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PURIFICATION OF TRITIUM-LABELED PROSTAGLANDINS BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Tritium-labeled prostaglandins can be purified rapidly and inexpensively by silica gel high performance thin-layer chromatography. Liquid scintillation counting of tritium-labeled samples from preparative high performance thin-layer plates indicates that the method is a practical alternative to high performance liquid chromatography for purification of prostaglandins. Recoveries of prostaglandins were greater than 98%.

INTRODUCTION

Prostaglandins, the major cyclooxygenase products of arachidonic acid, are potent mediators of cellular responses to injury by chemicals. In an effort to develop a probe for cellular toxicity, we have begun using endothelial cells, cultured *in vitro* to serve as a model to assess toxic effects of test substances. Prostaglandins are present in picomolar concentrations in tissue culture.

We wished to study the extraction efficiency and recovery of prostaglandins over a range of concentrations in cell culture media fortified with unlabeled and tritium-labeled prostaglandins, and during our initial work we had need of non-destructive analytical techniques. Such a technique is high performance liquid chromatography (HPLC), but we did not wish to dedicate an expensive liquid chromatograph and capillary columns only to future analyses of radioactive materials, and too, we wanted to avoid the difficulties associated with disposing of large volumes of radioactive column eluate. In addition, HPLC with ultraviolet absorbance detection is unable to detect prostaglandins at low levels (1 nmole/mL). The analyses were complicated further by the instability of tritium-labeled prostaglandins to heat; and therefore, separations had to be conducted at room temperature.

In the past five years there has been a resurgence of interest in thin-layer chromatography (TLC) as a powerful tool for separation and detection of a variety of compounds, including prostaglandins. Improvements in instruments, materials, and techniques of TLC have led to wide acceptance of TLC, and particularly high performance TLC (HPTLC), as a fast, accurate, inexpensive, and precise method that is a legitimate alternative to HPLC for many analyses. We report here the application of TLC and HPTLC to the purification of some tritium-labeled prostaglandins.

MATERIALS AND METHODS

New England Nuclear Products, Boston, MA, supplied solutions of the tritiated prostaglandins, prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-K-$PGF_{1\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The corre-

sponding unlabeled prostaglandins were purchased from Upjohn Diagnostics, Kalamazoo, MI. The scintillation cocktail, Opti-Fluor®, was supplied by United Technologies, Packard Chemical Co., Downers Grove, IL. Solvents were of spectrophotometric quality. Other chemicals were reagent grade.

Phosphomolybdic acid reagent was prepared by heating 25.0 g of phosphomolybdic acid in 250 mL of 95% ethanol until dissolution of the acid was complete.

To prevent adsorption of the prostaglandins on glass surfaces, volumetric flasks used to contain the prostaglandin solutions were treated for 30 minutes with 5% dimethyldichlorosilane in toluene, followed by rinses with dry toluene and absolute methanol.

Solutions of unlabeled PGE_2 and $PGF_{2\alpha}$ (1 mg/mL) were prepared in 70% ethanol-water. A solution of 6-K-$PGF_{1\alpha}$ (1 mg/mL) was prepared in acetone. Solutions of tritium-labeled prostaglandins were prepared by diluting the stock solutions obtained from the manufacturer. Both PGE_2 and $PGF_{2\alpha}$ were supplied as solutions in 70% ethanol-water. A dilution of 0.50 mL of PGE_2 and $PGF_{2\alpha}$ (9×10^{-5} mg prostaglandin) to 10 mL yielded an activity of 11,000 DPM/μL. Tritium-labeled 6-K-$PGF_{1\alpha}$ was supplied in 90% acetonitrile-water. An aliquot (0.25 mL, 5.8×10^{-5} mg) of 6-K-$PGF_{1\alpha}$ was diluted to 5.00 mL giving a solution with an activity of 11,000 DPM/μL. All prostaglandin solutions were stored at –20°C.

The purities of unlabeled and tritium-labeled prostaglandins were determined by thin-layer chromatography using visual detection for unlabeled prostaglandins and liquid scintillation counting for tritium-labeled prostaglandins. Silicic acid-impregnated, glass microfiber TLC plates (Toxi-Grams®A) with special sample application disks were a gift of Analytical Systems, Laguna Hills, CA. Channeled, straight-phase silica gel, high performance (HPTLC) plates (type LHP-KD) were purchased from Whatman Chemical Separations, Inc., Clifton, NJ.

Three solvent systems were used for separation: System I, ethyl acetate:acetone:acetic acid, 90:5:1; System II, chloroform:isopropyl alcohol:ethanol:formic acid, 45:5:0.5:0.3; and System III, toluene:dioxane:acetic acid, 20:10:1.

A small (3.5 mm) sample application disk was placed into the well of a spot plate; a solution of an unlabeled prostaglandin (5 μL, 1 mg/mL) was concentrated onto the disk by evaporation. The glass microfiber TLC plates were inoculated by inserting the dried sample application disk into one of the six holes located near the lower edge of the TLC plates. The plates were developed to 100 mm in System I, II, or III. Detection was accomplished by dipping the plates into 10% phosphomolybdic acid, followed by heating. In a separate experiment, a glass microfiber TLC plate was inoculated as follows: with sample disks prepared as described above, with sample disks spotted with the solvent mixture used to dissolve the prostaglandins, and with a sample disk to which nothing was added. This TLC plate was developed in System I and then treated with the phosphomolybdic acid reagent, as previously described.

A solution of each unlabeled prostaglandin (5 μL, 1 mg/mL) was applied to the preadsorbent area of channeled HPTLC plates. The plates were developed in System I, II, or III, until the solvent front had reached 60 mm. Detection was accomplished by spraying the plates with 10% phosphomolybdic acid, followed by heating.

An aliquot (5 μL, 55,000 DPM) from each solution of tritium-labeled prostaglandin was applied to the preadsorbent area of a channeled HPTLC plate. An adjacent channel was spotted with 5 μL (0.02 mg) of the corresponding unlabeled prostaglandin. After migration in System I, only the channel containing unlabeled prostaglandin was sprayed with phosphomolybdic acid solution and heated. The channel containing tritiated prostaglandin was removed from the plate in 2-mm cuts using a razor blade. The silica gel samples were added to scintillation vials and vortexed with 1 mL of 70% ethanol. Ten mL of Opti-Fluor® was subsequently added to each sample and the solutions were counted using either a Tri-Carb® Model 460 or Model 4530 Liquid Scintillation Counter, Packard Instrument Co., Downers Grove, IL. Counts were corrected for quench.

To determine what effect the presence of a cold-carrier might have on R_f, a series of studies was conducted in which both a tritium-labeled (~50 pg) and a corresponding unlabeled prostaglandin (~5 μg) were applied to the preadsorbent area in the same channel of HPTLC plates. After migration in System I, the channels were removed from the plates in 2-mm cuts, and the silica gel samples were analyzed as before.

Tritium-labeled PGE_2 was purified using preparative thin-layer chromatography. Nine mL of a solution containing approximately 8×10^{-5} mg PGE_2 (~45 μCi) were concentrated to about 3 μL. Concentration was accomplished by adding portions of the solution,1.5 mL at a time, to a silanized, conical 3-mL vial. The solvent was removed at room temperature using a Speed Vac® Concentrator, Savant Instruments, Inc., Farmingdale, NY. The 3-μL sample was applied to an HPTLC plate and air dried. The vial was washed with 3-μL portions of ethanol and the washings applied to the plate. After development in System I, an 8 mm region of silica gel known to encompass the tritium-labeled PGE_2 was scraped from the plate. The R_f of tritium-labeled PGE_2 was established by co-migration of unlabeled material in

an adjacent channel, followed by visualization using phosphomolybdic acid. The labeled PGE_2 was eluted from the silica gel sample with ethanol and the solution filtered through a Pasteur pipette containing a small plug of glass wool. The filtrate and washings were diluted to 5.00 mL with 70% ethanol-water. The PGE_2 concentration was calculated from liquid scintillation data using the value for specific activity provided by the supplier.

Results and Discussion

The R_f values of 6-K-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ in Systems I, II, and III, on glass microfiber TLC plates and HPTLC plates are in Table I. Though many migrations solvents were tried, only these three resulted in good separations.

Table I. R_f Values of Prostaglandins

	R_f					
	TLC (Toxi-Gram)			HPTLC (LHP-KD)		
Prostaglandin	I[a]	II[b]	III[c]	I[a]	II[b]	III[c]
PGE_2	0.55	0.58	0.65	0.25	0.26	0.33
$PGF_{2\alpha}$	0.40	0.38	0.46	0.13	0.12	0.20
6-K-$PGF_{1\alpha}$	0.48	0.42	0.40	0.10	0.08	0.17

[a] System I: ethyl acetate:acetone:acetic acid (90:5:1)
[b] System II: chloroform:isopropyl alcohol:ethanol:formic acid (45 : 5 : 0.5 : 0.3)
[c] System III: toluene:dioxane:acetic acid (20:10:1)

Linear HPTLC plates provided reproducible retention factors in chromatography of prostaglandin samples. Solvent System I resulted in the lowest band diffusion (about 2.5 mm). The detection limit of the three prostaglandins with phosphomolybdic acid reagent was 1 µg of prostaglandin on the HPTLC plates.

We had hoped to use the glass microfiber TLC plates for purification and recovery of both unlabeled and tritium-labeled prostaglandins because these plates are easy to cut into sections or strips with scissors. They can also be dipped directly into the phosphomolybdic acid detection reagent; this is more convenient than spraying the reagent onto the plate. There were several impurities on the sample application disks, however, which migrated near the prostaglandins and stained with the phosphomolybdic acid reagent. These spots interfered with detection of the prostaglandins. The plates also have a greater variation in silica gel particle size and layer thickness than have the HPTLC plates, and the distribution of the prostaglandins after development was therefore more diffuse (about 7-mm diameter spots).

Commercially prepared tritium-labeled $PGF_{2\alpha}$ and 6-K-$PGF_{1\alpha}$ contained less than 5% radio-chemical impurity (Figures 1 and 2). Since 6-K-$PGF_{1\alpha}$ consists of a mixture of open and different hemiketal forms, sometimes streaking occurs during TLC (Figure 2). We did not use ethanol to dissolve 6-K-$PGF_{1\alpha}$ because this compound reacts easily in alcohols forming the ketal. Only the tritiated PGE_2 sample required purification. The histogram in Figure 3 shows a labeled impurity at R_f 0.5. After purification by preparative HPTLC (Figure 4), the concentration of tritium-labeled PGE_2 was calculated using the specific activity (200.0 Ci/mmol): 5-µL samples gave 63,600 DPM; conc. = 10 pg/µL. As the histograms show, slightly larger R_f values are obtained when the amount of material applied to the plates is increased. This may be attributed to adsorption effects of the silica gel, the importance of which increase as the sample size decreases. Recovery of total counts from the plates was always greater than 98%, as determined by liquid scintillation counting.

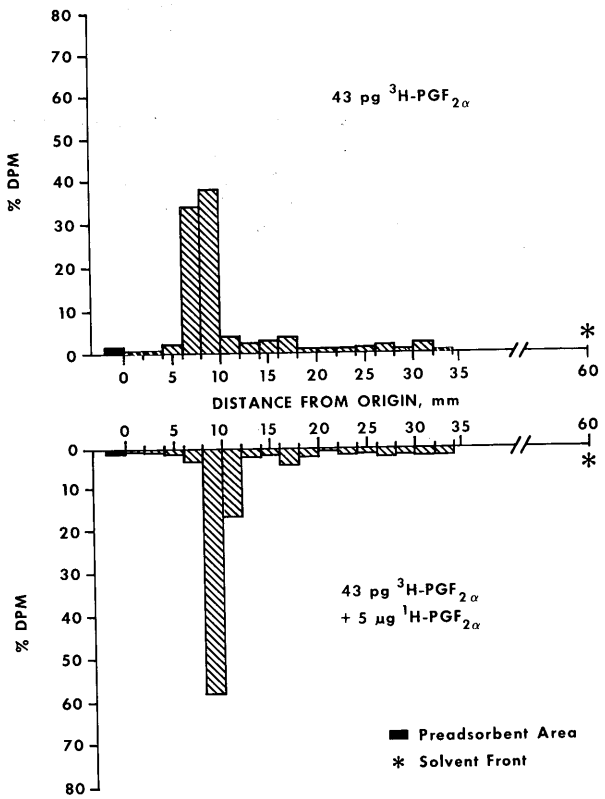


Figure 1. HPTLC separation of ^{3}H-$PGF_{2\alpha}$ from radiochemical impurities (upper histogram). The same separation performed again with the inclusion of 5 µg of unlabeled $PGF_{2\alpha}$ (lower histogram) shows the effect of cold carrier. Both migrations were in System I on LHP-KD plates.

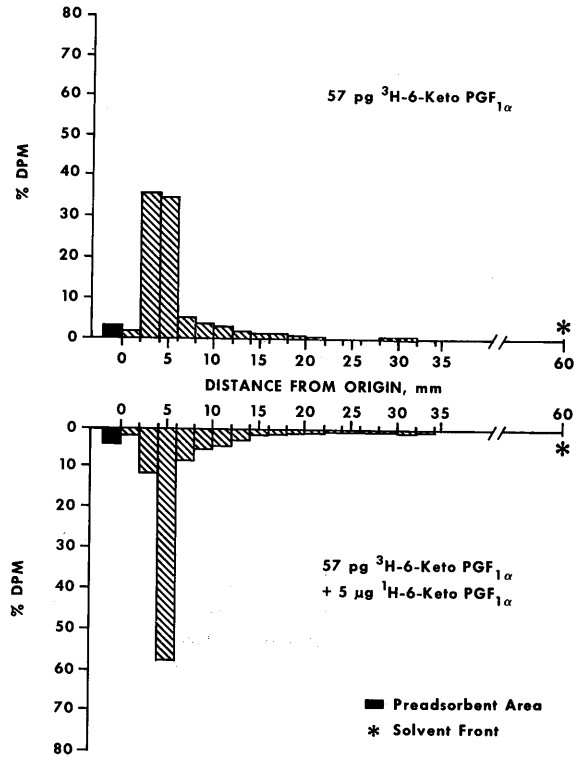


Figure 2. HPTLC separation of ^{3}H-6-K-PGF$_{1\alpha}$ from radiochemical impurities (upper histogram). Streaking above R_f 0.08 results from hemiketal forms. The lower histogram shows the migration pattern in the presence of unlabeled 6-K-PGF$_{1\alpha}$. Migrations were in System I on LHP-KD plates.

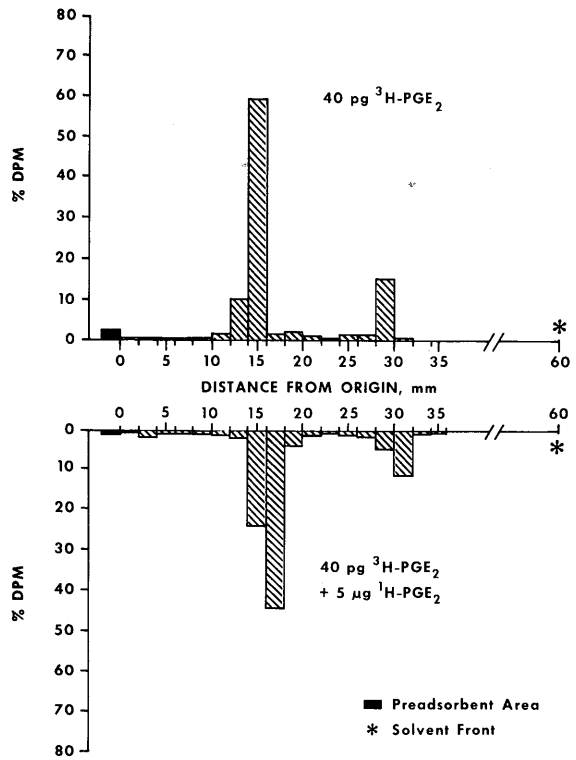


Figure 3. HPTLC separation of ^{3}H-PGE$_2$ from a radiolabeled impurity at R_f 0.5 (upper histogram). The lower histogram shows migration in the presence of additional cold material. Migrations were in System I on LHP-KD plates.

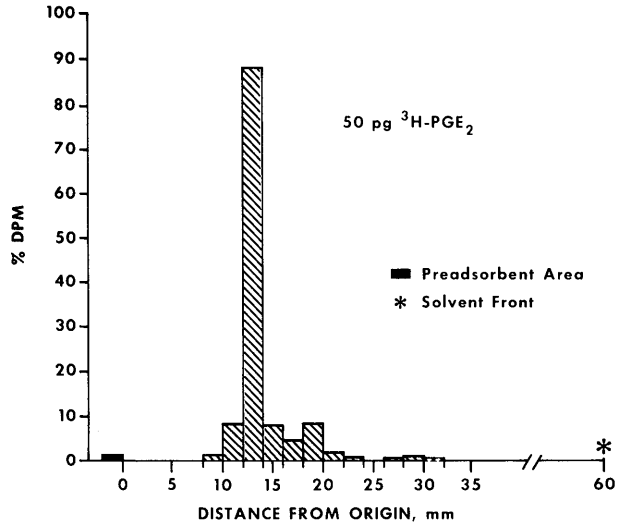


Figure 4. A histogram presentation of the chromatogram obtained from analysis of purified ^{3}H-PGE$_2$. The absence of radiolabeled impurity at R_f 0.5 (Figure 3) shows the success of purification by preparative HPTLC. Migration was in System I on LHP-KD plates.

with the pump valves, which necessitated the removal of the tubing, the well was deepened a second time (Safford, 1869; Ashley, 1912).

The Douglass well, drilled only 75 feet from the Newman well, produced about 80 barrels per day from a maximum depth of 22 feet. This well ceased production at the same time the Newman well first failed and apparently was never operated again (Safford, 1869).

A third well of note in the Spring Creek field was the Hoosier well, which was located about 250 feet from the Douglass well. This well, drilled in 1867, encountered oil at a depth of 35 feet and produced about 5000 barrels before failing in the fall of 1868. It was deepened to about 70 feet and again produced oil for some time.

Another significant early discovery of oil in Tennessee was the Hudson well on Jones Creek, in Dickson County, drilled in 1867. This well initially produced oil from a depth of 132 feet, but was later deepened to at least 340 feet (Safford, 1869).

In 1891, drilling in Scott County yielded oil in several wells from depths greater than 1000 feet. Wells were drilled just west of Glenmary, roughly centered in the present-day "oil patch" of Tennessee. The Scott County wells were some of the earliest wells to produce hydrocarbons from relatively deep holes (Ashley, 1910).

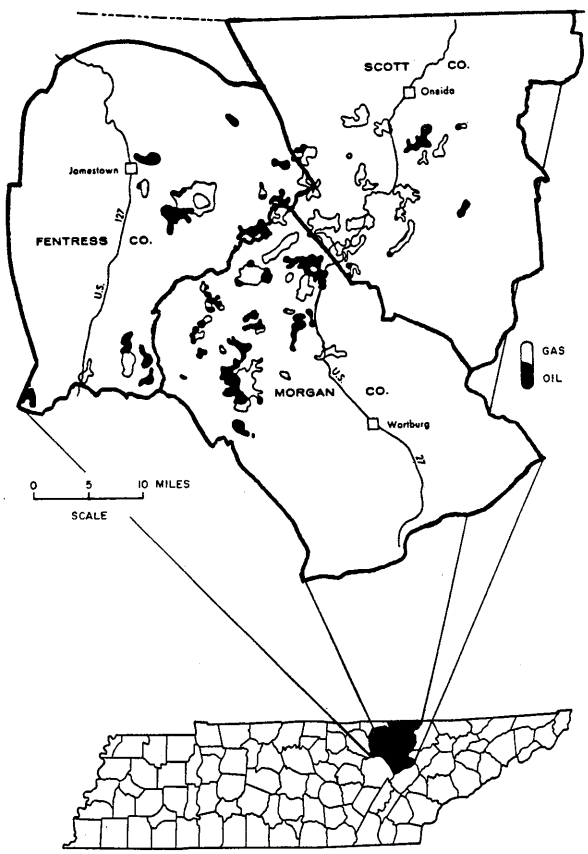
In January, 1896, a well at Bob's Bar near Riverton in Fentress County came in at 50 barrels per hour from a depth of 276 feet. After only 14 hours of operation, the oil caught fire and burned the rig. Ten months later the well was put back into production and by 1900, three years later, made over 20,000 barrels. Many wells were drilled in the Riverton area but few with any success at all (Ashley, 1910). A gas seepage has been observed near the Bob's Bar location, suggesting the possibility of a natural depressurization of the producing zones in the subsurface.

Another interesting occurrence of hydrocarbons in Tennessee is the area of gas seeps on an island several miles north of Memphis. These gas seepages have been known since the 1880s, and possibly earlier. In 1909, an organized attempt was made to test the potential of the oil and gas on Old Hen Island, where the majority of the gas seeps were located. Four wells were drilled eventually, yielding only noncommercial amounts of oil and gas. A number of additional wells were later drilled in the Memphis area as water wells, but no trace of oil or gas was ever reported from any of them (Munn, 1912).

During the early years of the petroleum industry in Tennessee, gas production was minor. There were occurrences of natural gas in many wells, but rarely did it exist in usable quantities. In a few instances a landowner could light and heat his home with gas from a well on his property.

Early oil and gas explorationists in Tennessee were intrigued with the search for hydrocarbons and apparently were not deterred by the lack of local geologic information. After early drilling results were studied, an association between the occurrences of oil and gas and the Chattanooga Shale was made (Ashley, 1910). Almost all of the oil and gas discoveries had been from zones that were stratigraphically close to the black shale. Using the available information, it seemed a safe assumption that the Chattanooga Shale was a possible source of oil or gas under the Highland Rim and the Cumberland Plateau.

OIL AND GAS FIELDS OF TENNESSEE

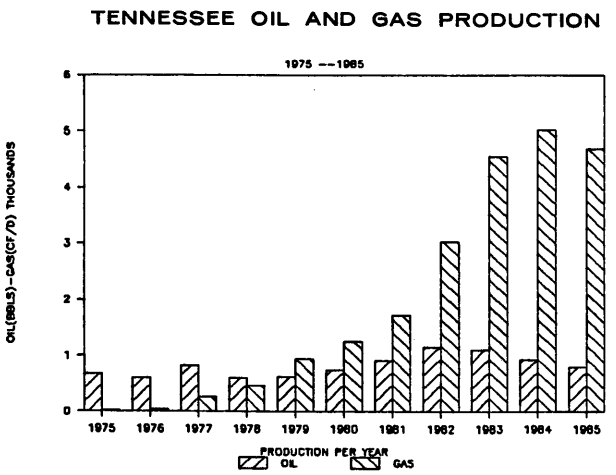


Eventually, as drilling information and methods improved, the search for oil and gas spread throughout most of Tennessee. Although there were numerous wells drilled between 1900 and 1960, a large percentage were dry. State regulation of the oil and gas industry in Tennessee began in 1968, followed shortly by the establishment of a well data system. This system contains drilling and completion information as well as production data, all of which are furnished by operators and petroleum purchasers. In 1985 there were over

10,000 wells on file at the Tennessee Division of Geology in Nashville. Because of insufficient data from operators, over a thousand wells still remained unclassified by industry standards.

In 1969, Tennessee's first million-barrel field was discovered in Scott County. Oneida West field, an early Mississippian Ft. Payne carbonate play, spurred a significant increase in the state's drilling activity and production. Numerous additional Ft. Payne discoveries were made on the northern Cumberland Plateau; many are stratigraphic traps and not related to structure. The better Ft. Payne fields are gas cap and gas-solution drive reservoirs (Statler, 1975).

Another Ft. Payne discovery of note was the Indian Creek field, Morgan County, in late 1973. The operators of Indian Creek initiated a gas re-injection and stripper program to further enhance the production capabilities of the reservoir (Statler, 1975). At the end of 1985, Indian Creek field had produced over 1.7 million barrels of oil. During the search for the Ft. Payne pays, numerous gas zones were encountered in Upper Mississippian Monteagle and Bangor Limestones. Marketed gas, in the 1970s, remained insignificant because of insufficient pipeline facilities despite the abundance of shut-in gas wells (Lindau, 1980).



Moderate drilling continued through the 1970s until 1979, when activity increased significantly. In May, 1979, a Morgan County well came in at an estimated 5000 BOPD (barrels of oil per day) with a substantial but unmeasurable amount of associated gas. This well, on the Luchin lease within Douglas Branch field, apparently was from fracture porosity in the Upper Ordovician Nashville Group. After producing an undetermined amount and selling over 5000 barrels of oil in five months, the Luchin well became idle amid rumors of paraffin causing its demise.

Just two months later, the John Billings well in Overton County initially tested at 260 BOPD out of the Middle Ordovician Lebanon Limestone. At the end of 1985 this well had yielded over 120,000 barrels of oil.

September 1980 saw one of the most prolific gas discoveries in Tennessee's history. Dixie-Shamrock's Brimstone No. 1 in Scott County encountered fractured zones in the Bangor and Monteagle Limestones, both Upper Mississippian in age. This well tested at nearly 3.25 million cubic feet of gas per day. Drilling continued to be active until 1983, when the international oil glut and fluctuations in world crude prices brought domestic drilling numbers to record lows.

During the 1979–1983 upsurge in drilling activity, large leases were made by major oil companies interested in deep drilling in the eastern overthrust area of the southern Appalachians. Very little drilling was actually accomplished, resulting in only two gas discoveries of moderate significance at depths of greater than 4000 feet.

The potential for hydrocarbon discoveries in Tennessee still exists. There are numerous areas and zones on the Cumberland Plateau that are yet untested. A more extensive usage of secondary recovery technology and more conservation in the maintenance and production of oil and gas might insure the continued existence of the oil and gas industry in Tennessee.

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