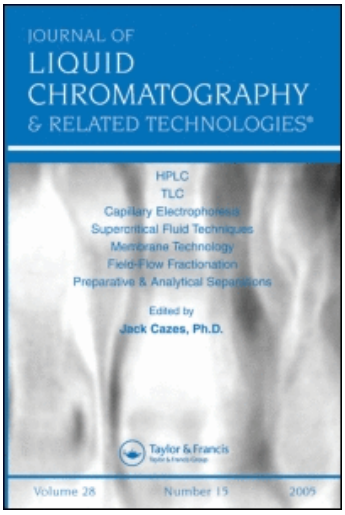

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RAPID SEPARATION OF PROSTAGLANDINS BY LINEAR HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A continuous development technique using silica gel linear high performance TLC plates is described for the separation of prostaglandins 6-keto-$F_{1\alpha}$, $F_{2\alpha}$, E_2, 13,14-dihydro-15-keto-$F_{2\alpha}$, 13-14-dihydro-15-keto-E_2, and thromboxane B_2. Complete separation of all six prostaglandins was achieved with a solvent system of ethyl acetate/acetone/acetic acid (90:5:1). The method is simple, rapid and provides excellent resolution of plasma prostaglandins prior to quantitation by gas chromatography-mass spectrometry.

INTRODUCTION

Various solvent systems have been described for the thin layer chromatographic (TLC) separation of prostaglandins on silica gel (1-4). With many of these systems, however, complete separation of certain prostaglandins is not achieved, and a second chromatography step is required for further resolution. In addition, conventional TLC development in a closed tank can be very time consuming. We describe here a continuous development technique using silica gel linear high performance TLC plates which provides simple, rapid, and complete separation of prostaglandins 6-keto-$F_{1\alpha}$,

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$F_{2\alpha}$, E_2, 13,14-dihydro-15-keto $F_{2\alpha}$, 13,14-dihydro-15-keto-E_2, and thromboxane B_2.

MATERIALS AND METHODS

Silica gel linear high performance (Type LHP-K) thin-layer plates (20 x 10 cm) were obtained from Whatman, Inc. (Clifton, NJ 07014). Prostaglandin standards, PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 (TxB_2) were obtained from either Upjohn (Kalamazoo, MI) or Cayman Chemical (Denver, CO 80205). Standards for the 13,14-dihydro-15-keto metabolites of E_2 (PGEM) and $F_{2\alpha}$ (PGFM) were the generous gifts of Dr. John E. Pike (Upjohn). All solvents used were glass-distilled HPLC-grade reagents obtained from Bodman Chemicals (Media, PA 19063).

Prostaglandin standard mixtures containing 1-15 µg of each PG were applied to the preabsorbent area of the TLC plate in 20 µl aliquots using a micropettor (Scientific Manu. Ind., Berkeley, CA 94710). The plate was developed in ethyl acetate/acetone/acetic acid (90:5:1) (5) for 15 minutes using a short-bed/continuous development chamber (SB/CD chamber) (Regis Chemical Co., Morton Grove, IL 60053).

The SB/CD chamber consists of a glass tank (24 x 10.5 x 3 cm), a glass cover plate, and two teflon wings. The bottom of the plate has 5 stop positions, ranging from most vertical (position 1) to most horizontal (position 5). The TLC plate is placed in the chamber against one of the stop positions and leaned against the tank wall, leaving the upper part of the plate extended beyond the chamber. The cover plate and teflon wings are used to seal

the top of the chamber and hold the TLC plate in place. During chromatography the developing solvent advances up the plate until it reaches the top of the chamber where it evaporates. This results in a constant and continuous flow of solvent up the plate. With conventional TLC, which is done in a closed chamber, the solvent velocity decreases exponentially as the solvent rises up the plate. Consequently, the development time with the SB/CD chamber is considerably less than that required in the conventional closed system. A more thorough description of continuous development is presented elsewhere (6,7).

For prostaglandin separations, TLC plates were placed in position 5 (most horizontal). Plates were developed as described above, and air dryed. Prostaglandin standards were visualized by spraying with a 10% phosphomolybdic acid solution in ethanol and heating.

For routine analysis of a human plasma sample, 1 ng of the deuterated prostaglandin analog was added to 20 ml of plasma and the sample acidified to pH 3.5 - 4.0 with formic acid. The acidified plasma sample was applied to a C_{18} reverse phase SEP-PAK cartridge preconditioned with acetonitrile (10 ml) and HPLC-grade water (20 ml). The SEP-PAK was washed first with 10 ml of water, then 4 ml of 15% acetonitrile/85% 0.0025 M phosphoric acid, and the prostaglandins eluted with 10 ml of acetonitrile. The solvent was evaporated and the residue resuspended in 200 µl of benzene/ethyl acetate/methanol (60:40:10). One ml of benzene/ethyl acetate (90:10) was added, and the solution applied to a silica

SEP-PAK cartridge preconditioned with 10 ml of methanol and 20 ml of benzene/ethyl acetate (90:10). The SEP-PAK was washed with 5 ml of benzene/ethyl acetate (60:40) and prostaglandins were eluted with 10 ml of methanol. The solvent was evaporated, the residue resuspended in 1 ml of methanol, and the solvent evaporated again. The final residue was taken up in 100 µl of methanol, applied to a TLC plate in 20 µl aliquots and developed as described. Prostaglandin standards were run on each end of the plate and visualized by spraying and heating as above. Lanes containing plasma extracts were not sprayed. Zones corresponding to the prostaglandin of interest were scraped off the plate, eluted with 3 ml of methanol, the silica removed by centrifugation, and the supernatant evaporated. The residue was resuspended in 300 µl of methanol, transferred to a 1 ml reacti-vial, and solvent evaporated under argon.

Prostaglandins were quantitated as their pentaflurobenzyl ester-methoxime-trimethyl silyl ethers using capillary column gas chromatography-negative ion chemical ionization mass spectrometry. For derivatization, the plasma extract was dissolved in 30 µl of acetonitrile, and then reacted with 10 µl of 35% pentafluorobenzyl bromide (PCR Research Chemicals, Inc., Gainesville, FL 32602) in acetonitrile and 10 µl of 10% diisopropylethylamine (Aldrich Chemical Co., Inc., Milwaukee, WI 52233) in acetonitrile at 40°C for 15 min. The reaction mixture was evaporated and the residue dissolved in 50 µl of 2% methoxylamine hydrochloride in pyridine (Pierce Chemical, Rockford, IL 61105) and heated at 70°C for 1 hr. Solvent was evaporated and the residue silylated with 50 µl of acetonitrile

and 20 µl of BSTFA (Pierce) at 60°C for 15 min. The solvent was evaporated and the residue resuspended in 10 µl of tetradecane. A 2-3 µl aliquot was injected onto the GC/MS column and prostaglandins quantitated by selective ion monitoring (SIM) of the $M-C_7F_5H_2$ ion (8).

RESULTS AND DISCUSSION

The thin layer chromatographic separation of various prostaglandin standards using linear high performance silica gel plates is shown in Fig. 1. The corresponding R_f values for these compounds, calculated from the preabsorbant area - silica gel boundary to the secondary solvent front, are listed in Table I. Separation of all 6 PGs examined was achieved with the ethyl acetate/acetone/acetic acid (90:5:1) solvent system. The excellent band resolution obtained can be attributed both to the linear, high performance

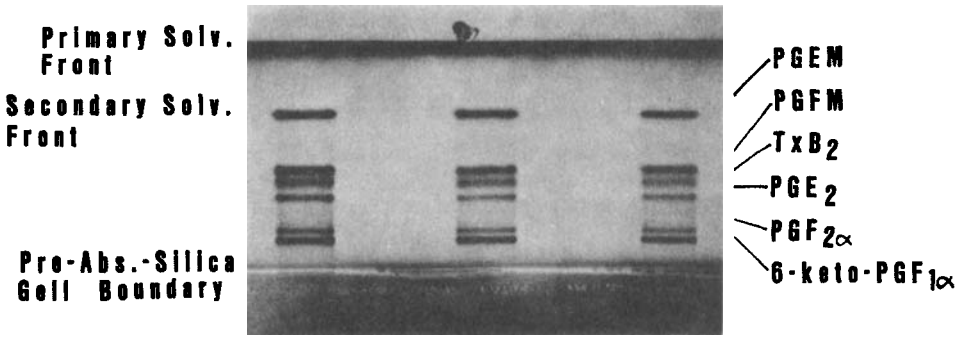

Figure 1. Prostaglandin standard chromatogram. The compounds were applied as a mixture in 20 µl aliquots. From left to right, the total volume applied to each lane was 60 µl, 40 µl, and 20 µl. Each 20 µl aliquot contained 1 µg each of 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and TxB_2, and 5 µg each of PGFM and PGEM. Prostaglandin bands were visualized by spraying with 10% phosphomolybdic acid and heating.

TABLE I

R_f* Values for Prostaglandins

Compound	R_f Value
6-keto-$PGF_{1\alpha}$	0.19
$PGF_{2\alpha}$	0.24
PGE_2	0.46
TxB_2	0.57
PGFM	0.64
PGEM	1.00

* R_f values are calculated from the pre-absorbant area - silica gel boundary to the secondary solvent front.

TLC plates used for chromatographic separation and to the SB/CD chamber used for plate development. The preabsorbant layer of the TLC plate concentrates the sample into a sharp band at the preabsorbant-silica gel boundary prior to separation on the silica gel layer. Rapid solvent development over a short distance, as well as the use of high performance silica gel, minimized spot diffusion, which also contributed to more compact bands.

This technique has been used in our laboratory for the separation of PGs in human plasma extracts prior to quantitation by GC/MS (8). Plasma concentrations calculated for PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2 were 2.7 $\pm$ 2.3, 3.0 $\pm$ 1.4, and 9.3 $\pm$ 6.5 pg/ml, respectively.

The need for separating PGs prior to quantitation has been well established (9). Linear high performance TLC offers a less expensive, simpler and more rapid method of separation than high performance liquid chromatography without compromising resolution.

By allowing the chromatography of several samples at once, sample throughput is also increased.

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