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Determination of femtomole/milliliter concentrations of enprostil acid in human plasma using high-performance liquid chromatography–laser-induced fluorescence detection

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ABSTRACT

This paper describes the use of multiple-column high-performance liquid chromatography (HPLC) combined with laser-induced fluorescence for the determination of femtomole/milliliter concentrations of enprostil acid, a prostaglandin analogue, in human plasma. The drug is isolated from plasma by phenyl solid-phase extraction and fluorescently labeled at its carboxyl functional group with a large excess of 2-bromoacetyl-6-methoxynaphthalene. A multi-column method using both normal- and reversed-phase chromatography is necessary to separate the labeled drug from the unreacted reagent. Post-column dilution of the mobile phase with water after the reversed-phase chromatography allows on-line concentration of the labeled analyte onto a guard column prior to the microbore HPLC. A loop guard column device provides a simple way to inject up to 1.0 ml of sample solution onto a microbore column without significantly reducing the column efficiency. A 325-nm He Cd laser is used to excite the labeled drug, and fluorescence emission is monitored at 450 nm. Using this system, we are able to derivatize, detect, and quantify 5 pg of the prostaglandin analogue in 1.0 ml of plasma.

INTRODUCTION

The determination of trace quantities of compounds in biological fluids is an important and challenging task in the pharmaceutical industry. Therapeutic drugs that have high pharmacological activity are given to patients in doses as low as micrograms. The concentrations of the drug and its metabolites *in vivo* are often no more than picograms per milliliter (10^{-11} *M*). This situation presents the analyst with severe difficulties in performing an assay.

Enprostil is a prostaglandin analogue that is under investigation for the treatment of gastro-intestinal disease. After administration of enprostil to humans, its ester group is rapidly hydrolyzed to the corresponding acid form. Plasma levels of enprostil acid are usually no higher than a few hundred picograms per milliliter. Analytical methods that are able to determine such concentrations of prostaglandins in human plasma include gas chromatography with mass spectrometric de-

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tection [1–3], radioimmunoassay (RIA) [1,4,5], and high-performance liquid chromatography (HPLC) with fluorescence detection [6].

Laser-induced fluorescence detection (LIFD) combined with liquid chromatography has been shown to give improved mass detection limits compared to fluorescence detectors with incoherent sources [7,8]. The mass detection limits become even more impressive when laser-induced fluorescence is combined with microcolumn separation techniques [9–14], because of the improved mass handling characteristics of the microbore columns and because the laser radiation can be focused entirely into the nanoliter to picoliter-sized detection volumes. However, all of these low-mass detection limits have been obtained by using either naturally fluorescent compounds or by derivatizing large amounts of compound with fluorescent label and then diluting the sample before analysis.

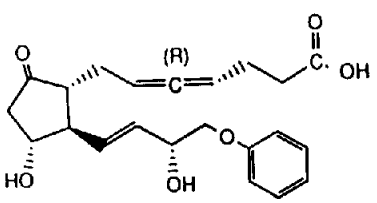
The use of laser-induced fluorescence techniques for the determination of picogram amounts of drugs in *ca.* 2 ml of plasma involves additional difficulties. Non-fluorescent compounds must first be extracted from plasma and then derivatized with a suitable fluorescent tag. Usually a large excess of the fluorescent reagent is required to completely label picogram levels of drug within a reasonable amount of time at moderate temperatures. Since the luxury of diluting the sample (which also dilutes the interferents) is not practical in this case, all of the unreacted reagent and all of the coextracted impurities that either are naturally fluorescent or have been labeled with the reagent (such as fatty acids) must be completely removed before quantification by the ultrasensitive LIFD method. We believe that the successful development of such a sensitive method depends on a highly efficient purification procedure and a well designed, low-noise LIFD system. For example, excellent results were obtained by Pullen and Cox [6] with a multiple column-switching technique in normal-phase HPLC and conventional-source fluorescence detection for prostaglandin analysis of clinical samples. A quantification limit (signal-to-noise ratio = 5) of 10 pg/ml was observed when a 3-ml volume of plasma was processed. In other examples, a carefully designed LIFD system, which included reduction of sample volume, spatial and spectral filtering to reduce background noise, and the use of high-intensity excitation energy allowed the detection of individual molecules on a microscope slide [15] and in the liquid phase [16].

Our laboratory is interested in achieving detection limits lower than 30 pg per 3 ml to support clinical trials of compounds such as enprostil acid. We therefore developed the following method, which incorporates fluorescent derivatization, multiple column-switching HPLC separation, and LIFD.

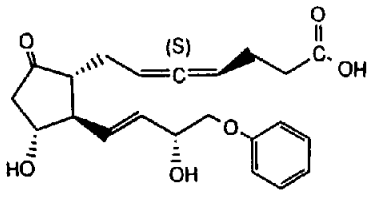
EXPERIMENTAL

Materials

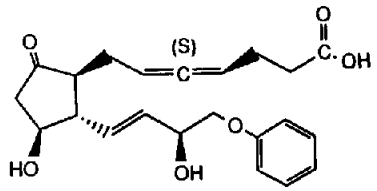
Enprostil acid, *RS*-86505-007 acid, tritiated enprostil acid and *RS*-86505-007 acid (specific activity 40 Ci/mmol), and 2-bromoacetyl-6-methoxynaphthalene



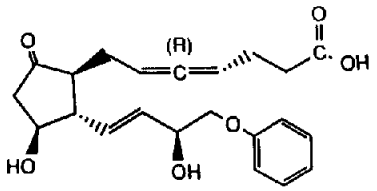
RS-86505-007 acid



RS-86812-007 acid

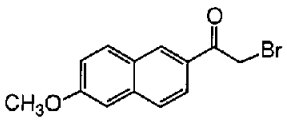


RS-86505-008 acid



RS-86812-008 acid

(I)



(II)

Fig. 1. Structures of (I) the four isomeric components of enprostil acid (*R* and *S* denote absolute configurations) and (II) 2-bromoacetyl-6-methoxynaphthalene.

(BMN) (Fig. 1) were prepared by the Institute of Organic Chemistry, Syntex; glacial acetic acid, sodium acetate, sodium sulfate anhydrous, and potassium carbonate were obtained from Mallinkrodt (Paris, KY, U.S.A.). All solvents used for chromatography and for derivatization were HPLC grade from Burdick & Jackson (Muskegon, MI, U.S.A.), except for water obtained from a Millipore filtration unit. 18-Crown-6–acetonitrile complex was from Eastman Kodak (Rochester, NY, U.S.A.). Phenyl bonded-phase (Bondesil 40 μm, Analytichem International, Harbor City, CA, U.S.A.) was packed into empty mini-extraction columns (Model 2033-4, 5 ml capacity; E & K Scientific Products, Saratoga, CA, U.S.A.) in 100- and 150-mg amounts.

Preparation of standard solutions

Tritiated *RS*-86505-007 acid standard solutions were prepared in acetonitrile at concentrations equivalent to 10, 25, 50 and 100 pg per 100 μl. Enprostil acid standard solutions were prepared in acetonitrile at concentrations of 10, 25, 50, 100 and 200 pg per 100 μl. Tritiated enprostil acid used as internal standard was dissolved in acetonitrile at 1000 cpm per 100 μl.

Extraction

For method development, blank plasma samples (1.0 ml) were spiked with tritiated *RS*-86505-007 acid at concentrations equivalent to 10–100 pg. For the enprostil study, study samples (2.0 ml) were spiked with tritiated enprostil acid (500–1000 cpm) as internal standard. For constructing calibration curves, blank plasma samples (2.0 ml) were spiked with enprostil acid at concentrations of 10–200 pg and also with tritiated enprostil acid (500–1000 cpm) as internal standard. A plasma sample was filtered through a 20-μm disc (Model 2034-4, E and K Scientific Products) and then passed through a 150-mg phenyl-phase extraction column that had been washed with 2 ml of methanol, 2 ml of water and 1 ml of 0.02 *M* sodium acetate buffer (pH 3.0) before use. To remove residual plasma and some of the polar impurities, the column was washed with 0.3 ml of pH 3 buffer, 1 ml of methanol–water (40:60, v/v), 0.3 ml of 0.3% acetic acid and 1 ml of water. The analyte was then eluted from the column with 1.5 ml of methanol–water (60:40, v/v), into a clean 10-ml screwtop test tube, and the solvent was evaporated under nitrogen before derivatization.

Derivatization

To the dried analyte were added 125 μl of acetonitrile containing 125 μg of BMN and 100 μl of acetonitrile containing 3.46 mg of 18-crown-6. The solvent was then evaporated under nitrogen. Single crystals (~2 mg each) of Na_2SO_4 and of K_2CO_3 were added to the test tube. Next, 100 μl of acetonitrile (dried over anhydrous Na_2SO_4) were added. The test tube was tighly capped and shaken gently at room temperature for 1 h. The solvent was evaporated under nitrogen, and the residue was redissolved in 250 μl of dichloromethane (if redissolved in aqueous solution for different procedures, the crystals must be removed first). This solution was used for the purification step described below.

Determination of retention time and recovery

Prior to purification, the retention time of the analyte was determined by manually injecting tritiated analyte (1000–2000 cpm) onto individual normal- or reversed-phase columns. The eluate was collected in 1-min fractions by a fraction collector and checked for radioactivity by a liquid scintillation counter (Tri-Carb, Packard Instrument, Laguna Hills, CA, U.S.A.). The recovery of a procedure was determined by comparison of recovered cpm with the cpm that were spiked initially.

Normal-phase purification

In the normal-phase purification system (Fig. 2), all HPLC components were interlinked through a six-port switching valve, SV (Model EC6W, Valco Instruments, Houston, TX, U.S.A.). The valve position was initially set at position 1 (Fig. 2a). For the purification, 200 μl of the dichloromethane solution from the derivatization above were injected onto the first normal-phase silica column, NC1

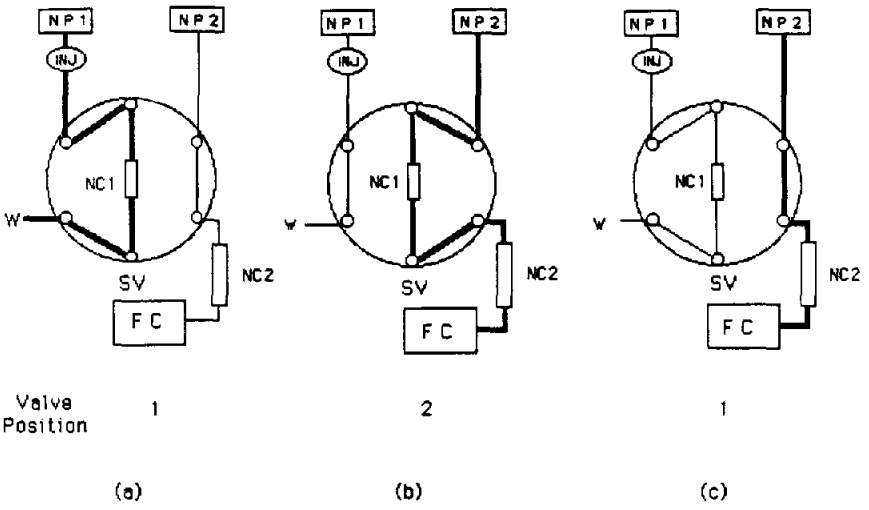


Fig. 2. Schematic diagram of normal-phase purification system: NP1 and NP2, HPLC pumps; INJ, injector; NC1 and NC2, 10- and 22-cm columns; SV, switching valve; FC, fraction collector; W, waste. The bold lines denote flows of interest (see text for details).

(Spheri-5 silica, 5 μm, 100 mm × 4.6 mm I.D.; Brownlee, Santa Clara, CA, U.S.A.), by an autoinjector (712 WISP, Waters Assoc., Milford, MA, U.S.A.). The analyte was eluted through the column by a mobile phase of dichloromethane–acetonitrile (20:70, v/v) delivered by an HPLC pump, NP1 (Rabbit-HPX, Rainin Instrument, Woburn, MA, U.S.A.). A control program (Dynamax HPLC method manager, Rainin Instrument) loaded in a computer (Macintosh SE, Apple, Cupertino, CA, U.S.A.) used the retention times determined above to switch the valve to position 2, 30 s before the analyte peak was to emerge from the column (Fig. 2b) and switched it back to position 1, 30 s after the analyte peak had left the first column (Fig. 2c). This position switching allowed only material in the analyte peak to pass to the second column, NC2 (Sheri-5 silica, 5 μm, 220 mm × 4.6 mm I.D.; Brownlee), and the contaminants before and after the peak were diverted to waste. The analyte was eluted through the second normal-phase column by the same mobile phase used in the first column and delivered by a second Rainin HPLC pump, NP2. A fraction collector, FC (Frac 100 Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.), collected the analyte peak eluted from the second normal-phase column. The collected eluate was evaporated under nitrogen, and the residue was redissolved in 1 ml of dichloromethane and loaded onto the silica cartridge of an AASP (advanced automated sample processor) cassette (Analytichem International) for reversed-phase purification.

Reversed-phase purification

The reversed-phase purification system consisted of three chromatography units serially linked with switching valves (Fig. 3). Full automation was achieved as follows. A Macintosh computer equipped with the same control software used above controlled the flow-rate of two Rainin pumps P1 and P2 and controlled the positions of one Valco six-port switching valve, SV2, and one Valco ten-port switching valve, SV3. Another Valco six-port valve, SV1, was controlled by the AASP, a cartridge injector, (Varian, Walnut Creek, CA, U.S.A.). A control module (System Controller, Waters Assoc.) was used to control the flow-rate of pumps WP1 and WP2 (Model 6000, Waters Assoc.). The computer, AASP, and control module were electronically interlinked. Initially, all valves were set at position 1 (Fig. 3a). The purification started when the AASP switched SV1 to position 2 (Fig. 3b). At this position, the mobile phase MP1 (methanol–water–acetic acid, 40:60:01, v/v) from P1 eluted the analyte from the cartridge onto C1 (Chemcosorb 7CN, 7 μm, 250 mm × 4.6 mm I.D.; Dychrom, Sunnyvale, CA, U.S.A.) at a flow-rate of 1.0 ml/min. During that time the AASP signaled both control systems (the computer and the control module) to initiate the preprogrammed sequences that would control all the following time events.

For clarity, each important stage, which may consist of one or more time events, is described sequentially in a separate paragraph below. The flow path of each stage is highlighted with bold lines (Fig. 3).

–1 min after injection, the AASP switched SV1 back to position 1 (Fig. 3c).

–2 min before the analyte peak was to emerge from C1, WP1 was activated to supply water (2.0 ml/min) to join MP1 at tee T1; 30 s before the analyte peak was to emerge from C1, SV2 was switched to position 2. At this position, the analyte in the water-diluted mobile phase was diverted to CC1 (Spheri-5 C_{18}, 5 μm, 250 mm × 4.6 mm I.D.; Brownlee) on which it was retained (Fig. 3c).

–30 s after the analyte peak eluted from C1, WP1 was turned off and SV2 was switched back to position 1. This position allowed the mobile phase MP2 (methanol–water–acetic acid, 50:50:0.1, v/v) from P2 to elute the analyte from CC1 onto C2 (Hypersil C_{18}, 5 μm, 250 mm × 1.0 mm I.D.; Keystone Scientific, Bellefonte, PA, U.S.A.) at a flow-rate of 50 μl/min for further purification (Fig. 3d).

–Similarly, 2 min before the analyte peak was to emerge from C2, WP1 was activated to supply water (150 μl/min) to join MP2 at T2. At the same time, P1 was turned off and remained off until WP1 was turned off; 30 s before the analyte peak was to elute from C2, SV3 was switched to position 2. At this position, the analyte in the water-diluted mobile phase was diverted to a 600-μl loop, LP, for temporary storage (Fig. 3e).

–30 s after the analyte peak had eluted from C2, WP1 was turned off and SV3 was switched back to position 1. Meanwhile, WP2 was activated to supply water (100 μl/min) to drive the analyte in the loop onto CC2 (Hypersil C_{18}, 5 μm, 30 mm × 1.0 mm I.D.; Keystone Scientific), on which it was retained (Fig. 3f).

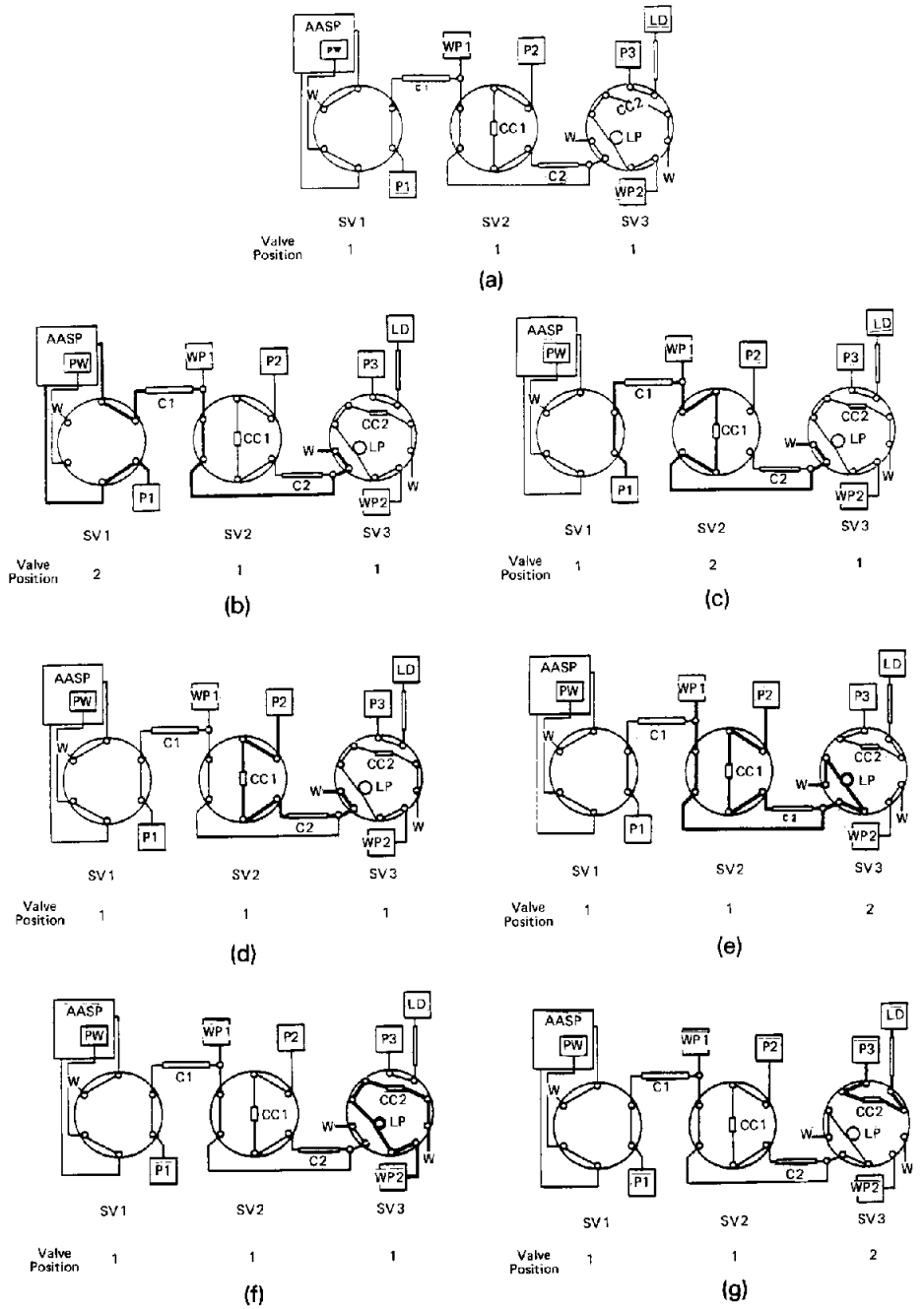


Fig. 3. Schematic diagram of reversed-phase purification system: AASP, advanced automated sample processor; PW, internal purge pump; P1, P2, WP1 and WP2, HPLC pumps; P3, positive displacement pump; C1, cyano column; C2 and C3, 25 and 15-cm microbore columns; CC1, 1-cm hand-packed microbore guard column; CC2, 3-cm microbore guard column; SV, switching valve; LP, 600-μl loop; LD, laser detector; W, waste. The bold lines denote flows of interest (see text for details).

-8 min later, WP2 was turned off and SV3 was switched back to position 2. At this position, mobile phase MP3 (methanol–water, 40:60, v/v) from P3 eluted the analyte from CC2 onto C3 (Hypersil, C_{18}, 3 μm, 150 mm × 1.0 mm I.D.; Keystone Scientific) at a flow-rate of 50 μl/min for final chromatographic purification and then to the LIFD for quantification (Fig. 3g).

-1 min after the analyte had eluted from CC2, SV3 was switched to position 1. All valves had then assumed the original position and were ready for the next injection (Fig. 3a).

When not in use during the run, the reversed-phase columns and guard columns were washed with their own respective mobile phases as long as the washing flow did not interfere with the purification flow. For example, immediately after SV3 was switched from position 2 to position 1 (Fig. 3f) for the last stage purification, P1 and P2 were turned on to wash C1, CC1, and C2.

Fluorescence detector

The equipment used for fluorescence detection is shown in Fig. 4. Linearly polarized light (15 mW, 325 nm) from a He–Cd laser (Liconix, Sunnyvale, CA, U.S.A.) is reflected by a mirror (Newport, Fountain Valley, CA, U.S.A.) and focused by a 100-mm focal length quartz lens (Melles Griot, Irvine, CA, U.S.A.) into the sample cell. Plasma emission lines from the laser are blocked with a colored glass filter. The sample cell is mounted inside a light-tight box. The sample cell is a ~1 cm length of uncoated, fused-silica tubing with a diameter of 500 μm (all fused silica supplied by Alltech, Deerfield, Il, U.S.A.). Each end of the cell is attached to short lengths (2–3 cm) of 100 μm diameter fused-silica tubing. One end is connected to an HPLC column and the other end is connected to waste by means of unions. The cell is mounted at about a 59° angle relative to the ordinarily polarized laser beam. This position reduces the amount of laser light scattered at the air glass and glass mobile phase interfaces. Fluorescence emission is collected 90° relative to the excitation beam by a microscope system (Melles Griot) consisting of a 16× objective, a 10× eyepiece, and a microscope body. A spatial filter located inside the eyepiece at the objective second conjugate plane serves to

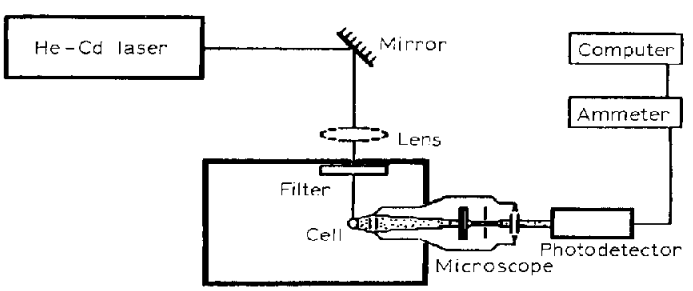


Fig. 4. Schematic diagram of the laser induced fluorescence detector.

reject light scattered at the flow cell. Two spectral filters are placed inside the microscope body: the first (closest to the objective) is an interference filter (Corion S40-450), and the second is a colored-glass, long-pass filter (Corion LL-400). The fluorescence is detected by a Centronix Model Q4249B photomultiplier tube. The signal is amplified by a current amplifier that was built by the Syntex Electronics Shop. Data are collected by a laboratory computer (Nelson Analytical, Cupertino, CA, U.S.A.). The entire system is mounted on an optical table (Syntex Machine Shop) and uses relatively massive optical mounts to minimize the effect of vibration on system alignment.

RESULTS AND DISCUSSION

The diastereoisomers in enprostil acid can be partially separated by a C_{18} column and form a fused peak with a split apex. This sometimes caused confusion with interference peaks during the early stage of development. For the sake of simplicity, tritiated *RS*-86505-007 acid, one of the four isomers of enprostil acid (Fig. 1), was selected as the model compound for method development. Besides showing a single peak in chromatogram, the radioactive isomer provided both the appropriate picogram level amounts for quantification as well as a radioactive label for procedural recovery correction. Otherwise, the method worked equally well for enprostil acid and *RS*-86505-007 acid.

Plasma extraction

Phenyl solid-phase extraction of 5–200 pg of analyte from 1–2 ml of plasma gave an average recovery of 90%, which is similar to the 81% reported by Pullen and Cox [6] for prostaglandin E_2-type extraction from acidified plasma. In our study, the plasma was not acidified because acidification usually resulted in the retention of additional contaminants from plasma onto the column. Instead of plasma acidification, we equilibrated the phenyl columns with 0.02 *M* sodium acetate buffer pH 3.0 before and after loading the plasma samples and after washing the columns with 40% aqueous methanol. The pre-column loading equilibration retained more than 90% of the analyte (but minimal contaminants) during the loading of the plasma, the post-column loading equilibration helped prevent the analyte from being washed of the column by 40% (v/v) methanol in water, and the post-wash equilibration helped retain some yellowish contaminants that were otherwise eluted with the analyte by 60% (v/v) methanol in water. The acidic buffer equilibration and the narrow elution window resulted in a clean eluate that was suitable for direct derivatization without further purification, and thus reduced procedural losses.

Derivatization

BMN was synthesized at Syntex. Compared to commercially available 4-bromomethyl-7-methoxycoumarin (Regis, Morton, Grove, IL, U.S.A.), BMN was

slightly more reactive toward carboxylic acids, although the quantum yields of their prostaglandin derivatives were similar. However, the much wider Stokes shift of the BMN derivative [$\lambda_{ex(max)}$ at 260 nm, $\lambda_{em(max)}$ at 460 nm] (Fig. 5) allowed excellent optical filtering of scattered light from the source lamp. The major advantage of BMN over some of the other fluorescent reagents for our LIFD system was that the BMN derivatives had a broad secondary excitation peak with a maximum at 315 nm (Fig. 5). This characteristic allowed very efficient excitation of the BMN derivative by the 325 nm He–Cd laser.

The derivatization procedure was similar to that reported by Voelter *et al.* [17]. A major improvement was made by reducing the amount of K_2CO_3 from 20 mg to approximately 2 mg, which was equivalent to the weight of a single grain of K_2CO_3. This reduction greatly increased the yield (Table 1) and enabled quantitative labeling of low picogram levels of compounds in biological extracts. Similar but more prominent results were obtained with laidlomycin. In that case, the yield was increased from 10% to about 95% simply by decreasing the amount of K_2CO_3 [18]. The reason for the improvement is unknown although part of it may be due to small amounts of water in the K_2CO_3. Other parameters studied to improve the yield included the purity of the analyte, the volume of the reaction vial, the reaction temperature, the type and volume of the reaction medium, the amount of BMN, and the type of base. However, in actual analysis less than optimal conditions were employed in order to minimize procedural losses and operational inconvenience. For example, if the plasma extract was further purified by HPLC as previously reported [6], the amount of BMN required for quantitative labeling was reduced from 125 to 30 μg, but the additional purification would require an additional HPLC system and also cause an additional 20–30% loss of the analyte. Using the reaction conditions reported above, we were able to label quantitatively in 1 h at room temperature 1 pg of enprostil acid from a plasma extract.

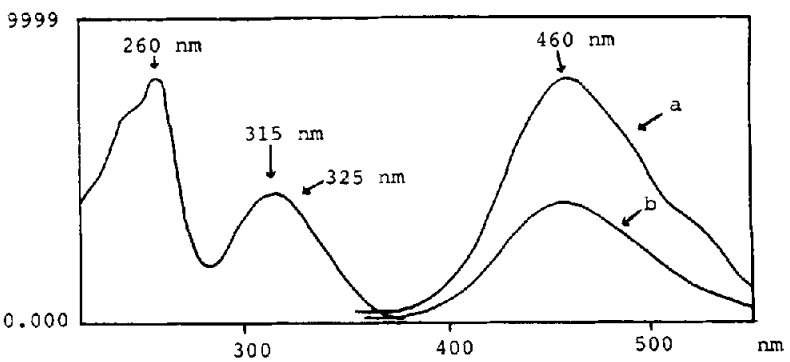


Fig. 5. Fluorescence excitation and emission spectrum of the BMN derivative of *RS*-86505-007 acid: (a) emission spectrum obtained by excitation at 260 nm; (b) emission spectrum obtained by excitation at 325 nm.

TABLE I

EFFECT OF THE AMOUNT OF POTASSIUM CARBONATE ON THE YIELD OF THE DERIVATIZATION OF *RS*-86505-007 ACID WITH BMN

Amount of K_2CO_3 (mg)	Yield (%)
20	54
15	61
10	72
5	82
2	92

Normal-phase HPLC purification

The primary reason for the normal-phase purification was to remove as much of the large excess of unreacted BMN as possible before the final purification by the reversed-phase HPLC columns. On the normal-phase column, BMN eluted faster than the labeled drug with the mobile phase stated above. During the chromatography in the short (10 cm) normal-phase column, the large excess of BMN overlapped the analyte and was only partially removed, although the column-switching techniques diverted only the analyte region to the 22-cm normal-phase analytical column. About 90% of the BMN was removed at this step. The 10-cm column was washed after each injection by the mobile phase. The subsequent purification by the 22-cm analyical column removed most of the remaining BMN as well as some contaminants from plasma. The overall purification efficiency, determined by a fluorimeter, was over 99% (Fig. 6). The effluent containing the analyte peak was collected, the solvent was evaporated, and the dichloromethane-reconstituted residue was loaded onto a normal-phase AASP cartridge for reversed-phase HPLC purification.

AASP cartridge injection

The AASP is a cartridge injector designed to elute the analyte directly from the cartridge onto the HPLC column without leaving the system. Since it required some time to elute the analyte completely from cartridge onto the column, the resulting HPLC peaks were usually broader than those obtained by a conventional injector. This effect became even more pronounced in our study when reversed-phase cartridges were used, because the relatively weak mobile phase employed in the first reversed-phase HPLC column also eluted the analyte from the cartridges (Fig. 3b). The problem was solved by loading the analyte from the normal-phase purification onto a silica cartridge. The weak (for reversed-phase cartridge) water-containing mobile phase then acted as a very strong one for the normal-phase cartridge. It eluted the analyte from the cartridge very rapidly and generated sharp HPLC peaks.

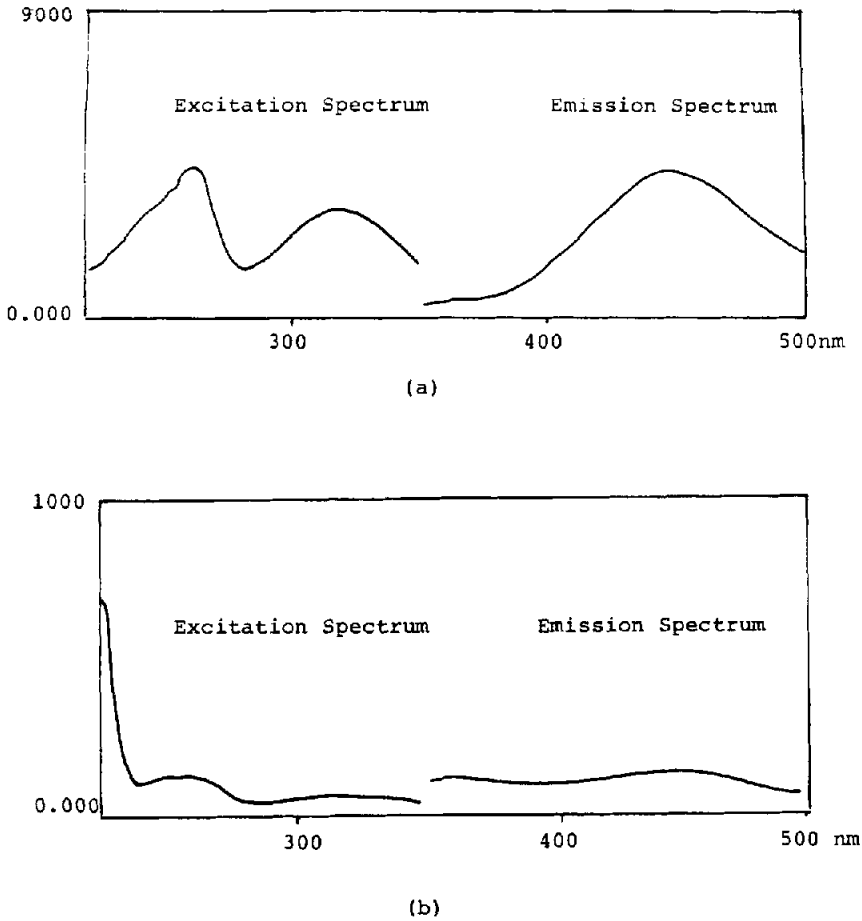


Fig. 6. Fluorescence intensity of (a) the reaction solution (dried and reconstituted with 1.0 ml of methanol) before the normal-phase purification and (b) the analyte-containing effluent collected from the 22-cm normal-phase column (dried and reconstituted with 1.0 ml of methanol). Note the different scales of y-axes.

Reversed-phase purification

A conventional cyano column as a reversed-phase cartridge was selected for major purification because toward prostaglandins it had HPLC characteristics complementary to those of phenyl and C_{18} columns. A 25-cm C_{18} microbore column was used for additional purification. A final 15-cm C_{18} microbore column packed with 3-μm particles was used for final purification and quantification. The system was designed such that the analyte from a previous column was directly concentrated onto the following guard column and later automatically injected from the guard column onto the next column. A similar idea with a different approach was reported by Pullen and Cox [6,19]. In their system, a 2-ml loop was used to temporarily store the analyte from a previous column. Upon the

switching of a valve, the analyte was eluted from the loop to the next column. This design required the mobile phase of the first column be weaker than that of the second one, otherwise the analyte peak from the second column would be severely broadened. In our design, a guard column was used to concentrate the analyte eluted from the previous column. The programmed on-line addition of water to the eluate that contained the analyte peak from the first HPLC purification column weakened the mobile phase and ensured the retention of the analyte by the short (1.5 cm) guard column. This method provides the equivalent of a concentration factor of more than 100, because the analyte was retained in a very thin band on top of the guard column. This band of analyte remained sharp as the mobile phase was changed to elute it onto the second HPLC column for further purification.

The on-line dilution approach was not feasible for the microbore column system, however. After the addition of water to the effluent, the combined flow-rate of 200 μl/min generated a back-pressure of nearly 413 bar on the 3-cm microbore guard column. The high back-pressure caused irreproducible retention times and poor and irreproducible recoveries of analyte. To avoid this problem, a 600-μl loop was inserted between these two columns to temporarily store the water-diluted analyte peak, and the guard column was held off-line. After the entire analyte peak was collected in the loop, the guard column was switched on-line, and the material in the loop was delivered to the guard column by a water pump at a flow-rate of 50–100 μl/min. This novel technique thus achieved the same effective concentration of the analyte from the 25-cm microbore column as was seen with the first reversed-phase HPLC column, while it avoided the generation of high back-pressure and its associated problems. This methodology can also be used independently as a way to inject up to 1 ml of sample solution onto a microbore column without broadening the peak significantly. Usually the optimal injection volumes for the microbore column were less than 10 μl. A positive displacement pump accurately delivered mobile phase to elute the analyte through the 15-cm microbore column to the LIFD cell for quantification. Since washing the columns with their own respective mobile phases removed all significant interferents, a separate washing cycle was not necessary. The entire reversed-phase run required 45–60 min to complete, which allowed us to process fifteen to twenty samples a day.

Laser-induced fluorescence detector

This detector incorporates features which are designed to maximize the amount of fluorescence collected, minimize scattered light, and provide good chromatographic resolution. The design of this apparatus is similar to those of LIFD systems used for capillary electrophoresis [10,20]. The entire optical system is mounted on an in-house-constructed optical table. The optics are mounted on sufficiently massive structures to minimize vibration. The flow-cell is mounted on a three-axis translation stage for easy adjustment of the sample cell position, and

is located in a light-tight box. The excitation radiation (325 nm) passes through a colored glass filter which screens plasma radiation emitted from the laser. The cell design incorporates a 500 μm I.D. fused-silica flow cell [21], which provides a long path length while it preserves chromatographic resolution. The cell is tilted at Brewster's angle relative to the ordinary polarized laser excitation beam to minimize scattered light at the air–glass and glass–liquid interfaces. The 500 μm I.D. flow cell is connected to the end of the microbore column with a piece of 100 μm I.D. fused-silica tubing to minimize band broadening. The microscope body provides a convenient alignment system for the fluorescence collection optics. The fluorescence is collected with a 16× microscope objective, which combines good light collection (numerica aperature, NA = 0.45) with a reasonable working distance (4.2 mm) for easy alignment of the sample cell in the field of view of the microscope. The spectral filters are mounted in the interior of the microscope body, with the interference filter preceding the absorption filter to prevent excitation of fluorescence from the absorption filter by scattered light. A spatial filter is placed at the microscope second conjugate plane, which is located within the eyepiece at the reticle stop and is constructed to reject light scattered at the air–glass and glass–liquid interfaces of the flow cell, while accepting the maximum amount of sample fluorescence. The signal is measured by an efficient photomultiplier tube monitored by a low-noise ammeter.

Day-to-day operation of the detector requires little maintenance or adjustment. The detector is simple to align; this task can be completed in less than 5 min. The 325-nm He–Cd laser is convenient to use, although it is not an ideal light source. We have noticed slow but steady deterioration of the laser power due to damage of the thin film coating of the UV mirrors. Over the course of several months, the laser output power decreased from 15 mW to about 6 mW under heavy use (the quantification limit of 5 pg/ml reported here was obtained at 10 mW).

TABLE II

EVALUATION OF THE CALIBRATION CURVE OBTAINED BY TRIPLICATE DETERMINATIONS OF THE PLASMA SAMPLES SPIKED WITH *RS*-86505-007 ACID

Quantity spiked (pg/ml)	Mean quantity found (n = 3) (pg/ml)	Recovery (mean ± S.D.) (%)	C.V. (%)
10	10.3	103 ± 1.53	14.7
25	23.8	95.2 ± 1.04	4.4
50	54.3	108.4 ± 0.57	1.1
100	102.7	102.7 ± 0.57	0.6

Coefficient of correlation 0.999

Linearity, reproducibility and accuracy

Linearity, reproducibility, and accuracy of the method were evaluated by triplicate analysis of plasma samples spiked with tritiated *RS*-86505-007 acid at concentrations equivalent to 10, 25, 50 and 100 pg/ml . The results (Table II) showed that, over the concentration range 10 100 pg/ml of plasma, the method was linear ($r > 0.9999$), reproducible (coefficient of variation, C.V. $< 14.7\%$) and accurate ($\rho < 8\%$). The representative chromatograms are shown in Fig. 7.

Detection and quantification limit

The on-column detection limit at a signal-to-noise ratio of 10 was 2 pg of *RS*-86505-007 acid. The quantification limit, defined as the concentration of *RS*-86505-007 acid that produced a signal-to-noise ratio of 10, was 5 pg/ml of plasma when 2 ml of plasma was used (Fig. 2b). Additional possible methods to improve the assay quantification limit include reducing the procedural loss by simplifing the purification procedures, designing a more sophisticated LIFD sys-

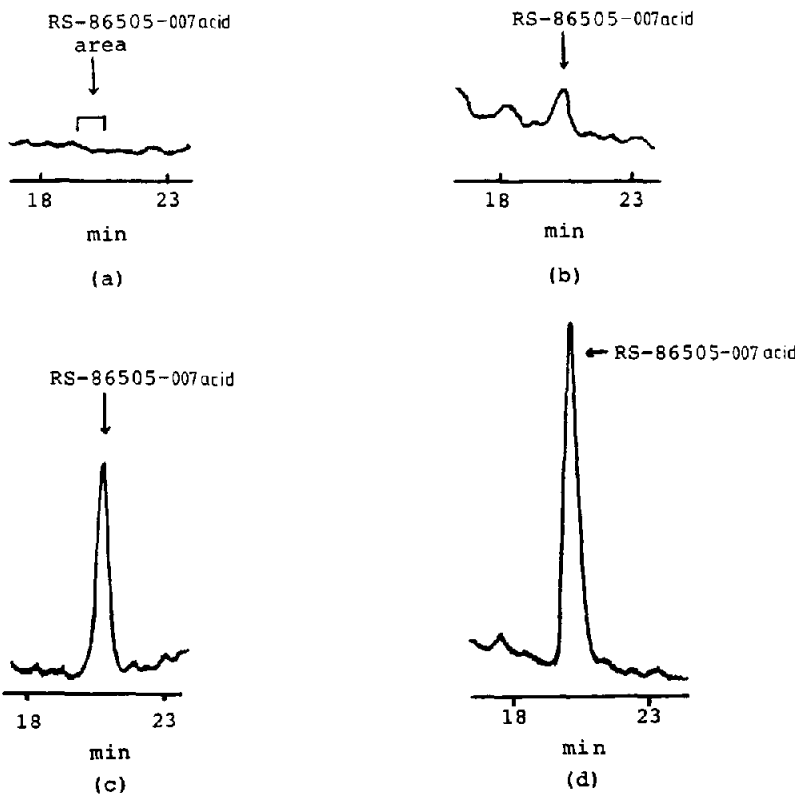


Fig. 7. Representative chromatograms of (a) 1 ml of plasma spiked with 0 pg, (b) 2 ml of plasma spiked with 10 pg, (c) 1 ml of plasma spiked with 50 pg, and (d) 1 ml of plasma spiked with 100 pg of *RS*-86505-007 acid.

tem, using capillary column chromatography and processing larger sample volumes. However, the latter two options are not generally suitable for routine analysis due to highly restricted injection volumes in capillary column chromatography and limited plasma volumes of study samples.

Recovery

The overall procedural recovery of the analyte was 40–60%, which corresponds to individual recoveries of 80–90% for each of the following steps: extraction, derivatization, normal-phase HPLC, and reversed-phase HPLC. Therefore, overall improvement in quantification limit is unlikely to be achieved by the improvement of the recovery of any one step.

Application

The method was used to assay enprostil acid in human plasma samples obtained from subjects who had been given an oral dose of 70 μg of enprostil. Tritiated enprostil acid was used as internal standard to correct for procedural recoveries; however, the mass of the added internal standard was significant compared to the mass of the enprostil acid in the sample initially. To correct the mass deviation, the small peak area generated by the added internal standard was subtracted from the corresponding total peak area of each sample (the internal standard peak area was calculated for each sample from a reference peak generated by pure tritiated enprostil acid that had been injected in the same injection run as the study samples). For each run, the corrected peak area from spiked samples were used for constructing the calibration curves, and the corrected peak areas from unknown samples were used for calculating the concentrations of enprostil acid in those unknown samples. A typical calibration curve of this assay is linear over the range of 10–200 pg of enprostil acid spiked into 2 ml of plasma samples ($r = 0.998$, $y = 540 + 142x$). Although the samples had been stored in a freezer for about two years prior to analysis by the method described in this report, the results shown in Table III correlated reasonably well with those previously obtained by an HPLC–RIA method [5] that had a detection limit of 25 pg/ml of

TABLE III

CONCENTRATIONS OF ENPROSTIL ACID IN HUMAN PLASMA OBTAINED FROM SUBJECTS RECEIVING A SINGLE ORAL DOSE (70 μg) OF ENPROSTIL

BQL means below the quantification limit (25 pg for HPLC–RIA; 5 pg for HPLC–LIFD).

Method	Concentration (pg/ml)						
	Pre-dose	15 min	30 min	1 h	1.5 h	2 h	3 h
HPLC RIA	BQL	BQL	123	86.5	37.5	BQL	BQL
HPLC–LIFD	BQL	24	113	27	21	7	BQL

plasma for a sample of 2 ml. The significant difference in the concentration of enprostil acid determined by the current LIFD method and by the previous HPLC–RIA method for the 1-h sample may be due to decomposition of the analyte during the previous assay or storage. It is noteworthy that the current method was able to quantify enprostil acid in the 15 min and 2-h samples, whereas those two samples were below the quantification limit of the previous HPLC–RIA method. The additional data provide more defined bioavailability profiles for pharmacokinetic studies.

CONCLUSION

We have demonstrated that the use of a microbore column with LIFD enabled the quantification of 5 pg of enprostil acid per ml of plasma when 2 ml of plasma was processed (signal-to-noise ratio = 10). It is better than a three-fold improvement over the procedure reported by Pullen and Cox [6], which had a quantification limit of 10 pg/ml for a 3-ml plasma sample and used a conventional detector (signal-to-noise ratio = 5). The automated heterocolumn system (silica, cyano, and C_{18} phases) efficiently removed the unreacted, large excess of BMN and interferents coextracted from plasma. In addition, the extraction procedure that we developed produced extracts that were cleaner and more suitable for derivatization, the BMN derivatization technique enabled the quantitative labeling of 1 pg of analyte in crude plasma extracts, the on-line-post-column addition of water provided on-line concentration of analyte onto a guard column without leaving the HPLC system, and the loop guard column combination allowed the injection of up to 1 ml of analyte solution onto a microbore column without significantly broadening the peak. A sensitive LIFD system was developed that determined fluorescent compounds eluted from a 1 mm diameter microbore column. The method was successfully used to determine the concentration of enprostil acid in plasma samples from a clinical study and produced useful data that were not obtainable by the HPLC–RIA method. Application of the methodology to other prostaglandin analogues is underway. Further study is also under investigation to lower the quantification limit and to simplify the purification procedures so that the method can be conveniently applied to routine assays.

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