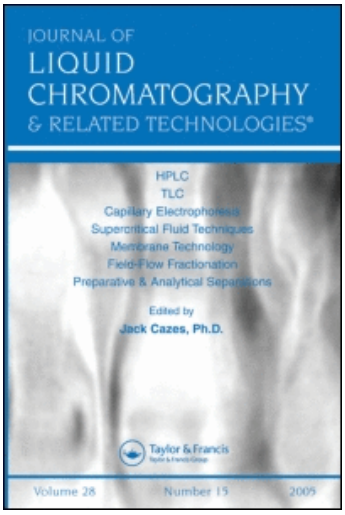

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PURIFICATION OF A WATER-SENSITIVE NATURAL PRODUCT WITH AN APROTIC CPC SOLVENT SYSTEM

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ABSTRACT

The partition coefficients of arachidonic acid (AA), prostaglandin (PG) H_2 and PGE_2 in the binary acetonitrile-hexane two-phase solvent system can be favorably modified by addition of diethyl ether. In the ternary two-phase solvent system acetonitrile-hexane-diethyl ether the partition coefficients of AA and the prostaglandins diverge as the ratio of ether to acetonitrile-hexane (1:1) is increased from 0.00 to 0.15 while the partition coefficients of PGH_2 and PGE_2 diverge as the ratio of ether to acetonitrile-hexane (1:1) is increased from 0.15 to 0.30. Isolation by CPC of PGH_2 from the AA bioconversion reaction mixture is described.

INTRODUCTION

The prostaglandin endoperoxide PGH_2 is a water-sensitive intermediate in the biosynthesis of prostaglandins, e.g. PGE_2, from arachidonic acid (AA). Isolation and purification of PGH_2 is complicated by the fact that it decomposes with a half life of 5 min in the aqueous environment of its biosynthesis producing a 4:1 mixture of prostaglandins and levuglandins, e.g. LGE_2 (1). Extraordinary instability in protic

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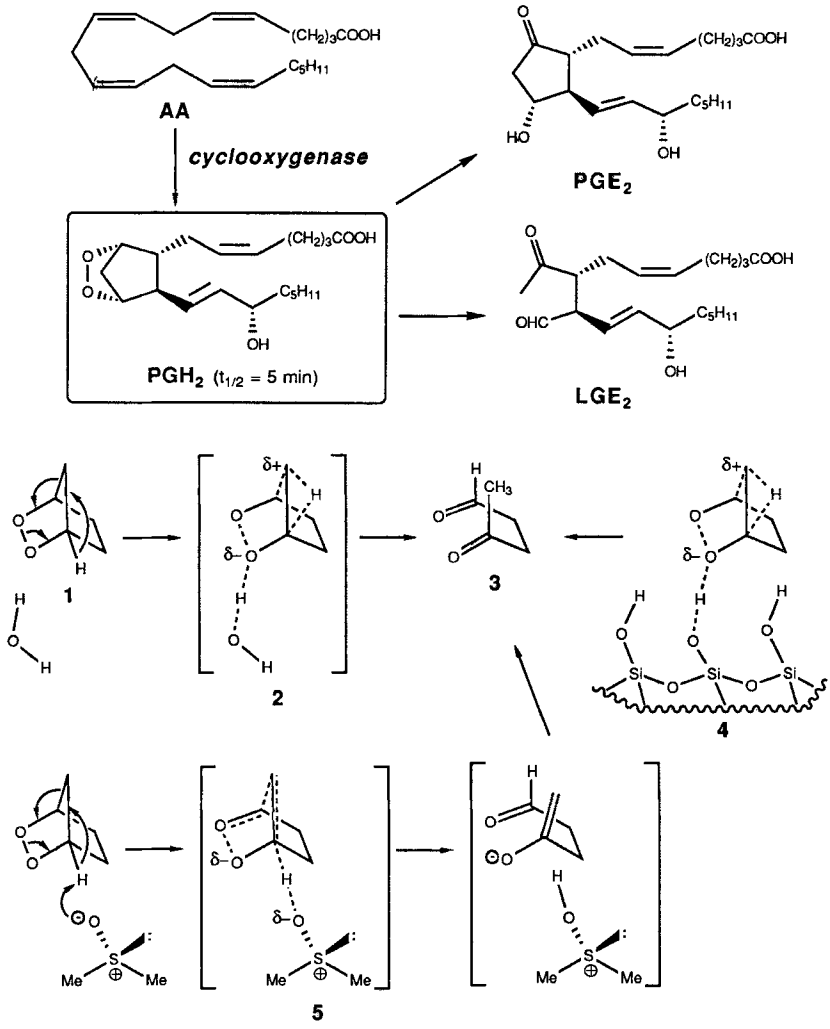

solvents was shown to be a characteristic of the 2,3-dioxabicyclo-[2.2.1]heptane nucleus **1** of PGH_2 (2). Isotopic labeling and kinetic studies revealed that protic solvents foster decomposition to give levulinaldehyde (**3**) by migration of a bridgehead hydrogen and simultaneous cleavage of carbon-carbon and oxygen-oxygen bonds (3). Apparently hydrogen bonding of protic solvents with a dipolar transition state as in **2** promotes the rearrangement of **1** to **3**. Silica-gel also catalyzes decompositions of PGH_2 (4,5) and **1.** Hydrogen bonding as in **4** may play a role in this catalysis. Dimethylsulfoxide (DMSO) promotes rearrangement of PGH_2 to form levuglandins virtually exclusively (3). This dipolar aprotic solvent apparently abstracts a bridgehead proton from the 2,3-dioxabicyclo[2.2.1]heptane nucleus of PGH_2 resulting in simultaneous cleavage of C-H, C-C and O-O bonds as in **5**.

Purification of PGH_2 by chromatography on silica-gel (4,6) or countercurrent partitioning between two-phase solvent systems containing *protic solvents or DMSO* is hampered by chemical instability. However, we now report that purification is readily achieved by centrifugal partition chromatography (CPC) using acetonitrile-hexane-ether, an *aprotic* two-phase solvent system.

EXPERIMENTAL

HPLC grade acetonitrile and n-hexane were used. Reagent grade ethyl ether was purified by distillation from lithium aluminium hydride under dry nitrogen immediately prior to use. After mixing by vigorous shaking, two phase solvent systems were allowed to stand overnight before separation of the phases.

Centrifugal Partition Chromatography

CPC separations were performed with a Sanki Engineering Limited model NMF centrifugal partition chromatograph equipped with 6 type 250W partition cartridges with a 1200 rpm rotor speed at 19°C. Solvents were pumped with a Waters model 6000A solvent delivery system equipped with extended flow heads. Solvent switching and sample injection was accomplished with a Sanki model FCU II valve module. The effluent was monitored with an Instrumentation Specialties (ISCO) model 1840 absorbance monitor at 220 nm.

CPC Purification of PGH_2

PGH_2 was prepared as described previously (3) by incubation of arachidonic acid (AA, 40 mg) in the presence of lyophilized ram seminal vesicle microsomes (1.8 g) in 0.1M pH 7.5 phosphate buffer (320 mL). With a rotor speed of 1200 rpm, the CPC cells were filled with the lower phase of an acetonitrile-hexane-ether (1.0:1.0:0.4 v/v/v) solvent system. The solvent flow was set at 2.2 mL/min, and the UV detector at 0.1% absorbance. The upper phase was eluted (ascending mode). After 15 minutes, one fourth of the crude bioconversion reaction product mixture (15 mg) in the upper phase (1 mL) was injected and the upper phase eluted for 165 min. The flow was then reversed and the lower phase was eluted (descending mode). After 19 min PGH_2 eluted as a sharp UV-active peak which responded positively to a peroxide test with acidic $Fe(NH_4)_2(SO_4)_2/NH_4SCN$. Solvent was removed by rotary evaporation below room temperature to deliver pure PGH_2 (6.9 mg) which was 96% pure according to ^1H NMR comparison of the integral areas of the olefinic hydrogen absorptions at δ5.33 - 5.48 (4H) with the bridgehead hydrogen absorptions at δ4.52 (H, s) and δ4.41 (H, s).

Partition Coefficients

The ^1H NMR method employed to determine partition coefficients is exemplified for PGE_2 for an 0.2:1.0 ratio of ether to acetonitrile-hexane (1:1). The two phases which form after allowing a mixture of dry ether (40.0 mL), acetonitrile (100.0 mL), and n-hexane (100.0 mL) to equilibrate at 23°C were separated. The top phase (10.0 mL) and bottom phase (9.9 mL) were placed in a small seperatory funnel and PGE_2 (0.3 mg) in the bottom phase (0.1 mL) was added. The mixture was shaken vigorously for 5 min and then allowed to stand 10 min. The two phases were then separated and the solvent was removed by rotary evaporation under water aspirator vacuum. The residue from each phase was dissolved in a solution (750 μL) of benzene (0.1% v/v) as internal standard in $CDCl_3$. ^1H NMR spectra of these solutions were recorded with a Varian XL-200 FT instrument. The ratio of the integral intensities for the olefinic hydrogen absorptions of PGE_2 (δ4.6 - 4.9) and the benzene hydrogen absorption (δ7.2 - 7.4) were determined. The top phase gave 0.0199 while the bottom phase showed 0.6936. The partition coefficient was then calculated to be K = 0.0287 for [top phase]/[bottom phase].

Ternary Solvent Phase Diagram

The molar ratio of components of each phase at 23°C or 6°C was determined by ^1H NMR analysis as illustrated for acetonitrile-hexane-ether (1.0:1.0:0.4 v/v/v). A spectrum was recorded for an aliquot of the top phase (100 μL) in $CDCl_3$ (600 μL). The composition of the mixture was calculated to be 19.9% ether, 13.2% acetonitrile, and 66.9% n-hexane from the relative integral intensities of the absorptions at δ3.56 (4H, q, J = 7.03 Hz) for ether, δ1.38 (3H, s) for acetonitrile, and δ0.95-1.09 (6H, m) for n-hexane. The bottom phase was analogously determined to contain 10.6% ether, 79.4% acetonitrile, and 10.0% n-hexane.

RESULTS AND DISCUSSION

The 2,3-dioxabicyclo[2.2.1]heptane nucleus **1** of PGH_2 is most stable in nonpolar solvents, i.e. with dielectric constants $\kappa \approx 2$, such as benzene or cyclohexane (2). Although the rate of decomposition in water ($\kappa \approx 80$) is about a thousand times greater, the rate of decomposition in acetonitrile ($\kappa \approx 28$) is only four times that in nonpolar solvents (2,3). This suggested the possibility of purifying biosynthetic PGH_2 by countercurrent partitioning between *aprotic* two-phase solvent systems such as acetonitrile and a hydrocarbon (7). As a model for the complex reaction product mixture from the biosynthesis of PGH_2, we determined partition coefficients $K = [solute]_{stationary}/[solute]_{mobile}$ for AA, PGH_2, and PGE_2 in acetonitrile-hexane (1:1). In the descending mode K was 0.225, 0.037, and 0.026 respectively. In the ascending mode K is the inverse of these values, i.e. 4.4, 27, and 38 respectively. These partition coefficients are satisfactory for separation of AA in either the ascending or descending mode but not satisfactory for separation of PGH_2 and PGE_2.

Ternary Acetonitrile-Hexane-Diethyl Ether Solvent Systems

Addition of diethyl ether to the acetonitrile-hexane solvent system was then examined since this would increase the solubilities of the relatively polar prostaglandins in the less polar upper phase. A phase diagram was constructed for the ternary acetonitrile-hexane-diethyl ether solvent system (figure 1). Mixtures containing up to 26% diethyl ether separate into two phases at 23°C. The diethyl ether additive is distributed approximately 60% into the less polar upper phase and 40% into the more polar lower phase. The temperature dependence of the

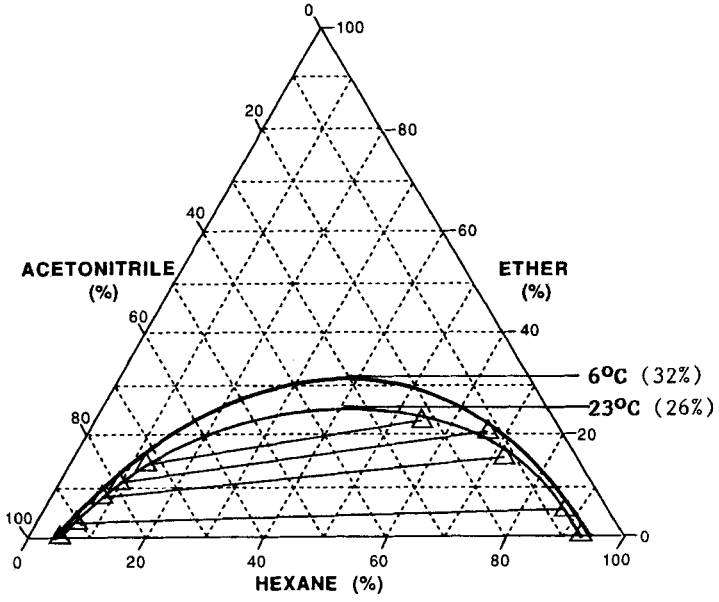

Figure 1. Phase diagram for acetonitrile-hexane-diethyl ether at 23°C (lower curve) and binodal curve at 6°C (upper curve).

plait point is evident from a comparison of binodal curves for this system at 23° and 6°C (figure 1). At 6°C two phases are obtained with diethyl ether comprising as much as 32% of the solvent mixture. Thus, phase volumes and compositions are expected to be especially sensitive to temperature for mixtures containing high ratios of diethyl ether versus acetonitrile-hexane. This should have a corresponding influence on the reproducibility of CPC separations using solvent mixtures near the plait point, solvent compositions which are best suited for separation of prostaglandins (*vide infra*).

Dependence of Partition Coefficient on Solvent Composition

Partition coefficients were determined for AA, PGH_2, and PGE_2 in two phase solvent mixtures prepared from acetonitrile-hexane (1:1) and various amounts of diethyl ether. A plot of these data (figure 2) shows that the partition coefficients of AA and the prostaglandins diverge as

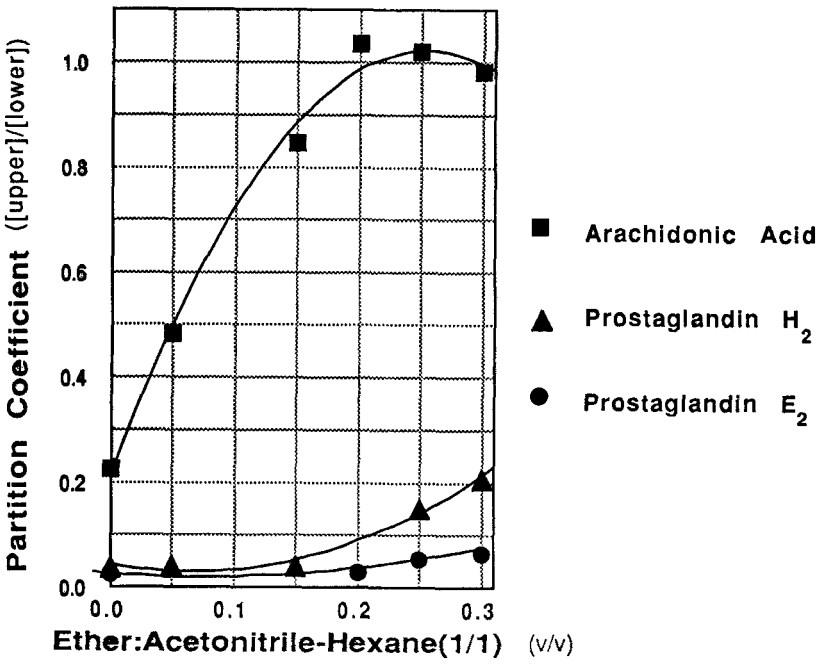

Figure 2. Partition of AA, PGH_2, and PGE_2 in 1:1 hexane-acetonitrile solvent systems containing various amounts of ether (v/v).

the ratio of diethyl ether to acetonitrile-hexane (1:1) is increased from 0.00 to 0.15. Most importantly, the partition coefficients of PGH_2 and PGE_2 diverge as the ratio of diethyl ether to acetonitrile-hexane (1:1) is increased from 0.15 to 0.30 where ether comprises 23% of the solvent mixture. Further improvement is limited by the solvent composition of the plait point, 26% ether at 23°C (see figure 1).

CPC Purification of PGH_2

PGE_2 and PGH_2 are not resolved by CPC with acetonitrile-hexane (1:1). Although nonpolar impurities are removed during an initial two hour extraction in the ascending mode, all polar components of the mixture including PGH_2 and PGE_2 exit the system immediately upon reversal of flow (figure 3 top). Isolation of pure PGH_2 can be achieved with acetonitrile-hexane (1:1)/ether 1:0.15 (figure 3 middle) or 1:0.25

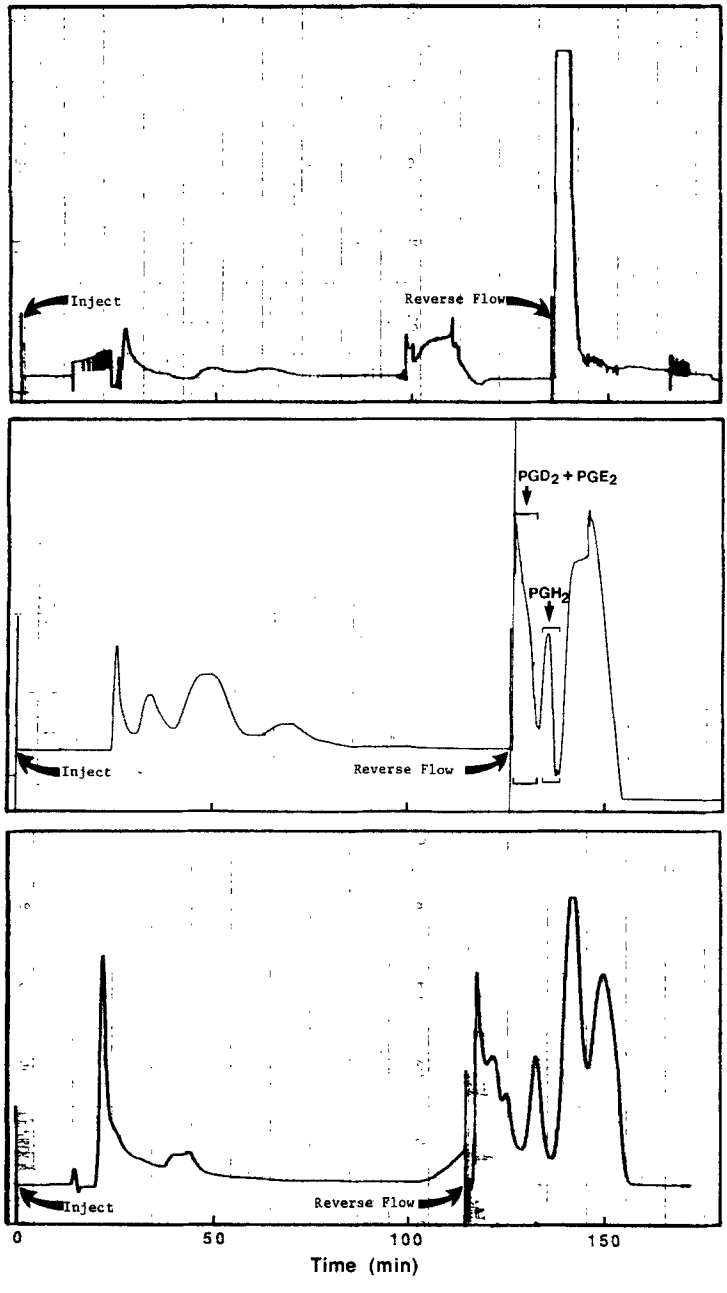

Figure 3. CPC purification of PGH_2 from AA bioconversion.

(figure 3 bottom). With the 1:0.15 solvent system, a mixture of polar byproducts including PGE_2 exit the system *immediately* upon reversal of flow. PGH_2 elutes after the more polar prostaglandins and is followed by a large peak containing less polar impurities. With the most polar 1:0.25 solvent system (figure 3 bottom), the peak containing prostaglandins E_2 and D_2 elutes *a few minutes after* reversal of flow from the ascending to the descending mode and shows some resolution as do the less polar impurities which elute after PGH_2. Thus, acetonitrile-hexane (1:1)-ether mixtures close to the limiting diethyl ether content of the plait point provide optimum resolution of the reaction mixture from bioconversion of arachidonic acid.

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