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A chromatographic method for the quantification of prostaglandin E_1 and prostaglandin A_1 encapsulated in an intravenous lipid formulation

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Abstract

Peripheral vascular disease is a common ailment of the aged and diabetic communities. As the numbers of these individuals increase, the need for therapeutic interventions will continue to grow. One of the possible therapies is the use of prostaglandins (PGE_1, prostacyclin and Iloprost) to decrease the vascular tone and increase vascular blood flow. Due to the hydrophobicity of the prostaglandins and prostaglandin analogues, various vehicles have been utilized to maintain the active pharmaceutical ingredient in a stable solution, e.g. α-cyclodextrin (Alprostadil, Edex™) or emulsified lipid vehicles. In our laboratory, we designed a method for separating and assaying lipid-encapsulated PGE_1. Utilizing organic extraction, automated solid-phase extraction and precipitation techniques, we validated the measurement of the PGE_1 and PGA_1 content of the clinical drug formulation in the microgram per milliliter concentration range with an high performance liquid chromatography (HPLC) assay.

Keywords: Prostaglandin E_1; Prostaglandin A_1; Encapsulated intravenous lipid formulation; HPLC

1. Introduction

Prostaglandin E_1 (PGE_1) is a naturally occurring prostanoid which may be used as an intravenous therapeutic agent for the treatment of peripheral vascular occlusive disease and other disorders [1–3]. PGE_1 appears to limit necrotic damage caused by restricted circulation and subsequent loss of tissue in, for example, Fontaine stage IV patients [1,2]. However, the lipophilicity and stability of PGE_1 necessitates its encapsulation in a lipid emulsion vehicle for intravenous application. Thus, the vehicle presents a barrier for the quantitative determination of encapsulated PGE_1 and its primary degradation product, prostaglandin A_1 (PGA_1).

The lipid vehicle, composed of a mixture of soybean oil, lecithin, oleic acid and glycerol, re-

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quired a novel approach for measuring the concentration of PGE_1 and PGA_1 contained within the vehicle. The vehicle's large mass of oils and phospholipids, compared with the mass of the prostanoids, dominated the separation chemistry and was a major source of interference in quantification. In addition, PGA_1 which is the major degradation contaminant, results from the conversion of PGE_1 to PGA_1 in the presence of water at temperatures above 5 °C, and is the limiting factor for product stability [4]. At higher temperatures (e.g. 60 °C), the conversion of both PGE_1 and PGA_1 continues to prostaglandin B_1 (PGB_1) in the presence of hydroxide ion.

This report details a rapid automated procedure for simultaneously quantitating the concentrations of PGE_1 and PGA_1 within a lipid vesicle formulation. Quantitative determination of PGE_1 and PGA_1 was performed by reversed-phase high-performance liquid chromatography (HPLC) after organic extraction and automated normal-phase solid phase extraction (SPE). The assay is linear and reproducible within a range of 2.5–6.0 μg/ml for PGE_1 and 1.0–3.0 μg/ml for PGA_1.

2. Materials and methods

Lipid encapsulated PGE_1 (lot number 014S1) and placebo (lot number 014N1) were obtained from Taisho Pharmaceutical Company, Ltd., Tokyo, Japan. Alprostadil (USP standard 01600) was obtained from the United States Pharmacopoeia, Rockville, MD, USA. PGA_1 and PGB_1 were obtained from Cayman Chemical Company, Ann Arbor, MI, USA. HPLC grade organic solvents were obtained from Burdick and Jackson (Muskegon, MI) with the exception of absolute ethanol that was obtained from Quantum Chemical Company (Tuscola, IL). PGB_1, used as an internal standard, was made by dissolving 1 mg of PGB_1 into 50 ml of absolute ethanol. The resulting solution (20 μg/ml) was stored at −20 °C for up to 1 month. All extractions were performed using borosilicate glassware when appropriate. SPE cartridges containing 1 g of normal silica in a 3 cc SPE cartridge housing were from Waters Corporation (Milford, MA).

2.1. Organic extraction of samples

Encapsulated PGE_1 or placebo samples (0.5 ml) were added to borosilicate glass centrifuge tubes in triplicate. Ethyl acetate (5.0 ml), ethanol (0.3 ml), PGB_1 internal standard (0.1 ml) and 1.0 N HCl (0.05 ml) were sequentially added to each tube to break the emulsion and trap the prostanoids in the organic layer. Each tube was capped, vortexed for 15 s and then centrifuged at $500 \times g$ for 15 min in a Beckman TJ-6 tabletop centrifuge (Fullerton, CA), to separate the phases. Following the centrifugation, each upper organic layer was transferred to a separate 16×100 mm borosilicate centrifuge tube into which 0.2 ml of 0.155 M sodium phosphate (pH 7.7) was added. The contents of the tubes were dried by a stream of nitrogen in a Turbovap® LV (Zymark Corporation, Hopkinton, MA) at 25 °C. The initial drying sequence consisted of a 10 psi nitrogen stream for 10 min, followed by a 20 psi nitrogen flow for 50 min. Care was taken to ensure that the liquid contents of each tube were not blown out of the tube and that the contents remained immersed in the 25 °C water bath. The tubes containing the resulting residue were then transferred to the BenchMate® II (Zymark Corporation) for SPE.

2.2. Solid phase extraction

The automated SPE of dried samples was performed with the use of a Zymark BenchMate® II equipped with the 3 cc SPE cartridge housing. The SPE cartridges were custom produced by Waters Corporation to contain 1 g of normal silica in a 3 cc SPE cartridge. The BenchMate® II is designed to measure the liquid volume dispensed into each tube by measuring the mass of the tube and liquid. The temperature of the laboratory was monitored to ensure minimal losses of liquid during extended extraction procedures. The BenchMate® II is capable of processing up to 100 SPE samples in a single load.

The BenchMate® II was configured to (1) sequentially remove the SPE cartridge and secure it in its appropriate retainer; (2) measure the weight of the sample; (3) remove sample and load the

SPE cartridge; (4) extract the SPE cartridge and collect the effluent; (5) discard the SPE cartridge; (6) weigh the captured effluent; (7) secure the extracted sample; and (8) repeat the process for the next sample with a new SPE cartridge and effluent tube.

Using the BenchMate® II, 5 ml of hexanes was added to the sample tube and vortexed. The SPE cartridge was conditioned with 10 ml of hexanes at a flow rate of 0.25 ml/s. The resuspended sample was applied to the SPE cartridge at 0.01 ml/s. The sample tube was rinsed with an additional 5 ml of hexanes which was then applied to the SPE cartridge as above. The SPE cartridge was cleansed of non-prostaglandin components by an application of 10 ml of diethyl ether/hexanes (3:1) at a flow rate of 0.25 ml/s. The wash eluate was then discarded. Prostaglandins were eluted from the SPE cartridge by sequential application of diethyl ether, methanol, and acetonitrile in 4 ml volumes at 0.01 ml/s. Following elution of the prostaglandins, the SPE cartridge was ejected and the system (tubing and syringes) was automatically cleaned and prepared for the next sample by the sequential washing with DMSO in water (20% v/v), methanol and hexanes in 5 ml volumes.

The completed samples were removed from the BenchMate® II and dried by a nitrogen stream in the Turbovap® LV at 25 °C in two stages. The first stage consisted of a 5 psi stream of nitrogen for 10 min followed by a second stage of a 20 psi stream of nitrogen for 50 min. The resulting residue was resuspended in mobile phase/ethanol (9:1) and transferred to a microcentrifuge tube. The samples were centrifuged for 1 min (not less than $10\,000 \times g$) and the resulting supernatants were transferred to brown glass HPLC autosampler vials and capped.

2.3. HPLC analysis

A Beckman System Gold HPLC (Beckman Instruments, Fullerton, CA), running GOLD software version 8.1, was used for the development and validation of this method. The HPLC hardware necessary for assaying each extracted prostanoid included a 507 autosampler (maintained at 5 °C), a 168 diode array detector (wavelength set at 278 nm), two 126 analytical pumps and a Waters post-column reaction system (Milford, MA), consisting of a column heating block set at 60 °C and a Teflon® coil. Two 15 cm Beckman Ultrasphere C18 columns preceded by a Beckman 2 cm guard column (all columns at 4.6 mm diameter and 5 μm particle size) were connected in series and placed within the column oven. Following the analytical columns, a post column Teflon® coil was connected and kept at 60 °C. The post column reaction mixture consisted of 1 M potassium hydroxide flowing at 0.5 ml/min.

The analytical mobile phase consisted of a mixed cation phosphate buffer prepared as follows. One gram of potassium phosphate monobasic (anhydrous) and 0.21 g of sodium phosphate dibasic (anhydrous) were dissolved in 700 ml of distilled water. The pH of the solution was adjusted to 6.3 using solid sodium phosphate dibasic (anhydrous), and the solution was brought to a final volume of 1.0 l. The mobile phase was completed by addition of acetonitrile to the phosphate buffer (3:7), filtered through a 0.2 μm nylon-66 disc filter and degassed under vacuum with sonication.

Samples and standards maintained at 5 °C in light protected HPLC vials in the autosampler were injected (100 μl) in triplicate and data was collected for 15.25 min. The quantity of PGE_1 was determined by measuring the identified peak area resolved by the HPLC against peak areas resolved from known quantities of a USP Alprostadil reference standard. The quantities of PGA_1 and PGB_1 were determined in a similar manner by measuring the identified peak areas against known quantities of reference standards obtained from Cayman Chemical Corporation. All standard curves ranged from 0 to 12 μg/ml. The quantity of PGA_1 was normalized according to the percentage recovery of PGB_1 used as an internal standard (Eq. (1)).

$$\mathrm{PGA_1}\ (\mu\mathrm{g/ml}) = \mathrm{PGA_1\ measured}\,\frac{\mathrm{PGB_1\ internal\ standard\ measured}}{2.0\ \mu\mathrm{g/ml}} \quad (1)$$

3. Results

3.1. Accuracy

The accuracy of the PGE_1 assay was determined by spiking known quantities of a USP Alprostadil standard and a PGA_1 standard into a prostaglandin-free vehicle emulsion. Ten tested concentration groups ranged from 0.6 to 12.0 μg/ml of PGE_1 and 0.6 to 6.2 μg/ml of PGA_1 (Table 1). Samples in groups 4 through 9 were designed to contain amounts of PGE_1 and PGA_1, which would be similar to those amounts expected in the final drug product. Thus, group 4 represents the PGE_1 and PGA_1 content expected in freshly manufactured encapsulated PGE_1, while groups 5 through 7 represent product that has acceptable stability levels of PGE_1 and PGA_1, and groups 8 through 10 represent various degrees of unacceptable, out of specification levels of PGE_1 and PGA_1. These concentrations of prostaglandins were chosen to determine the accuracy of the assay at both high and low concentrations of PGE_1 and PGA_1.

For each group, three separate samples were prepared, extracted and each assayed in triplicate, for a total of nine HPLC assays per group. The mean, standard deviation (S.D.) and correlation of variation (CV) were calculated from the data for the nine injections for each group. The assay percent recovery was calculated as the percentage of the mean group value recovered from the known spiked value (measured/spike). The percent recovery data presented in Table 1 show that the assay is 93.9–100.3% accurate for PGE_1 in the range of 4.00–6.20 μg/ml and 96.8–100.8% accurate for PGA_1 in the range of 1.00–2.50 μg/ml. These ranges represent the acceptable minimum

Table 1
Accuracy of the automated assay method

Group (#)	Analyte	Theoretical (μg/ml)	Measured (±S.D.) (μg/ml)	CV (%)	Percent recovery (%)
1	PGE_1	12.00	11.76 ± 0.15	1.3	98.0
	PGA_1	0.00	0.00	N.D.	N.D.
2	PGE_1	9.00	8.98 ± 0.08	1.0	99.8
	PGA_1	0.00	0.00	N.D.	N.D.
3	PGE_1	6.80	6.52 ± 0.13	2.0	95.9
	PGA_1	0.00	0.00	N.D.	N.D.
4	PGE_1	6.20	5.82 ± 0.07	1.2	93.9
	PGA_1	0.60	0.74 ± 0.02	2.7	123.3
5	PGE_1	5.80	5.80 ± 0.04	0.7	100.3
	PGA_1	1.00	1.08 ± 0.02	1.9	100.8
6	PGE_1	5.40	5.32 ± 0.05	0.9	98.5
	PGA_1	1.40	1.36 ± 0.02	1.5	97.1
7	PGE_1	4.30	4.18 ± 0.09	2.2	97.2
	PGA_1	2.50	2.42 ± 0.02	0.8	96.8
8	PGE_1	4.00	3.88 ± 0.07	1.8	97.0
	PGA_1	2.80	2.80 ± 0.07	2.5	100.0
9	PGE_1	2.00	1.90 ± 0.05	2.6	95.0
	PGA_1	4.80	4.82 ± 0.06	1.2	100.4
10	PGE_1	0.60	0.64 ± 0.02	3.1	106.7
	PGA_1	6.20	6.28 ± 0.06	1.0	101.3
11	PGE_1	0.00	0.00	N.D.	N.D.
	PGA_1	0.00	0.00	N.D.	N.D.

The table presents the results of the recovered PGE_1- and PGA_1-spiked lipid emulsion. The amount of each analyte spiked is described as the theoretical value while that measured is presented with the calculated S.D. All samples were subjected to organic extraction, followed by automated SPE and precipitation of non-analyte materials prior to HPLC analysis. For each group, three samples were extracted and each sample extraction analyzed by HPLC in triplicate. N.D., not determined; CV, correlation of variation (S.D./mean).

Table 2
Recovery factors for quantification of PGE_1 in the final drug product

Prostaglandin	Recovery factor	Valid assay range for correction factor (μg/ml)
PGE_1	1.03	4.00–6.00
PGA_1	1.00	1.00–2.50

The calculation of the recovery factor is based upon the data presented in Table 1 with the understanding that the expected concentrations of the analytes in the final drug product are 4.00–6.00 and 1.00–2.50 μg/ml for PGE_1 and PGA_1, respectively. In the case of PGA_1, the concentration was corrected by the internal standard, PGB_1.

Table 3
Precision and reproducibility of measured PGE_1 and PGA_1 concentrations in lipo PGE_1 (lot number 014S1)

Analyst	Analyte	Mean ($\pm$S.D.) (μg/ml)	CV (%)
A	PGE_1	5.36 ± 0.19	3.5
	PGA_1	1.28 ± 0.06	4.7
B	PGE_1	5.39 ± 0.22	4.1
	PGA_1	1.23 ± 0.05	4.1

The table presents the data obtained after extraction by two analysts. Each analyst extracted three samples, and each sample extraction was analyzed by HPLC in triplicate.

stability values of the drug after storage at 5 °C and the maximum acceptable values of the drug after manufacture, respectively. The precision of the assay, for all tested concentrations, as measured by the CV, respectively, ranged from 0.7 to 3.1% and 0.8 to 2.7% for PGE_1 and PGA_1.

A recovery factor for PGE_1 was calculated from the accuracy data in the expected measurement range, 4.00–6.00 μg/ml (Table 1). The percent recovery for PGE_1 in group 8 were utilized for normalization of the prostaglandin content to the minimum specification limit of 4.00 μg/ml for PGE_1 in the final formulated drug product. The recovery factors to be used in the calculation of the assay results for the prostaglandins are presented in Table 2. The recovery factors were multiplied by the mean result for each sample. The recovery factor of PGE_1 was valid within the corrected assay ranges of 4.00–6.00 μg/ml, the respective low and high specifications of PGE_1 encapsulated drug. For PGA_1 (1.00–2.50 μg/ml), recovery was based upon the inclusion of a known quantity of an internal standard (PGB_1) that was used to normalize the data (Eq. (1)).

Since the percent recovery for PGA_1 in group 8 was 100%, no further correction was required.

3.2. Precision and reproducibility

Estimates of the precision and reproducibility of the assay were performed by measuring PGE_1 and PGA_1 in two separate randomly selected samples of formulated drug (lot number 014S1). The corrected mean, S.D. and CV (%) results of nine injections (three sample extractions with three injections each) are shown in Table 3. Significant differences between the two groups were calculated using paired two-tailed t-tests ($\alpha = 0.05$). PGE_1 values were not statistically different for the two tests ($P = 0.7$). However, PGA_1 values were statistically different in the two tests ($P = 0.04$). This significant difference was due to the very low variance (S.D.^{2}) of the two test groups, 4.0 and 2.0 ng/ml, respectively, and is not a reflection of a relevant difference between groups. Thus, it was determined that no real significant differences occurred between the two test groups.

3.3. Linearity

The linearity of the automated extraction procedure and HPLC assay, within the concentration ranges of 0.6–12.0 μg/ml for PGE_1 and 0.6–6.2 μg/ml for PGA_1, were evaluated. The data from Table 1 are presented graphically in Fig. 1A and B. Visual inspection of the figures clearly shows the linearity of the assay in the concentration ranges tested. For PGE_1, the slope of the line from 0 to 12 μg/ml is 1.0175 with a y-intercept of 0.0308 and a correlation coefficient (r) of 0.9995. For PGA_1, the slope of the line from 0 to 6.2 μg/ml is 0.9984 with a y-intercept of -0.0210 and a correlation coefficient (r) of 0.9994. These linear regression values support the finding that the assay is sufficiently linear in the assay range tested.

3.4. Specificity

The assay method is specific for prostaglandins E_1, A_1 and B_1. Components of the lipid emulsion do not elute at the retention times of the analytes. Representative chromatograms of placebo (Fig. 2A) and PGE_1, PGA_1 and PGB_1 standards (Fig. 2B) separated by HPLC after sample preparation are presented below. Each analyte is completely resolved and free of competing interferences.

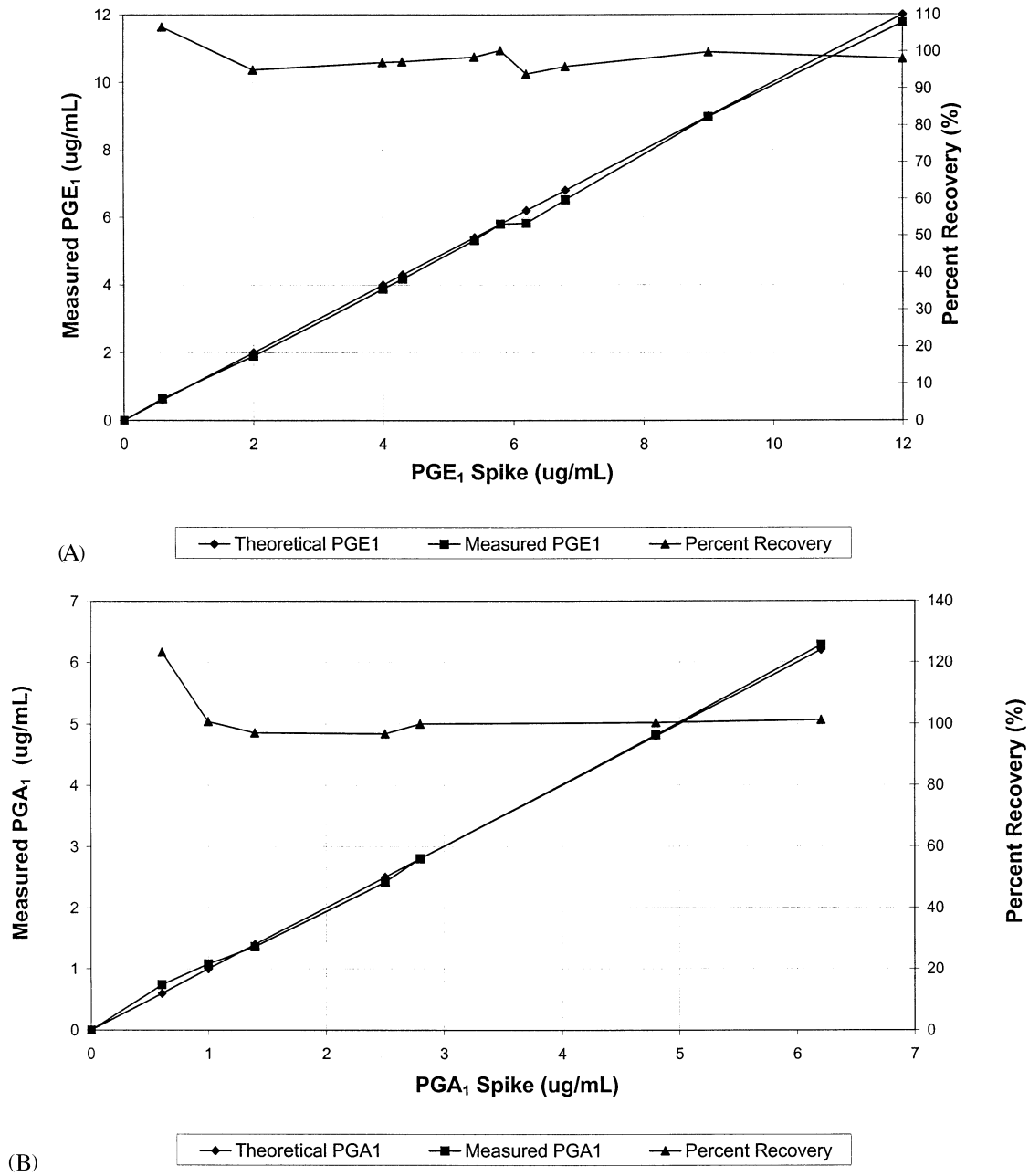

Fig. 1. (A) Linearity of assayed PGE_1. (B) Linearity of assayed PGA_1.

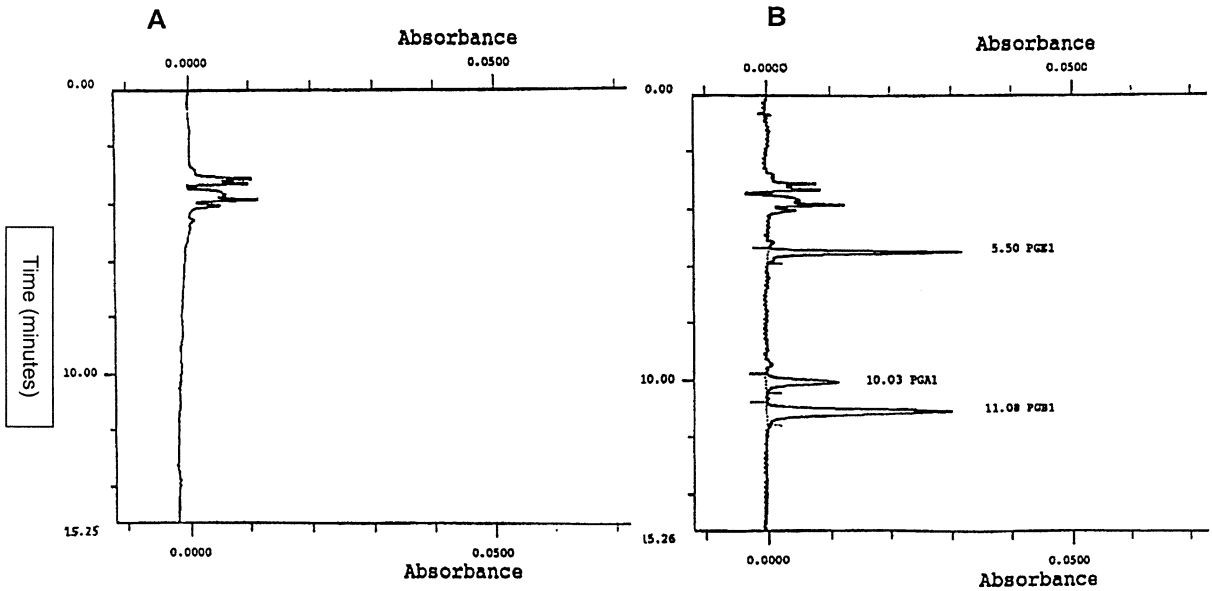

Fig. 2. Representative chromatograms of placebo (A) and placebo spiked with PGE_1, PGA_1 and PGB_1 standards (B) separated by HPLC.

3.5. Limits of quantitation

The sensitivity of the assay was evaluated from the data presented in Table 1. The assay was sufficiently precise at concentrations as low as 0.6 μg/ml (CV of 3.1 and 2.7% for PGE_1 and PGA_1, respectively). Further attempts to decrease the limit of quantitation were not performed due to the deviation from linear recovery seen with PGA_1 at 0.6 μg/ml (Fig. 1B). As described above, PGA_1 recovery at 0.6 μg/ml exceeds 100% when corrected to the recovery of the internal standard (PGB_1). Thus, the lowest limit of quantitation was 0.6 μg/ml for both PGE_1 and PGA_1. Due to the fact that 12 μg/ml of PGE_1 was 240% of the labeled concentration of the manufactured PGE_1 drug, no further attempt was made to increase the upper limit of quantitation in this lipid emulsion environment.

4. Discussion

This assay demonstrates the utility of an automated SPE technique to create a highly precise and accurate method for the quantitation of PGE_1 and PGA_1 encapsulated in a lipid emulsion. Since the formulated drug is labile, with an estimated shelf life of 6–12 months at 5 °C, a highly precise and rapid assay was required to adequately determine the stability profile of the product. Thus, the addition of an automated SPE step reduced both the variation in sample preparation and processing time, and allowed for rapid quantitative characterization of the emulsified product.

This assay effects the separation of PGE_1, PGA_1, and PGB_1 from a complex mixture of phospholipids using normal phase chromatography during the SPE prepatory step. Attempts to use reversed phase chromatography described by others [5–9] were inadequate. Use of reverse phase SPE typically resulted in the inability to separate the prostaglandins from the soybean oils and resulted in multi-phasic samples for HPLC injection and poor accuracy and precision. The normal phase separation of prostaglandins from oils and phospholipids was presented in detail by Hirsh and Ahrens [10]. Methods for the determination of PGA_1 and PGB_1 were not capable of

simultaneously determining PGE_1 [4,11], nor were these methods sensitive enough to measure all the prostaglandins within the range of expected values [4,12]. Additionally, the USP method for measurement of Alprostadil® relies on normal phase chromatography for the quantitation of PGE_1. However, this method neither separated the complex mixture of phospholipids from PGE_1, nor did it separate PGE_1 from its major degradation product, $PGA_{1,}$ at the concentrations tested. In contrast, the method described here was sufficiently sensitive for the measurement of prostaglandins within the range of expected values.

In conclusion, the method described here represents a major advancement over the current USP method for the measurement of PGE_1 in a lipid-emulsified vehicle.

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