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CONSTANT-HUMIDITY CHROMATOGRAPHY OF PROSTAGLANDINS AND THEIR METABOLITES ON A NEUTRAL SILICIC ACID-GLASS MICROFIBER MATRIX

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SUMMARY

Solvents have been developed for separating phospholipids, prostaglandins E (PGEs), PGFs, 15-keto-PGFs, 15-keto-PGEs, 15-keto-13,14-dihydro-PGEs, 15-keto-13,14-dihydro-PGFs, PGAs, fatty acids and triglycerides by a single 15.5-cm migration on a nominally 650-μm thick sheet of neutral silicic acid-glass microfiber matrix in a sandwich chamber. The various classes of PGs and metabolites can then be separated according to unsaturation by cutting off a strip of the first chromatogram which contains one or two PG classes and allowing the compounds from this strip to migrate onto a silver nitrate-treated sheet of the silicic acid-glass microfiber matrix. Such a double development permits the isolation of PGs with less than 0.009% of labeled fatty acid precursor (100–200 dpm of 0.5–1.0 μCi) remaining as a contaminant of the isolated PG.

The relative humidity was found to have a profound effect upon the separation. The range of humidity between 15 and 40% gave the best separations with the greatest reproducibility in the absence of $AgNO_3$ treatment, while 60–70% humidity was best with $AgNO_3$-treated sheets.

INTRODUCTION

While studying the synthesis of a wide spectrum of prostaglandins (PGs) by a variety of ocular epithelial tissues, we found that published chromatographic procedures did not meet our needs[1–4]. We wanted to estimate the relative quantities of PGs synthesized during short incubation times (10–60 sec) by microgram quantities of tissue. The reliable determination of the small quantities of PGs obtained under these conditions required incubation with high levels of radioactive precursor and isolation techniques which would reduce precursor contamination of the PGE and PGF compounds to less than 0.009%. Methods which allow us to satisfy these requirements while processing more than 80 samples simultaneously are described below.

EXPERIMENTAL

Authentic non-radioactive prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, A_1 and D_2 as well as the derivatives, 15-keto-PGE_1, 15-keto-PGE_2, 13,14-dihydro-15-keto-PGE_2, 15-keto-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 (TXB_2) were gifts from Upjohn (Kalamazoo, Mich., U.S.A.). PGB_1 (for marker purposes) was formed from PGE_1 by adjusting an aqueous solution to pH 12–12.5 and heating at 95° for 5 min. Trieicosenin, arachidonic acid, cholesterol and phosphatidyl ethanolamine standards were purchased from Applied Science Labs. (State College, Pa., U.S.A.). The following radioactive prostaglandins were obtained from New England Nuclear (Boston, Mass., U.S.A.) and Amersham/Searle (Arlington Heights, Ill., U.S.A.): [5,6-^{3}H(N)]PGE_1, [5,6,8,11,12,14,15-^{3}H(N)]PGE_2, [5,6-^{3}H-(N)]$PGF_{1\alpha}$, and [5,6,8,9,11,12,14,15-^{3}H(NN)]$PGF_{2\alpha}$. [1-^{14}C]8,11,14-Eicosatrienoic acid was also obtained from New England Nuclear. All solvents used were reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.) used without redistillation. ChromAR®-500 and ChromAR® sheets (neutral silicic acid-glass microfiber matrix) were purchased from Mallinckrodt. The ChromAR sheet had a nominal thickness of 650 μm[5] and will be designated as ChromAR-650 in this report to distinguish it from the ChromAR-500. ChromAR sheets (18.0 × 18.5 cm) were spotted 3 cm from the bottom with 10 samples, using 5-μl aliquots so there was one sample every 1.7 cm with 1.3-cm margins. The sheets were then equilibrated for 10 min in a closed glove box over saturated salt solutions selected to give the desired humidities. Saturated salt solutions were chosen for approximate humidities at 23° as follows: $H_3PO_4 \cdot \frac{1}{2}H_2O$, 9%; $LiCl \cdot H_2O$, 15–20%; $CaCl_2 \cdot 6H_2O$, 30–35%; $Mg(NO_3)_2 \cdot 6H_2O$, 52–60%; $NaNO_2$, 63–67%; $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 65–72%; $Na(C_2H_3O_2) \cdot 3H_2O$, 72–76%; $(NH_4)_2SO_4$, 75–81%; $Na_2SO_4 \cdot 10H_2O$, 93–94% ref. 6). The humidity within the box was checked with an Abbecon Certified Hydrometer, Model HTAB169, and by a closed circuit circulation across wet-bulb–dry-bulb thermometers. Changes in water content of the ChromAR were determined by weighing a 2 × 3 cm section of the sheet with a Cahn recording electrobalance model RG 2000. ChromAR sheets were assembled into a sandwich chamber (Brinkmann, Westbury, N.Y., U.S.A.) while still under controlled humidity. All data presented in this report were obtained with only a single sheet in each chamber. Sheets were separated from the chamber walls by Teflon® matting (Chemfluour, VWR Scientific, San Francisco, Calif., U.S.A.) and Teflon mesh (1-mm openings, Spectrum Medical Industries, Los Angeles, Calif., U.S.A.). After withdrawing the assembled sandwich chamber from the humidity-controlled box, 50 ml of the appropriate solvent was added and development continued to the top of the sheet. After development, the sheets were air dried under a blower for 10 min. PGB_1 (2 μg), PGA_1 (10 μg), 15-keto-PGE_2 (2.8 μg) and 15-keto-$PGF_{2\alpha}$ (2.8 μg) were visible under shortwave UV light at this point.

If it was desirable to further visualize the location of standards after development and drying, the chromatograms were sprayed lightly with a solution of silver nitrate made by dissolving 1 g of $AgNO_3$ in 1 ml of water and diluting the mixture to 100 ml with absolute ethanol. The chromatograms were then heated at 120° for 5–10 min until a light tan background was visible. PGA_1 and PGEs could occasionally be visualized as uncolored spots against a tan background under these conditions. If urethan had been present in the developing solvent or was added at a concentration

of 1% to the $AgNO_3$ spray, all compounds present in quantities exceeding 3 μg could be visualized after heating as uncolored areas with a tan background. When the $AgNO_3$ spray was followed by a light spray of 3% cupric acetate in 15% aqueous phosphoric acid and the chromatograms were reheated at 120° for 10–20 min, all the compounds were visualized as dark spots on a light background. The pre-spray with silver nitrate allowed better visualization by the cupric acetate spray but was not helpful when sheets were treated with silver nitrate prior to chromatography.

Maximum purification of PGE and PGF compounds was obtained by a two-development procedure. The initial development employed a 15.5-cm migration of a solvent system which would move the fatty acids and metabolites ahead of PGE_1 and PGE_2 while moving $PGF_{1\alpha}$ and $PGF_{2\alpha}$ just enough to leave the phospholipids at the origin. The strip of the developed chromatograph containing the PGE and PGF compounds was then cut out and positioned with 2-mm overlaps between a wick and a 12.2-cm section of ChromAR which had been pretreated with silver nitrate. Overlapped positions of the wick were maintained by light pressure from strips of Teflon matting and mesh. The spliced assembly was then equilibrated at 70% humidity before closing the sandwich chamber for development. Sheets pretreated with silver nitrate were prepared by allowing a 1.5% aqueous silver nitrate solution to develop up the sheets in a closed sandwich chamber. Sheets with the same characteristics could also be produced by enclosing the ChromAR in a folded mat of Teflon (Chemfluour) and dipping them for 30 sec in 1.5% aqueous silver nitrate. The treated sheets were then air dried at 35° for 30 min followed by 30 min at 120°.

Development onto the silver nitrate treated paper separated the PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ compounds into four distinct spots.

Radioactivity was determined by cutting out and counting specific areas whose position was estimated by the movement of known standards. The areas were dispersed in 1 ml of a mixture containing 10 m*M* dithiothreitol and 22.5 m*M* EDTA adjusted to pH 7 with Tris base. Scintillation cocktail (9 ml) was then added and samples were counted (standard error <5%) using a Beckman LS-250 scintillation counter operating in the automatic quench correction mode. The scintillation cocktail contained 28% Triton X-100, 2,5-diphenyloxazole (5.35 mg/ml) and 1,4-bis-2(5-phenyloxazolyl)-benzene (0.5 mg/ml) dissolved in toluene. The buffer dithiothreitol–EDTA mixture stabilized the quench ratio at about 75–90% of that obtained with water-free argon-purged standards. Neither silver nitrate nor cupric acetate treatment of the ChromAR reduces the quench ratio to less than 40%. The scintillation counter was set for double isotope counting such that standard carbon-14 and tritium efficiencies were 70% and 58%, respectively, for unquenched standards.

Four solvent systems were developed for primary isolation of major classes of PGs and lipids. Solvent P-1 contained dichloroethane–methanol–acetone–glacial acetic acid–water (94.8:3:1:1:0.2, by vol.). Solvent P-2 contained benzene–acetone–glacial acetic acid–water (80.8:18:1:0.2, by vol.). Solvent P-3 contained benzene–*p*-dioxane–glacial acetic acid (82:17:1, by vol.). Solvent P-4 contained benzene–*p*-dioxane–urethan–glacial acetic acid (86:11:2:1, v/v/w/v).

The solvents for secondary development on silver nitrate treated sheets were solvent P-5 which contained chloroform–urethan–glacial acetic acid–methanol (88:10:1:1, v/w/v/v) and solvent P-6 which contained chloroform–urethan–2-methyl-

2-butanol–glacial acetic acid–water, (82.5:10:6:1:0.5, v/w/v/v/v). Another secondary solvent (P-5K) containing benzene–acetone–urethan–glacial acetic acid (86:12:1:1, v/v/w/v) was occasionally used to separate 15-keto-PGs on silver nitrate-treated sheets. All solvents contained a 1 m*M* final concentration of 2,6-di-*tert.*-butyl-*p*-cresol (BHT) as an antioxidant.

RESULTS AND DISCUSSION

We chose silicic acid-glass microfiber matrix (ChromAR-500 or -650) as our chromatographic support medium because previous experience[7] had convinced us that it had the properties we desired for processing large numbers of samples. The development time is short (16–29 min for a 15.5-cm front with the solvents employed here) and the final spots are easily cut out and dispersed in scintillation fluid with very little radioactive quenching. This support also handles like filter paper in that any area of the chromatogram can be cut off and used with a wick for development onto a second sheet.

Initially we found poor reproducibility of chromatographic R_F values. This variability was traced to fluctuations in room humidity from day to day. In agreement with the reports of Dallas[8], Geiss *et al.*[9] and De Zeeuw[10] we found that R_F values became reproducible when the chromatography sheets were allowed to equilibrate in a constant humidity chamber immediately prior to closure of the sandwich chamber and development with solvent.

Because of the rapid rate at which silicic acid equilibrates with atmospheric moisture (50% of the weight change due to water loss or uptake occurred within 1.5 min following abrupt changes in humidity from 9 to 70% or *vice versa*[8,9,11]), it was necessary to spot materials prior to humidity equilibration and to place the front cover of the sandwich chamber in position before removing the assembly from the constant humidity box.

Although the manufacturer states that both ChromAR-500 and -650 have 70% silicic acid by weight, the water vapor-absorption isotherms at 23° (see Fig. 1) would

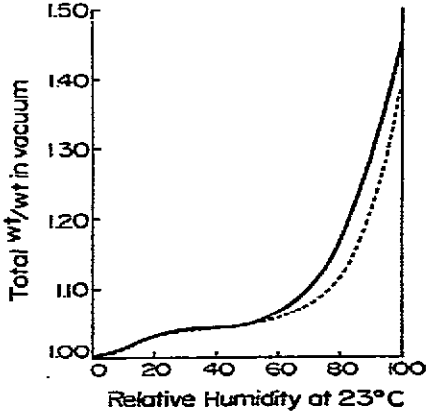
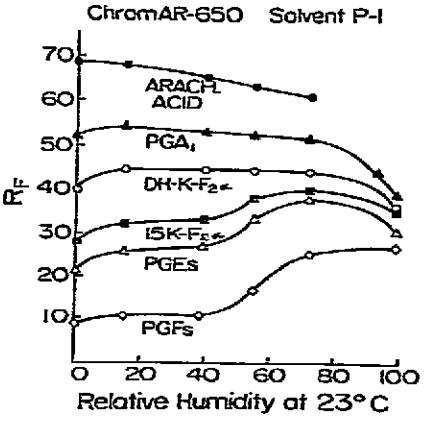


Fig. 1. Water vapor-absorption isotherms for (———) ChromAR-650 and (— — —) ChromAR-500 at 23°.

indicate either a higher silicic acid content or a more porous product in the ChromAR-650. The manufacturer has also found the ChromAR-650 to be more active[5]. The rapid increase in relative moisture content above 50% humidity (Fig. 1) corresponds to the inflection point in the R_F values observed with both solvent P-1 and solvent P-2 (see Figs. 2 and 3). The down-turn in R_F values observed at higher humidities has been observed in other mixed solvent systems and is thought to result from solvent demixing[10]. Solutes below this demixing zone have depressed R_F values as the zone drops with increasing water vapor. Solutes above the demixing zone show R_F values which increase as the moisture content of the sheet increases.



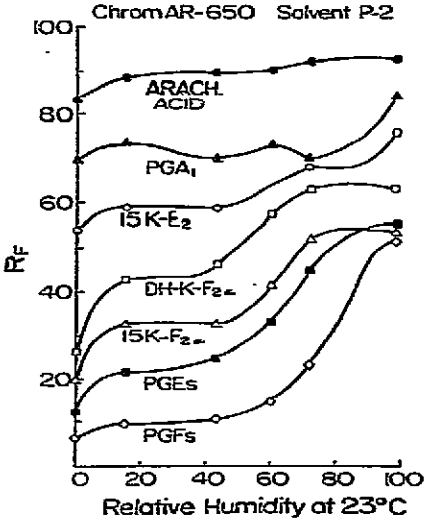


Fig. 2. Effect of humidity on the R_F values of prostaglandins and their metabolites using ChromAR-650 and solvent P-1.

Fig. 3. Effect of humidity on the R_F values of prostaglandins and their metabolites using ChromAR-650 and solvent P-2.

The best overall separation for the first development seems to occur in the 15–40% humidity range. Although solvent P-1 gives greater purification of triglycerides from the free fatty acids this was found to have limited value in our study of ocular epithelial tissue incorporation using dihomo-γ-linolenic acid as the labeled precursor[11]. Very low levels of activity were incorporated into the triglyceride fraction. As was expected[12], preincubation with [1-^{14}C]8,11,14-eicosatrienoic acid resulted in more incorporation into PGE_1 than into PGE_2. The greatest portion of activity (aside from the phospholipids and free fatty acids) co-chromatographed with the 15-keto-PGE metabolites and was probably thromboxane B_1 or PGD_1.

The solvents P-2, P-3 and P-4 were developed to give better separations in the regions of the 15-keto metabolites (Fig. 4A, Table I). Appropriate sections of the primary chromatogram containing the PGE and PGF compounds or, for example, the 15-keto-PGE compounds, could then be subjected to a secondary development onto a $AgNO_3$ treated sheet using solvent P-6 to separate PGE_1, PGE_2,

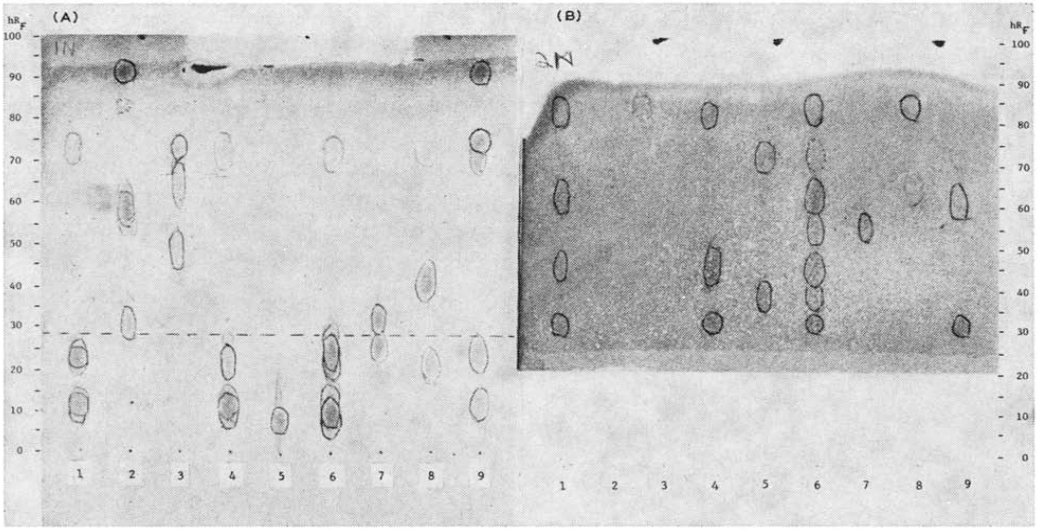


Fig. 4. (A), Primary development; ChromAR-650; solvent, benzene–*p*-dioxane–urethan–glacial acetic acid (87:10:2:1, v/v/w/v); humidity, 34.3%; temperature, 22.2°; 4 mm chamber thickness. hR_F averages (K = keto, DH = dihydro): 6K-$PGF_{1\alpha}$ (8), $PGF_{1\alpha}$ (11), $PGF_{2\alpha}$ (12), PGE_1 (21), PGE_2 (23), TXB_2 (25), 15K-$PGF_{1\alpha}$ (32), 15K-$PGF_{2\alpha}$ (32), PGD_2 (41), 13,14-DH-15K-$PGF_{2\alpha}$ (48), 15K-PGE_1 (58), 13,14-DH-15K-PGE_2 (65), PGB_1 (72), PGA_1 (73), arachidonic acid (91). Tracks (* indicates visibility with short-wave UV): (1) $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2, PGB_1*; (2) 15K-$PGF_{1\alpha}$*, 15K-PGE_1*, (unknown)*, arachidonic acid; (3) 13,14DH-15K-$PGF_{2\alpha}$, 13,14DH-15K-PGE_2, PGA_1*; (4) $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGB_1*; (5) 6K-$PGF_{1\alpha}$; (6) 6K-$PGF_{1\alpha}$, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2, TXB_2, PGB_1*; (7) TXB_2, 15K-$PGF_{2\alpha}$*; (8) PGE_1, PGD_2, (unknown); (9) $PGF_{2\alpha}$, PGE_2, (unknown below PGA_1), PGA_1*, arachidonic acid; (10) contained marker dyes and is not shown. (B), Secondary development using the duplicate of the section below the dotted line of Fig. 4A; 1.5% $AgNO_3$-impregnated ChromAR-650; solvent, P-6; humidity, 67.4%; temperature, 22.6°; 7 mm chamber thickness. hR_F averages: $PGF_{2\alpha}$ (32), 6K-$PGF_{1\alpha}$ (39), $PGF_{1\alpha}$ (45), TXB_2 (55), PGE_2 (62), 6K-$PGF_{1\alpha}$-(hemiketal?) (72), PGE_1 (82). Tracks: (1) $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, PGE_1; (2) and (3) no compounds but a spray artifact between the tracks; (4) $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_1; (5) 6K-$PGF_{1\alpha}$, 6K-$PGF_{1\alpha}$ (hemiketal?); (6) $PGF_{2\alpha}$, 6K-$PGF_{1\alpha}$, $PGF_{1\alpha}$, TXB_2, PGE_2, 6K-$PGF_{1\alpha}$ (hemiketal?), PGE_1; (7) TXB_2; (8) spray artifact, PGE_1; (9) $PGF_{2\alpha}$, PGE_2.

$PGF_{1\alpha}$ and $PGF_{2\alpha}$ (see Fig. 4B) or solvent P-5K to separate 15-keto-PGE_1 (R_F 0.52) from 15-keto-PGE_2 (R_F 0.36). The best separation using solvents P-5 and P-6 on $AgNO_3$-treated sheets occurred at a humidity of 70% but the recoveries were variable with solvent P-5 —apparently related to effects caused by variations in sheet thickness. Reproducible chromatograms were obtained with solvent P-6 even when the unit weight of the sheet was 30% greater than normal. If standards are spotted directly on $AgNO_3$-treated ChromAR and developed with solvent P-5 as the first and only development (Table I), PGE_1 is not separated from 15-keto-$PGF_{2\alpha}$ and 15-keto-13,14-dihydro-$PGF_{2\alpha}$.

The recoveries of radioactive prostaglandins are shown in Table II. Pace-Asciak and Wolfe[13] state that thin-layer chromatography of labeled arachidonic acid left about 3% of the radioactivity distributed non-specifically throughout the chromatogram. We have found that when labeled dihomo-γ-linolenic acid was re-chromatographed there still was a maximum of 0.12% of the label trailing in the combined

TABLE I

hR_F VALUES FOR PROSTAGLANDINS AND RELATED COMPOUNDS ON CHROMAR SHEET

Values in parentheses equal the total size of the spots expressed as an hR_F value.

Compound	*Sheet type*	*Solvent mixture used at 30% humidity*				*70% Humidity*	
		P-1	*P-2*	*P-3*	*P-4*	*P-5**	*P-6***
Trieicosenin	500	80 (4)	—	—	91 (2)	—	—
	650	—	—	97 (6)	—	—	—
Arachidonic acid	500	71 (2)	—	—	84 (5)	—	—
	650	66	88 (7)	93 (8)	90 (8)	90	—
Cholesterol	500	69 (2)	—	—	82 (6)	—	—
	650	—	—	91 (10)	—	—	—
PGA_1 or PGB_1	500	55 (4)	—	—	71 (6)	—	—
	650	54	72 (8)	77 (9)	76 (10)	84	—
PGA_2	500	55 (4)	—	—	68 (5)	—	—
	650	—	72 (9)	75 (8)	74 (6)	—	—
13,14-Dihydro-15-keto-PGE_2	500	—	—	—	61 (5)	—	—
	650	52	65 (7)	67 (8)	70	—	—
15-Keto-PGE_1	650	—	—	—	64 (11)	80	—
15-Keto-PGE_2	500	48 (4)	—	—	54 (7)	—	—
	650	50	61 (8)	61 (12)	62 (10)	78	
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	500	36 (4)	—	—	38 (7)	—	—
	650	45	44 (9)	46 (13)	47 (10)	61	—
15-Keto-$PGF_{2\alpha}$	500	26 (5)	—	—	26 (7)	—	—
	650	33	31 (9)	32 (12)	34 (9)	57	—
PGE_1	500	24 (4)	—	—	18 (6)	—	—
	650	27	22 (8)	22 (10)	22 (8)	60 (8)	82 (9)
PGE_2	500	24 (4)	—	—	14 (5)	—	—
	650	25	21 (7)	18 (7)	20 (8)	45 (8)	65 (10)
$PGF_{1\alpha}$	500	9 (5)	—	—	8 (4)	—	—
	650	10	9 (7)	11 (6)	11 (6)	34 (7)	46 (10)
$PGF_{2\alpha}$	500	9 (5)	—	—	5 (4)	—	—
	650	9	8 (6)	7 (6)	9 (6)	23 (6)	30 (7)
Phosphatidyl	500	1 (3)	—	—	1 (3)	—	—
Ethanolamine	650	2	—	1 (3)	—	—	—

* ChromAR impregnated with 1.5% $AgNO_3$ prior to development in P-5 solvent.

** Secondary development onto ChromAR impregnated with 1.5% $AgNO_3$ (hR_F assumes 15.4 cm solvent path).

PGE and PGF area. This trailing was reduced to 0.10% if the labeled fatty acid was spotted for chromatography in the presence of phosphatidyl ethanolamine (20 μg, Table III). After the secondary chromatography on $AgNO_3$-treated sheets using solvent P-5 to separate compounds according to the number of double bonds, contamination from precursor was a minimum of 13-fold less. This degree of purification (0.009% of original precursor activity) is desirable when short time control incubations with small amounts of tissue may yield levels of incorporation into $PGF_{2\alpha}$ as low as 60 dpm.

If extraction from the chromatogram is planned, the P-2 solvent might be preferred because of possible peroxide formation with the dioxane of solvents P-3 and P-4[14]. However, we did not observe any significant reduction in recoveries of PGs after re-chromatography when solvent P-4 was used for the first chromatography

TABLE II

AVERAGE PERCENT RECOVERIES OF PURIFIED PROSTAGLANDINS ON CHROMAR-650

Re-chromatography: the initial spot was placed in a tube and extracted with 1 ml of water-saturated acid ethyl acetate (EA, pH 3.5 with acetic acid), mixed and centrifuged. 1 ml of saturated NaCl was then added and the tube mixed and centrifuged. The EA layer was sequentially transferred through two 1-ml washings with double distilled water with mixing and centrifugation each time. After the final water wash, the EA layer was placed in a clean tube. To the original tube containing the spot with NaCl solution an additional 1 ml of acid EA was added, and the EA layer mixed, centrifuged, and sequentially transferred through the same water washes used for the first EA layer. The EA layers were pooled. After vacuum drying, the material was taken up with 50 μl of methanol–EA (30:70) and spotted in superimposed 5-μl aliquots under a nitrogen stream. The tubes were washed out by adding another 25 μl of spotting solvent and spotted in the same manner. The percent recovery statement represents the actual amount of activity spotted for the re-chromatography chromatograms and represents 85–95% of the total activity spotted on the initial chromatogram. The loss of material is distributed among the residue or the first chromatogram, the 3 extraction tubes, and residues left in the drying tube after the spotting solvent had been removed. The percentages are the average of 2–8 determinations.

Compound	*Solvent mixture at 30% humidity (no Ag)*				*70% Humidity 1.5% $AgNO_3$-impregnated ChromAR*		
	P-2		*P-4*		*P-5**		*P-6**
	Initial	*Re-chromatography*	*Initial*	*Re-chromatography*	*Wicked P-2*	*Wicked P-4*	*Wicked P-2*
PGE_1 (H^3)	95.6	95.4	95.2	92.5	90.8	89.3	95.3
PGE_2 (H^3)	96.0	94.8	94.4	91.5	91.8	89.4	95.3
$PGF_{1\alpha}$ (H^3)	97.5	97.1	97.5	95.3	72.8–95.5	72.0–96.2	97.5
$PGF_{2\alpha}$ (H^3)	98.7	97.8	96.6	95.9	97.1	96.2	94.7

* Unlabeled PGs (3 μg each of PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$) were used as carriers only in the double-development study.

TABLE III

EFFECT OF PHOSPHATIDYL ETHANOLAMINE (PE) UPON TRAILING OF [H³]DIHOMO-γ-LINOLENIC ACID (DHGL)

Compound	*Chromatography with P-4 solvent**					*Chromatography with P-5 solvent***		
	hR_F range (bottom to top)	*Recovery (%) after pre-spotting with*				*hR_F range (bottom to top)*	*Recovery (%) after pre-spotting with*	
		0 μg PE	*5 μg PE*	*10 μg PE*	*20 μg PE*		*0 μg PE*	*20 μg PE*
Above DHGL	95–100	0.04	0.01	0.02	0.01			
DHGL	85–95	98.23	98.38	98.51	98.58		0.044	0.041
13,14-DH-15K-PGA	80–85	0.08	0.13	0.14	0.20			
PGA***	72–80	0.27	0.29	0.30	0.33			
13,14-DH-15K-PGE	68–72	0.06	0.07	0.05	0.05			
15K-PGE***	55–68	0.23	0.19	0.20	0.17			
13,14-DH-15K-PGF	40–55	0.11	0.11	0.10	0.09			
15K-PGF***	28–40	0.10	0.08	0.07	0.07			
PGE_1***	16–28	0.06	0.06	0.05	0.04	53–67	0.007	0.009
PGE_2***	PGE_1 value includes PGE_1 and PGE_2					40–53	0.000	0.000
$PGF_{1\alpha}$***	3–16	0.06	0.06	0.06	0.06	29–40	0.000	0.000
$PGF_{2\alpha}$***	$PGF_{1\alpha}$ value includes $PGF_{1\alpha}$ and $PGF_{2\alpha}$					17–29	0.005	0.007
Origin	−5 to +3	0.77	0.61	0.50	0.40			

* Approximately 50,000 dpm [H³]dihomo-γ-linolenic acid spotted per sample.

** Wick cut from P-4 chromatogram which includes the PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ and chromatographed in P-5 solvent onto 1.5% $AgNO_3$-impregnated ChromAR.

*** These unlabeled compounds were also spotted (3 μg each).

(Table II). Solvent P-4 does seem to give slightly better placement of the metabolites and has been our choice for one-step chromatography. Solvent P-4 also makes it easier to detect low levels of 13,14-dihydro-15-keto-PGs. These compounds, in our experience, are less reactive to cupric acetate spray but their positions show up readily on a urethan treated sheet when sprayed lightly with $AgNO_3$ and heated.

When the thinner sheets of ChromAR-500 were developed with solvent P-1 or P-4 the migration of the various standards was lower than when the thicker ChromAR-650 was used (Table I). This is in accord with the findings of Jänchen[15] for development in an unsaturated chamber.

CONCLUSIONS

The chromatographic methods we have described are being utilized in studying the synthesis of a wide spectrum of PGs in a variety of ocular epithelial tissues. Solvent P-4 gives slightly better separations of PGEs, PGFs, 15-keto-PGFs, 15-keto-PGEs, 15-keto-13,14-dihydro-PGEs, 15-keto-13,14-dihydro-PGFs, PGAs, and the fatty acid–triglyceride fraction while leaving the phospholipids at the origin. The various classes of PGs and metabolites may then be further separated by cutting off a strip of the ChromAR sheet which contains one or two PG classes and allowing the compounds from this strip to migrate onto a silver nitrate-treated sheet of the silicic acid-glass microfiber matrix. We have obtained best results by using P-6 solvent for the second development. Under these conditions, recoveries have generally been in excess of 95% for the PGEs and PGFs and contamination by labeled fatty acid precursor has been reduced to less than 0.009% of the amount spotted. Since it requires less than 30 min to develop one of the chromatograms, it has been routine for one technician to process 80 samples through both the primary and secondary developments in less than 8 h by utilizing 8 sandwich chambers and 4 solvent troughs.

Although the relative humidity does have a profound effect upon the separations obtainable with a silicic acid-glass microfiber matrix, they became quite reproducible when the humidity and temperature were carefully controlled. This control has been obtained by the simple expedient of equilibrating the spotted sheets for 10 min in a glove box where the humidity is controlled by saturated salt solutions. The sandwich chambers were then closed prior to leaving the glove box. A glove box containing saturated calcium chloride gives a relative humidity of 33–34% at 23° which is suitable for the first development. A saturated solution of magnesium acetate equilibrates with a relative humidity of 67–69% at 23° which has worked well for the second development.

These methods have given more complete and reproducible purification of PG compounds than others we have used.

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