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GLASS CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

SEPARATION OF PROSTAGLANDINS

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SUMMARY

Glass capillary gas chromatography of the prostaglandins was performed on a system including an all-glass, solventless injector; thermostable methylphenylpolysiloxane glass capillary columns; and a conventional electron-capture detector fitted with a make-up gas tee. The principal stable metabolites of prostaglandin endoperoxide were separated as perfluorinated derivatives in 35 min. Detection limits equal or exceed those obtained for packed column separations and electron-capture detection. Prostaglandin endoperoxide metabolic profiles from mammalian cell cultures were obtained using this system. These profiling studies are not possible with other chromatographic methods because of inferior resolution and sensitivity.

INTRODUCTION

Electron-capture detection, after glass capillary gas chromatography (GC), seems ideal for biochemical trace analysis problems. The detector sensitivity complements the operating conditions of efficient capillary columns, and suitable halogenated derivatives exist for different classes of biochemicals. Yet electron-capture detection is used infrequently with capillary columns. Most researchers apparently believe the large dead volume of typical electron-capture detectors (ECDs) destroys the resolution gained by capillary columns. Recently, attempts to wed conventional ECDs with capillary columns have successfully contradicted this notion[1–5]. Insufficient detector sensitivity, an often cited[6–7] limitation to the widespread application of glass capillary GC for biochemical analysis, may no longer be

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a serious obstacle. Naturally, confirmation of this point resides in the successful application of glass capillary GC–ECD to formidable problems. The separation and analysis of the prostaglandins represents one such problem.

We describe a system for glass capillary GC–ECD using a solventless injection device[8], thermostable methylphenylpolysiloxane glass capillary columns[9,10], and an ordinary ^{63}Ni ECD provided with a make-up gas tee. This system separates halogenated derivatives of closely related prostaglandins without adverse influences from the detector dead volume when a properly regulated make-up gas flow augments the carrier gas flow through the detector. Sensitivity equals or exceeds that for packed column separations and electron-capture detection. The high resolution of the capillary columns, and the sensitivity of the ECD permit *in vitro* cell metabolism studies of prostaglandin endoperoxides. These profiling studies are not possible with other chromatographic methods because of incomplete metabolite resolution and inadequate detection limits.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5713A gas chromatograph with a 15-mCi ^{63}Ni ECD was modified for glass capillary GC. An all-glass, solventless injector[8] was mounted in the heated injector block. A needle valve, instead of a capillary restrictor regulates the column/vent gas flow ratio in our system[11]. Glass capillary columns were mounted in aluminum mandrils (Perkin-Elmer, Norwalk, Conn., U.S.A.). The mandrils have a low volume tee at the outlet side to add, argon–methane (90:10) make-up gas to the column effluent gas before its entry into the detector. The columns were connected as previously described to the injection system[11] and to the detector[2]. Thermostable methylpolysiloxane and methylphenylpolysiloxane (95/5) glass capillary columns were prepared according to Rigaud *et al.*[9] and Madani *et al.*[10].

Chromatographic conditions

Helium and argon–methane (90:10), were purified over a desiccant ($CaCl_2$ and 4-Å molecular sieves (Applied Science Labs., State College, Pa., U.S.A.). The helium flow was optimized with a homologous series (C_{12}–C_{28}) of saturated fatty acid pentafluorobenzyl esters. An inlet pressure of 10 p.s.i.g., *i.e.* a linear carrier gas velocity of 22.5 cm/sec (approximately 1 ml/min), gave maximum column efficiency with a 25-m methylphenylpolysiloxane (95/5) glass capillary column. The injector block and detector were at 250 and 300°, respectively. The column was at 250°, isothermal, unless otherwise noted. Relative retention data were determined according to Woodford and VanGent[12] with a homologous series (C_{12}–C_{28}) of fatty acid pentafluorobenzyl ester standards for electron-capture sensitivity.

Reagents

Hexane, methanol (Burdick & Jackson, Muskegon, Mich., U.S.A.); anhydrous ether (Mallinckrodt, St. Louis, Mo., U.S.A.); bis(trimethylsilyl)acetamide (BSA), *o*-methylhydroxylamine hydrochloride, *o*-pentafluorobenzylhydroxylamine hydrochloride (Applied Science Labs.); N-methyl-N-nitroso-N-nitroguanidine (Eastman,

Rochester, N.Y., U.S.A.); pentafluorobenzylbromide, diisopropylethylamine (Aldrich Milwaukee, Wisc., U.S.A.); potassium hydroxide solution, 45% (w/v), and potassium hydroxide pellets (Baker, Phillipsburg, N.J., U.S.A.) were used as received. Silylation-grade pyridine (Pierce, Rockford, Ill., U.S.A.) was stored over 4-Å molecular sieves during use. Stable prostaglandins and thromboxanes were supplied by the Experimental Chemistry Laboratories of the Upjohn Company. Prostaglandin endoperoxide (PGH_2) was prepared biosynthetically[13]. Other reagents cited were the highest purity available.

Esterification

Protaglandin, thromboxane and fatty acid standards or the residues from ether extracted cell incubation medium were esterified. Pentafluorobenzyl esters were prepared according to Wickrema Sinha *et al.*[14]. Methyl esters were prepared by treatment with anhydrous ethereal diazomethane (1.0 ml) for 10 min at 25°. The diazomethane was prepared immediately before use. After esterification, excess reagents were evaporated at 25° with nitrogen.

Oximation

Prostaglandin and thromboxane pentafluorobenzyl esters were converted to methoximes by warming (45°, 2 h) the residue left after esterification with a saturated solution (0.2 ml) of *o*-methyl-hydroxylamine hydrochloride in anhydrous pyridine[15]. Prostaglandin or thromboxane methyl esters were converted to *o*-pentafluorobenzyloximes as previously described[16]. Pyridine was evaporated at 25° with nitrogen; and excess derivatizing reagent was removed by partitioning twice between hexane (2.0 ml) and water (1.0 ml, pH 3.0). The combined hexane layers were desiccated with Na_2SO_4 and evaporated with nitrogen. Prostaglandin and thromboxane ester oximes were converted to trimethylsilyl esther by heating (40°, 1 h) with BSA (20 μl). The final residue was dissolved in hexane (10.0 ml) and 1 μl was chromatographed.

PGH_2 metabolism in cell preparations

Balb 3T3 and balb 3T12 cells (ATCC Repository, Bethesda, Md., U.S.A.) were grown to confluency in 35-mm wells containing Eagle's minimum essential medium (MEM) and 10% fetal calf serum (FCS). At confluency (10^6 cells per well) cells were washed with 3 × 1.0 ml of serum free MEM, and then incubated for 10 min at 37° with PGH_2 (1 μg) in MEM (0.4 ml). The incubation medium from six wells was pooled and the wells were washed once with 0.4 ml of MEM. The fluid (total volume = 4.8 ml) was acidified with 100 μl of 2 *M* citric acid, and extracted with ether (3 × 5 ml). The ether was evaporated with nitrogen and PGH_2 metabolites in the residue were converted to pentafluorobenzyl ester methoxime trimethylsilyl ethers, or methyl ester pentafluorobenzyloxime trimethylsilyl ethers before chromatography.

Mouse splenic and thymic lymphocyte cultures were prepared according to Ford[17] and incubated and analyzed as above.

RESULTS

Thermostable methylphenylpolysiloxane (95/5) glass capillary columns separate structurally similar F series prostaglandins as pentafluorobenzyl ester trimethylsilyl ethers. Table I lists relative retention data for these compounds. For capacity factors between 11 and 17, the column (25 m) shows 26,000 to 34,000 theoretical plates. Higher plate numbers were already reported[8] for these columns with F series prostaglandin methyl ester trimethylsilyl ethers. Thus, diffusion and band broadening within the ECD lowered the resolution, but not so severely to impair the practical value of the system.

TABLE I

RELATIVE RETENTION DATA FOR PENTAFLUOROBENZYL ESTER TRIMETHYLSILYL ETHERS OF F SERIES PROSTAGLANDINS

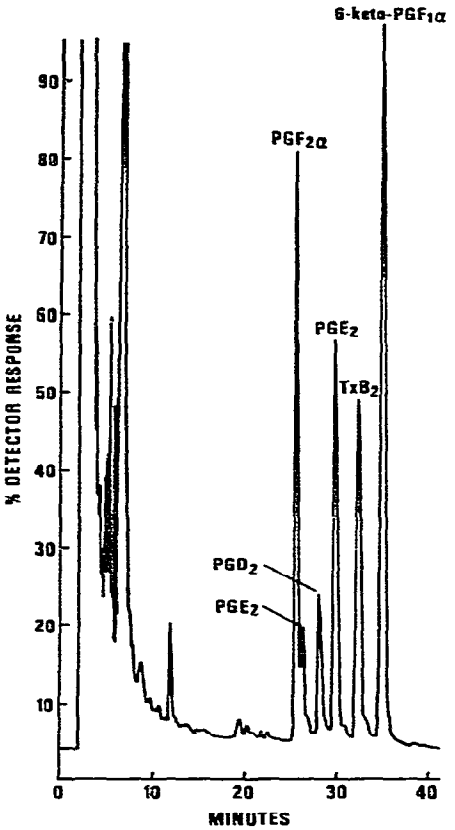
Column: 25 m × 0.3 mm I.D. methylphenylpolysiloxane (95:5). Carrier gas: helium, 22.5 cm/sec. Make-up gas: argon–methane (90:10), 15 ml/min. Temperatures: column, 250°; detector, 300°; injector, 250°. Detector: ^{63}Ni, 15 mCi. Instrument: Hewlett-Packard Model 5713. Injector: Vanden-Berg all-glass solventless injector.

Compound	*k'**	*Carbon number***
$PGF_{1\alpha}$	14.06	23.76
$PGF_{1\beta}$	12.26	23.27
$PGF_{2\alpha}$	12.39	23.31
$PGF_{2\beta}$	11.04	22.90
13,14-Dihydro-$PGF_{1\alpha}$	14.24	23.82
13,14-Dihydro-15-keto-$PGF_{1\alpha}$	15.82	24.19
15-keto-$PGF_{1\alpha}$	16.92	24.43
13,14-Dihydro-$PGF_{2\alpha}$	12.48	23.34
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	13.80	23.70
15-keto-$PGF_{2\alpha}$	14.60	23.90
6-keto-$PGF_{1\alpha}$	14.75	23.94

* $k' = (V_r - V_0)/V_0$.

** Carbon numbers are related to the capacity factors (k') of a homologous series of saturated fatty acid pentafluorobenzyl esters (C_{15}–C_{28}).

The PGF compounds are a difficult test mixture but they have limited biological significance. The five biochemically important PGH_2 metabolites; $PGF_{2\alpha}$, PGD_2, PGE_2, thromboxane B_2 (TXB_2) and 6-keto $PGF_{1\alpha}$, are baseline resolved as pentafluorobenzyl ester methoxime trimethylsilyl ethers (Fig. 1). Pentafluorobenzyl esters impart EC sensitivity to all prostaglandin metabolites with carboxylic acid functionality[14,18]. Since some prostaglandins have β-ketol functionality and thromboxanes have acetal functionality, they require oximation before chromatography, thus pentafluorobenzyloximes are also suitable for ECD. The methylphenylpolysiloxane column did not completely resolve the methyl ester pentafluorobenzyloxime trimethylsilyl ethers of PGD_2, PGE_2, TXB_2 and 6-keto $PGF_{1\alpha}$; however, they were resolved sufficiently to assign peak identities (Fig. 2). Formation of both types of functionally distinct perfluorinated derivatives corroborates peak identities in unknown mixtures. Table II lists relative retention data for several prostaglandins and thromboxane pentafluorobenzyl esters or pentafluorobenzyloximes. Multiple values



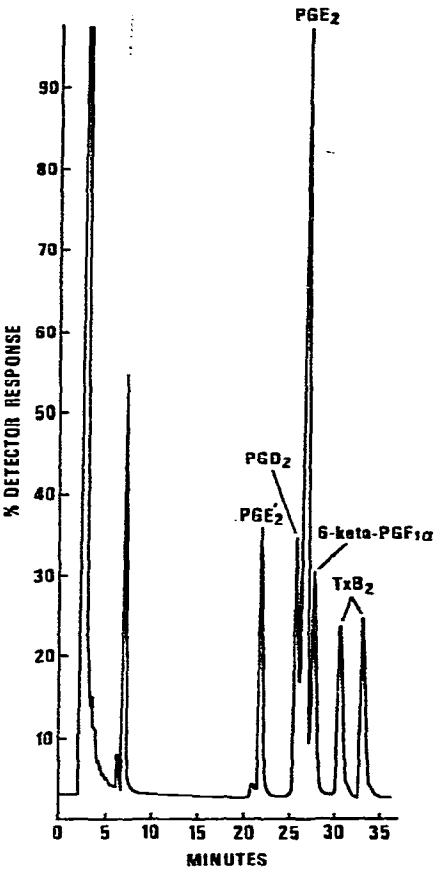


Fig. 1. Separation of principal stable metabolites of PGH_2. Column: 25 m methylphenylpolysiloxane (95:5) 250° isothermal. Carrier gas: helium, 22.5 cm/sec. Make-up gas: argon–methane: (90:10), 15 ml/min. Detector: ^{63}Ni ECD, 300°, attenuation 256 ×, approximately 500 pg on column. Injector: VandenBerg solventless probe injector, 250°. Chart: 0.1 in./min, 1-mV recorder. Compounds were chromatographed as pentafluorobenzyl ester methoxime TMS ethers.

Fig. 2. Separation of principal stable metabolites of PGH_2 as methyl ester pentafluorobenzyloxime TMS ethers. Conditions as in Fig. 1.

for single, authentic prostaglandins indicate the resolution of *syn-* and *anti-* isomers of the oximes.

ECD limits with glass capillary columns equal or exceed those reported for packed columns[14,15,18]. In practice, manipulation and derivatization steps before chromatography limit the attainable sensitivity as much, or more, than column and detector performance do. We prepared calibration curves by derivatizing 100 to 400 ng of PGH_2 metabolites. The derivatives were dissolved, finally, in 1.0 ml of hexane and 1 μl was deposited on the injector probe. The nanograms derivatized and the picograms injected correspond. At 32 × attenuation with a 1-mV recorder, 100 pg "on-column" could be quantitated. We draw attention to the fact that 32 × represents a conservative, but realistic attenuation setting, and we arbitrarily defined 20:1 as the minimally acceptable signal-to-noise ratio for practical quantitation at this

TABLE II

RELATIVE RETENTION DATA OF PROSTAGLANDINS AND THROMBOXANES

Chromatographic conditions as in Table I.

Compound	*Pentafluorobenzyl ester methoxime trimethylsilyl ethers*		*Methyl ester pentafluorobenzyloxime trimethylsilyl ethers*	
	k'	*Carbon number*	*k'*	*Carbon number*
PGA_1	11.04	22.89	8.17/10.25	21.78/22.57
PGA_2	9.91/9.91	22.20/22.50	7.80/9.64	
PGB_1	14.63	23.91	14.10	23.74
PGB_2	13.98	23.75	14.52	23.85
PGD_1	14.86/15.88	23.97/24.20	11.13/13.38	22.87/23.55
PGD_2	13.84	23.71	10.04/11.98	22.50/23.15
PGE_1	14.00/15.96	23.74/24.22	10.39/13.06	22.62/23.46
PGE_2	12.88/14.67	23.45/23.92	9.94/12.38	22.46/23.26
Dihomo-PGE_2	23.65/27.13	25.65/26.14	17.23/21.50	24.48/25.29
13,14-Dihydro-PGE_1	14.63/16121	23.91/24.28	10.88/13.06	22.79/23.46
13,14-Dihydro-PGE_2	13.63/15.02	23.60/24.00	10.46/12.33	22.65/23.25
15-keto-$PGF_{1\alpha}$	15.00	24.00	11.92/12.85	23.13/23.40
15-keto-$PGF_{2\alpha}$	13.08	23.51	10.77/11.71	22.76/23.06
13,14-Dihydro-15-keto-$PGF_{1\alpha}$	15.11	24.02	11.81/12.65	23.09/23.34
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	13.29	23.57	10.67/11.50	22.72/23.00
TXB_2	16.03	24.24	14.33/15.58	23.80/24.11
6-keto-$PGF_{1\alpha}$	17.42	24.54	12.31/12.88	23.25/23.41
13,14-Dihydro-6,15-diketo-$PGF_{1\alpha}$	19.08	24.87	34.41/35.87/37.33/33.58	27.01/27.16/27.31/27.43
6,15-Diketo-$PGF_{1\alpha}$	18.42	24.74	33.17/35.04/36.19/38.27	26.88/27.07/27.15/27.40
13,14-Dihydro-15-keto-TXB_2	18.50	24.76	43.90/46.50/47.64/50.67	27.90/28.11/28.20/28.43
15-keto-PGE_1	14.83/15.67/15.92/17.33	23.96/24.16/24.21/24.53	33.79/36.60/38.17/41.70	26.94/27.23/27.39/27.72
15-keto-PGE_2	14.33/15.79	23.84/24.19	31.19/33.90/38.06/41.81	26.65/26.96/27.38/27.72

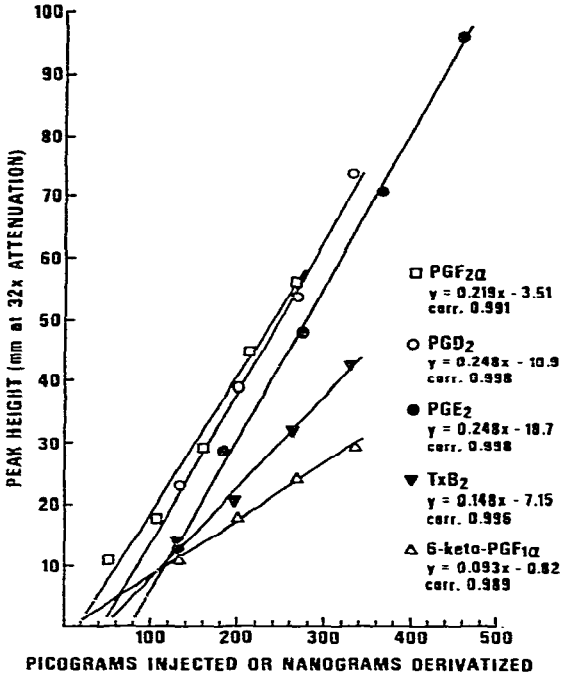


Fig. 3. Calibration curves for principal stable metabolites of PGH_2. Compounds were chromatographed as pentafluorobenzyl ester methoxime TMS ethers as in Fig. 1. Note: the attenuation was held constant at 32× and the minimally acceptable signal-to-noise ratio was arbitrarily fixed at 20:1 to obtain a conservatively realistic limit of quantitation, distinct from the limit of detection.

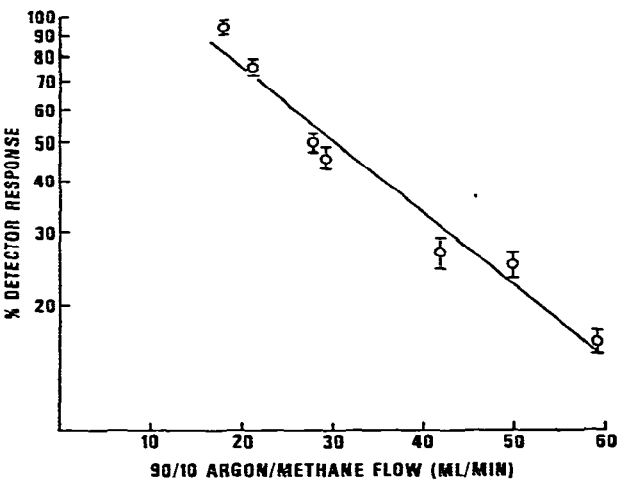


Fig. 4. Detector response characteristics as a function of make-up gas flow-rate. The carrier gas (helium) flow was fixed at 22.5 cm/sec. Points represent the mean response ± the relative standard deviations for nine different compounds examined. The relative standard deviation in the detector response for individual compounds was ±2–4%.

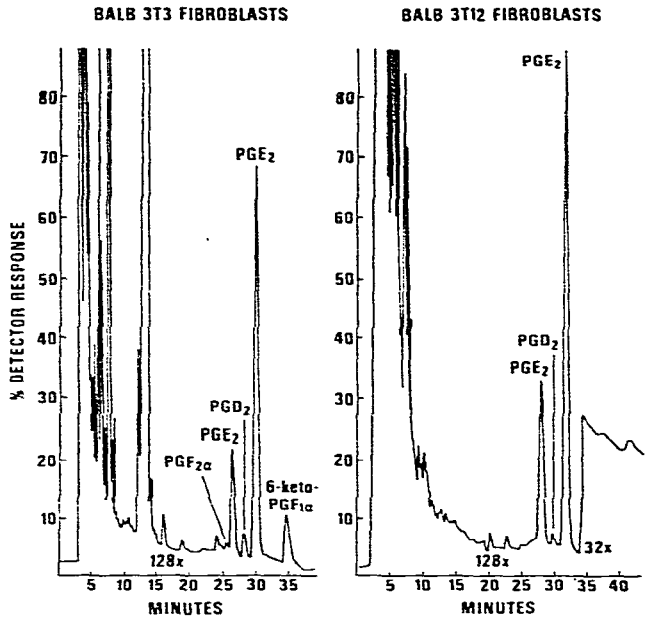


Fig. 5. Metabolism of PGH_2 by mammalian fibroblasts. Conditions as in Fig. 1. Attenuation adjusted as required.

attenuation (Fig. 3). At lower attenuations (4–8 ×) and at lower signal-to-noise ratios (5:1), on column "detection" of 1–10 pg is possible.

The ECD is a flow sensitive device. We characterized the detector response as

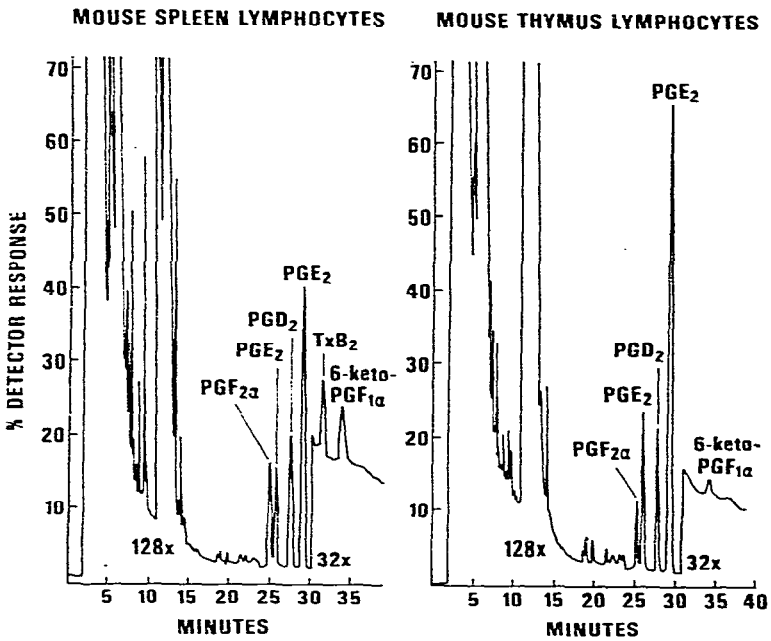


Fig. 6. Metabolism of PGH_2 by lymphocytes. Conditions as in Fig. 1. Attenuation adjusted as required.

a function of make-up gas argon–methane, 90:10) flow at a constant carrier gas flow. Nine different compounds (five fatty acid pentafluorobenzyl esters and four prostaglandin methyl ester pentafluorobenzyloxime trimethylsilyl ethers) were injected and the detector response was monitored at different argon–methane make-up gas flow-rates. Flow-rates of 12–15 ml/min gave the maximum detector response without distorting chromatographic resolution by mixing in the EC cell. Detector response declined logarithmically with increasing flow-rates for all compounds tested (Fig. 4). At flow exceeding 60 ml/min, back-pressure in the EC cell altered retention times.

We monitored the metabolic profiles of PGH_2 in intact cell preparations with glass capillary GC–ECD. Typical results are shown in Figs. 5 and 6.

DISCUSSION

Our results are in concordance with those reported by others on the problem of glass capillary GC linked with ECDs[1,4,5]. Schomburg *et al.*[1] have shown unequivocally, that peak tailing occurs in conventional ECDs with capillary columns. Our results agree. While our system may not have the maximum efficiency theoretically possible, it has the efficiency needed to solve a difficult biochemical problem: the characterization of prostaglandin endoperoxide metabolism in intact cell preparations. For example, GC methods using packed columns cannot resolve the prostaglandins sufficiently without other supplementary chromatographic steps[15,18,19] or unless specific ion monitoring is employed[20]. A high-performance liquid chromatographic technique reported by Turk *et al.*[21] appears to be the only other chromatographic technique with sufficient intrinsic resolution to rival glass capillary chromatography for prostaglandin separations[22,23].

The system described should be applicable to other analytical problems demanding high-sensitivity detection and high-resolution GC. Despite its utility, demonstrated improvements are possible. For example, Brechbühler *et al.*[24] developed a low dead volume ECD for capillary columns which should permit higher chromatographic efficiencies, such as those obtained with "zero" volume ionization detectors. The Brechbühler detector is not yet adaptable to many commercial chromatographs.

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