatographic conditions as in Fig. 2.

labelled amounts, showed high recovery values for some formulations, suggesting some interference. Such an effect was particularly observed when aged (product A) and low-dosage (products C and D) formulations were analysed. It was shown that the spectrophotometric procedure, based on the direct dilution of the sample followed by reaction with isoniazid, may be affected by interference from the oily vehicle [6]. This was confirmed in the present work: the analysis of synthetic preparations of nandrolone phenylpropionate in peanut oil (10 mg ml^{-1} of steroid ester) gave recovery values of $\simeq$104% by the USP method. This interference, however, was found to be not significant

for synthetic preparations containing a higher level ($\geq$25 mg ml^{-1}) of steroid ester, owing to higher dilution of the sample.

Benzaldehyde interference

Since the interference due to the oily vehicle does not completely account for the data obtained (Table 3), the presence of benzaldehyde, a product of oxidative degradation of the benzyl alcohol used in this type of formulation, was investigated as a possible source of errors. The benzaldehyde isonicotinyl hydrazone was synthesized and its UV-visible spectroscopic properties were examined. In Fig. 4 are reported the spectra of benzaldehyde isonicotinyl hydrazone in methanol–chloroform (1:1 v/v) and in acidic methanol–chloroform (1:1 v/v) solvent systems. The chosen ratio of methanol to chloroform corresponds to that of the final analytical solution in the USP assay and the acidic methanol (0.63 ml of hydrochloric acid in 500 ml of methanol) was the same used for the isoniazid reagent preparation according to USP. In neutral solution (spectrum A) the benzaldehyde isonicotinyl hydrazone exhibits an absorption maximum near 302 nm (log ϵ = 4.350) with no absorption at 380 nm, the selected measurement wavelength in the USP assay. In acidic medium, however, the spectrum profile was significantly changed (spectrum B) and a remarkable absorption (log ϵ = 3.40) was observed at 380 nm. Therefore, under the experimental conditions predicted by the pharmacopoeial procedure for the nandrolone esters determination, the benzaldehyde isonicotinyl hydrazone formed could be a source of error. This was verified analysing (isoniazid method) synthetic solutions in peanut oil, containing a known amount of nandrolone phenylpropionate, spiked with increasing amounts of benzaldehyde. The assay results (Table 4) showed that the benzaldehyde was responsible for a positive interference in the steroid ester analysis. Therefore, the presence of benzaldehyde in those commercial formulations containing benzyl alcohol was investigated by HPLC. The mobile phase composition was modified to achieve an optimum separation between benzyl alcohol, benzaldehyde and acetophenone (the internal standard) and an adequate resolution was obtained when methanol–water (52:48 v/v) was used as mobile phase (Fig. 5). Under these conditions the steroid esters were completely retained. The benzaldehyde peak in the chromatograms from the commercial samples was recognized on the basis of its

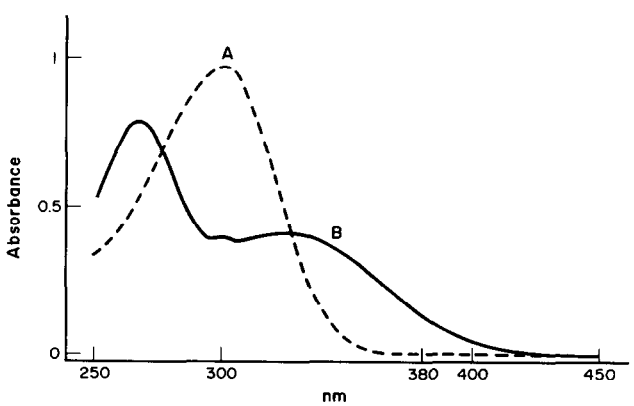

Figure 4
UV-visible spectrum of benzaldehyde isonicotinyl hydrazone in methanol–chloroform 1:1 (A) and in acidic methanol–chloroform 1:1 (B). Concentration = 4.4×10^{-5} M.

Table 4
Effect of added benzaldehyde on the spectrophotometric USP assay of nandrolone phenylpropionate in synthetic peanut oil solutions

Benzaldehyde added (mg ml^{-1})	Steroid ester (mg ml^{-1})	Absorbance	Found (%) (steroid ester)
—	—	0.030	—
0.50	—	0.056	—
1.00	—	0.078	—
1.50	—	0.108	—
—	10	0.433	103.1
0.50	10	0.456	108.5
0.75	10	0.468	111.4
1.00	10	0.484	115.2

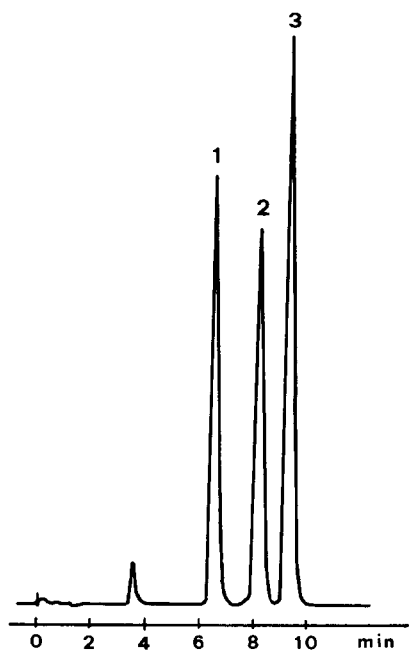

Figure 5
Separation of benzyl alcohol (1), benzaldehyde (2) and acetophenone (the internal standard) (3). Column: LiChrosorb RP-8 (7 μm); mobile phase: methanol–water (52:48) at a flow rate of 1 ml min^{-1}. Detection: 240 nm, 0.05 a.u.f.s.

retention time (R_t = 8.1 min) and the benzaldehyde identity was verified by determining the peak height ratio at two different wavelengths (283 and 252 nm). Indeed, benzaldehyde in 50% methanol exhibits absorption maxima at 252 nm (log ϵ = 4.00) and 283 nm (log ϵ = 3.20), while benzyl alcohol (λ_{max} at 258 nm; log ϵ = 2.33) shows no absorbance at 283 nm [24]. Thus, the peak height ratio (283/252) for the peak at R_t = 8.1 in the chromatograms from the sample extracts was compared with the same ratio obtained from a standard solution of benzaldehyde. No differences were found, thereby indicating that benzaldehyde was present in the examined commercial formulations.

For the quantitative determination of benzaldehyde the detector was adjusted to 240 nm in order to obtain a reduced peak from benzyl alcohol, and acetophenone was used as the internal standard. The concentration of benzaldehyde in each of the examined products was calculated by interpolating the linear ($y = 0.880x + 0.0022$; $r = 0.9999$; n =

5) calibration curve (peak height ratios of benzaldehyde/acetophenone versus the respective concentration ratios) found over the concentration range of 2–8 $\mu g\ ml^{-1}$ (method A). Alternatively, trace amounts of benzaldehyde were determined by using the regression lines (Fig. 6; $r > 0.98$) obtained from sample extracts, spiked with varying amounts of benzaldehyde (method B). The results obtained, expressed as mg of benzaldehyde per ml of oily solution, were as follows: product A: 0.81 mg ml^{-1}; product B: 0.20 mg ml^{-1}; product C: 0.63 mg ml^{-1} and product D: 0.067 mg ml^{-1}. These data allow an interpretation of the discrepancies between the USP and HPLC assay results (Table 3) to be offered. The high recovery value from the USP assay for product A is mainly due (Table 4) to the presence of benzaldehyde (0.81 mg ml^{-1}) derived from benzyl alcohol, and to a slight extent this effect was also involved in product B. For product D the only interference was caused by the oily vehicle whereas both the oily vehicle and benzaldehyde interfered with the analysis of the product C.

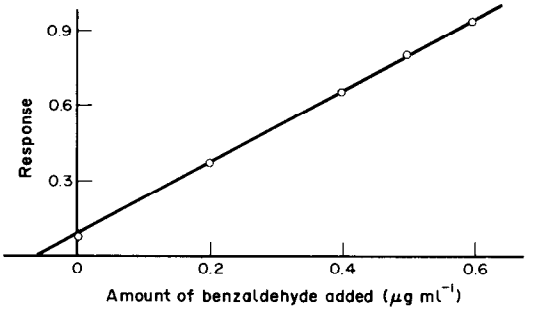

Figure 6
Plot of benzaldehyde/acetophenone peak height ratio versus amount of benzaldehyde added to the product D extract. The peak-height ratio determined by extrapolating the calibration plot to zero amount added, agreed with that obtained by direct analysis of the original extract. Chromatographic conditions as in Fig. 5.

Conclusion

The pharmacopoeial (USP, BP) method for the determination of nandrolone ester, based on the spectrophotometric determination of the hydrazone resulting from the reaction between steroid and isoniazid, was found to be of limited specificity when applied to the analysis of commercial oily formulations. The spectrophotometric response proved to be inflated because the oily vehicle and amounts of benzaldehyde, derived from the oxidative degradation of benzyl alcohol, provided a contribution to the absorbance values. The relative effect of these interferences was found to be dependent on the formulation type: the vehicle interference was significant for low-dosage formulations and that from benzaldehyde was high for aged formulations containing benzyl alcohol. In contrast, the proposed reversed-phase HPLC method proved to be rapid and specific for each nandrolone ester examined and suitable as a reliable monitor of drug content in commercial oily injections. The method may also be used as a simple test for the rapid identification of the various nandrolone esters with simultaneous detection of trace amounts of nandrolone alcohol (chromatographic conditions as in Fig. 2; detection limit: 0.2 $\mu g\ ml^{-1}$), as required by USP [4] and BP [5]. Moreover, by an appropriate adjustment of the mobile phase composition, the determination of trace benzaldehyde in those formulations containing benzyl alcohol as preservative may be performed.

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