Journal of Chromatography, 321 (1985) 353–362
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,392

SILVER-MODIFIED MOBILE PHASE FOR NORMAL-PHASE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROSTAGLANDINS AND THEIR 5,6-*TRANS* ISOMERS IN PROSTAGLANDIN BULK DRUGS AND TRIACETIN SOLUTIONS

L. D. KISSINGER* and R. H. ROBINS
Control Analytical Research and Development, The Upjohn Company, 7843-259-12 Kalamazoo, MI 49001 (U.S.A.)
(Received November 5th, 1984)

SUMMARY

A silver-modified, normal-phase, high-performance liquid chromatographic system has been developed for prostaglanding bulk drugs and triacetin solutions. Silver nitrate present in the mobile phase results in high selectivity for *cis/trans* isomers with conventional silica columns. Prostaglandins were esterified with α-bromo-2'-acetonaphthone prior to chromatography to provide high detectability at 254 nm. For dilute triacetin solutions, a sample preparation scheme based on gravity-flow chromatography with silica columns was developed to isolate the prostaglandin from triacetin prior to derivatization. The analytical technique was applied to triacetin solutions containing as little as 10 μg/ml arbaprostil [15-(*R*)-methyl-PGE_2].

INTRODUCTION

Prostaglandins are a family of fatty acids with a wide variety of biological activities. Pharmaceuticals based on prostaglandins have been developed for cardiovascular disorders, labor induction, and gastrointestinal disorders. Analytical methods for prostaglandins analysis in the pharmaceutical industry must be sensitive, selective, and precise. Prostaglandins are biologically potent; therefore, analytical procedures for pharmaceutical formulations of prostaglandins must provide the sensititivy necessary for the often small amounts of active ingredient. Selectivity of the analytical technique is necessary to assure the resolution of similar prostaglandins, which may have different biological activity. Accuracy and precision of the analytical method are necessary in the pharmaceutical industry to provide high confidence in the determined amount of prostaglandin.

Chromatography has been employed extensively for the determination of prostaglandins. Thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) can be directly employed for separation of prostaglandins, while derivatization is generally necessary to perform gas chromatography of the prostaglandins. Detection of the prostaglandins, especially at lower levels, is often a prob-

lem. Although TLC is useful for separation of prostaglandins, convenient quantitation methods are not available with sufficient sensitivity and precision. Reversed-phase (RP) and normal-phase (NP) HPLC have been performed on the free prostaglandins with detection by refractive index or low wavelength UV absorption[1,2]. To improve the detectability and chromatographic behavior of prostaglandins, derivatives have been formed with the following reagents: α-bromoacetophenone[3], *p*-bromophenacyl bromide[4], *p*-nitrophenacyl bromide[5], α-bromo-2′-acetonaphthone[6,7], and panacyl bromide[8]. The esters produced from the prostaglandin and these α-bromoketones all have strong chromophores for UV light. The general reaction for the derivatization reaction of carboxylic acids with α-bromoketones is shown in Fig. 1 with arbaprostil [15-(*R*)-methyl PGE_2], α-bromo-2′-acetonaphthone and N,N-diisopropylethylamine. A wide variety of NP- and RP-HPLC conditions can be employed for separation of the neutral prostaglandin esters prior to detection with the common 254 nm (mercury vapor source) absorbance detectors.

Even with the wide variety of columns and mobile phase modifiers available for HPLC, often times resolution of analogous prostaglandins may be incomplete. To improve the selectivity with respect to *cis/trans* isomers, silver has been employed in HPLC systems. The selectivity with respect to the *cis/trans* isomers is enhanced by the presence of silver in the chromatographic system, as a result of its preferred interaction with the *cis* isomer of compounds containing carbon–carbon double bonds. Silver complexation or argentation chromatography has been employed to resolve alkenes[9], unsaturated lipids[10,11], and prostaglandins[12,13]. The selectivity of the HPLC system may be modified by having silver present in either the mobile phase or the stationary phase. Water-soluble silver salts have been added to mobile phases for RP-HPLC[14]. For NP-HPLC, silver has been immobilized on ion-exchange stationary phases[13] or coated on stationary phases[15].

A silver-modified NP-HPLC system is described for the determination of prostaglandins as their 2′-naphthacyl esters in the presence of their 5,6-*trans* analogues. Silver is introduced to the NP-HPLC system by dissolving a small amount of silver nitrate in the mobile phase. By having silver present in the mobile phase, an equilibrium with respect to silver on the stationary phase is established. This analytical

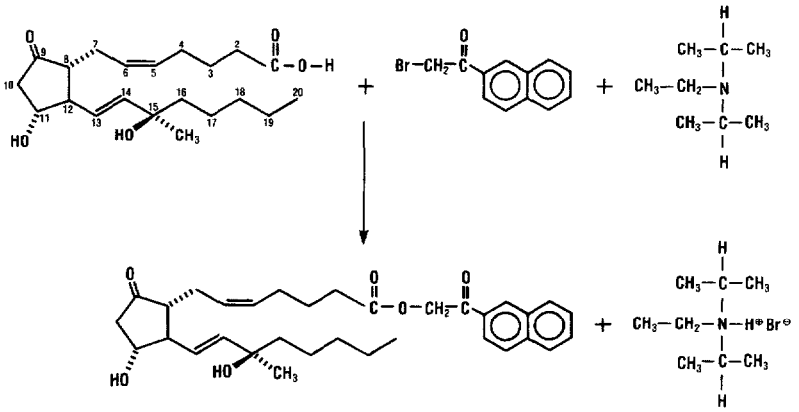

Fig. 1. Derivatization reaction for arbaprostil [15-(*R*)-methyl PGE_2] with α-bromo-2′-acetonaphthone in the presence of N,N-diisopropylethylamine.

approach was applied to several prostaglandins and to triacetin (glycerine triacetate) solutions of arbaprostil. Arbaprostil in triacetin solutions and soft elastic capsules filled with triacetin solutions are stable drug delivery systems for these normally unstable E-type prostaglandins[16]. Since arbaprostil has high activity for gastrointestinal disorders, validation of the method down to a concentration of 10 μg/ml was necessary.

EXPERIMENTAL

HPLC conditions

A modular HPLC consisting of an Altex 100 A pump, Waters WISP autosampler, and Laboratory Data Control UV Monitor III (254 nm absorbance) detector was used. The chromatographic column was a Dupont Zorbax SIL, 250 × 4 mm I.D. The mobile phases were prepared by mixing the appropriate volumes of water, acetic acid, and acetonitrile, then dissolving the appropriate amount of silver nitrate in this solution. This solution was then diluted with the appropriate volume of methylene chloride. Unless otherwise noted, the amounts of methylene chloride, acetonitrile, water, acetic acid, and silver nitrate are: 875 ml, 125 ml, 4 ml, 2 ml and 1.7 g, respectively. Mobile phase flow-rate and injection volume were 1.0 ml/min and 10 μl, respectively, unless otherwise noted.

Derivatization reagents

N,N-Diisopropylethylamine (DIPEA) and α-bromo-2′-acetonaphthone (BAN) were obtained from the Aldrich. Activated carbon treatment of acetone solutions and recrystallization from carbon tetrachloride was performed to purify supplies of BAN before use. Distilled-in-glass acetonitrile (Burdick & Jackson) was employed to prepare solutions of 20 mg/ml BAN and 15 μl/ml DIPEA. These derivatization solutions were prepared fresh daily.

Standard and bulk drug preparation

Approximately 2 mg of prostaglandin were accurately weighed and dissolved in 100.0 ml of methylene chloride. A 1.0 ml aliquot of this solution was transferred to an appropriate container, *i.e.*, a 15-ml conical centrifuge tube for evaporation with a gentle stream of nitrogen. For arbaprostil, an internal standard solution containing 80 μg of prednisone in methylene chloride was added prior to evaporation of the solvent.

Arbaprostil triacetin solutions

Glass columns for gravity-flow chromatography were packed with a 6 × 0.6 cm I.D. bed of Grade 40 silica gel from E. Merck. The columns were conditioned by passing 10 ml of an acetone solution containing 10% ethanol and 0.2% water through the column. A portion of the triacetin solution which contains *ca.* 20 μg of arbaprostil (but not more than 1.0 ml) was added simultaneously with 10 ml of water-saturated carbon tetrachloride to the column. After allowing the solvent to elute from the column, the column was eluted with a 10-ml portion of 20% ethyl acetate in methylene chloride. The prostaglandin was collected in an appropriate container (*i.e.* 15-ml conical centrifuge tube) by eluting the column with 10 ml of an acetone solution

containing 10% ethanol and 0.2% water. To the eluent, an internal standard solution containing 80 μg of prednisone in methylene chloride was added. The solvent was then removed with a gentle stream of nitrogen.

Procedure

To each preparation, 100 μl of each of the following were added: BAN solution, DIPEA solution, and acetonitrile. The bottom tip of the centrifuge tubes were heated for 1 h at 45°C. The acetonitrile from the preparations was then removed with a gentle stream of nitrogen and the residue reconstituted with 5 ml of methylene chloride. These solutions were chromatographed according to the described conditions. Quantitation of arbaprostil was performed by peak height or peak area ratio to the internal standard *versus* the ratio obtained for the standard.

RESULTS AND DISCUSSION

The chromatographic behaviors of several prostaglandins and their 5,6-*trans* isomers as 2′-naphthacyl esters were determined with this silver modified NP-HPLC system. Separation of several of these derivatives is demonstrated by the chromatogram in Fig. 2. Although the prostaglandins are polar and generally yield asymmetric peaks from silica gel columns, the 2′-naphthacyl esters are less polar and symmetric peaks are regularly obtained. With the 6-μm particle size column, chromatographic efficiencies of 10,000 to 15,000 theoretical plates were routinely obtained for the 2′-naphthacyl esters.

Sensitivity for the 2′-naphthacyl esters is high because the molar absorptivities at 254 nm are *ca.* 37,000. UV spectra of α-bromo-2′-acetonaphthone and the 2′-naphthacyl ester of arbaprostil in Fig. 3 demonstrate the detection is being performed

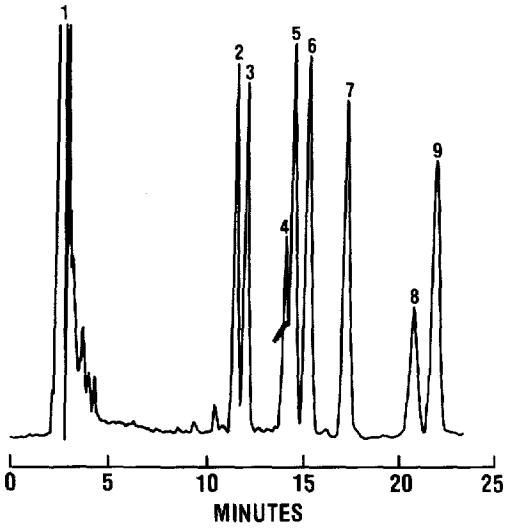

Fig. 2. Chromatogram of several prostaglandin 2′-naphthacyl esters. Peaks: 1 = excess BAN; 2 = 5,6-*trans*-16,16-dimethyl PGE_2; (3) 5,6-*trans*-15-(*R*)-methyl PGE_2; 4 = 16,16-dimethyl PGE_2; 5 = arbaprostil [15-(*R*)-methyl PGE_2]; 6 = 5,6-*trans*-9-deoxo-16,16-dimethyl-9-methylene PGE_2; 7 = 5,6-*trans*-PGE_2; 8 = meteneprost (9-deoxo-16,16-dimethyl-9-methylene PGE_2); 9 = dinoprostone (PGE_2).

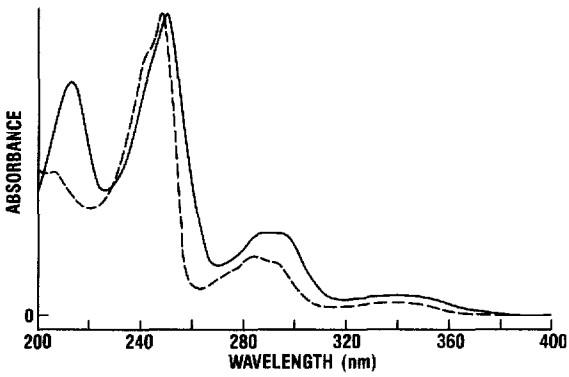

Fig. 3. UV spectra of α-bromo-2′-acetonaphthone (———) and 2′-naphthacyl ester of arbaprostil (----).

approximately at the absorbance maximum. For an injection containing 7 μg of arbaprostil, which has been derivatized to form its 2′-naphthacyl ester, the chromatographic peak has a maximum absorbance of one. Since the chromophore is common for each of the prostaglandin derivatives, peak area responses are equal on a molar basis.

The chromatographic retention behaviors of the 2′-naphthacyl esters of several prostaglandins were determined. As expected with a polar stationary phase, the less polar prostaglandins eluted prior to the more polar prostaglandins. Having hydroxyl groups at the 9 and 11 positions, F-type prostaglandins, result in high capacity factors. A methylene group attached to the 9-carbon results in a higher polarity molecule, as indicated by the retention times, than the analogous E-type prostaglandin which has the 9-keto function. The A-type prostaglandins result from E-type prostaglandins by dehydration of the 11-hydroxyl to form a 10,11-double bond. In turn,

TABLE I

RETENTION OF VARIOUS PROSTAGLANDINS AS THEIR RESPECTIVE NAPHTHACYL ESTERS

Compound	*Retention time (min)*	*k′*
Void (α-BAN)	2.25	0
15-(*R*)-Methyl PGA_2	< 4	< 1
15-(*R*)-Methyl PGB_2	< 4	< 1
8-*epi*-15-(*R*)-Methyl PGE_2	10.8	3.81
5,6-*trans*-16,16-Dimethyl PGE_2	11.5	4.11
5,6-*trans*-15-(*R*)-Methyl PGE_2	12.1	4.38
16,16-Dimethyl PGE_2	14.1	5.27
Arbaprostil [15-(*R*)-methyl PGE_2]	14.5	5.44
15-(*S*)-Methyl PGE_2	16.3	6.26
5,6-*trans*-9-Deoxo-9-methylene-16,16-dimethyl PGE_2	15.4	5.84
5,6-*trans*-PGE_2	17.3	6.67
Meteneprost (9-deoxo-9-methylene-16,16-dimethyl PGE_2)	20.8	8.24
Dinoprostone (PGE_2)	22	8.7
15-(*R*)-Methyl $PGF_{2\alpha}$	>30	>10

the B-type prostaglandin can result from migration of the 10,11-double bond of the A-type prostaglandin to the 8,12-position. The less polar A- and B-type prostaglandins elute near the void volume of the chromatogram. For a series of prostaglandins, the order or increasing polarity and retention, according to prostaglandin type is: B, A, E, F. The A- and B-type prostaglandins elute near the void volume while the F-type prostaglandins generally have capacity factors greater than ten.

The high selectivity of the chromatographic system is ideal for testing pharmaceutical products which contain prostaglandins. Data on arbaprostil and its analogues in Table 1 demonstrate the specificity of the assay in the presence of potential process and degradation impurities. The 5,6-*trans* isomer, which is a common impurity in bulk prostaglandins, is, of course, well resolved using the silver-modified HPLC conditions. Epimers at the 8 and 15 positions differ only at the chiral carbon and their respective 2′-naphthacyl esters are baseline resolved from arbaprostil. The probable decomposition products via dehydration of the 11-hydroxyl and rearrangement to the A and B analogs of arbaprostil are well resolved from arbaprostil. All potential impurities from synthesis and degradation of arbaprostil are resolved, which assures the specificity of the assay.

Silver nitrate in the mobile phase acts as a polar modifier, resulting in reduced retention of the solutes. If the 1.7 g/l silver nitrate were removed from the mobile phase used in Fig. 2, the acetonitrile content to obtain similar retention times must be increased from 125 to *ca.* 300 ml/l. The reduced retention of the prostaglandin 2′-naphthacyl esters is a result of either one or both of the following mechanisms. First, the silver nitrate could be adsorbed on the stationary phase reducing its surface area or its activity which would reduce the distribution coefficients of the prostaglandin 2′-naphthacyl esters. Alternately, the silver ion in the mobile phase could form a complex with the prostaglandin 2′-naphthacyl esters that has a lower distribution coefficient than the uncomplexed species. Of these two mechanisms, the former seems more probable. The change in the stationary phase is also supported by the relative retention of 5,6-*cis vs. trans* isomers of prostaglandin naphthacyl esters. For each prostaglandin, the 5,6-*trans* isomer eluted prior to their respective parent species. Since silver prefers complexation with the *cis* isomer and the *cis* isomer has a higher retention, the silver complex must have a higher distribution coefficient than the free ester. This conclusion is consistent with the fact that the silver ion complex would be more polar than the free ester, which would indicate greater retention with silver present. Therfore, silver nitrate in the mobile phase reduces the retention of these species by altering the stationary phase and resolves *cis*/*trans* isomers by selective complexation with the *cis* isomer, which increases the retention of the *cis* isomer relative to its *trans* isomer. Reduction of the silver nitrate in the mobile phase results in reduced resolution of prostaglandins from their 5,6-*trans* isomers. Without silver nitrate in the mobile phase, no separation of the 2′-naphthacyl ester of arbaprostil and its 5,6-*trans* isomer occurs. Further selectivity for the separation is limited by the *ca.* 2 g/l solubility of silver nitrate in the mobile phase.

Since the equilibrium content of silver on the stationary phase is dependent on mobile phase composition, injecting solutions of different composition from the mobile phase results in perturbation of the silver equilibrium. Addition of 1.7 g/l silver nitrate introduces an absorbance to the mobile phase of 2.9 at the maximum of 240 nm. At 254 nm the absorbance of the mobile phase is *ca.* 0.3; therefore, small changes

of the silver nitrate concentration on the mobile phase will have a significant effect on the baseline. Samples were dissolved in a solvent similar to the mobile phase (*i.e.* methylene chloride) and small injection volumes (*i.e.* 10 μl) were employed. Although the silver equilibrium could be maintained by using mobile phase as the solvent for samples, a precipitate forms when the derivatized samples are reconstituted with mobile phase.

Retention times of prostaglandin 2′-naphthacyl esters were quite reproducible with these chromatographic conditions. To equilibrate the column packing material with the silver ion concentration in the mobile phase, it was necessary to pump the mobile phase through the column for a few hours. After a steady baseline was obtained, the retention times were reproducible for several hours during automated runs. During an 18-h run with arbaprostil bulk drug, the retention time of its 2′-naphthacyl ester was reproduced with a relative standard deviation of 0.7%.

The silver modification of the stationary phase was found to be reversible. Silver can be purged from the system by pumping several hundred milliliters of acetonitrile through the column. After the acetonitrile wash, the column functions normally with mobile phases that do not contain silver. The silver modified mobile phase performed well with several silica HPLC columns, such as Waters Microporasil®, Merck LiChrosorb® SI-100 and the DuPont Zorbax® SIL columns.

The detector operated normally with the silver-modified mobile phase. After several hundred hours of operation with silver-modified mobile phases, no silver deposits were observed in the flow cell. Apparently, at this concentration in a flowing stream, photodeposition is minimal. Other researchers have demonstrated the compatibility of RP systems containing silver with absorbance detectors[9,14]. Special care should be exercised in using the silver-modified mobile phases; for example, deposition of silver on a brass union in the waste line of the liquid chromatograph blocked

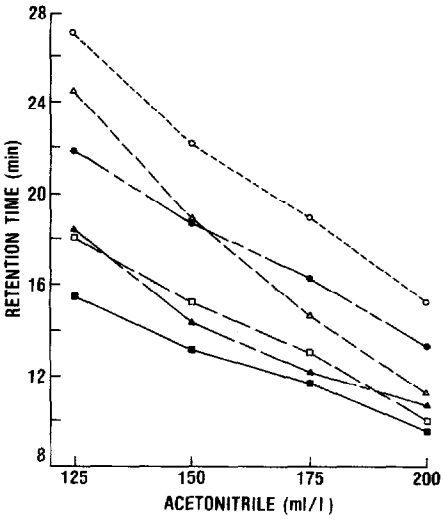

Fig. 4. Retention times as a function of acetonitrile content of mobile phase for 2′-naphthacyl esters of various prostaglandins. Curves: ○ = dinoprostone (PGE_2); ● = 5,6-*trans*-PGE_2; △ = meteneprost (9-deoxy-9-methylene-16,16-dimethyl PGE_2); ▲ = 5,6-*trans*-9-deoxy-9-methylene-16,16-dimethyl PGE_2; □ = arbaprostil [15-(*R*)-methyl PGE_2]; ■ = 5,6-*trans*-15-(*R*)-methyl PGE_2.

the flow of mobile phase thus causing a leak in the detector. No deposition has been observed with HPLC components made of stainless steel, PTFE, and quartz. Mobile phase leaks result in silver deposits that are difficult to remove. With appropriate care, these mechanical difficulties were manageable in a routine laboratory environment.

The derivatization scheme was optimized for rapid reaction with small amounts of the labile prostaglandins. Other researchers have demonstrated that the esterification reaction occurs most rapidly in polar aprotic solvents such as the acetonitrile which was employed[3,6]. Reaction rates were also kept rapid by performing the reaction with high concentrations of the reactants, α-bromo-2′-acetonaphthone and N,N-diisopropylethylamine, in a small volume. Further acceleration of the reaction was achieved by elevating the temperature to 45°C. Since decomposition of many prostaglandins is rapid at high temperature and in the concentrated basic solutions, further acceleration of the derivatization reaction resulted in extra peaks in the chromatograms and reduced precision for the quantitative determination.

Reaction rates for esterification of prostaglandins are much slower in triacetin than in acetonitrile. To analyze dilute triacetin solutions it was necessary to separate the prostaglandin from this solvent prior to the derivatization step. The gravity-flow chromatographic conditions listed above were effective for the separation of arbaprostil from triacetin solutions. After dilution of the triacetin solution with carbon tetrachloride, the arbaprostil can be retained on the small silica gel column. A moderate polarity solvent, 20% ethyl acetate in methylene chloride, is used to elute all of the triacetin from the silica gel. Elution of the prostaglandin from the column can be performed with a small volume of the more polar acetone solution. The gravity-flow chromatographic separation results in quantitative recovery of arbaprostil, 15-(*R*)-methyl PGA_2, 15-(*R*)-methyl PGB_2, 15-(*R*)-methyl $PGF_{2\alpha}$, and dinoprostone (PGE_2). After evaporation of the solvent, the prostaglandin can be derivatized in the absence of triacetin in an acetonitrile solution. This sample preparation scheme has been routinely used for analysis of arbaprostil triacetin solutions from 50 to 200 μg/ml.

The dynamic range of the chromatographic analysis is shown in Fig. 5. From 3.3 ng to 2.3 μg injected, response for arbaprostil is linear. From the plot, sensitivity for arbaprostil is *ca.* 0.2 absorbance units per microgram injected. The smallest sample, 3.3 ng of arbaprostil injected, is equivalent to derivatization of 1.5 μg of arbaprostil. Assuming a peak of $2 \cdot 10^{-4}$ absorbance units is detectable, 800 pg of arbaprostil can be observed by this method. Precision and accuracy of this scheme for determination of submicrogram amounts of prostaglandins is compromised by the inherent instability of these compounds. For samples greater than a milligram, concentrations of the derivatization reagents are significantly reduced by the reaction and lower yield of the derivative results. The upper end of the dynamic range can be extended by taking a smaller fraction of the sample or increasing the concentration of the derivatization solutions.

An example of the chromatography of a preparation from a 50 μg/ml triacetin solution of arbaprostil is shown in Fig. 6. In this example, a higher flow-rate of 1.5 ml/min is employed for shorter analysis time with no significant loss of chromatographic performance. At this high detector sensitivity, several peaks are observed early in the chromatogram from the derivatization step. Prior to the arbaprostil

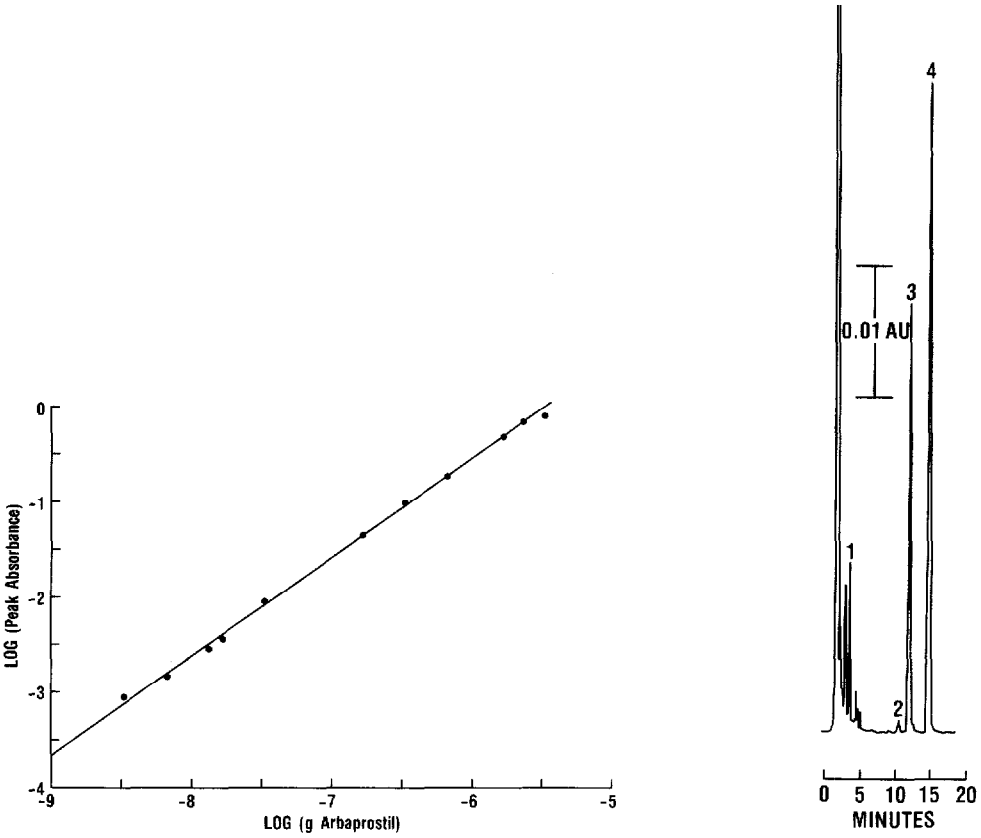

Fig. 5. The logarithm of peak absorbance for naphthacyl ester of arbaprostil as a function of the logarithm arbaprostil weight injected.

Fig. 6. Chromatogram of sample prepared from 50 μg/ml triacetin solution of arbaprostil (flow-rate, 1.5 ml/min). Peaks: 1 = excess BAN; 2 = 5,6-*trans*-15-(*R*)-methyl PGE_2; 3 = arbaprostil; 4 = prednisone (internal standard).

derivative peak, a peak corresponding to the *ca.* 2.5% 5,6-*trans* isomer in arbaprostil is clearly observed. The response for the 5,6-*trans* isomer is a result of 1.2 ng of this analog in the injection. Over a three-month period, 38 assays were performed by several analysts on a 50 μg/ml triacetin solution with the relative standard deviation for these determinations of 2.9%. Triacetin solutions of arbaprostil at 10 μg/ml have been assayed by this procedure, but reproducibility of results is reduced.

No chromatographic separation of arbaprostil from triacetin was necessary for solutions containing more than 200 μg/ml. For these more concentrated solutions, a sample containing *ca.* 20 μg of arbaprostil (40 μl for a 500 /ml solution) can be reacted directly with the derivatization reagents. The small amount of triacetin present in the derivatization solution does not have a significant effect on the reaction rate.

Because of the large molar absorptivity from the two aromatic rings, the derivatization reagent and derivatized samples should be protected from intense light.

If the derivatization solution is exposed to light for extended periods of time, a yellow color results and extra peaks are observed in the chromatogram. Photodecomposition of the 2'-naphthacyl esters of prostaglandins often results in isomerization to form the 5,6-*trans* analogue.

CONCLUSIONS

The described sample preparations and chromatographic conditions provide precise and accurate results for the determination of prostaglandins *versus* reference samples. The silver-modified mobile phase results in high chromatogrtaphic selectivity to resolve the *cis/trans* isomers of the prostaglandin 2'-naphthacyl esters. Dilute triacetin solutions of prostaglandins can be analyzed by these HPLC conditions after a small-scale chromatographic preparation and a derivatization step. Many variations of this analytical scheme are feasible for the determination of prostaglandins or other long-chain carboxylic acids in a variety of matrices.

REFERENCES

1 R. K. Lustgarten, *J. Pharm. Sci.*, 65 (1976) 1533.
2 K. Nagayo and N. Mizuno, *J. Chromatogr.*, 178 (1979) 347.
3 H. D. Durst, M. Milano, E. J. Kikta, S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
4 J. P. Roggero and S. V. Coen, *J. Liq. Chromatogr.*, 4 (1981) 1817.
5 W. Morozowich and S. L. Douglas, *Prostaglandins*, 10 (1975) 19.
6 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.
7 L. W. Brown and B. E. Carpenter, *J. Pharm. Sci.*, 69 (1980) 1396.
8 W. D. Watkins and M. B. Peterson, *Anal. Biochem.*, 125 (1982) 30.
9 G. Schomberg and K. Zegarski, *J. Chromatogr.*, 114 (1975) 174.
10 H. Rakoff and E. H. Emken, *J. Amer Oil Chem. Soc.*, 55 (1978) 564.
11 C. R. Scholfield, *J. Amer. Oil Chem. Soc.*, 57 (1980) 331.
12 W. S. Powell, *Methods Enzymol.*, 86 (1982) 530.
13 M. V. Merritt and G. E. Bronson, *Anal. Chem.*, 48 (1976) 1851.
14 B. Vonach and G. Schomberg, *J. Chromatogr.*, 149 (1978) 417.
15 R. Aigner and H. Spitzy, *Anal. Chem.*, 48 (1976) 2.
16 S. H. Yalkowsky and T. J. Roseman, *J. Pharm. Sci.*, 68 (1979) 114.