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QUANTITATIVE DETERMINATION OF PROSTAGLANDINS A_1 AND B_1 IN ALPROSTADIL (PGE_1) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An absorption high-performance liquid chromatography assay tor the quantitative determination of prostaglandins A_1 and B_1 in alprostadil (prostaglandin E_1) and in Prostin VR Pediatric® Sterile Solution was developed. Prostaglandins A_1 and B_1 have been shown to be the major degradation products of prostaglandin E_1. The adsorption system provided baseline resolution of the 2-naphthacyl esters of prostaglandin A_1 from prostaglandin B_1. Derivatization conditions providing maximal response for prostaglandins A_1 and B_1 while minimizing the conversion of prostaglandin E_1 to prostaglandins A_1 and B_1 were established.

INTRODUCTION

To control the quality of any bulk chemical or pharmaceutical formulation it is necessary to quantitate the major component and any related compounds present. In a previous paper[1] the authors described a high-performance liquid chromatography (HPLC) method for the quantitation of alprostadil, prostaglandin E_1, and several related compounds. The major degradation pathway of alprostadil is dehydration to prostaglandin A_1 (PGA_1) which subsequently rearranges to prostaglandin B_1 (PGB_1)[2,3]. A method of quantitating the levels of PGA_1 and PGB_1 in alprostadil and pharmaceutical formulations is essential. Structures of the compounds of interest are shown in Fig. 1. This report describes a method of accomplishing this for the bulk drug and for Prostin VR Pediatric® Sterile Solution which was recently approved by the United States Food and Drug Administration for use in maintaining the patency of the ductus arteriosus in neonatal infants until corrective surgery can be performed.

A variety of analytical methods such as gas chromatography, gas chromatography–mass spectrometry, radioimmunoassay and HPLC[4,5] have been used to quantitate prostaglandins. Of these methods HPLC is currently the method of choice except where very high sensitivity for biological samples is required. Prostaglandins are normally derivatized to enhance sensitivity and improve chromatographic behavior[6–10]. Since the naphthacyl ester derivatives are less polar than most of the other commonly used derivatives it was anticipated that baseline separation of these very similar compounds could be achieved.

There is an extensive body of literature on the HPLC of prostaglandins, but only a few papers have explicitly directed themselves toward the separation of A and B prostaglandins. Uekama *et al.* separated prostaglandins E_1, A_1 and B_1 on an anion-exchange column with cyclodextrin in the mobile phase[11]. Morozowich employed a TEAE-cellulose ion-exchange column to separate prostaglandins A_2 and B_2[12]. Merritt and Bronson separated prostaglandins A_2 and B_2 and a variety of other prostaglandins as their *p*-nitrophenacyl esters on a silver loaded cation-exchange resin[13]. A variety of prostaglandins including A_1, B_1, A_2, and B_2 were chromatographed on a reversed-phase system with detection at 208 nm by Inayama *et al.*; however, this system was not capable of separating A_1 from B_1 or A_2 from B_2[14].

EXPERIMENTAL

HPLC conditions

A modular HPLC system consisting of an Altex Model 110 pump, a loop injection valve, a Tracor Model 770 autosampler or an in-house designed and built autosampler, and an LDC UV Monitor III Model 1203 fixed-wavelength detector was employed. Detection was performed at 254 nm. A Brownlee LiChrosorb Si-100 column (25 × 4.6 nm I.D.) was used. The mobile phase consisted of 1000 ml of methylene chloride, 7.5 ml of *tert.*-amyl alcohol and 1.0 ml of deionized water. The mobile phase was filtered and degassed before use. The flow-rate was 2.0 ml/min and the injection volume was 25 μl.

Reagents and chemicals

Samples of PGA_1, PGB_1 and 13,14-dihydro-PGE_1 were obtained from F. H. Lincoln, Experimental Science I, The Upjohn Company. Methylene chloride was Burdick & Jackson distilled-in-glass solvent. *tert.*-Amyl alcohol was obtained from Fisher, Aldrich and from Riedel-De Haen (Seelze-Hannover) through Crescent Chemical. The majority of lots of *tert.*-amyl alcohol obtained from Fisher and Aldrich (regular and Gold label) were contaminated by UV-absorbing impurities. These lots were unsuitable for use in the mobile phase as they produced background absorption so high it could not be balanced out by the detector. Only two out of seven lots obtained from Fisher or Aldrich were acceptable. The alcohol from Riedel-De Haen was much cleaner and gave no background problems. Attempts at removing these impurities were unsuccessful. Analysis by vapor phase chromatography show the presence of several impurities. The identity of these impurities and a method to remove them is under investigation.

N,N-Di-isopropylethylamine (DIPEA) was obtained from Aldrich and was used as received. α-Bromo-2'-acetonaphthone (α-BAN) was obtained from Aldrich and was recrystallized from carton tetrachloride with carbon treatment before use. α-BAN and DIPEA were used as solutions in acetonitrile at 20 mg/ml and 10 μl/ml, respectively, unless otherwise stated. In all instances 200 μl of the α-BAN solution and 100 μl of the DIPEA solution were used. These solutions were prepared fresh daily. For the derivatization studies all points are an average of three or four samples.

Internal standard solution

Prepare a solution containing 0.02 mg of methyltestosterone per milliliter in methylene chloride.

Standard preparation

Accurately prepare a 0.1 mg/ml solution of PGA_1 in methylene chloride. Transfer a volume equivalent to 0.2 mg to a suitable container and gently evaporate to dryness under a stream of nitrogen.

Sample preparation: bulk drug

Accurately weigh *ca.* 2 mg of alprostadil, transfer to a suitable container, dissolve in *ca.* 2 ml of absolute ethanol, and gently evaporate to dryness under a stream of nitrogen.

Sample preparation: sterile solution

Transfer an accurately known volume equivalent to *ca.* 2 mg of alprostadil to a suitable container, and gently evaporate to dryness under a stream of nitrogen.

Procedure

Add *ca.* 200 μl of acetonaphthone solution to each standard and sample vial and swirl to wash down the sides. Add 100 μl of di-isopropylethylamine solution and swirl again. Allow to stand at room temperature for at least 90 min than evaporate to dryness under a stream of nitrogen. Redissolve the sample preparation in 2.0 ml of internal standard solution and the standard preparations in 20.0 ml of internal standard solution. Chromatograph the solutions using the conditions previously outlined. (Samples should be chromatographed within one day of derivatization.)

Calculations were based on peak height ratios (PHR) of prostaglandin to internal standard. The PGA_1 standard was used to quantitate both PGA_1 and PGB_1.

RESULTS AND DISCUSSION

PGA_1 and PGB_1 are structurally very similar (see Fig. 1) and are of similar polarities. PGE_1 on the other hand is significantly more polar than PGA_1 and PGB_1. Hence, any isocratic HPLC system capable of separating PGA_1 from PGB_1 either retains the PGE_1 very strongly (absorption) or elutes it in the void volume (reversed

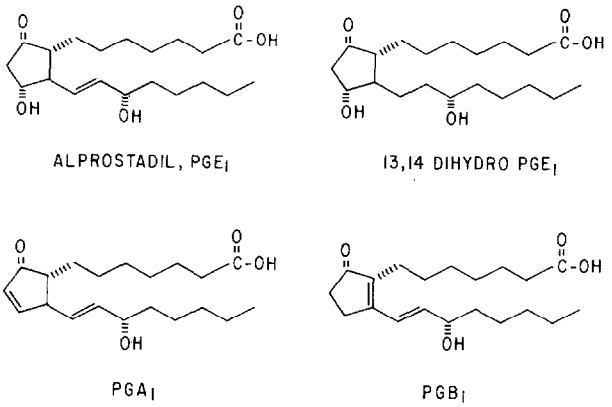

Fig. 1. Structures of PGE_1 (separation factor, S.F. = *ca.* 25), PGA_1 (S.F. = 1.0), PGB_1 (S.F. = 1.18), and 13,14-dihydro-PGE_1 (S.F. = 1.25).

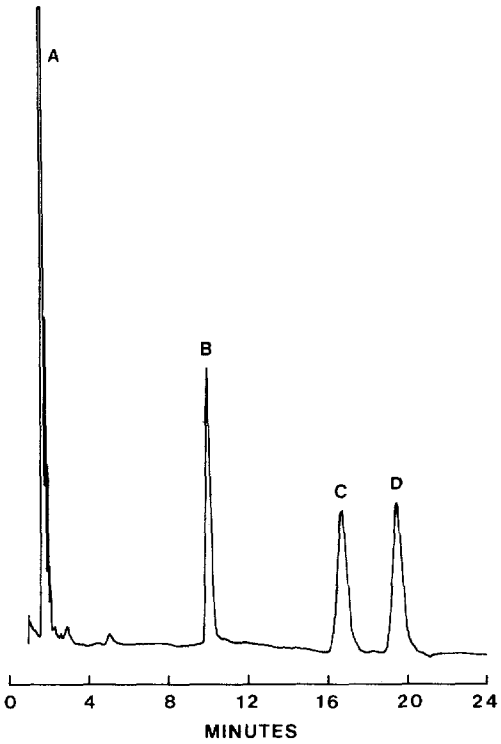

Fig. 2. Chromatogram of PGA_1 and PGB_1 in alprostadil. Peaks: A = excess derivatizing reagent; B = internal standard, methyl testosterone; C = PGA_1; D = PGB_1.

phase). In the adsorption system described in this paper alprostadil has a retention time *ca.* 25 times that of PGA_1. Generally, 20 to 22 injections can be made before interference by alprostadil occurs. This system provides baseline resolution of PGA_1 from PGB_1 as shown in Fig. 2. An analog of PGE_1, 13,14-dihydro-PGE_1, elutes after PGB_1, but is not completely resolved from it. These peaks can be separated by reducing the polarity of the mobile phase; however, to achieve baseline separation the run time becomes excessively long. Further, increasing the run time increases the detection limit

TABLE I

PEAK HEIGHT RATIO FOR PGA_1 STANDARDS DERIVATIZED WITH VARIOUS AMOUNTS OF DIPEA

Derivatized at room temperature, 21–23°C.

Reaction time (min)	*Concentration of DIPEA (μl/ml acetonitrile)*		
	10	*15*	*20*
30	1.23	1.24	1.23
60	1.23	1.24	1.23
90	1.22	1.22	1.22
120	1.22	1.22	1.22

TABLE II

PEAK HEIGHT RATIO FOR TRACE AMOUNTS (0.5%) OF PGA_1 in PGE_1 DERIVATIZED WITH VARIOUS AMOUNTS OF DIPEA

Derivatized at room temperature, 21–23°C.

Reaction time (min)	*Concentration of DIPEA (μl/ml acetonitrile)*		
	10	*15*	*20*
30	0.185	0.219	0.234
60	0.233	0.250	0.274
90	0.241	0.265	0.295
120	0.241	0.260	0.277

and decreases the sensitivity. Reporting the sum of PGB_1 + 13,14-dihydro-PGE_1 is adequate, since 13,14-dihydro-PGE_1 is not a degradation product of PGE_1.

Two considerations complicate the selection of optimal conditions for the derivatization. First, the desired limit of detection for PGA_1 and PGB_1 corresponds to less than one thousandth of the amount of PGE_1 present. Insuring complete derivatization in the presence of this large excess of PGE_1 which competes for the derivatizing reagent is a potential problem. Second, PGA_1 and PGB_1 are degradation products of PGE_1 and could form during the derivatization reaction. It is known that this degradation proceeds more rapidly in acidic or alkaline environments[2,3]. Since DIPEA is used as a catalyst for the derivatization reaction, the amount used must be carefully controlled to minimize the formation of PGA_1 and PGB_1 during the derivatization process.

The effect of the reaction temperature, time and the concentrations of reagent (α-BAN) and catalyst (DIPEA) on the derivatization reaction were examined. Table I illustrates the effect of varying the concentration of DIPEA and reaction time on the derivatization of PGA_1 standards. The PHRs of the PGA_1 peak to the internal standard peak approached a constant value after 30 min and were identical for all three DIPEA concentrations employed, indicating that complete derivatization was obtained.

This experiment was repeated on PGE_1 which contained 0.5% PGA_1. When these samples were derivatized under the same conditions two interesting observations were noted (Table II). For all concentrations of DIPEA used, maximum response was not reached until 90 min. Additionally, the PHRs at different DIPEA concentrations did not converge to a single value but increased as the DIPEA concentration increased. The longer reaction time required for complete derivatization was due to competition

TABLE III

PERCENTAGE RECOVERY OF PGA_1 SPIKES (1.0%) DERIVATIZED WITH VARIOUS CONCENTRATIONS OF α-BAN

90-min derivatization for all samples, at room temperature, 21–23°C.

Concentration of α-BAN (mg/ml acetonitrile)	*Recovery of PGA_1 in the presence of excess PGE_1 (%)*
20	84.7
40	89.8
60	91.9
80	91.5
100	93.0

with the 200-fold excess of PGE_1 present. The data in Table I for PGA_1 alone demonstrated the additional PGA_1 observed was due not to more complete derivatization but rather to dehydration of PGE_1 to PGA_1. At higher DIPEA concentrations, a slight decrease in the PHR was noted at 120 min. The reason for this decrease is not known. This study demonstrated that the concentration of DIPEA used should be kept to a minimum to minimize the dehydration of PGE_1 to PGA_1.

The effects of the concentration of α-BAN on the recovery of PGA_1 from PGE_1 was determined by spiking PGE_1 samples with PGA_1 (1.0%) and derivatizing them with α-BAN solutions with concentrations ranging from 20 to 100 mg/ml at room temperature for 90 min. Unspiked PGE_1 samples were run under each condition to determine the amount of PGA_1 initially present in the PGE_1 and this amount was subtracted from each sample. Results are summarized in Table III. The data indicates that complete derivatization is not achieved with any of the α-BAN concentrations used (for a 90-min derivatization) although increasing the α-BAN concentration does help improve recoveries somewhat.

To determine the effect of temperature on the derivatization reaction six samples of PGE_1 spiked with PGA_1 were derivatized at room temperature and six at 45°C. At room temperature (21–23°C) the mean PGA_1 result was 0.46% with a relative standard deviation of 8%. The mean PGA_1 result after derivatization at 45°C was 0.76% with a relative standard deviation of 13%. In light of the data already presented, it is evident that this apparent increase in PGA_1 is not due to more complete derivatization but to the formation of PGA_1 due to the elevated temperature.

On the basis of this data, the following derivatization conditions are recommended for quantitating prostaglandins A_1 and B_1 in alprostadil: 200 μl of 20 mg/ml α-BAN solution, 100 μl of 10 μl/ml DIPEA solution, and derivatize for 90 min at room temperature. These conditions provide the best compromise between maximal derivatization and generation of PGA_1 by degradation of alprostadil. While it could be argued that using a higher concentration of α-BAN should improve the recovery of PGA_1 slightly, it was also found to give larger relative standard deviations for replicate samples, *i.e.* it was less reproducible. When the sample was blown to dryness after the derivatization, the excess derivatizing reagent often formed a crusty material on the side of the vial. This material occasionally does not completely redissolve in methylene chloride even after vigorous shaking and sonication. Drug could be occluded in this material resulting in low recoveries. The more α-BAN used, the more noticeable this problem became. In the author's opinion the loss in precision is not worth the slight improvement in recovery that increasing the concentration of α-BAN might afford.

The overall precision of this assay was acceptable for the low percentages of material being determined. Six replicate assays on a sample of alprostadil gave a mean PGA_1 result of 0.46% with a relative standard deviation of 8.0%.

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