High-Performance Liquid Chromatographic Analysis of Prostaglandins Formed during *In Vitro* Incubations with Prostaglandin 15-Dehydrogenase

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Abstract □ Dinoprost, dinoprostone, and prostaglandin E_1 were each separated from their major 15-keto metabolites by high-performance liquid chromatography on a microparticulate, bonded, reversed-phase column after conversion to their *p*-bromophenacyl esters. Detection and simultaneous quantitation of prostaglandins in 1.0 ml of a 5-μM solution is possible. The method was applied to monitor prostaglandins formed during *in vitro* incubations with prostaglandin 15-dehydrogenase from monkey lungs. The advantages of this technique for assessing enzyme purity and activity are discussed.

Keyphrases □ Prostaglandins—formed during *in vitro* incubation with prostaglandin 15-dehydrogenase, high-performance liquid chromatographic analysis □ Dinoprost and dinoprostone—derivatives formed during *in vitro* incubation with prostaglandin 15-dehydrogenase, high-performance liquid chromatograpic analysis □ High-performance liquid chromatography—analysis, prostaglandins formed during *in vitro* incubation with prostaglandin 15-dehydrogenase □ Oxytocic agents—dinoprost and dinoprostone derivatives formed during *in vitro* incubation with prostaglandin 15-dehydrogenase, high-performance liquid chromatographic analysis

A significant strategy for prolonging pharmacological effects of prostaglandins focuses on inhibiting their metabolic inactivation. The first stage in the metabolism of most prostaglandins is dehydrogenation of the secondary hydroxyl group at the C-15 position along with concomitant formation of a 15-keto derivative. Several investigators recently isolated and characterized prostaglandin 15-dehydrogenase enzyme in different tissues from various species including pig, beef, and monkey lungs (1–3); dog heart (4); pig and monkey kidneys, and human placenta (5–8). With the availability of highly active, purified prostaglandin 15-dehydrogenase preparations, further advances should occur in the development of prostaglandin analogs resistant to metabolic inactivation and also in the development of drugs capable of direct *in vivo* inhibition of the enzyme (9).

A prominent obstacle remaining is the lack of analytical methodology capable of accurately quantitating substrate and metabolic conversion products from prostaglandin 15-dehydrogenase incubations *in vitro*. Enzyme activity is usually measured indirectly by fluorometric or spectrophotometric determination of the rate of formation of NADH, the product of the NAD-linked reaction. Another widely used spectrophotometric method, based on the alkaline-catalyzed formation of a transient chromophore (λ_{max} 500 nm) from the enzymatic oxidation products of prostaglandin E_1, has the drawback of limited utility, except with prostaglandin E-type substrates, and poor reproducibility relative to NADH measurements. The validity of the analytical results for both approaches depends greatly on the purity of the enzyme preparation, which is often judged by electrophoretic homogeneity.

Characterizations of enzymes by indirect methods

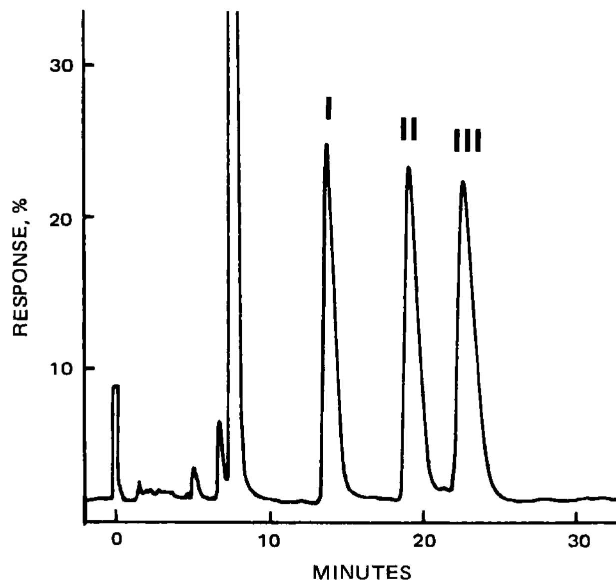

Figure 1—*Separation of* p-*bromophenacyl esters of dinoprostone (I), 15-keto derivative (II), and 13,14-dihydro-15-keto derivative (III).*

must always be regarded with some skepticism. Except for the limited use of radiometric TLC (10) and a radioimmunoassay procedure for the 15-keto derivative of dinoprost (8), virtually no specific analytical methods have been employed to determine 15-keto derivatives from *in vitro* prostaglandin 15-dehydrogenase incubations. This report describes the development of a reversed-phase, high-performance liquid chromatographic (HPLC) system for the separation of several prostaglandins from their principal 15-keto metabolites after conversion to *p*-bromophenacyl esters. Quantitative analysis is easily performed on the basis of peak height measurements for 1 ml of solution containing prostaglandins in concentrations greater than 5 μM.

EXPERIMENTAL

Apparatus—A high-performance liquid chromatograph[1] with a pneumatic drive, positive displacement pump, and a single-wavelength (254 nm) UV detector was used. The chromatographic column was operated at ambient temperature (25 ± 3°), and injections were made into a stopped-flow injection port with a 10-μl syringe[2]. Chromatograms were recorded on a single-pen recorder[3], 0–1 mv full-scale.

Chromatographic Column—A prepacked, pretested, microparticulate, reversed-phase column[4] was used as received. Low dead volume fittings were easily constructed to adapt this column to the stopped-flow injection port of the chromatograph. A mobile phase

[1] Chromatec model 5100, Tracor Instruments, Austin, Tex.
[2] Hamilton model 701.
[3] Varian A-25.
[4] μBondapak C18, Waters Associates, Milford, Mass.

Table I—Prostaglandin Recovery Experiments

Dinoprostone		Dinoprostone 15-Keto Derivative		Dinoprost		Dinoprost 15-Keto Derivative	
μg/0.5 ml	% Recovery	μg/0.5 ml	% Recovery	μg/0.5 ml	% Recovery	μg/0.5 ml	% Recovery
112.2	97.9 ± 4.5	105.0	97.8 ± 5.6	122.2	102.7 ± 4.0	80.4	101.5 ± 8.9
56.1	93.7 ± 2.2[a]	52.5	94.9 ± 5.4[a]	61.2	88.9 ± 3.7	40.2	93.2 ± 4.3
22.4	92.6 ± 2.8	21.0	96.6 ± 5.7	24.4	83.5 ± 4.5	16.1	93.8 ± 4.9
				12.2	88.3 ± 6.9	8.04	99.2 ± 8.7

[a] $n = 3$; in all other cases, $n = 6$ determinations.

composition of acetonitrile–water (50:50) at a flow rate of 1.2 ml/min (1000 psig) eluted the prostaglandins in less than 30 min.

Reagents[5]—Distilled-in-glass acetonitrile[6], methanol[6], methylene chloride[6], and α,*p*-dibromoacetophenone[7] were used as received. Diisopropylethylamine[8] was redistilled (bp 127°) before use.

Procedure—*Derivatization*—Prostaglandin *p*-bromophenacyl esters were prepared by a modification of the procedure of Morozowich and Douglas (11). Briefly, the prostaglandin (500 μg or greater) was dissolved in 1.0 ml of anhydrous acetonitrile containing a threefold molar excess of α,*p*-dibromoacetophenone. Two microliters of diisopropylethylamine was added to catalyze the reaction, which was >95% complete in 1 hr at 25°. For 1–100 μg of prostaglandin, the volume of acetonitrile should be reduced to 0.1–0.2 ml and the amount of diisopropylethylamine should be reduced to 0.5 μl.

A threefold molar excess of reagent gave complete conversion without an unduly large reagent peak. For extracts from enzymatic incubations, an amount of derivatizing reagent corresponding to a threefold molar excess of initial prostaglandin substrate should be added.

Enzyme Incubation—Purified prostaglandin 15-dehydrogenase[9]

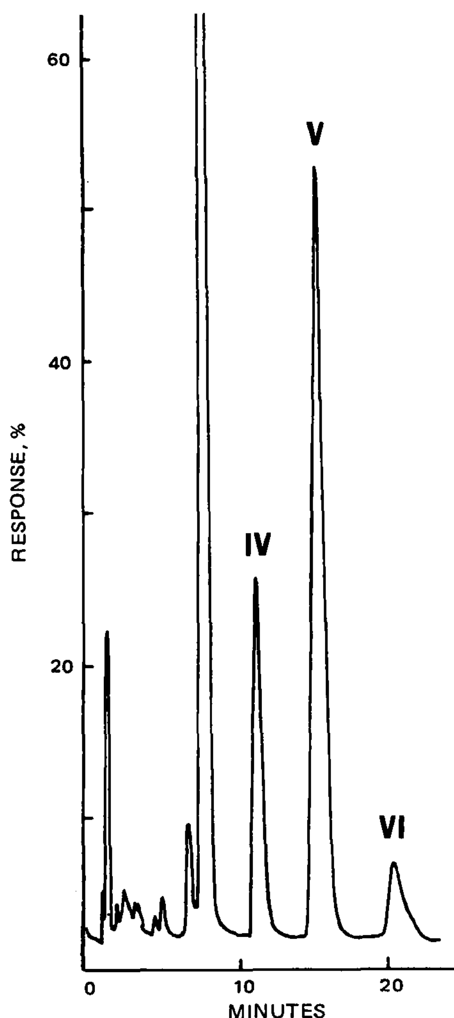

Figure 2—*Separation of* p-*bromophenacyl esters of dinoprost (IV), 15-keto derivative (V), and 13,14-dihydro-15-keto derivative (VI).*

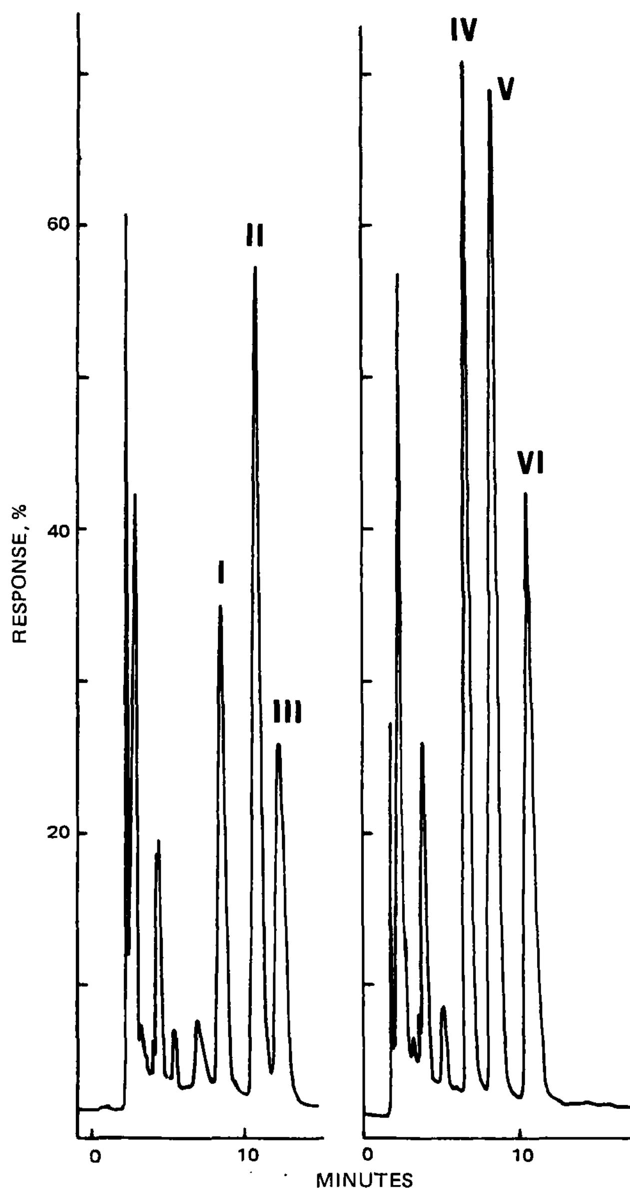

Figure 3—*Separation of* p-*nitrophenacyl esters of I–III and IV–VI.*

[5] All prostaglandins were supplied by the Experimental Chemistry Laboratories, The Upjohn Co.
[6] Burdick & Jackson, Muskegon, Mich.
[7] Eastman Kodak, Rochester, N.Y.
[8] Aldrich Chemical Co., Milwaukee, Wis.
[9] Supplied by J. Mc Guire, Experimental Biology Unit, The Upjohn Co.

Table II—Incubation and Analysis Reproducibility

	50 μl of Enzyme					25 μl of Enzyme			
	Dinoprostone		Dinoprostone 15-Keto Derivative			Dinoprostone		Dinoprostone 15-Keto Derivative	
Minutes	A[a]	B[a]	A	B	Minutes	A	B	A	B
0	209[b]	216	0	0	0	196	196	0	0
2	199	201	12	16	5	186	180	16	20
5	184	198	26	37	10	181	183	31	34
7.5	183	191	38	52	30	127	120	62	65
10	170	173	53	54	45	101	97	73	79
15	148	149	62	66	60	87	87	85	96
20	121	129	64	83	90	69	64	94	84
30	97	106	76	105	120	50	43	91	77
60	56	56	102	119	180	41	41	84	69
180	39	33	149	131					

[a] A and B represent completely separate incubations and assays. [b] Values given are micrograms per 0.5 ml, the assay volume.

enzyme was isolated from monkey lungs. The soluble enzyme present in the high speed supernate of a tissue homogenate was obtained by standard procedures (12).

A volume of enzyme preparation from 10 to 50 μl, depending upon the activity desired, was added to 5.0 ml of 0.1 *M* phosphate buffer (pH 7.4), 180 mg of NAD, and 2.5 μl of mercaptoethanol in a 75 × 130-mm disposable tube. The mixture was preincubated to a temperature of 37°, and the enzymatic reaction was initiated by adding 200 μl of prostaglandin (100–2000 μg) dissolved in 0.1 *M* phosphate buffer (pH 7.4) at 37° and then vortexing.

Samples (0.5 ml) were withdrawn for analysis at appropriate time intervals and immediately quenched in a 12 × 75-mm tube containing 1.0 ml of methanol. Usually, 0-, 2-, 5-, 7.5-, 10-, and 15-min samples provided sufficient data points to characterize the initial reaction rate adequately. The contents of the tube were quantitatively transferred to a 60-ml separator containing 4 ml of 0.1 *M* citric acid and 500 mg of anhydrous sodium sulfate.

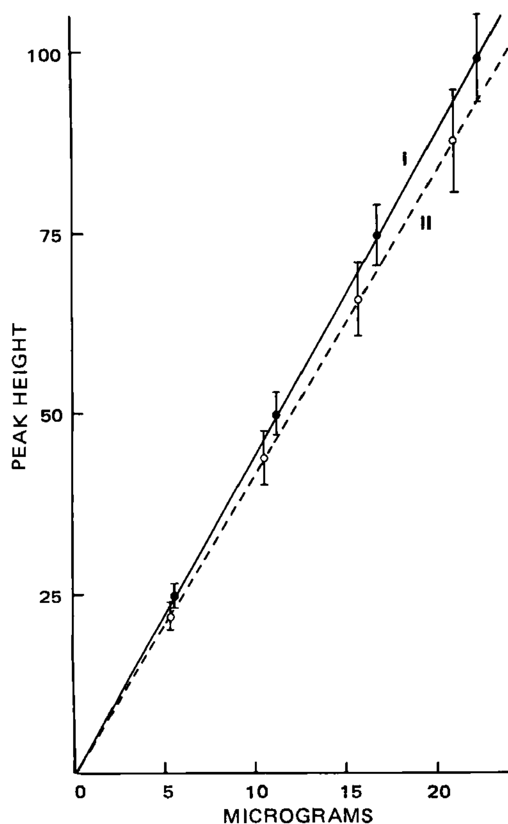

Figure 4—*Calibration curve based on peak height* versus *micrograms derivatized. Key: I, dinoprostone; and II, 15-keto derivative.*

For prostaglandin E_1 and dinoprostone[10], the aqueous phase was extracted twice with 5 ml of methylene chloride; for dinoprost[11], it was extracted three times with 5 ml. The pooled organic phases were desiccated by passage through a funnel containing about 1 g of anhydrous sodium sulfate and then evaporated to dryness under nitrogen. The residue concentrated in the bottom of the tube was derivatized, evaporated to dryness, and reconstituted in 200 μl of methanol for analysis. Ten-microliter aliquots were chromatographed.

RESULTS AND DISCUSSION

Chromatography—The principle of forming *para*-substituted phenacyl esters of prostaglandins to improve the detection limit with a 254-nm UV detector was described previously, and separations were reported by adsorption chromatography on microparticulate silica gel (11). The rationale for using reversed-phase, liquid chromatography as the basis for a quantitative assay on the *in vitro* enzyme system is due to the fact that the 15-keto metabolites are less polar than their parent compounds and elute close to the chromatographic void volume with only partial resolution on a microparticulate silica gel adsorption column, thereby presenting difficulties for quantitative analysis. On a reversed-phase column, the metabolites are retained more strongly than the parent prostaglandins but still elute in a reasonable time with good chromatographic resolution.

Figures 1 and 2 show the separation of dinoprostone and dinoprost from their respective 15-keto metabolites. Resolution of the Δ^{13},15-keto derivative from the 13,14-dihydro-15-keto derivative is

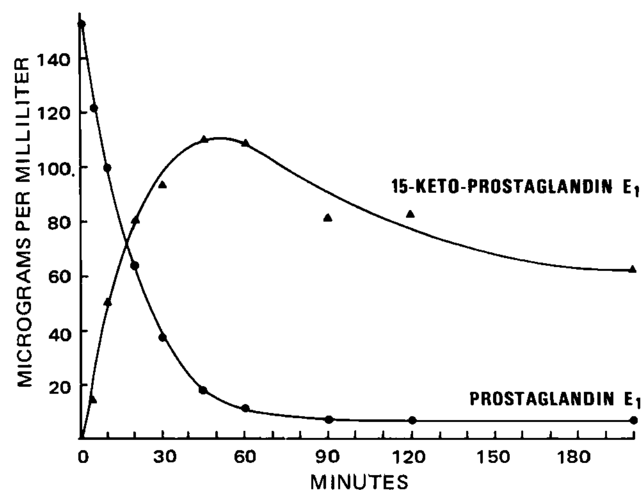

Figure 5—*Concentration* versus *time for the prostaglandin E_1 substrate. Enzyme = 25 μl. Total incubation volume = 5.2 ml; samples assayed = 0.5 ml.*

[10] Prostaglandin E_2.
[11] Prostaglandin $F_{2\alpha}$.

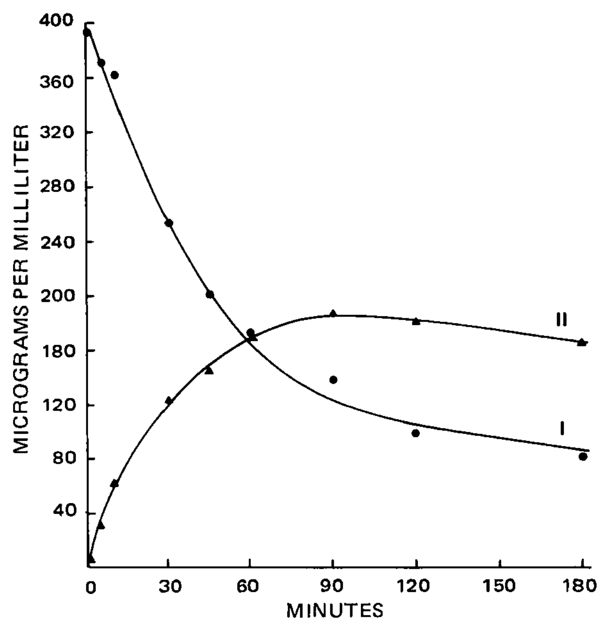

Figure 6—*Concentration* versus *time. I = dinoprostone substrate. II = 15-keto derivative. Enzyme = 25 μl. Total incubation volume = 5.2 ml; samples assayed = 0.5 ml.*

essential to determine the purity of the enzyme preparation, since crude preparations generally contain Δ^{13}-reductase activity. The *p*-nitrophenacyl esters of these prostaglandins (Fig. 3) are also easily separated; however, for routine quantitation of less than 50 μg, the *p*-bromophenacyl esters were preferred because of a lower reagent blank.

Calibration—Typical calibration curves based on peak height response for dinoprostone and its 15-keto derivative are shown in Fig. 4, with the 95% confidence limits predicted by linear regression analysis. Similar results were obtained for dinoprost and its 15-keto metabolite. An internal standard could have been selected from a variety of prostaglandin analogs; however, the prostaglandin 15-dehydrogenase enzyme is water soluble, so quantitative transfers in steps prior to chromatography are simple. With the use of external standard calibration curves, 15–20 samples/day can be assayed.

Sample Analysis—Accuracy and precision were verified by several recovery experiments. Phosphate buffer (pH 7.4), spiked with varying amounts of prostaglandin substrate and the corresponding 15-keto derivatives, was incubated under conditions identical to an active enzyme preparation for 15 min before extraction and chromatographic analysis (Table I). Recovery was nearly quantitative for all prostaglandins except dinoprost. This disadvantage can be compensated for by running the calibration curve for dinoprost after extraction from buffer or by applying a correction factor based on a single recovery experiment.

Application of Method—Figures 5–7 show the time course for the prostaglandin 15-dehydrogenase reaction under identical conditions for three substrates, prostaglandin E_1, dinoprostone, and dinoprost. The corresponding 13,14-dihydro-15-keto derivative, prostaglandin A, and prostaglandin B can also be determined if present. Replicate incubations with dinoprostone (Table II) at two different enzyme concentrations showed reasonable reproducibility, and a comparative radioimmunoassay analysis for the dinoprost 15-keto derivative provided additional authentication of the chromatographic method (Table III). High values at the last two time intervals may reflect cross-reactivity with small amounts of unidentified material formed during the incubation.

Table III—Comparative Analysis for Dinoprost 15-Keto Derivative by Radioimmunoassay (RIA) and HPLC

	Minutes						
Method	0	2	5	10	15	20	60
HPLC, μg/0.5 ml	3.7	16.2	34.0	49.2	80.9	82.6	76.0
RIA, μg/0.5 ml	<8	36	38	48	82	112	116

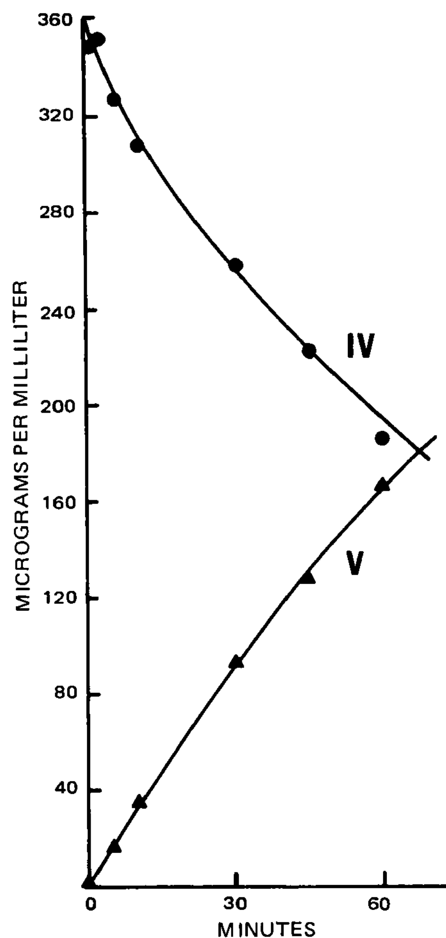

Figure 7—*Concentration* versus *time. IV = dinoprost substrate. V = 15-keto derivative. Enzyme = 25 μl. Total incubation volume = 5.2 ml; samples assayed = 0.5 ml.*

Figures 8 and 9 show the application of the method to kinetic studies. Rate constants for substrate decline can be compared with product appearance to verify enzymatic purity.

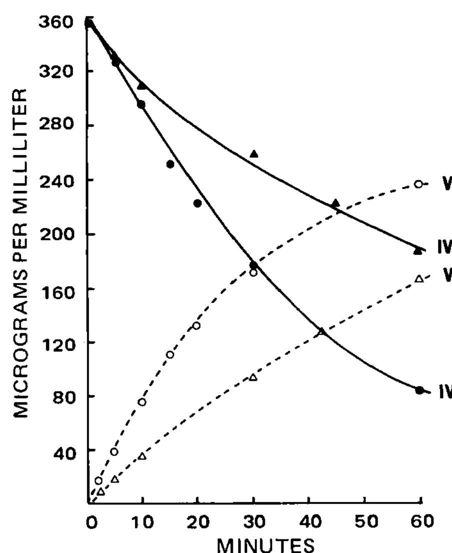

Figure 8—*Concentration* versus *time for two concentrations of prostaglandin 15-dehydrogenase. Key: Δ, ▲, 25 μl; ○, ●, 50 μl; —, dinoprost (IV); and - - -, 15-keto derivative (V). Total incubation volume = 5.2 ml; samples assayed = 0.5 ml.*

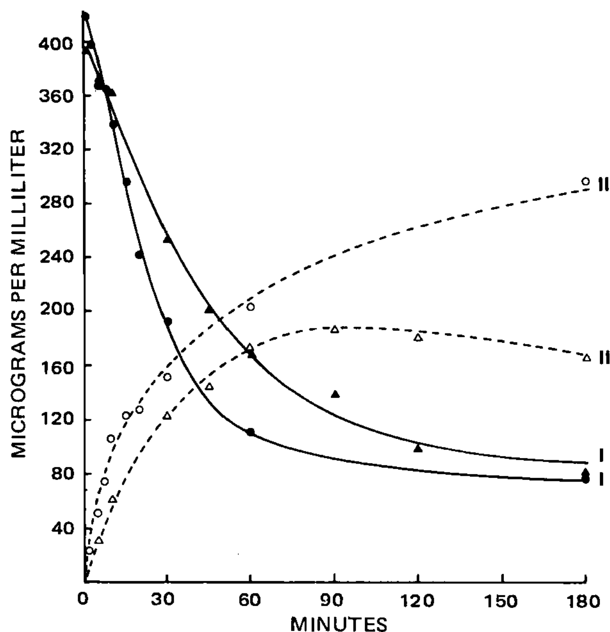

Figure 9—*Concentration versus time for two concentrations of prostaglandin 15-dehydrogenase enzyme. Key:* Δ, ▲, *25 μl;* ○, ●, *50 μl; —, dinoprostone (I); and - - -, 15-keto derivative (II). Total incubation volume = 5.2 ml; samples assayed = 0.5 ml.*

CONCLUSIONS

An HPLC method was developed, allowing the simultaneous determination of substrate and metabolic conversion products from *in vitro* incubations with prostaglandin 15-dehydrogenase. The specific nature of the assay provides vital information for a complete characterization of enzyme quality and activity. Preliminary results pointed toward differences in *in vitro* enzyme kinetics for different substrates. Dinoprost, while showing the lowest initial reaction rate (*cf.*, Figs. 5–7), is completely consumed while prostaglandin E_1 and dinoprostone reach an asymptote with time.

The method may be useful as a: (*a*) screen for prostaglandin 15-dehydrogenase inhibitors, (*b*) reference method for questionable results from other screening assays, and (*c*) control method to ensure that the inhibitor screening assay is employing a satisfactorily specific and active enzyme.

If reasonably high activities can be obtained, then HPLC probably may be used to characterize other prostaglandin-metabolizing enzymes. With the availability of superfused organ techniques, it also may be possible to compare, by an accurate physical–chemical technique, the effect of inhibitors on the enzyme in this system and *in vitro* enzymes to distinguish differences.

REFERENCES

(1) H. Vonkeman, D. H. Nugteren, and D. A. van Dorp, *Biochim. Biophys. Acta*, **187**, 581(1969).

(2) S. A. Saeed and A. C. Roy, *Biochem. Biophys. Res. Commun.*, **47**, 96(1972).

(3) H. S. Hansen, *Prostaglandins*, **8**, 95(1974).

(4) C. J. Limas and J. N. Cohn, *Proc. Soc. Exp. Biol. Med.*, **142**, 1230(1973).

(5) H. H. Tai, C. L. Tai, and C. S. Hollander, *Biochem. Biophys. Res. Commun.*, **57**, 457(1974).

(6) J. Jarabak, *Proc. Nat. Acad. Sci. USA*, **69**, 533(1972).

(7) W. Schlegel, L. M. Demers, H. E. Hildebrandt-Stark, H. R. Behrman, and R. O. Greep, *Prostaglandins*, **5**, 417(1974).

(8) S. C. Lee and L. Levine, *J. Biol. Chem.*, **250**, 548(1975).

(9) L. J. Lerner, P. Carminali, F. Luzzani, and A. Soffientini, presented at the International Conference on Prostaglandins, Florence, Italy, 1975, Session 13.

(10) C. Pace-Asciak, *J. Biol. Chem.*, **250**, 2789(1975).

(11) W. Morozowich and S. Douglas, *Prostaglandins*, **10**, 19(1975).

(12) F. F. Sun, S. B. Armour, and V. R. Bockstanz, presented at the International Conference on Prostaglandins, Florence, Italy, 1975, Session 10.

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Preparation and Properties of Solid Dispersion System Containing Citric Acid and Primidone

M. P. SUMMERS and R. P. ENEVER [x]

Abstract ☐ Solid dispersions containing 1–32% (w/w) primidone were prepared by fusing the drug with citric acid and rapidly cooling the melt. The solidified dispersions were clear glasses which devitrified on aging or when stored at 60° for up to 3 days. The phase diagram of the devitrified system indicated that the drug may exist as a solid solution at 1–3% (w/w) concentrations but that a eutectic mixture is formed at higher concentrations. The solubility of primidone increased in the presence of citric acid. Preliminary dissolution studies showed that the dissolution rate from the solid dispersion was greater than that of the pure drug or the physical mixture.

Keyphrases ☐ Solid dispersion systems—primidone with citric acid, effect of concentration, aging, and temperature ☐ Primidone—solid dispersion with citric acid, effect of concentration, aging, and temperature ☐ Citric acid—solid dispersion with primidone, effect of concentration, aging, and temperature ☐ Anticonvulsant agents—primidone, solid dispersion with citric acid, effect of concentration, aging, and temperature

Sekiguchi and Obi (1) first demonstrated the use of a eutectic mixture of a sparingly soluble drug and a water-soluble compound in increasing the rates of dissolution and absorption of the drug. This type of formulation, a "solid dispersion system," has undergone much investigation and has been extended to include