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DETERMINATION OF (15*R*)- AND (15*S*)-15-METHYLPROSTAGLANDIN E_2 IN HUMAN PLASMA WITH PICOGRAM PER MILLILITER SENSITIVITY BY COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*,**

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SUMMARY

(15*R*)-15-Methylprostaglandin E_2 (PGE_2) is a pro-drug under evaluation for the treatment of acute upper gastrointestinal hemorrhage and gastrointestinal cytoprotection. It is converted in acid (e.g., gastric fluid) to its active 15*S* epimer. Both epimers are found in human plasma at low pg/ml levels following oral dosing. A high-performance liquid chromatographic (HPLC) method was developed for the simultaneous analysis of (15*R*)- and (15*S*)-15-methyl-PGE_2 in human plasma. The method combined off-line solid-phase extraction and reversed-phase HPLC clean-up with panacyl bromide derivatization and subsequent analysis using a heteromodal column-switching technique. Assay linearity was demonstrated over a range of 10—200 pg/ml for both 15-methyl-PGE_2 epiemers ($r \geq 0.995$). There were no significant inter-day differences in assay results for either epimer at 50 and 25 pg/ml ($p > 0.05$), with pooled estimates of precision at these levels producing relative standard deviations of $\leq$ 8% and $\leq$ 12%, respectively. The method quantitation limit (signal-to-noise ratio 5:1) for both epimers was 10 pg/ml when processing 3 ml of plasma. The analysis procedure was shown to be useful for quantifying at or below 10% of the (15*R*)-15-methyl-PGE_2 maximum plasma concentration following a 50-µg oral dose in three human volunteers. For the same three subjects, however, the plasma concentration of (15*S*)-15-methyl-PGE_2 did not exceed the quantitation limit of 10 pg/ml.

INTRODUCTION

Arbaprostil, (15*R*)-15-methylprostaglandin E_2 (PGE_2) (Fig. 1) is a pro-drug

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**The generic name of (15*R*)-15-methylprostaglandin E_2 is arbaprostil, the active ingredient in Arbacet soft elastic capsules and oral solution (The Upjohn Company, Kalamazoo, MI, U.S.A.).

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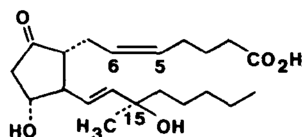

Fig. 1. Structure of (15*R*)-15-methyl-PGE_2.

under evaluation for the treatment of acute upper gastrointestinal hemorrhage and gastrointestinal cytoprotection. Administered orally at doses of 10—100 μg, it is epimerized in acid (e.g., gastric fluid) [1, 2] to the more biologically active 15*S* epimer, which exhibits both gastric cytoprotective and gastric antisecretory activities [3—5]. In order to support clinical studies with arbaprostil, it was of interest to develop a plasma assay for both (15*R*)- and (15*S*)-15-methyl-PGE_2. Based on work with radiolabeled (15*R*)-15-methyl-PGE_2 in humans [6], clinical doses of arbaprostil were predicted to result in plasma concentrations for both epimers in the low pg/ml range.

Analytical methods with pg/ml sensitivity for prostaglandins in physiological fluids are limited to radioimmunoassay [7] or gas chromatography—mass spectroscopy (GC—MS) [8, 9]. We recently described a high-performance liquid chromatographic (HPLC) method for the simultaneous quantitation of (15*R*)- and (15*S*)-15-methyl-PGE_2 in solution standards as fluorescent derivatives with picogram level sensitivity [10]. The present report describes the application of this method to the analysis of the 15*R* and 15*S* epimers in human plasma.

EXPERIMENTAL

Materials

Abraprostil [(15*R*)-15-methyl-PGE_2], (15*S*)-15-methyl-PGE_2 and 5,6-*trans*-(15*R*)-15-methyl-PGE_2, the internal standard (for structures see Fig. 1), ^{3}H-labeled (15*R*)-15-methyl-PGE_2-11β (specific activity of 3.48 mCi/mg), ^{3}H-labeled (15*S*)-15-methyl-PGE_2-11β (specific activity of 3.82 mCi/mg) and panacyl bromide [*p*-(9-anthroyloxy)phenacyl bromide] were supplied by the Pharmaceutical Research and Development Laboratories of The Upjohn Company (Kalamazoo, MI, U.S.A.). Hexane, methanol, isooctane, water, toluene, ethyl acetate, acetonitrile, tetrahydrofuran, ethylene dichloride, methylene chloride and 2-propranol were UV or HPLC grade and were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Liquid scintillation cocktail was Aqueous Counting Scintillant (ACS) (Amersham, Arlington Heights, IL, U.S.A.). N,N-Diisopropylethylamine, 98%, and formic acid, 95—97%, were obtained from Aldrich (Milwaukee, WI, U.S.A.). High-purity-grade nitrogen was purchased from Union Carbide Corporation (New York, NY, U.S.A.). Bond-Elut® C_{18} 200 mg per 3.0 ml and 100 mg per 1.0 ml extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Plasma standard preparation

The plasma for standard samples was obtained from Plasma Alliance (Knoxville, TN, U.S.A.). Blood was collected from individual donors in 1200-

ml lots using plastic containers and sodium citrate anticoagulant. The plasma was separated and stored at −20°C. To prepare plasma standards, an entire lot was thawed and filtered through glass wool. An additional filtration step was performed by passing the plasma through a Nalgene® sterilization filter unit (Nalge, Rochester, NY, U.S.A.) that contained 8 g of diatomaceous earth to act as a large particle trap. The filtered plasma was kept on ice during the sample preparation process.

Accurately weighed amounts of (15*R*)-15-methyl-PGE_2 and its (15*S*)-15-methyl epimer were dissolved in acetonitrile to produce a stock concentration for each of 75.0 μg/ml. These solutions were diluted serially to produce standard working solutions. All dilutions were performed using polypropylene materials. Aliquots (3.0 ml) of filtered plasma in 10-ml polypropylene tubes were then spiked with the standard solutions, keeping the acetonitrile concentration of the resulting plasma standards less than 1% (v/v). Seven standards were prepared over a concentration range of 10—200 pg/ml. After mixing, the plasma standards were capped and stored at —20°C.

Pooled plasma standards were prepared to assess stability and inter- and intra-day precision and accuracy. They were prepared at 50 and 25 pg/ml by spiking an appropriate amount of standard solution into a plastic beaker containing 55.0 ml of plasma, then aliquoting 3.0-ml samples to tubes for storage. Blank, pooled controls were also aliquoted.

Plasma extraction

Bond-Elut C_{18} columns (200 mg per 3.0 ml) were placed in a vacuum manifold (J.T. Baker, Phillipsburg, NJ, U.S.A.) and prepared for the plasma extraction by washing with 4 ml of methanol followed by 4 ml of water. The plasma blanks, standards, and unknowns were all thawed at room temperature and spiked with internal standard (I.S.) (12 μl of a 32.5 ng 5,6-*trans*-(15*R*)-15-methyl-PGE_2 (U-67205) per ml acetonitrile solution) using a Varimetric Micropipetter (Labindustries, Berkeley, CA, U.S.A.). After mixing, the samples were acidified to pH 4 by adding 0.3 ml of a 5% formic acid solution. The samples were again vortexed then centrifuged at 1500 *g* for 15 min at 4°C. The clear plasma samples were applied to the extraction columns and vacuum applied (660 mmHg internal manifold pressure). The columns were washed with 2 ml of water, twice, 2 ml of 10% methanol in water, twice, and 2 ml of toluene. The analytes were eluted with 1 ml of ethyl acetate and collected in 4-ml polypropylene test tubes.

Extract purification and derivatization

The ethyl acetate eluent was evaporated to dryness under nitrogen in a 40°C water bath. The samples were reconstituted by adding 0.61 ml of methanol, vortexing for 30 sec, then adding 1.42 ml of 0.01% formic acid (aqueous). The samples were then transferred to 2-ml polypropylene microtubes (Sarstedt, Princeton, NJ, U.S.A.) for injection onto a reversed-phase HPLC system.

Reversed-phase HPLC instrumentation and conditions. This automated reversed-phase HPLC system (Fig. 2) injected 1.8 ml of the 2.0-ml sample, backflushed the guard column, and collected a 1.2-ml fraction of the analytical column eluent that contained both (15*R*)- and (15*S*)-15-methyl-PGE_2, and

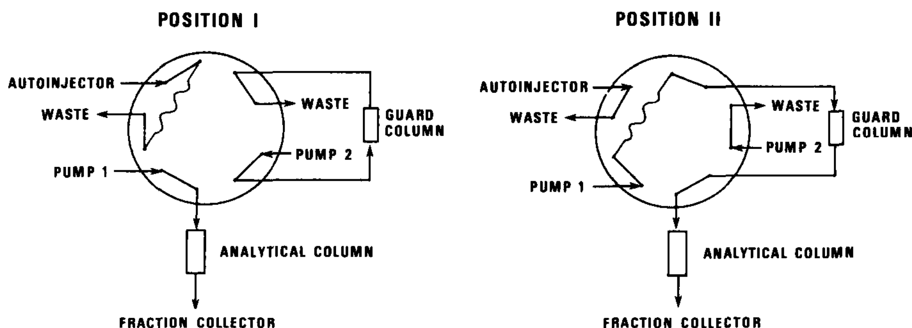

Fig. 2. Schematic representation of the apparatus and switching valve configurations used in the reversed-phase HPLC system. Program: 0—1 min, position I loads the sample loop; 1—3.5 min, position II injects the sample and elutes the analytes onto the analytical column; 3.5—9.5 min, position I permits guard column backflushing and elutes the analytes from the analytical column.

the internal standard. The HPLC conditions were a modification of conditions employed by Stolle and Hsi [11] to determine the purity of radiolabeled (15*R*)-15-methyl-PGE_2. Samples were injected using an Upjohn MicroAS autosampler (Upjohn, not commercially available) equipped with an LDC minipump® (Laboratory Data Control, Riviera Beach, FL, U.S.A.) as the sample pump. The mobile phase composition was water—acetonitrile—formic acid (60:40:0.01). Pump 1 was a Beckman 112 solvent delivery module (Beckman Instruments, Berkeley, CA, U.S.A.) and pump 2 was a Milton Roy minipump (St. Petersburg, FL, U.S.A.). Both pumps were operated at a flow-rate of 2 ml/min. Mobile phase routing was performed with a Valco ten-port air-actuated (4 bar) switching valve (Valco Instruments, Houston, TX, U.S.A.). The guard column was a Brownlee RP-8, 5 μm particle size, 3 cm × 4.6 mm I.D. cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.). The analytical column was a Supelcosil® LC-18, 5 μm particle size, 250 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). Samples were collected using a Foxy® fraction collector (Isco, Lincoln, NE, U.S.A.), which was activated by a Beckman 421 system controller.

The switching valve positions and program were as described in Fig. 2. The switching valve position was controlled by the autosampler, which was linked to the Beckman 421 controller via a pressure switch (Bulletin 836, Allen-Bradley, Milwaukee, WI, U.S.A.) to the controller's remote input. Injection of the sample was associated with a pneumatic pulse which initiated a controller program for sample collection. A circuit closure event in the program actuated the fraction collector to deliver the desired eluent fraction from the analytical column to a polypropylene tube.

The retention time of (15*R*)- and (15*S*)-15-methyl-PGE_2 was determined daily by injecting 1.4 ng (10 000 dpm) of a ^{3}H-labeled (15*RS*)-15-methyl-PGE_2-11β standard mixture and collecting 0.2-ml fractions. The fractions were transferred to scintillation vials and mixed with 10 ml of ACS, then counted on a Mark III liquid scintillation system, Model 6880 (G.D. Searle, Des Plaines, IL, U.S.A.) using automatic external standardization to correct for quenching. The proper collection time was then written into the file on the Beckman 421

controller. The typical retention time of the analytes was 6.5–7.1 min, which permitted a total run time of 9.5 min per sample.

Solvent exchange step. The fraction collected from the HPLC clean-up was diluted 1:1 (v/v) with water and applied to a 100 mg per 1.0 ml Bond-Elut C_{18} column (prepared by washing with methanol and water as previously described). After the sample was applied and aspirated, the columns were washed with 1 ml of hexane. The analytes were eluted with two 0.5-ml washes of ethyl acetate and collected in polypropylene tubes. The eluent was evaporated to dryness under nitrogen in a 40°C water bath in preparation for derivatization.

Derivatization. The samples were reconstituted in 0.25 ml of panacyl bromide solution (25 μg in tetrahydrofuran–acetonitrile, 1:4, v/v) by vortexing for 30 sec. They were then transferred to polypropylene microvials (0.3-ml volume) and derivatized as described previously [10]. After derivatization, the samples were evaporated at 40°C under a stream of nitrogen and reconstituted in 0.23 ml of isooctane–ethylene dichloride–2-propanol (70:30:1, v/v). The vials were capped with an aluminum seal containing a 0.25 mm (0.01 in.) thick PTFE disc (SCI/SPEC, Randallstown, MD, U.S.A.) and the samples were sonicated for 10 min in an immersion bath (Branson Cleaning Equipment, Shelton, CT, U.S.A.). The samples were then placed in the auto-sampler for injection onto the analytical chromatography system.

Analytical high-performance liquid chromatography

Instrumentation and conditions. The analytical chromatography system was a modification of the apparatus reported by Cox and Pullen [10]. It consisted of two isocratic systems (1 and 2) linked serially via a 2.2-ml sampling loop which permitted the direct injection of system 1 eluent into system 2 (Fig. 3). Mobile phase 1 was hexane–methylene chloride–2-propanol (70:30:1, v/v). Mobile phase 2 was hexane–methylene chloride–tetrahydrofuran–2-propanol (60:20:20:1, v/v). Samples (200 μl) were injected with an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.). Pumps 1A, 1B and 2 were Beckman 112 solvent delivery modules and mobile phase routing was performed via two Valco ten-port air-actuated switching valves. The guard column (10 cm × 4.6 mm I.D.) was dry-packed with Whatman Co:Pell PAC (Whatman, Clifton, NJ, U.S.A.). Analytical column 1 was a Zorbax CN 15 cm × 4.6 mm I.D. cyanopropylsilane bonded-phase column and analytical column 2 was a Zorbax SIL 24 cm × 4.6 mm I.D. silica column (both packing materials are spherical particles, 6 μm diameter) (DuPont, Wilmington, DE, U.S.A.). Detector 1 was an LDC UV (254 nm) monitor III, Model 1203A. Detector 2 was a Perkin-Elmer 650S fluorescence detector with the following settings: excitation wavelength, 375 nm; emission wavelength, 470 nm; slit width (excitation and emission) 20 nm; response time, 6 sec. All connections between columns, detectors, and switching valves were made with 1.6 mm O.D. × 0.254 mm I.D. stainless-steel tubing and low dead volume connectors.

Switching valve actuation. Switching valve positions were controlled by external flags from the Beckman 421 controller which was interfaced to the valves by a solenoid valve/solid-state relay device (Upjohn; not commercially available). The program in the controller was initiated by a contact closure on

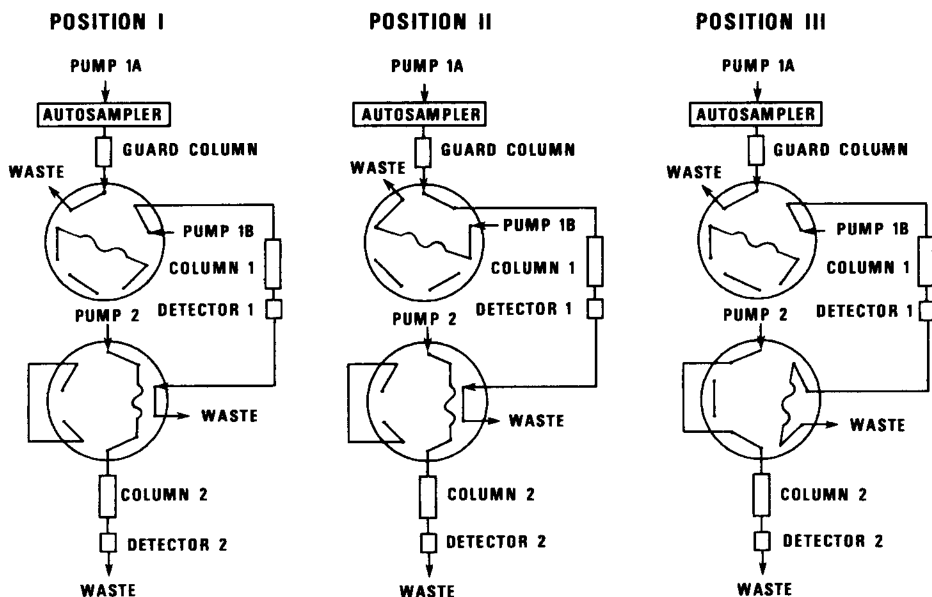

Fig. 3. Schematic representation of the apparatus and switching valve configurations used in the analytical chromatography system. Program: 0—3.0 min, position I injects the sample with the guard column vented to waste: 3.0—4.5 min, position II injects the guard column eluent onto column 1; 4.5—12.0 min, position I directs the guard column eluent back to waste and vents eluent from column 1 to waste; 12.0—14.0 min, position III fills the sampling loop with column 1 eluent; 14.0—43.0 min, position I injects the peak of interest from system 1 into system 2 for separation and quantitation.

the ISS-100 to the controller's remote input. Three switching valve configurations were used in the analysis (Fig. 3).

The controller program provided for an initial vent to waste from the guard column to remove the majority of the unreacted panacyl bromide [10]. An eluent fraction taken from the guard column was routed to the primary analytical column and then the guard column eluent was routed back to waste. An eluent fraction was then taken from the primary analytical column by collecting the desired window in a 2.2-ml loop. Finally, this loop was switched online to system 2 and the analytes were separated and quantitated by fluorescence detection on the silica analytical column.

The proper guard column eluent fraction was determined daily by injecting 1.4 ng (10 000 dpm) of a ^{3}H-labeled (15*RS*)-15-methyl-PGE$_2$-11β panacyl ester standard mixture. The elution volume of the derivative was determined by collecting 0.1-ml fractions from the guard column and counting them for radioactivity (see above). Typically, the switching times were set at 3.0 and 4.5 min (flow-rate 1 ml/min). The eluent fraction from column 1 was determined by injecting 1 ng of a (15*RS*)-15-methyl-PGE$_2$ panacyl ester standard mixture and noting the baseline-to-baseline elution times by UV detection (typically 12—14 min at 1 ml/min).

Data acquisition. Collection and analysis of the fluorescence detector output was performed with the UPACS II automated chromatography system 3 (Upjohn) on a Harris 500 computer. The chromatograms were computer-

smoothed using the Savitsky and Golay [12] least-squares procedure. Output from both the fluorescence and UV detectors was recorded on a strip chart recorder.

Quantitation method. Data were analyzed by unweighted linear regression best-fit of peak height ratio [(15*R*)- or (15*S*)-15-methyl-PGE_2/I.S.] versus concentration.

In vivo studies

Human plasma samples were collected from subjects given oral doses of arbaprostil as part of a drug interaction study designed to determine the influence of arbaprostil on diazepam intravenous pharmacokinetics. Apparently healthy male volunteers between the ages of 18 and 45 years and having a fasting gastric pH of less than 3 received 50 μg of abraprostil dissolved in a triacetin vehicle in a soft elastic capsule four times per day 0.5 h before meals for seven days. At 0.5 h after the morning dose of abraprostil on day 8, the subjects received 10 mg of diazepam by constant intravenous infusion over 10 min, and were not given a meal until 4 h after the administration of diazepam. Blood samples for abraprostil analysis were collected just prior to arbaprostil dosing on day 8 and at 30, 45, and 60 min post-dose. The blood was collected by venipuncture using all plastic syringes and immediately transferred to polypropylene tubes containing 0.25 ml of 80 mg/ml Na_2EDTA. The tubes were mixed and stored on ice until they were centrifuged at 4°C to separate the plasma. The plasma was drawn off using a plastic-tipped P-5000 pipettor (Rainin Instrument, Woburn, MA, U.S.A.) and transferred to another polypropylene tube for storage at −20°C.

RESULTS AND DISCUSSION

Plasma extraction and purification

The internal standard for this analysis was the 5,6-*trans* isomer of (15*R*)-15-methyl-PGE_2. It was added to the sample plasma immediately after thawing and prior to extraction. Calculation of an HPLC relative weight response factor (peak area ratio/(15*R*)-15-methyl-PGE_2 concentration/I.S. concentration) indicated that the overall recovery of the I.S. was not significantly different from the recovery of (15*R*)-15-methyl-PGE_2 ($p > 0.05$, $n = 7$). Experiments in which the I.S. was individually carried through the extraction/purification procedure demonstrated that the analysis conditions did not promote significant isomerization of the 5,6 double bond.

Using the C_{18} Bond-Elut extraction columns, the absolute recovery of 190 pg of a ^{3}H-labeled (15*RS*)-15-methyl-PGE_2-11β standard mixture from 3 ml of plasma acidified to pH 4 with formic acid was 81%. This is similar to the C_{18} extraction column recovery of 74% reported by Luderer et al. [13] for PGE_2 in acidified plasma. After application of the plasma, the columns were washed with water and 10% methanol to elute polar components, and then toluene to elute neutral lipids and to remove residual water from the columns. The 15-methyl-PGE_2 isomers were then eluted with ethyl acetate.

When derivatized and analyzed directly by HPLC, the crude extract was unsuitable for 15-methyl-PGE_2 quantitation. Further purification was

attempted with a C_{18}/SIL column sequencing extraction technique described for prostaglandin fractionation [14], but this procedure also failed to provide an acceptable chromatographic baseline. However, an acceptable baseline was obtained with a reversed-phase (C_{18}) HPLC system designed so that both the 15-methyl-PGE_2 epimers and the I.S. coeluted in a single fraction. Since the prostaglandins do not have a chromophore for sensitive UV detection, the HPLC retention time was determined by injecting a radiolabeled (15*RS*)-15-methyl-PGE_2 mixture and collecting and counting the eluent. The system was automated and the 1.2-ml fraction containing the analytes and internal standard was collected with a fraction collector. A guard column backflushing step was instituted to prevent the late eluting components of previous injections from eluting in subsequent 15-methyl-PGE_2 fractions. The on-column recovery for approximately 165 pg of a radiolabeled (15*RS*)-15-methyl-PGE_2 mixture in a C_{18} plasma extract was 88%. Ninety per cent of the sample was injected on-column so that actual recovery for the HPLC purification step was 79%.

In order to prepare the 15-methyl-PGE_2 fraction for derivatization and analysis, it was exchanged from the aqueous mobile phase into ethyl acetate. This was accomplished by diluting the sample with water and applying it to another C_{18} Bond-Elut column. After washing with hexane to dry the column, the 15-methyl-PGE_2 isomers were eluted with 90% recovery by two 0.5-ml ethyl acetate washes. The overall recovery for the extraction and purification procedure was 58 ± 5% (S.D., n = 6) when processing a 190-pg ^{3}H-labeled (15*RS*)-15-methyl-PGE_2-11β plasma standard. Linearity studies indicate that this recovery is a constant proportion of the sample mass over a range of 30—600 pg for each analyte. The time for one person to process twenty plasma samples from thawing to placement on the autoinjector for overnight HPLC analysis was 9 h.

Analytical high-performance liquid chromatography

Plasma extracts were derivatized to form fluorescent panacyl esters of carboxylic acids with panacyl bromide and quantified using a modification of a previously described column-switching HPLC system [10]. After injection, an eluent fraction from the cyano-amino guard column was transferred to a cyano analytical column, from which another fraction was transferred to a silica analytical column (Fig. 3). Derivatives of the three 15-methyl-PGE_2 isomers coeluted on the guard and cyano analytical columns, but were baseline-resolved by the silica column for quantitation by fluorescence detection (Fig. 4). The switching times for the guard column were determined for a batch of HPLC mobile phase by injecting radioactive (15*R*)- and (15*S*)-15-methyl-PGE_2 derivatives and collecting and counting the eluent. The switching times for the cyano analytical column were determined daily by injecting a concentrated solution standard and monitoring the output from a UV detector placed between the column terminus and the sampling loop. The switching times were chosen based on the baseline-to-baseline elution volume of a (15*RS*)-15-methyl-PGE_2 derivatized solution standard. The column switching times were then programmed into a system controller for attendant free operation.

This system incorporated several changes from the previously described

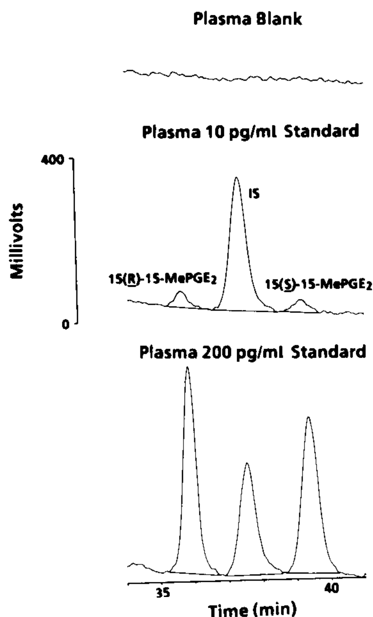

Fig. 4. Representative chromatograms (detector 2) from the analysis of 3-ml plasma standards for (15*R*)- and (15*S*)-15-methyl-PGE_2.

HPLC system [10] which were made in order to eliminate interferences from the plasma extract. The guard column was lengthened from 5 to 10 cm to provide greater separation efficiency and a cutting mechanism for the guard column eluent was installed. Previously, when analyzing solution standards [10], only the solvent front was vented to waste to dispose of excess panacyl bromide reagent. When analyzing plasma extracts, however, late eluting components from the guard column eventually eluted with the 15-methyl-PGE_2 isomers of subsequent injections and were transferred to the silica column. This resulted in broad interference peaks that appeared with regular injection periodicity but which were absent from the first several chromatograms. These interferences were eliminated by taking only a fraction of the guard column eluent. Interferences were also eliminated by instituting a wash step for the guard and cyano analytical columns after the analytes had been transferred to the silica column. The flow-rate to these columns was increased from 1 to 2—2.5 ml/min. None of these changes lengthened the overall chromatographic analysis time, which remained at 43 min.

It was discovered that recently manufactured lots of the guard column packing material (Whatman Co:Pell PAC) more strongly retained the panacyl esters of the 15-methyl-PGE_2 isomers than do lots manufactured prior to 1979 (Batch Nos. 100300 and 100295) which were used in this validation. Initial investigation has indicated that Zorbax BP-CN bulk packing material (cyano-bonded phase, 7—8 μm particle size; DuPont) may provide an acceptable alternative. Also, within lot variability in 15-methyl-PGE_2 derivative retention by the cyano analytical column (Zorbax CN, DuPont) was observed and some of these columns were unsuitable for this analysis without significant modification of the HPLC mobile phase.

Linearity

The linearity of fluorescence detector response versus the plasma concentration of 15-methyl-PGE_2 epimer standard curves was evaluated on three separate days. Plots of peak height ratio versus 15-methyl-PGE_2 plasma concentration had linear correlation coefficients of 0.995 or greater over a range of 10—200 pg/ml (10, 15, 25, 50, 100, 150 and 200 pg/ml). All curves were analyzed with a best-fit linear regression model. One of the six curves had a positive y-intercept (95% significance level) although no interference was detected in the plasma blank. Representative chromatograms of plasma standards are shown in Fig. 4.

Precision and accuracy

The intra- and inter-day precision and accuracy of the assay procedure were evaluated at 25 and 50 pg/ml plasma concentrations of both 15R and 15S analytes. Triplicate assays of pooled plasma standards were repeated on three successive days using independently prepared plasma standards (Table I). There was no significant inter-day difference in assay results ($p > 0.05$) for either analyte and so the data at each concentration level were pooled. The pooled estimate of the assay relative standard deviation (R.S.D.) at the 25 and 50 pg/ml concentrations was 8—12% and 6—8%, respectively, for both epimers.

The pooled assay results showed no significant bias (within the limits of assay precision, $p > 0.05$) at either the 50 or 25 pg/ml levels for (15R)-15-methyl-PGE_2, and no bias at the 50 pg/ml level for the 15S epimer (Table I). However, there was significant bias (33%) for the 15S epimer at the 25 pg/ml concentration ($p < 0.05$). The latter finding is difficult to explain since the plasma blanks did not show an interference peak near the 15S epimer and since the peak height ratio response factor for the 25 pg/ml plasma standards was not

TABLE I

INTER-AND INTRA-DAY ASSAY PRECISION AND ACCURACY

Actual concentration (pg/ml)	Assay results (mean ± S.D., pg/ml)			Significant inter-day difference ($p < 0.05$)	Pooled estimate of R.S.D. and accuracy (mean ± S.D.)
	Day 1	Day 2	Day 3		
(15R)-15-Methyl-PGE₂					
48.9	47.7 ± 3.2	51.5 ± 4.1	45.9 ± 3.6	No	48.4 ± 4.0
R.S.D. (%)	6.8	8.0	7.7		8.3
Error (%)	−2.5	5.3	−6.1		−1.0
24.3	26.7 ± 1.2	27.8 ± 0.4	26.0 ± 5.6	No	26.7 ± 3.2
R.S.D. (%)	4.4	1.3	21.7		11.9
Error (%)	9.9	14.4	7.0		9.9
(15S)-15-Methyl-PGE₂					
50.2	50.2 ± 2.3	55.7 ± 2.1	52.2 ± 3.6	No	52.7 ± 3.4
R.D.S. (%)	4.7	3.8	7.0		6.4
Error (%)	0	11.0	4.0		5.0
24.9	30.0 ± 1.0	35.0 ± 1.8	34.1 ± 1.9	No	33.2 ± 2.6
R.S.D. (%)	3.3	5.1	5.6		7.8
Error (%)	20.5	40.6	36.9		33.3

significantly greater than the response factor for the higher concentration standards. Errors in solution preparation were ruled out. Without a satisfactory explanation for this bias, the results indicate that the assay may overestimate (15*S*)-15-methyl-PGE_2 concentrations below 50 pg/ml.

Sensitivity

The assay quantitation limit was defined as the concentration of analyte which produced a signal-to-noise ratio of 5:1. This corresponded to 10 pg/ml (using 3 ml of plasma) for either (15*R*)- or (15*S*)-15-methyl-PGE_2. Since 86% of the derivatized sample was injected on-column, further gains in assay sensitivity will require either (a) modification of the chromatography system to improve the signal-to-noise ratio or (b) processing larger volumes of plasma. Neither possibility has been investigated in detail.

Stability

The stability of (15*R*)-15-methyl-PGE_2 at 25 and 50 pg/ml in plasma stored at −20°C was evaluated in a preliminary study by analyzing pooled plasma standard samples against fresh standards. There was no loss in potency after 35 days of storage. A more extensive stability study on both epimers is currently underway.

Application to human clinical samples

The utility of the assay method was tested by analyzing plasma samples

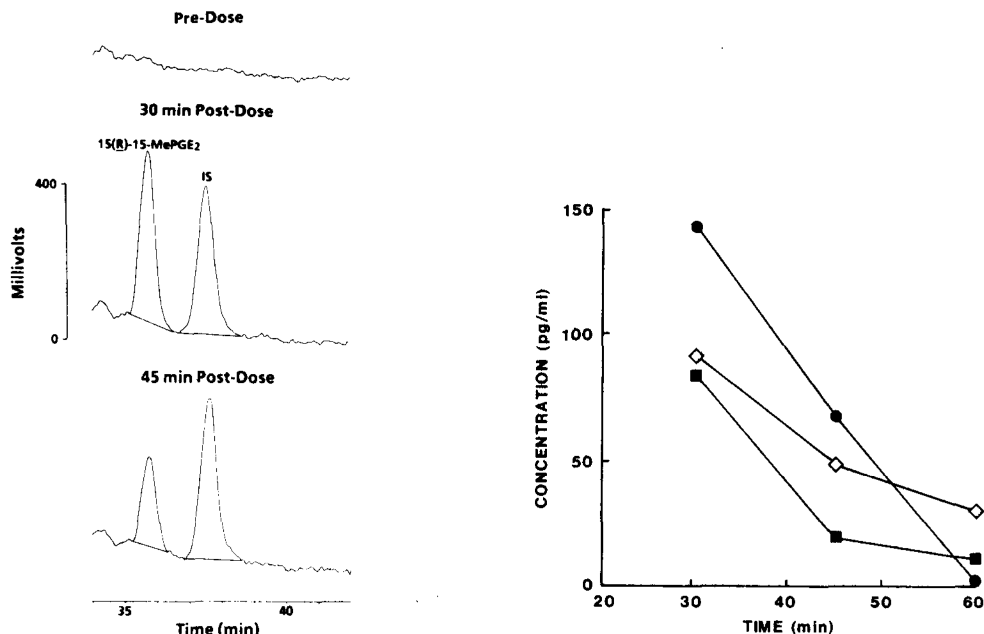

Fig. 5. Representative chromatograms (detector 2) from the analysis of 3 ml of human subject plasma for (15*R*)- and (15*S*)-15-methyl-PGE_2.

Fig. 6. Plasma concentration–time profile from three human subjects after a 50-μg dose of arbaprostil.

(3 ml) from three volunteers participating in a drug interaction study between diazepam and arbaprostil. The volunteers were administered arbaprostil (50 μg) orally four times a day for seven days. On the morning of day 8, plasma samples were collected before and at 30, 45, and 60 min after a 50-μg dose of arbaprostil. As part of the study, diazepam was administered intravenously at 30 min post-dose of arbaprostil.

Neither (15*R*)- nor (15*S*)-15-methyl-PGE_2 were detected in the pre-dose plasma samples, but measurable levels of (15*R*)-15-methyl-PGE_2 were detected in the post-dose samples (Figs. 5 and 6). At 30 min, the mean plasma concentration of (15*R*)-15-methyl-PGE_2 for the three volunteers was 106 pg/ml. With a quantitation limit of 10 pg/ml, the assay is therefore sufficiently sensitive to quantify (15*R*)-15-methyl-PGE_2 to at least 10% of the maximum plasma concentration (C_{max}) following a 50-μg oral dose. The plasma concentration of (15*S*)-15-methyl-PGE_2 was below the quantitation limit of the procedure (10 pg/ml) at all timepoints for the three volunteers.

CONCLUSION

The applicability of a heteromodal column-switching HPLC—fluorescence technique [10] was demonstrated for the quantitative analysis of (15*R*)- and (15*S*)-15-methyl-PGE_2 in human plasma. The internal standard for this analysis was the 5,6-*trans* isomer of (15*R*)-15-methyl-PGE_2. The samples were prepared for analysis by a combination of solid-phase extraction and automated reversed-phase (C_{18}) HPLC purification. The purified extracts were then derivatized to form fluorescent panacyl esters and analyzed by an automated HPLC system which performed sequential elution from cyano—amino, cyano, and silica columns. The three 15-methyl-PGE_2 isomers were copurified as the free acids through the reversed-phase HPLC clean-up step and as the panacyl esters through the cyano—amino and cyano columns in normal-phase systems. The three were finally separated for quantitation on the silica column. Satisfactory sample clean-up was dependent upon the combined use of four different HPLC modes of separation to maximize selectivity. Related E-type prostaglandin derivatives have been shown to be resolved from the 15-methyl-PGE_2 epimers on the analytical chromatography system [10].

The sensitivity of the method was 10 pg/ml (signal-to-noise ratio 5:1) for either epimer when processing 3 ml of plasma. The precision of the method at 25 pg/ml was ca. 10%, and linearity was demonstrated from 10 to 200 pg/ml. The sample preparation time for one person to process twenty samples from thawing to loading on the injector was 9 h. The analytical run time for twenty samples was 14 h (unattended).

The HPLC analysis procedure was shown to be useful for quantifying at or below 10% of the (15*R*)-15-methyl-PGE_2 plasma C_{max} following a 50-μg oral dose of the drug in three human volunteers. For the same three subjects, however, the plasma concentration of the (15*S*) epimer did not exceed 10 pg/ml at 30—60 min post-dose. This result is similar to the findings of Wickrema Sinha et al. [6], who reported that the (15*R*)-15-methyl-PGE_2 plasma concentration (using tritium-labeled drug) always exceeded the 15*S* epimer concentration at 9- and 48-μg oral doses, and that in three of six

volunteers at the 48-μg dose the (15*S*)-15-methyl-PGE_2 concentration did not exceed 10 pg/ml. Thus, these preliminary results indicate that the present analysis procedure may not be sufficiently sensitive for useful quantitation of the 15*S* epimer following a 50-μg oral dose of arbaprostil.

The only other physicochemical method with demonstrated capability of quantifying E-type prostaglandins at these plasma levels is GC—MS [8, 9, 15]. Using a two-stage solid-phase extraction procedure and capillary GC—MS with negative-ion chemical ionization (NICI), Waddell et al. [9] reported a PGE_2 sensitivity limit of 100—200 pg/ml when assaying 1 ml of platelet-rich plasma. By processing 20 ml of plasma and inserting a high-performance thin-layer chromatography clean-up step, Smith et al. [8] reported a PGE_2 sensitivity by GC—NICI-MS of at least 0.5 pg/ml. Thus, the sensitivity limit of the present HPLC analysis procedure for 15-methyl-PGE_2 in plasma is comparable to the sensitivity of GC—MS procedures for PGE_2 in plasma, and the precision of the GC—MS and HPLC procedures is comparable (13—21% R.S.D. at 10—20 pg/ml reported by Smith et al. [8] for GC—MS compared to 8—12% R.S.D. at 25 pg/ml for HPLC). Although other HPLC methods have been described with picogram level sensitivity for prostaglandin derivatives [16—18], none have been reported to be useful for the determination of low pg/ml levels of prostaglandins in physiological fluids.

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