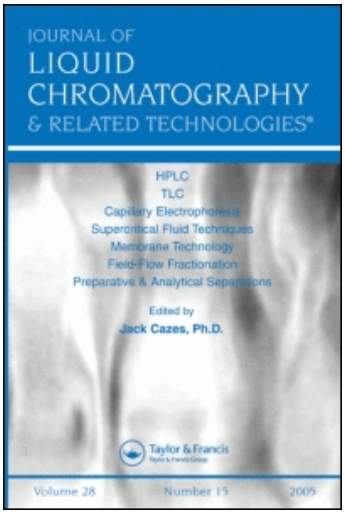

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DEVELOPMENT OF A HPLC METHOD FOR THE DETERMINATION OF 17β-ESTRADIOL-3-PHOSPHATE IN PHARMACEUTICAL PREPARATIONS

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ABSTRACT

A validated reversed-phase high-performance liquid chromatographic (HPLC) procedure for the analysis of 17ß-estradiol-3-phosphate is reported. In the development of this assay, several factors were evaluated including buffer ionic strength, mobile phase pH, ion-pairing concentration, organic composition, and column type. The described method is rapid and coupled with standard HPLC procedures leads to a selective, accurate, and reproducible assay. The peak area versus 17ß-estradiol-3-phosphate concentration is linear over the range of 0.1 - 100 μg/mL, with a detection limit of 0.02 μg/mL.

INTRODUCTION

An analytical method for the determination of the conjugated estrogen, 17ß-estradiol-3-phosphate (E_p), in the presence of 17ß-estradiol (E_2), estrone (E_1), and estriol (E_3) is reported. E_p can hydrolyze to E_2 and,

since E_2 is readily oxidized to E_1, which in turn can be hydrated to E_3 [1], the proposed method must be selective for these analogues.

The analyses of estrogen conjugates typically requires enzyme hydrolysis or solvolysis, which increases the time and cost of each analysis. Furthermore, following chemical cleavage, it is not possible to simultaneously measure both the conjugated and unconjugated species.

Several methods exist for the determination of estrogens in pharmaceutical preparations [2-12]. However, to our knowledge, no method exists for the determination of E_p in the presence of E_1, E_2, and E_3 and, moreover, that satisfies the USP XXII guidelines under Assay Category I [13]. Data elements required for Assay Category I include precision, accuracy, selectivity, range, linearity, and ruggedness. The method described herein for 17ß-estradiol-3-phosphate satisfies all of these requirements.

The development of this reversed-phase HPLC method required investigating several factors including buffer ionic strength, mobile phase pH, ion-pairing concentration, organic composition, and column type.

EXPERIMENTAL

Chemicals and Reagents

17ß-Estradiol-3-phosphate, disodium salt, was purchased from Research Plus (Bayonne, NJ, USA). Estrone, estriol, and 17ß-estradiol were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, 1 **N** hydrochloric acid, and ACS reagent grade potassium phosphate, monobasic, monohydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Tetrabutylammonium chloride hydrate (TBAC) was purchased from Aldrich (Milwaukee, WI, USA). The water was deionized

and distilled. All reagents were used without further purification.

Apparatus

The chromatographic system consisted of a Waters Model 600E system controller and pump, a WISP 712 autosampler, and a 486 variable-wavelength UV detector set at 220 nm (Waters Associates, Milford, MA, USA). Columns which were investigated included a Zorbax Rx C18 (4.6 x 250 mm, 5 μm, MAC-MOD Analytical, Inc., Chadds Ford, PA, USA), a Keystone CPS Hypersil-1 cyano (4.6 x 150 mm, 3 μm, Keystone Scientific, Inc., Bellefonte, PA, USA), a Keystone ODS Hypersil (4.6 x 250 mm, 5 μm) and an Alltech Adsorbosphere HS C18 (4.6 x 150 mm, 3μm, Alltech Associates, Inc., Deerfield, IL, USA). All columns were maintained at ambient temperature.

Mobile Phase

The mobile phase consisted of buffer-acetonitrile-methanol (35:15:50, V/V/V) adjusted to pH 3.0 (apparent) with 1**N** HCl. The buffer was comprised of 10 m**M** potassium phosphate, monobasic, monohydrate and 50 m**M** TBAC. The mobile phase was filtered through a 0.45 μm filter and degassed for 10 minutes and maintained under a helium atmosphere. The flow rate was 1.0 mL/minute with a typical operating pressure of **ca.** 93 bar.

Data Acquisition

The peak area of E_p was measured using a PE Nelson 900 series interface and down-loaded to a PE Nelson Turbochrom II workstation (Perkin-Elmer Corporation, Cupertino, CA, USA). The chromatographic data was automatically processed for peak area followed by a weighted (1/C) linear regression analysis.

Preparation of 17ß-Estradiol-3-Phosphate Solutions

An E_p stock solution was prepared at 100 µg/mL in water. Appropriate dilutions of the E_p stock solution were made with water to prepare standards ranging from 0.1 - 100 µg/mL.

System Suitability Requirements

The system suitability results are calculated according to Chromatography <621> of the USP XXII from typical chromatograms [14]. The instrument precision as determined by six successive injections of an E_p standard solution should provide a relative standard deviation (RSD) not greater than 1.0%. The tailing factor should not exceed 1.5 at 5% peak height. Finally, the resolution between the analyte peaks should be greater than 1.5.

RESULTS AND DISCUSSION

Chromatography

A reference solution containing E_p, E_1, E_2, and E_3 each at a concentration of 100 µg/mL in methanol/water (1:1, v/v) was used to verify that the method met all the suitability limits.

To obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of capacity factor (k') on mobile phase pH (Figure 2), KH_2PO_4 ionic strength (Figure 3), and TBAC concentration (Figure 4). By adjusting the organic composition from acetonitrile to methanol (Table 1), the dead time between E_1 and E_2 was reduced by approximately 4 minutes, while having little effect on E_p and E_3. However, increased band broadening was observed as the amount of methanol increased. Therefore, a compromise between the ratio of acetonitrile and methanol ensued. Finally, four different columns were evaluated in optimizing the method. The Keystone CPS Hypersil-1 cyano column was too

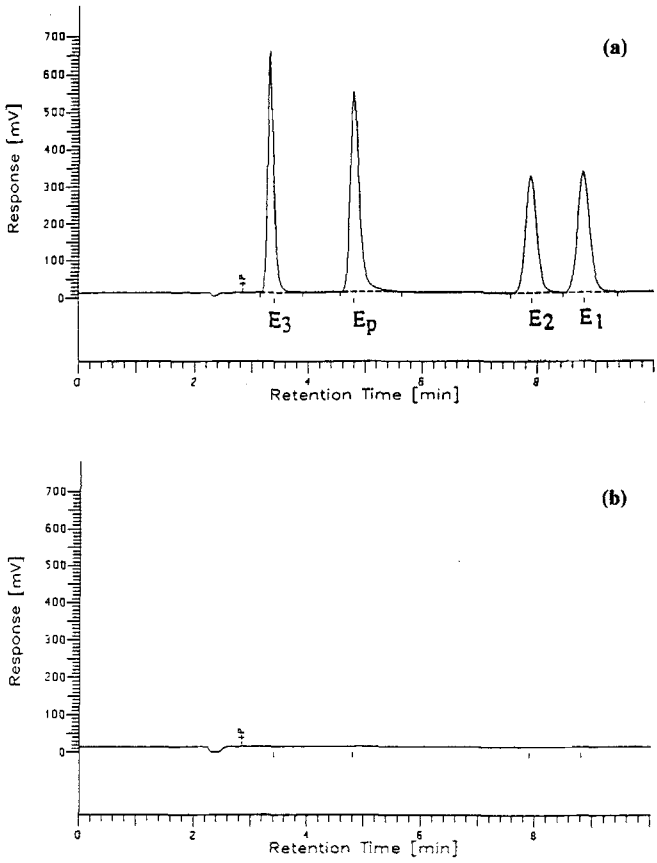

FIGURE 1. Typical chromatograms; (a) reference solution and (b) blank.

polar and did not provide sufficient retention of the analytes. The Keystone ODS Hypersil column provided adequate resolving power, but, severe peak tailing resulted with E_p. Both the Alltech Adsorbosphere HS C18 and Zorbax Rx C18 columns provided sufficient system suitability, however, the latter provided a shorter chromatographic run time and, consequently, was selected as the column of choice.

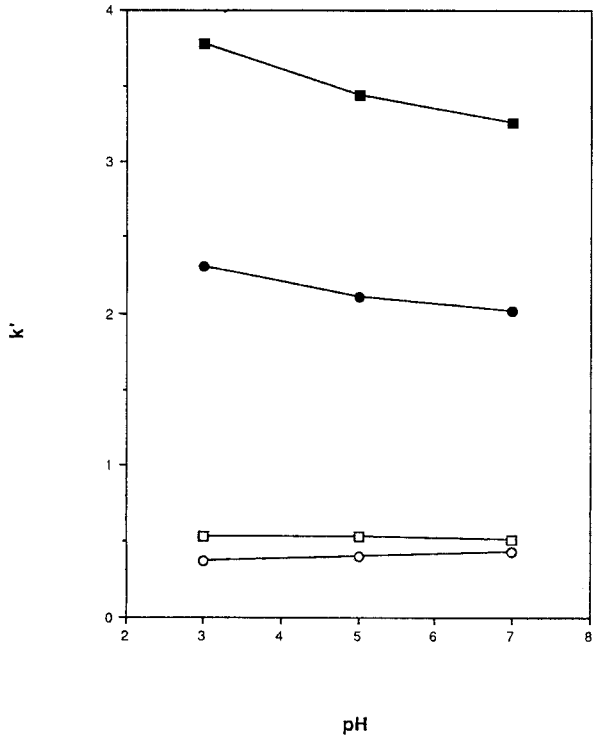

FIGURE 2. Effect of mobile phase pH on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

Typical chromatograms of a reference and blank solution are illustrated in Figure 1 using a Zorbax Rx C18 column and a 50 μL injection. The retention times of estriol, 17ß-estradiol-3-phosphate, 17ß-estradiol, and estrone were 3.3, 4.8, 7.9, and 8.8 minutes, respectively. The overall chromatographic run time was 10 minutes.

System Suitability

The column efficiency for E_p was 3856 theoretical plates. The tailing factor of E_p was 1.2. The resolution between E_3 and E_p, E_p and E_2, and E_2 and E_1 was 4.4,

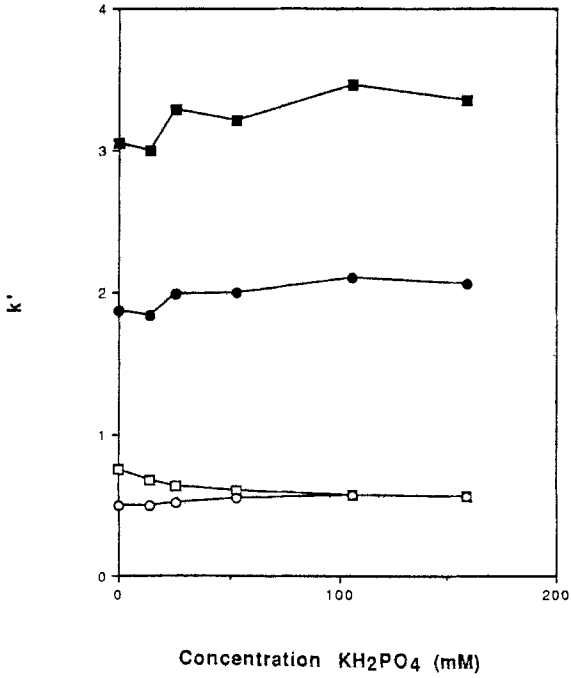

FIGURE 3. Effect of KH_2PO_4 ionic strength on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

7.8, and 2.0, respectively. The instrument precision, determined by 6 replicate injections of the E_p standard solution, exhibited a RSD of 0.3%.

Precision and Accuracy

The precision (RSD) and accuracy (relative error, RE) was determined by analyzing 17ß-estradiol-3-phosphate standards ranging from 0.1 - 100 μg/mL, in replicates of six (Table 2).

Linearity

A linear response in peak area for E_p over the range of 0.1 - 100 μg/mL was observed. The correlation coeffi-

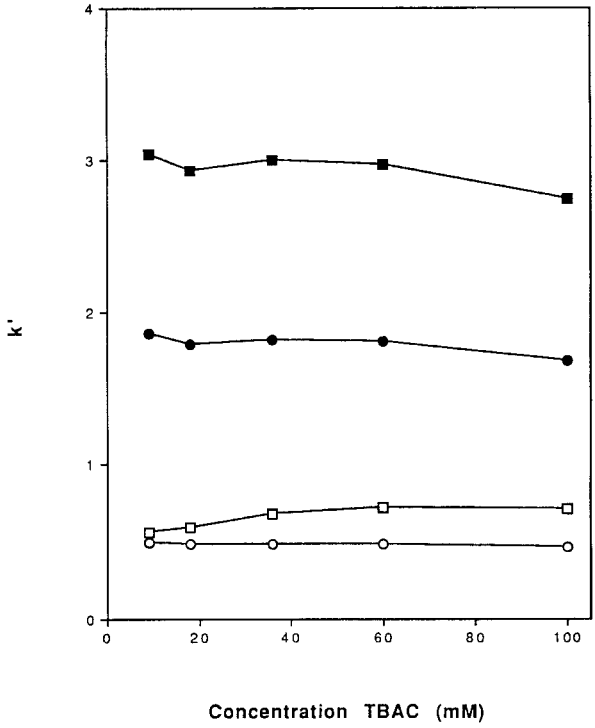

FIGURE 4. Effect of TBAC concentration on k': (■) E_1, (●) E_2, (□) E_p and (○) E_3.

TABLE 1

Effect of Mobile Phase Composition on Retention Time

Composition (%)			Retention Time (min)			
Buffer	ACN	MeOH	E_3	E_p	E_2	E_1
60	40	--	3.1	4.4	8.8	14.3
35	--	65	3.6	5.5	9.7	10.2
55	40	5	3.0	4.0	7.6	11.7
35	10	55	3.3	4.8	7.9	8.9
35	15	50	3.2	4.6	7.3	8.2

TABLE 2

Accuracy and Precision of 17ß-Estradiol-3-Phosphate

Nominal Conc. (μg/mL)	n	Mean Found Conc. (μg/mL)	%RSD	%RE
0.10	6	0.12	8.0	20.0
0.25	6	0.24	2.9	-4.0
0.50	6	0.48	1.8	-4.0
1.00	6	1.01	1.4	1.0
5.00	6	4.98	0.9	-0.4
10.00	6	10.20	1.0	2.0
50.00	6	49.83	0.5	-0.3
100.00	6	100.07	0.3	0.1

cients were 0.998 or better (n=6). The limit of detection for E_p, defined as 3 times the signal-to-noise ratio, was 0.02 μg/mL.

Conclusion

The described assay for the analysis of 17ß-estradiol-3-phosphate in the presence of estriol, 17ß-estradiol, and estrone is selective, sensitive, and robust. With exception of the limit of quantitation, the precision of the method is below 3.0%, while the accuracy is within 4.0%. The method is rapid and requires no sample pretreatment, resulting in **ca.** 100 samples being analyzed daily. More than 1000 injections can be made on a single analytical column with minimal loss in chromatographic integrity.

Furthermore, it is anticipated that this method could be used for the analysis of 17ß-estradiol-3-phosphate in pharmaceutical preparations designed for hormone replacement therapy.

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