(15) J. Autian, *ibid.*, **4**, 3(1973).
(16) L. S. Hillman, S. L. Goodwin, and W. R. Sherman, *N. Engl. J. Med.*, **292**, 381(1975).
(17) J. Mes, D. E. Coffin, and D. S. Campbell, *Bull. Environ. Contam. Toxicol.*, **12**, 721(1974).
(18) *Chem. Eng. News*, **Feb. 15, 1971**, 12.
(19) J. J. McNamara, M. D. Molot, and J. F. Stremple, *Ann. Surg.*, **172**, 334(1970).
(20) R. T. Solis and M. B. Gibbs, *Transfusion*, **12**, 245(1972).
(21) R. V. Moseley and D. B. Doty, *Ann. Surg.*, **171**, 329(1970).
(22) S. W. Kim, R. G. Lee, H. Oster, D. Coleman, J. D. Andrade, D. Lentz, and D. Olsen, *Trans. Amer. Soc. Artif. Int. Organs*, **20**, 449(1974).
(23) R. G. Lee and S. W. Kim, *J. Biomed. Mater. Res.*, **8**, 251(1974).
(24) R. G. Lee, C. Adamson, and S. W. Kim, *Thromb. Res.*, **4**, 485(1974).
(25) "Platelet Function and Thrombosis," P. M. Mannucci and S. Gorin, Eds., Plenum, New York, N.Y., 1972.
(26) "Platelet and Thrombosis," S. Sherry and A. Scriabine, Eds., University Park Press, Baltimore, Md., 1974.
(27) E. W. Salzman, *Fed. Proc.*, **30**, 1503(1971).
(28) M. A. Packham, G. Evans, M. F. Glynn, and J. F. Mustard, *J. Lab. Clin. Med.*, **73**, 686(1969).
(29) J. F. Mustard, M. F. Glynn, E. E. Nishizawa, and M. A. Packham, *Fed. Proc.*, **26**, 106(1967).
(30) R. G. Mason, M. S. Read, and K. M. Brinkhous, *Proc. Soc. Exp. Biol. Med.*, **137**, 680(1971).
(31) L. Vroman and A. L. Adams, *J. Biomed. Mater. Res.*, **3**, 43(1969).
(32) R. G. Lee and S. W. Kim, *ibid.*, **8**, 393(1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 7, 1975, from the *Department of Applied Pharmaceutical Sciences, College of Pharmacy, University of Utah, Salt Lake City, UT 84112*

Accepted for publication July 16, 1975.

Supported in part by Contract NIH-NHLI-73-2908-B and in part by Grant PO1-HL-13738-04 (National Heart and Lung Institute).

The authors thank Dr. H. Oster and Dr. D. Olsen for their contributions to this work.

[x] To whom inquiries should be directed.

High-Pressure Liquid Chromatographic Determination of the 15-Epimer of Dinoprost in Bulk Drug

T. J. ROSEMAN [x], S. S. BUTLER, and S. L. DOUGLAS

Abstract □ The *p*-nitrophenacyl esters of dinoprost and its 15-epimer are well resolved using high-pressure liquid chromatography. Quantitation was achieved using the internal standard technique. The specially synthesized diphenylurea ester of cholic acid was found to be a model internal standard. Graphs of peak height ratios of the prostaglandin to the internal standard were linear with respect to the amount of prostaglandin injected, with the lower detection limit of the 15-epimer being about 0.5%. Data are presented that demonstrate the usefulness of this analytical technique in determining the concentration of the 15-epimer present during studies on the kinetics of decomposition of dinoprost.

Keyphrases □ Dinoprost—high-pressure liquid chromatographic analysis of 15-epimer in bulk drug □ High-pressure liquid chromatography—analysis, dinoprost and its 15-epimer □ Prostaglandins—dinoprost and its 15-epimer, analysis, high-pressure liquid chromatography

Development of stability-indicating assays is necessary for evaluating the feasibility of pharmaceutical dosage forms before clinical testing programs are initiated. Prostaglandins, for example, are being developed in many different drug delivery systems (1), *e.g.*, solutions, suppositories, and tablets, where the spectrum of decomposition products depends upon the particular prostaglandin and dosage form. To obtain accurate rate constants for the decomposition process, more than one analytical technique may be needed to distinguish between various degradation products.

A case in point is the separation and quantitation of the 15-epimer of dinoprost in the presence of dinoprost. It was reported previously that dilute solutions of dinoprost are unstable in acidic media (2), with a major route of degradation being epimerization of the C-15 hydroxyl group[1]. To date, the 15-epimer has not been resolved from dinoprost by GLC (3), but it can be separated by TLC in a reported solvent system (4).

Recently, however, radioisotopically labeled methyl esters were used to determine the 15-epimer content in a prostaglandin closely related to dinoprost (3), while the 15-epimer content in bulk dinoprost was determined using a refractive index detector on a high-pressure liquid chromatograph (5). High-pressure liquid chromatography (HPLC) was used to detect and quantitate various prostaglandins (6).

Utilizing the discovery that prostaglandins can be rapidly converted to *p*-nitrophenacyl esters for HPLC (7), a procedure is presented that quantitatively separates the 15-epimer from naturally occurring dinoprost. The procedure involves the reaction of the carboxylic acid group of the prostaglandin with *p*-nitrophenacyl bromide to yield the UV-absorbing *p*-nitrophenacyl ester. The derivatives are well separated and are quantitated using the internal standard technique. This analytical procedure is not only useful in calculating the amount of 15-epimer in bulk dinoprost but also can be utilized to determine the concentration of the 15-epimer in solution during studies on the kinetics of decomposition of dinoprost.

[1] T. J. Roseman, unpublished data.

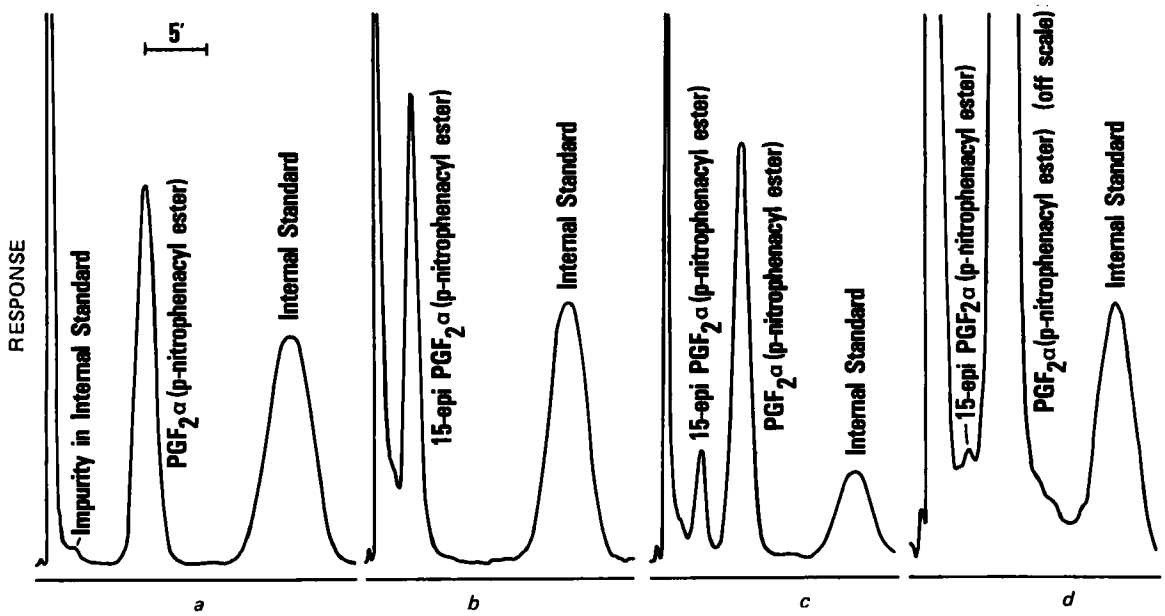


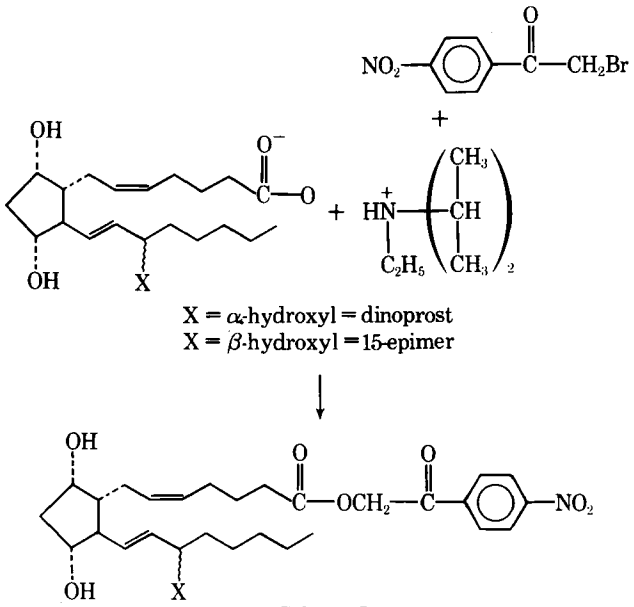
Figure 1—*High-pressure liquid chromatograms of* p-*nitrophenacyl esters of dinoprost ($PGF_{2\alpha}$) and its 15-epimer (15-epi $PGF_{2\alpha}$). (a and b) Concentration of internal standard = 3.75 mg/ml, attenuation = 16 × 10^{-2} aufs, and amount injected = 20 μg. (c) Concentration of internal standard = 1.25 mg/ml, attenuation = 16 × 10^{-2} aufs, and percent of 15-epimer = 16.7. (d) Concentration of internal standard = 0.42 mg/ml, attenuation = 2 × 10^{-2} aufs, and percent of 15-epimer = 1.0.*

EXPERIMENTAL

The prostaglandins[2], dinoprost, dinoprost tromethamine salt, and the 15-epimer were of high purity, being at least 99% pure by GLC and showing only one major zone by TLC. All of the solvents used were "distilled in glass" quality.

Preparation of Samples for Standard Curves—Samples for response curves were prepared from 5-mg/ml stock solutions of dinoprost (free acid) or its 15-epimer in chloroform. Aliquots of the stock solutions were pipetted into small vials and dried under nitrogen to yield the desired amount of prostaglandin.

The *p*-nitrophenacyl esters of dinoprost and its 15-epimer were prepared in the manner reported previously (7) as follows (Scheme I). Samples were dissolved in 0.5 ml of acetonitrile containing 12.2 mg/ml of *p*-nitrophenacyl bromide[3]. Then 5.6 μl of bis(isopropyl)ethylamine[4] (distilled one time) was added, and the mixture was allowed to react for 30 min at room temperature. Then 0.5 ml of the internal standard solution, 0.83–7.5 mg/ml of cholic acid diphenylurea ester in methanol, was added to the sample. Methanol was used to keep the internal standard in solution.



Scheme I

A 4-μl sample was injected into a high-pressure liquid chromatograph[5] within 3 hr under the following conditions: column, 1 m stainless steel (2.1 mm i.d.) packed with 20-μm silica gel[6]; detector, UV 254 nm; temperature, ambient; pressure, 2000 psi; flow rate, ~1 ml/min; and solvent mixture, acetonitrile–methylene chloride–water (50:50:1).

The internal standard concentration was reduced by a factor of three (to 1.25 mg/ml) when a concentration range of 5–20% (w/w) of the 15-epimer in dinoprost was quantitated. A further threefold reduction (to 0.42 mg/ml) was employed for levels below 5%. Samples were chromatographed within 3 hr after the reactants were added, since the ratio of derivatized prostaglandin to internal standard decreased gradually with time, *i.e.*, 15% over 3 days.

Extraction of Prostaglandins from Kinetic Experiments—To check for the presence of the 15-epimer under acidic and basic conditions using HPLC, aqueous solutions were first extracted and then derivatized as described. A 10-ml acidic sample of dinoprost (0.2 mg/ml as the tromethamine salt) from a kinetic run, stored for 30 days at 37° in pH 3.0 citric acid buffer, was pipetted into a 60-ml separator. The solution was then extracted with 3 × 15 ml of chloroform and evaporated to complete dryness with a stream of nitrogen. This extraction procedure yielded complete recovery of the prostaglandins. The dried extract was then derivatized as described.

For the study in the alkaline milieu, a solution containing 5 mg/ml equivalents of dinoprost as the tromethamine salt was stored for 10 months at 25° at pH 8.0. One milliliter was then placed in a separator, and the pH was lowered to 3 with citric acid buffer. The solution was extracted with 3 × 15 ml of chloroform and then reacted as described after the chloroform was dried under a stream of nitrogen. For this experiment, a reference sample was prepared by dissolving 5-mg equivalents of dinoprost as the tromethamine salt, in 1 ml of water and then extracted and analyzed as already described.

Synthesis of Internal Standard—The internal standard, cholic acid *p*-(3′-phenylureido)phenyl ester, commonly called the di-

[2] Obtained from the Pharmaceutical Research and Development Division, The Upjohn Co.

[3] 2-Bromo-4′-nitroacetophenone, practical grade, Eastman Organic Chemicals, Rochester, NY 14650

[4] Pfaltz-Bauer Co., Flushing, NY 11368

[5] Dupont 830 liquid chromatograph, DuPont Co., Wilmington, DE 19898

[6] LiChrosorb SI60, EM Laboratories, Inc., Elmsford, NY 10523. See Ref. 7 for the silica wash procedure.

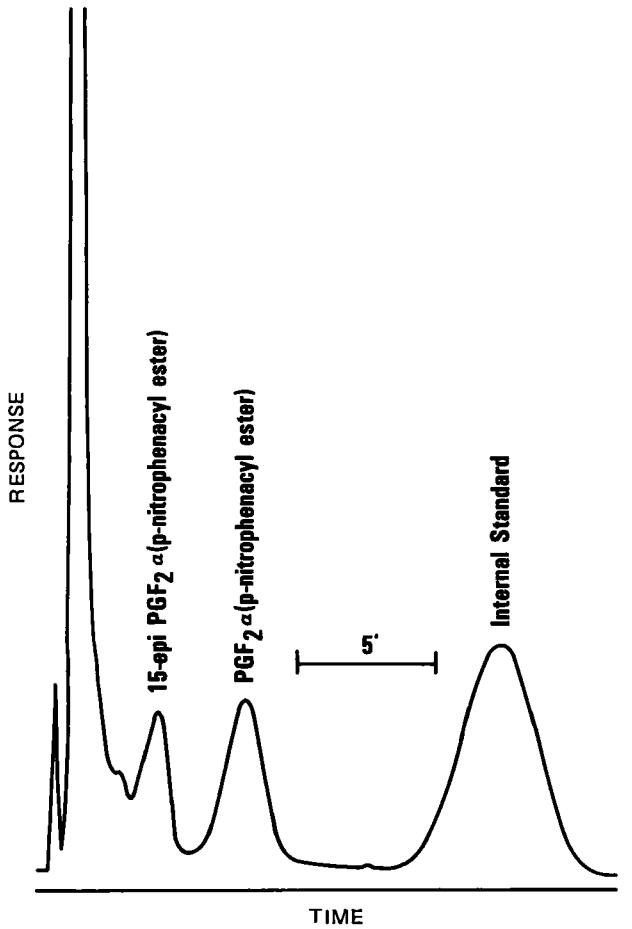


Figure 2—*Chromatogram of extracted sample from a kinetic experiment at pH 3.0. Internal standard concentration was 1.25 mg/ml, and the attenuation setting was 16 × 10^{-2} aufs.*

phenylurea ester of cholic acid, was prepared in the following manner.

A suspension of 4.0 g of cholic acid[7] in 50 ml of dry acetone was treated with 1.39 ml of triethylamine[8]. Immediate crystallization of the triethylamine salt occurred. While under a nitrogen atmosphere, the suspension was cooled to −10° and 1.35 ml of isobutyl chloroformate[9] was added. The mixture was maintained at −5–0° for 6 min before 2.2 g of *p*-hydroxydiphenylurea[10] in 15 ml of dry pyridine was added.

After 15 min at room temperature, the reaction mixture was diluted with 150 ml of ethyl acetate and washed with 2 × 150 ml of aqueous 5% citric acid followed by 2 × 100 ml of 0.1 *N* Na_2HPO_4[11] at pH 7.5. The organic phase was dried (sodium sulfate[11]) and evaporated to dryness. The compound was dissolved in 10 ml of chloroform–acetonitrile (3:7), filtered through 20 g of silica gel[12], and washed with 50 ml of the same solvent. The filtrate was concentrated and chromatographed over 300 g of silica gel 60.

The column was eluted with chloroform–acetonitrile (3:7) followed by chloroform–acetonitrile (1:4), acetonitrile, and, finally, acetonitrile–methanol (95:5). The first 1500 ml of eluent was discarded, and the product was found in the following 2000-ml fraction.

Evaporation of the solvent from the product fraction gave a white residue. This residue was crystallized from acetone by the addition of water, affording 0.85 g of product, mp 218–219°.

Anal.—Calc. for $C_{37}H_{50}N_2O_6$: C, 71.82; H, 8.14; N, 4.52. Found: C, 71.38; H, 7.61; N, 4.31.

[7] Nutritional Biochemicals Corp., Cleveland, OH 44128
[8] Matheson, Coleman and Bell, Norwood, OH 45212
[9] Eastman Organic Chemicals, Rochester, NY 14650
[10] Synthesized by reacting *p*-aminophenol with phenyl isocyanate, mp 218.8–220.3°.
[11] Analytical reagent, Mallinckrodt Chemical Works, St. Louis, MO 63160
[12] EM Laboratories, Elmsford, NY 10523

Table I—Peak Height Ratio (Prostaglandin–Internal Standard) of *p*-Nitrophenacyl Esters of Dinoprost and Its 15-Epimer as a Function of the Amount of Prostaglandin Injected

Micrograms Injected	Peak Height Ratio
Dinoprost	
1.22	0.098
4.12	0.342
12.4	0.982
20.8	1.77
15-Epimer	
0.40	0.039
2.00	0.210
4.00	0.350
12.0	1.08
20.0	1.84

Table II—Peak Height Ratio (Prostaglandin–Internal Standard) for the 15-Epimer (*p*-Nitrophenacyl Ester) in the Presence of Dinoprost (*p*-Nitrophenacyl Ester) for Low and High Concentrations of the 15-Epimer

Percent 15-Epimer Present	Peak Height Ratio
Low Concentration[a]	
0.50	0.056
1.0	0.125
2.0	0.273
High Concentration[b]	
2.0	0.150
5.7	0.413
9.1	0.652
16.7	1.21

[a] Internal standard concentration was 0.42 mg/ml, and attenuation was 2 × 10^{-2} aufs. [b] Internal standard concentration was 1.25 mg/ml, and attenuation was 16 × 10^{-2} aufs.

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms of dinoprost, its 15-epimer, and a mixture of the two. Baseline separation was achieved, with the epimer following directly after the solvent front. The internal standard, the diphenylurea ester of cholic acid, was nicely resolved after the dinoprost peak.

The use of an internal standard is quite helpful when quantitative studies are required. In this study, selection of an appropriate standard was a formidable task because (*a*) it should not decompose in the reaction medium, (*b*) it must be soluble in the reaction medium, and (*c*) it should have a retention time greater than dinoprost and yet elute in a reasonable time. This last requirement is necessary because the *p*-nitrophenacyl ester of the 15-epimer elutes before the ester of dinoprost and it would be difficult to resolve the internal standard peak in this region.

As a first attempt, many standard laboratory chemicals were chromatographed but did not meet these requirements. Since cholic acid has similar physicochemical properties as dinoprost (8), cholic acid derivatives were explored as possible internal standards. Commercially available compounds such as cholic acid, deoxycholic acid, and glycolic acid were unacceptable, so derivatives were synthesized. As shown in Fig. 1, the diphenylurea ester of cholic acid had sufficient polarity to yield an ideal retention time, and it did not degrade in the reaction medium. However, methanol was required to maintain it in solution.

Standard Curves—Table I lists the ratio of the peak heights of dinoprost and its 15-epimer to the internal standard height as a function of the amount of prostaglandin injected. Response curves were linear with respect to the amount injected in the regions under consideration. A plot of this ratio for the 15-epimer *versus* the percent of epimer present was also linear, as reflected by the data in Table II. The slope of this standard curve for the 15-epimer in the presence of dinoprost was comparable (within 13%) to that of the 15-epimer alone.

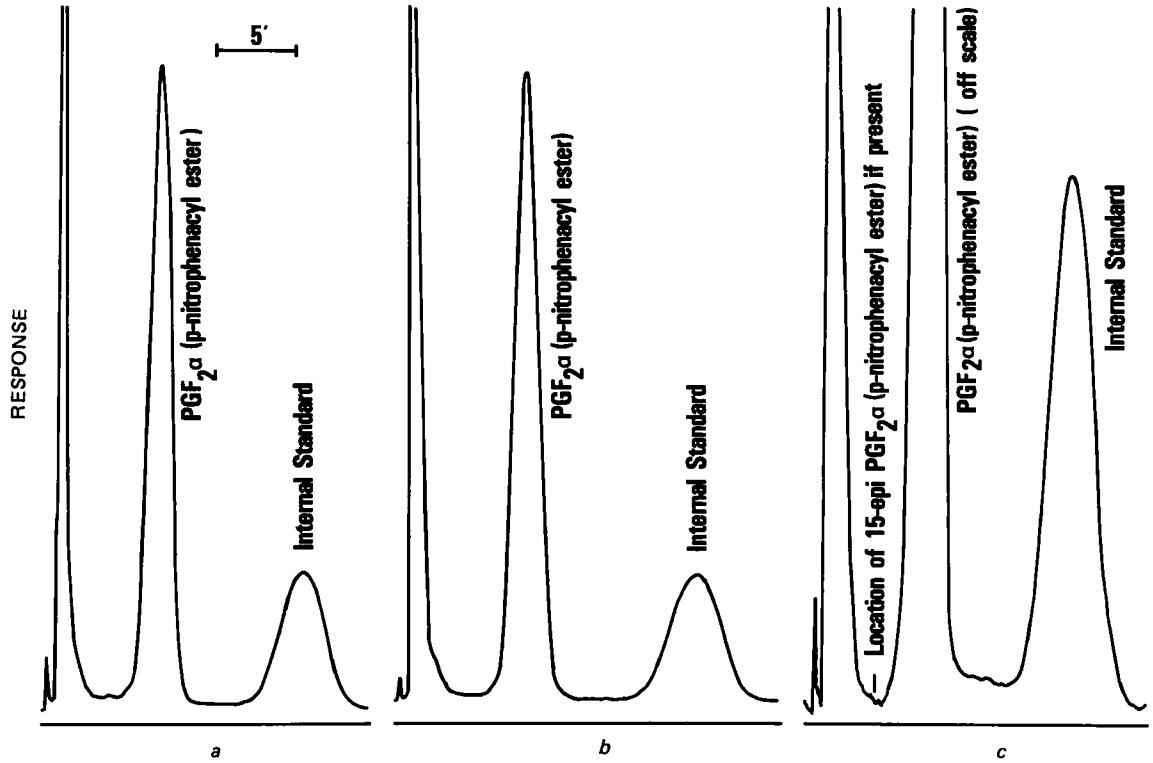


Figure 3—*High-pressure liquid chromatograms of the* p-*nitrophenacyl ester of dinoprost ($PGF_{2\alpha}$) from pH 8.0 solution stored for 10 months at 25°. Chromatograms do not show the presence of the 15-epimer (15-epi $PGF_{2\alpha}$). (a) Concentration of internal standard = 1.25 mg/ml, attenuation = 16 × 10^{-2} aufs, and sample was extracted standard of dinoprost tromethamine. (b) Concentration of internal standard = 1.25 mg/ml, attenuation = 16 × 10^{-2} aufs, and sample was extracted from solution stored at pH 8.0 for 10 months at 25°. (c) Concentration of internal standard = 1.25 mg/ml, attenuation = 4 × 10^{-2} aufs, and sample was extracted from solution stored at pH 8.0 for 10 months at 25°.*

To detect accurately 15-epimer concentrations of less than 2% (w/w) in dinoprost, it was necessary to lower the attenuation setting from 16 × 10^{-2} to 2 × 10^{-2} aufs and to lower the concentration of the internal standard. Linearity was achieved to a lower detectable limit of about 0.5% (Table II), which is an improvement over the level of detection reported previously (5). However, the slopes of the low and high concentration plots from the table were not equal (after accounting for differences in internal standard concentration). Presumably, this result was due to the significant baseline shifts that occur at high sensitivity settings (Fig. 1). Therefore, prostaglandin standards should be run under identical chromatographic conditions as the unknowns.

Determination of 15-Epimer Content in Acidic and Basic Dinoprost Solutions—Under acidic conditions, dinoprost forms the 15-epimer[1]. The epimer is not resolved by GLC but can be separated by TLC using the solvent system[13] of Hamberg and Samuelsson (4). One method to quantitate the epimer concentration requires the preliminary TLC separation followed by derivatization (silylation) for subsequent GLC analysis (9). This tedious process can be circumvented by using the one-step HPLC procedure described.

Figure 2 shows the chromatogram (HPLC) of an extracted dinoprost sample (after derivatization); the sample was stored at 37° for 30 days at pH 3.0. Two peaks were observed with retention times equal to dinoprost and its 15-epimer. Quantitation of the 15-epimer concentration showed that 40% conversion had taken place. In adsorption chromatography, it is expected that the less polar C-15 prostaglandin dehydration products, which also form under acidic conditions, elute in the solvent front.

In contrast to the solution stored under acidic conditions, the chromatograms in Fig. 3 show that at pH 8.0 no 15-epimer was detectable, even after 10 months of storage at 25°. In fact, when using this HPLC technique, dinoprost assayed at 103% of theory, indicating that dinoprost is quite stable under these circumstances.

The results of this investigation demonstrate that (*a*) the *p*-nitrophenacyl ester of 15-epimer of dinoprost can be used to determine its content in bulk dinoprost with HPLC and (*b*) this technique can be used to determine the concentration of the 15-epimer in dinoprost solutions stored for prolonged periods.

[13] Ethyl acetate–acetic acid–2,2,4-trimethylpentane–water (90:20:50:100); upper phase used.

REFERENCES

(1) T. O. Oesterling, W. Morozowich, and T. J. Roseman, *J. Pharm. Sci.*, **61**, 1861(1972).

(2) S. M. M. Karim, J. Devlin, and K. Hillier, *Eur. J. Pharmacol.*, **4**, 416(1968).

(3) M. F. Jones, *J. Pharm. Pharmacol.*, **25**, 900(1973).

(4) M. Hamberg and B. Samuelsson, *J. Biol. Chem.*, **241**, 257(1966).

(5) N. M. Weinshenker and A. Longwell, *Prostaglandins*, **2**, 207(1972).

(6) N. H. Andersen and E. M. K. Leovey, *ibid.*, **6**, 361(1974).

(7) W. Morozowich and S. L. Douglas, *ibid.*, **10**, 19(1975).

(8) T. J. Roseman and S. H. Yalkowsky, *J. Pharm. Sci.*, **62**, 1680(1973).

(9) T. J. Roseman, B. Sims, and R. G. Stehle, *Amer. J. Hosp. Pharm.*, **30**, 236(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 18, 1975, from the *Pharmacy Research Unit, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication July 16, 1975.

Valuable discussions with Dr. Walter Morozowich were appreciated.

[x] To whom inquiries should be directed.