Journal of Pharmaceutical & Biomedical Analysis
Vol. 3, No. 4, pp. 375–379, 1985
Printed in Great Britain

0731–7085/85 $3.00 + 0.00
Pergamon Press Ltd.

Short Communication

An HPLC method for the determination of the kinetics of hydrolysis of testosterone esters

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Keywords: *Testosterone; testosterone esters; reversed-phase high-performance liquid chromatography.*

Introduction

The employment of testosterone esters as pro-drugs has been examined by several authors [1, 2]. The esters are used to prolong hormone activity and are generally formulated in oily solution. Carignan *et al.* [3] have determined six testosterone esters in sesame oil by high-performance liquid chromatography (HPLC) using an RP8 column with methanol–tetrohydrofuran–water as the mobile phase. The authors believe that the stability of testosterone esters to *in vitro* hydrolysis should be an important criterion in the selection of a clinically useful compound and describe here an HPLC procedure suitable for investigations into the kinetics of the alkaline hydrolysis of testosterone esters. Previous workers [4] in stability studies of these compounds did not employ HPLC.

Experimental

Instrumentation

Chromatography was performed using a Perkin–Elmer model LC-65T liquid chromatograph equipped with a 2/2 pump module and fitted with a variable-wavelength UV detector and a Rheodyne injector model 7105. The detector was linked to a Perkin–Elmer data station, model 3600. Separations were performed on a 250 × 4.6 mm i.d. 10-μm LiChrosorb RP-18 column (E. Merck, Darmstadt, FRG). The mobile phase consisted of methanol–water (85:15, v/v) maintained at 40°C and at a flow-rate of 0.8 ml/min. The detector wavelength was 243 nm at an absorbance range of 0.01 a.u.f.s. The amount of sample injected was 10 μl in all cases, made in triplicate.

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Materials and reagents

Testosterone was obtained from Schering AG Berlin/Bergkaman. The acetate, benzoate, phenylacetate, phenylbutyrate and phenylvalerate esters of testosterone were synthesized by a method reported earlier [5]. The purity of the compounds were checked by HPLC [6] (one peak) and TLC [7] (one spot). HPLC grade methanol and dioxan were obtained from E. Merck, Darmstadt. Double-distilled water was used throughout. All other reagents were of analytical reagent quality.

Standard calibration solutions. Stock solutions (20 μg/ml in the mobile phase) of testosterone and each of the above esters were initially prepared separately. Each calibration solution was then prepared by mixing 5 ml of testosterone acetate (internal standard) stock solution and an appropriate aliquot of an ester stock solution and making up to 10 ml with the mobile phase. The calibrations were carried out using six concentrations of testosterone and each respective ester in the range of 1–8 μg/ml in the mobile phase.

Preliminary kinetic studies. Hydrolysis studies were carried out according to the method of Vesely *et al.* [4] with one-tenth of the quantity of the respective ester (0.0125 mmol) in 10 ml of dioxan–aqueous sodium hydroxide 0.025 M (70:30 v/v). The reactions were quenched at selected time intervals by the addition of 0.1 M HCl solution. The reaction mixture was made up to 50 ml with methanol. For each determination, 1 ml of the diluted solution was mixed with 10 ml of the internal standard solution (5 μg/ml of testosterone acetate in methanol) and diluted to 20 ml with methanol.

Results and Discussion

Separation

Figure 1(a) shows a representative chromatogram of the separations obtained for a 10 μl standard mixture containing 100 ng of each of the drugs studied quantitatively and the internal standard in the mobile phase. From the chromatograms it is clear that the components are well resolved and clearly separated from the internal standard. The overall chromatographic time of 18 min provides efficient assay capabilities. Peak shapes were improved by working at 40°C rather than at ambient temperature (25°C). Initial studies on the chromatographic behaviour of the steroidal esters indicated that the pH of the eluent exerted little influence on retentions. However, the percentage of methanol in the mobile phase was important and optimum separations were obtained using 85:15 v/v methanol–water mixture. The system described gives complete resolution of the compounds investigated.

Quantification

The concentrations of testosterone esters and testosterone were calculated by the peak area ratio of analyte to internal standard and comparison with calibration curves which were linear in the range 1–8 μg/ml injected (Table 1).

Precision and sensitivity

To examine the precision of the procedure ten determinations were performed on solutions of each of the above esters at a concentration of 5 μg/ml in the mobile phase. Relative Standard Deviations between 1.8 and 3.9% were obtained. Responses were

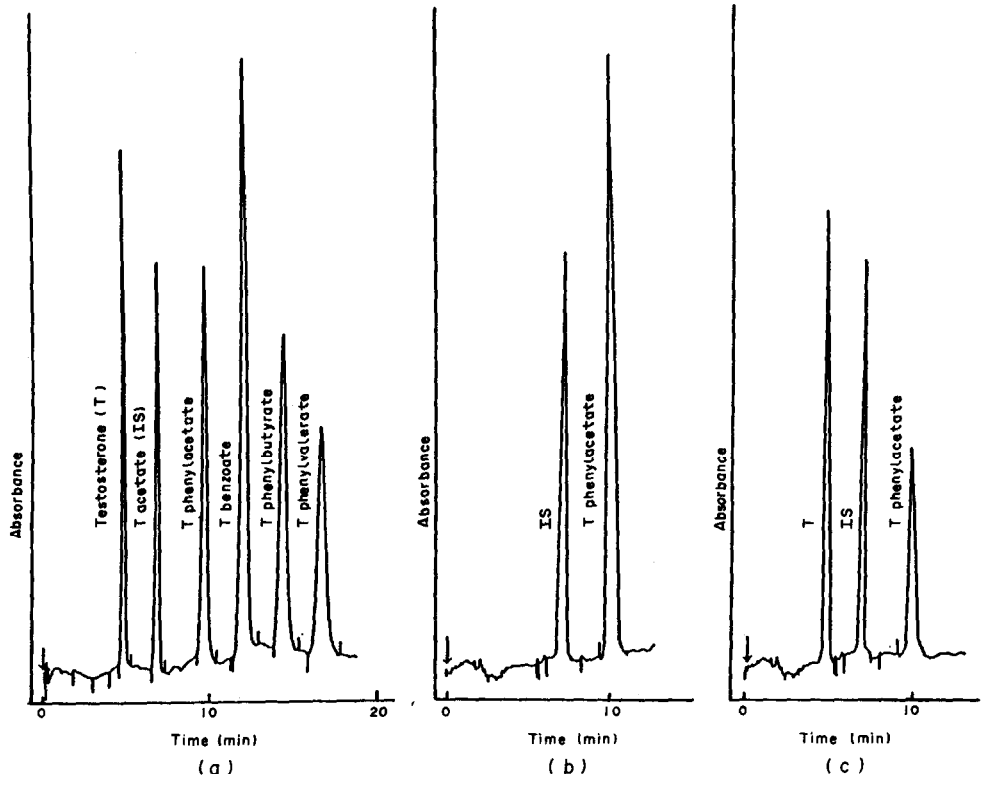

Figure 1
Representative chromatograms of (a) a mixture of testosterone and some of its esters, (b) a sample at the start ($t = 0$) of the kinetic studies on T. phenylacetate and (c) a sample when $t = 8$ h. Chromatographic conditions: see text.

Table 1
Regression data for calibration of testosterone and some of its esters

Compound*	Gradient $\times 10^4$	S.D. of gradient $\times 10^6$	Intercept $\times 10^2$	S.D. of intercept $\times 10^4$	r†
Testosterone (T)	2.28	2.95	−4.62	5.10	0.9998
T Phenylacetate	3.17	5.02	−2.19	6.19	0.9996
T Benzoate	2.18	3.43	−4.74	6.20	0.9997
T Phenylbutyrate	3.20	5.38	6.82	7.82	0.9993
T Phenylvalerate	3.61	12.11	−2.01	14.98	0.9982

*Linear range: 1–8 μg/ml.
†Correlation coefficient ($p = 0.95$; $n = 6$).

considered significant when the signal to noise ratio was greater than 3.0. In such conditions, using a detector sensitivity of 0.005 a.u.f.s., levels as low as 150 ng/ml (equivalent to 1.5 ng on-column) could be quantified. The regression data and correlation coefficients are listed in Table 1. For all cases the intercepts were not significantly different from zero ($p = 0.95$) and good linearity is observed.

Preliminary kinetic results

The suitability of the above chromatographic method to study the hydrolysis of testosterone esters was investigated using testosterone phenylacetate as the test substance. The first-order rate constants of alkaline hydrolysis of testosterone phenylacetate in dioxan–0.025 M NaOH (70:30, v/v) were determined at different temperatures, measuring the loss of ester and the appearance of testosterone, and are plotted in Fig. 2. Typical chromatograms obtained in the kinetic studies of this ester at $t = 0$ and $t =$ 8 h, respectively are shown in Figs 1(b) and (c). The Arrhenius plot for testosterone phenylacetate hydrolysis in dioxan–0.025 M sodium hydroxide (70:30, v/v) solvent system is shown in Fig. 3. The apparent activation energy and the first order rate constants were in good correlation with those obtained using the titration method described by Vesely *et al.* [4]. Thus the method that has been described permits the separation and determination of both testosterone and its ester derivatives within a single run and the total time required is minimal.

Further investigations into the kinetics of alkaline hydrolysis of testosterone esters are in progress.

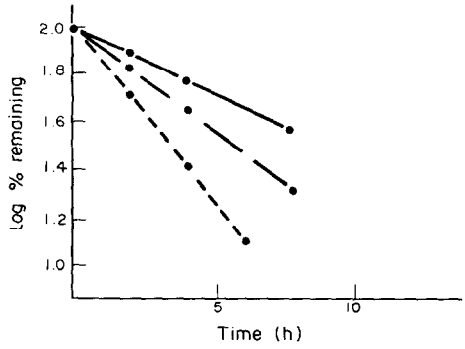

Figure 2
First-order plots for the degradation of testosterone phenylacetate in dioxan–0.025 M sodium hydroxide (70:30, v/v) solution at 30°C (———), 37°C (— —) and 45°C (– – –).

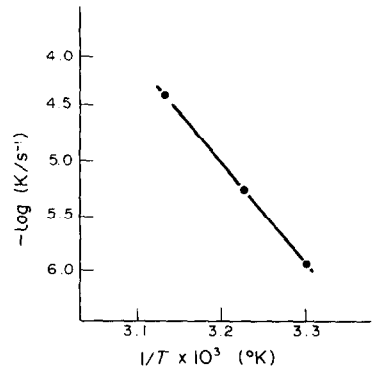

Figure 3
Arrhenius plot for testosterone phenylacetate hydrolysis in dioxan–0.025 M sodium hydroxide (70:30, v/v) solution.

Acknowledgement: This work was supported by a grant from the Singapore Turf Club.

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[*Received for review 24 May 1984; revised version received 19 July 1984*]