JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 6 NUMBER 7 1983

Special Section on
THIN LAYER CHROMATOGRAPHY

Edited by HALEEM J. ISSAQ
NCI-Frederick Cancer Research Facility
Frederick, Maryland

and

JACK CAZES
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CODEN: JLCHD8 6(7), i-x, 1171-1352 (1983)
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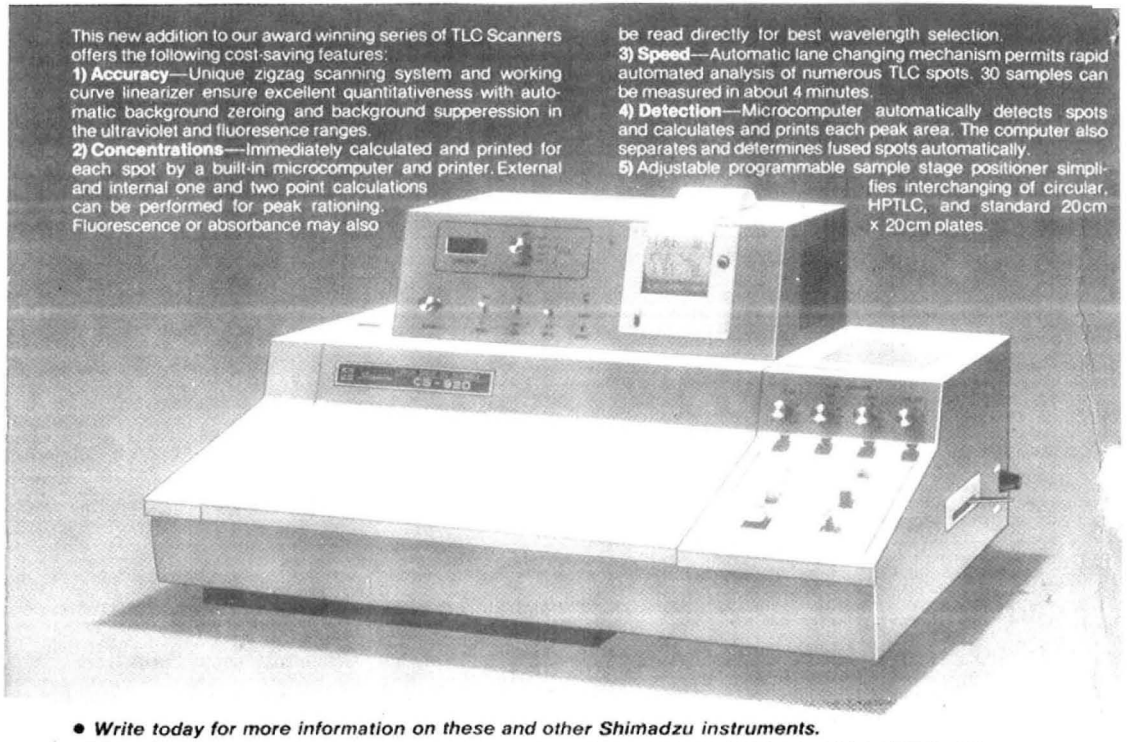

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JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

June 1983

Aims and Scope. The journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all modes of liquid chromatography.

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THIN-LAYER CHROMATOGRAPHY

Edited by

HALEEM J. ISSAQ
NCI-Frederick Cancer Research Facility
Frederick, Maryland

and

JACK CAZES
Fairfield, Connecticut

This is a special section of *Journal of Liquid Chromatography*, Volume 6, Number 7, 1983.

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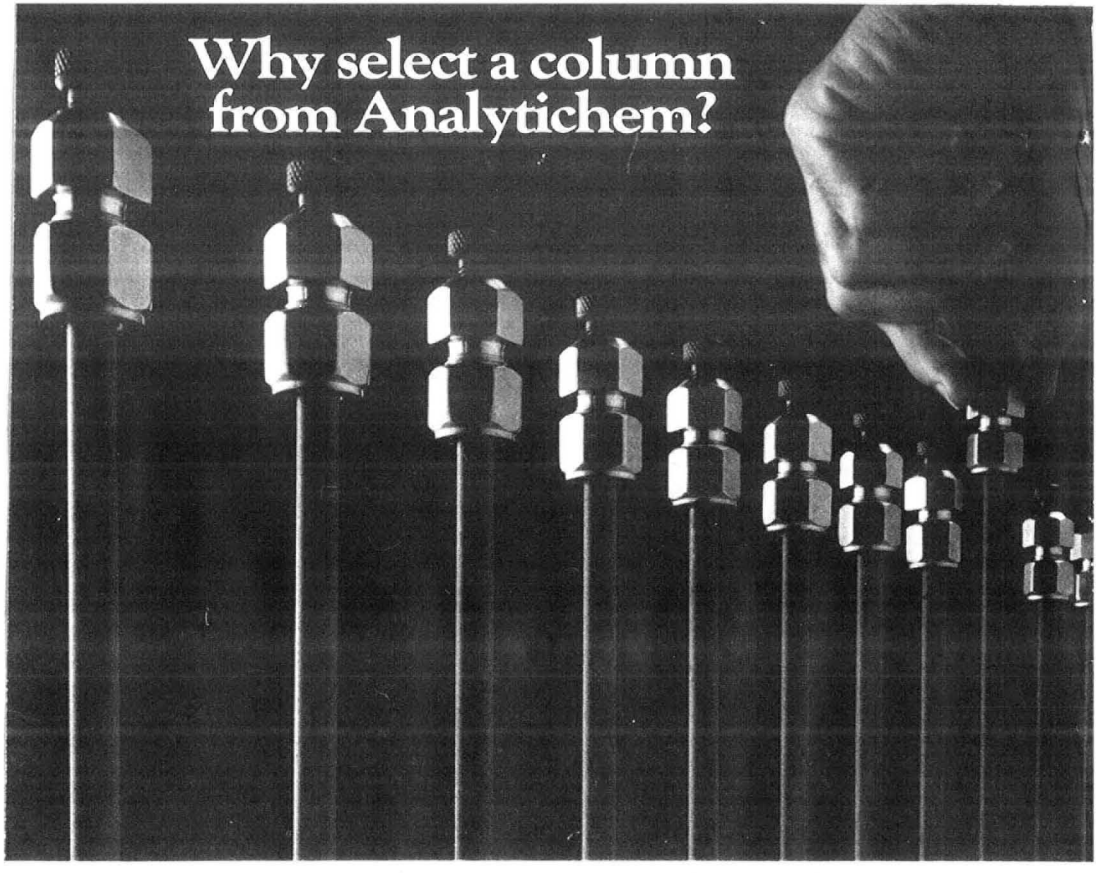

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Special Section on Thin-Layer Chromatography

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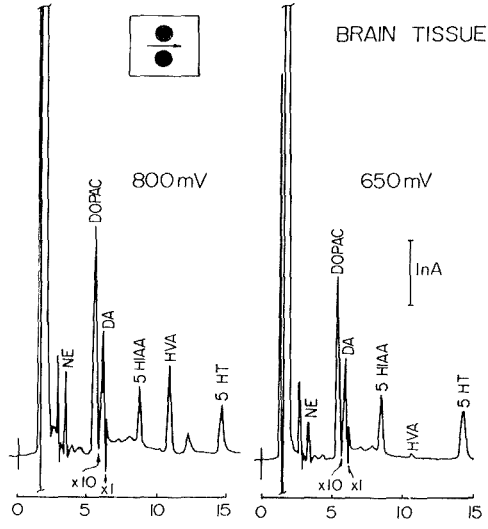

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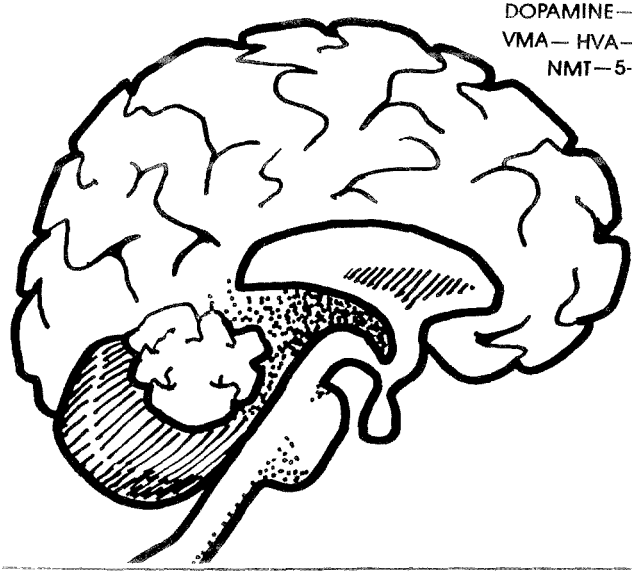

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1171-1174 (1983)

THE SEPARATION OF 2-, 3- AND 4-CYCLOPENTYLPHENOL BY THIN-LAYER CHROMATOGRAPHY

T.M. Atkins, D.G. Towles, and R.H. Bishara
Lilly Research Laboratories
Division of Eli Lilly and Company
Indianapolis, Indiana 46285

ABSTRACT

A TLC system is described for the resolution of the three positional isomers of cyclopentylphenol. Quantitation of the 3- and 4- isomers in 2-cyclopentylphenol at a sensitivity of 0.1% may be achieved.

INTRODUCTION

The widespread use of substituted cyclopentylphenols is well documented in the literature. Substituted cyclopentylphenols have been used as antioxidants for organic materials, e.g., polymers, rubbers, lubricants (1), as tough, hard, weather resistant coatings, e.g., on wood (2), as bactericides and fungicides (3,4), in the synthesis of a β-sympatholytic drug (5) and as diuretics which do not increase K^+ excretion as much as that of Na^+ (6). In addition, their phosphorus acid esters showed anti-corrosive and antioxidative properties as lubricating oil additives when tested at the 1% concentration (7). In the synthesis of the cyclopentylphenols, small

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quantities of isomeric impurities are also produced. In this laboratory, the isomeric purity of 2-cyclopentylphenol must be controlled to prevent the formation of undesired by-products. The chromatographic behavior of 4-cyclopentylphenol on paper (8), alumina-impregnated papers and thin layers of alumina (9) and by reverse-phase partition chromatography on home made thin layers of cellulose/ethyl oleate (10) has been investigated. No reference was found in the literature describing resolution of the three isomers of cyclopentylphenol. Using commercially available precoated silica gel plates, a variety of solvent systems was screened according to a previously described procedure (11). This paper describes a two-component TLC system that separates the 2-, 3- and 4-cyclopentylphenols.

EXPERIMENTAL

Silica gel 60, F254 TLC plates (EM Laboratories, Inc., Elmsford, N.Y.), 20 cm x 20 cm with 0.25 mm thick adsorbent layer and a conventional TLC chamber, 30 cm x 25 cm x 8 cm, of heavywall glass were used for all of the development work.

One-tenth, one-fifth and one percent solutions of the 3- and 4-isomers spiked in the 2-cyclopentylphenol standard at a concentration of 10 mg/ml were prepared. The 2-cyclopentylphenol sample to be tested was prepared at a concentration of 10 mg/ml. All solutions were prepared in methanol. Ten microliter aliquots were applied at 2.5 cm from the bottom of the plate. The developing solvent was a mixture of chloroform and ethyl acetate (95:5), v/v. The solvent front was allowed to travel 15 cm above the point of application. The developing time was approximately 90 minutes. The plate was allowed to dry in a ventilated hood and then placed in an iodine chamber (a conventional TLC chamber containing several grams of metallic iodine) for about 30 minutes. The spots were photographed under short-wavelength (254 nm) ultraviolet light.

RESULTS AND DISCUSSION

Viewing the developed, dried plate under short-wavelength ultraviolet light after exposure to iodine reveals the cyclopentylphenols as purple spots on a yellowish-green fluorescent background.

The R_f values were 0.65, 0.51, and 0.44 for 2-, 3-, and 4-cyclopentylphenol respectively. This is in agreement with the conventionally documented mobility for the 2-alkylphenol derivatives which are less strongly adsorbed due to the "ortho effect", than the 3- and 4-isomers (12) and hence move faster.

To quantitate any 3- or 4-isomers in the 2-cyclopentylphenol sample, the intensity of the extra spots with similar mobilities was compared to the lanes containing the spiked 0.1, 0.2 and 1% standards.

In summary, this simple method provides a relatively quick way to identify the isomers and to semi-quantitate the isomeric purity of 2-cyclopentylphenol.

ACKNOWLEGDEMENT

The authors wish to thank Mrs. K. R. Whitten (Lilly Research Laboratories) for supplying authentic samples of the isomeric cyclopentylphenols used in this work.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1175-1182 (1983)

DETERMINATION OF PULEGONE IN H. pulegioides AND PEPPERMINT OIL BY THIN LAYER CHROMATOGRAPHY WITH DENSITOMETRY

Barry P. Sleckman*, Joseph Sherma**, and
Lorraine C. Mineo*
Departments of Biology* and Chemistry**
Lafayette College
Easton, PA 18042

ABSTRACT

A method was developed for determination of the phytotoxic terpene pulegone using silica gel thin layer chromatography with detection by acetic acid-sulfuric acid-anisaldehyde reagent and densitometric scanning. The method was used to monitor levels of pulegone in the plant Hedeoma pulegioides and to assay the compound in a commercial peppermint oil. The storage of pulegone was found to be localized in the foliage and stem of the plant and to be highest in the spring.

INTRODUCTION

Pulegone is a phytotoxic terpene produced by a variety of plants, including the annual Hedeoma pulegioides (American Pennyroyal) (1,2). The present study involved the quantification of pulegone in the foliage, stem, roots, and flower of H. pulegioides at 30 day intervals during the 1982 growing season. The soil around the plants was also tested for the presence of pulegone at these times. The quantification of pulegone was carried out over a complete growing season in order to assess varying concentration levels for each plant organ during the growth cycle. This information was applied to apparent allelopathy in the field.

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In addition to its interest as a phytotoxic compound, the pulegone content of commercial products is of importance because of the toxicological effects on humans (3). Pulegone is found in many plant oils, such as peppermint oil, which are used as flavoring agents in the pharmaceutical, food, and confectionery industries (4). Because of its biological and commercial interest, methods have been devised for the determination of pulegone in the oils of various plants, most often based on gas-liquid chromatography. Separation and quantification of pulegone by GLC is made difficult by the presence of interfering compounds that overlap the pulegone peak. Therefore, elaborate extraction and derivatization methods and multiple GLC detectors are required for the analyses (4,5).

This paper describes a simple procedure employing thin layer chromatography with densitometry for the direct determination of pulegone in plants and plant oils. Because of the selectivity of the detection reagent, no extensive sample preparation was required. The method is shown to be accurate and sensitive to low ng amounts of pulegone.

EXPERIMENTAL

Plant and Soil Analysis

Standard solutions of pulegone were prepared in absolute ethanol at concentrations of 50.0-500 ng/µl by dilution of a 1.00 mg/ml ethanolic stock solution. Whole *H. pulegioides* plants and soil from around the plants were collected under similar environmental conditions at 30 day intervals from May 26, 1982, until September 28, 1982, from an area in northeast Pennsylvania. Composite samples were collected from both partial shade and fully sunny locations. The collected materials were dried at ambient temperature for one week prior to extraction. Three samples each of approximately 0.12 g of dried foliage and stem and 1.0 g of dried soil, roots, and flower were accurately weighed into screw-topped vials and extracted by soaking for 24 hours with

5.00 ml of absolute ethanol. At the end of this period the vials were shaken, the sample was allowed to settle, and the clear extract was applied for TLC.

Extracts and standards were applied at 1.0 µl levels with a Drummond Dialamatic microdispenser to origins located 2 cm from the bottom of Baker-Flex IB2 silica gel sheets (20 x 20 cm). The layers were developed for a distance of 10 cm with hexane-ethyl acetate (5:2 v/v) in a paper-lined, rectangular glass N-tank that was pre-equilibrated with the mobile phase for at least 10 minutes. Chromatograms were air dried and pulegone was detected by lightly spraying the layer with acetic acid-sulfuric acid-anisaldehyde (100:2:1 v/v) prior to heating in an oven at 100°C for 5 minutes.

Pulegone zones were scanned with a Kontes Chromaflex fiber optics densitometer equipped with a baseline corrector and strip chart recorder, using the single beam (5 mm head)-transmission mode and the visible wavelengths from the longwave UV source. Peak areas were calculated using the equation: area = height x width at half height, and calibration curves were plotted as peak area vs. ng of pulegone applied. The amount of pulegone in the extract spots was then determined by interpolation. If the peak area from the extract was not in the linear range (50-500 ng) of the pulegone calibration curve, the solution was quantitatively diluted prior to spotting. Mean pulegone concentrations were calculated from the triplicate analyses of each sample. A portion of foliage, stem, root, soil and flower from each collection was dried at 100°C for 24 hours in order to obtain a baseline dry weight to which the level of pulegone was related.

The identity of the chromatographic zone believed to be pulegone was confirmed using a Finnegan 4000 gas chromatograph/mass spectrometer with INCOS data system and 70eV electron impact source. The 1.8 m x 2mm 3% OV-101 column was programmed from 100 to 300°C at 15°C/minute. Sufficient pulegone for the MS analysis was obtained by preparative TLC of a scaled-up extract from a larger plant sample. The mass spectrum was compared to the library spectrum of pulegone and a spectrum of an injected pulegone standard.

Peppermint Oil Analysis

Exactly 2 ml of a commercial peppermint oil was diluted with ethanol to 100 ml in a volumetric flask. One µl of sample was spotted along with bracketing standards, and the amount of pulegone determined as described above. Standard addition was used to evaluate the accuracy of the assay. Identical aliquots of oil were measured and diluted, but 20 mg of pulegone standard was added to one of the samples before analysis. The difference between analyses was compared to the spike level to calculate recovery.

RESULTS AND DISCUSSION

Collections of plants and soil were made in 1982 on May 26 (day 0), June 25, August 7 and 21, and September 28. Flowers did not appear on the plants until late summer, and no pulegone was found in the flower extract from the single collection. Roots and soil from around the plants were analyzed from each collection; pulegone was never detected in these samples, even when 10 µl of extract was spotted. Based on the amount of sample extracted, the extract volume spotted, and the sensitivity of the detection reagent (50 ng/spot), the sensitivity limit of the assay was approximately 60 µg/g. Therefore, amounts of pulegone in flowers, roots, and soil below this value would not have been detected. If necessary, the sensitivity limit of the analysis could be improved by extraction of a larger sample, concentration of the extract to a small volume prior to spotting, and/or spotting a larger volume on the layer.

Figures 1 and 2 show the decreasing pulegone concentrations found in *H. pulegioides* stem and foliage over the period studied. Pulegone was found in greatest concentration in the foliage, which suggests that it is stored and possibly synthesized there, and may be released into the environment from this location. The concentration of pulegone in the foliage was greatest early in the season and decreased steadily until August, at which time the concentration remained at a low and relatively steady level throughout the rest

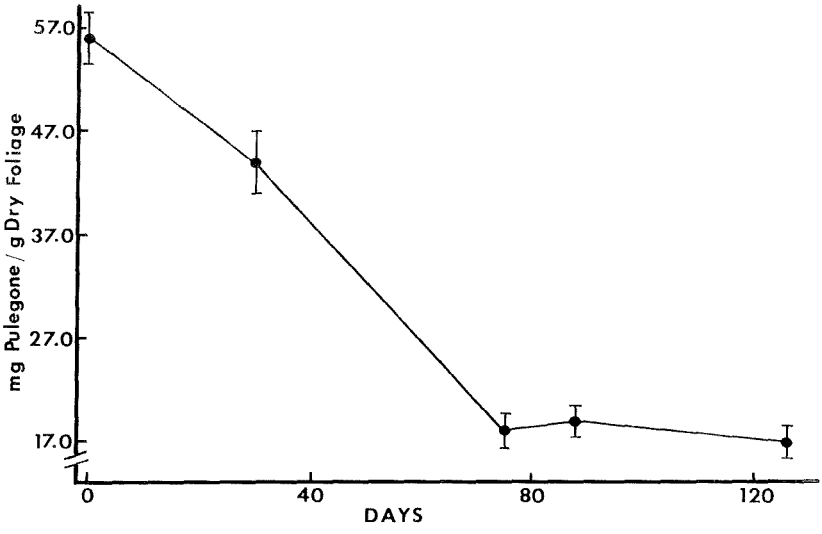

FIGURE 1. Graph of mean pulegone concentration in H. pulegioides foliage as a function of date of collection. The bars indicate the standard deviation of triplicate analyses.

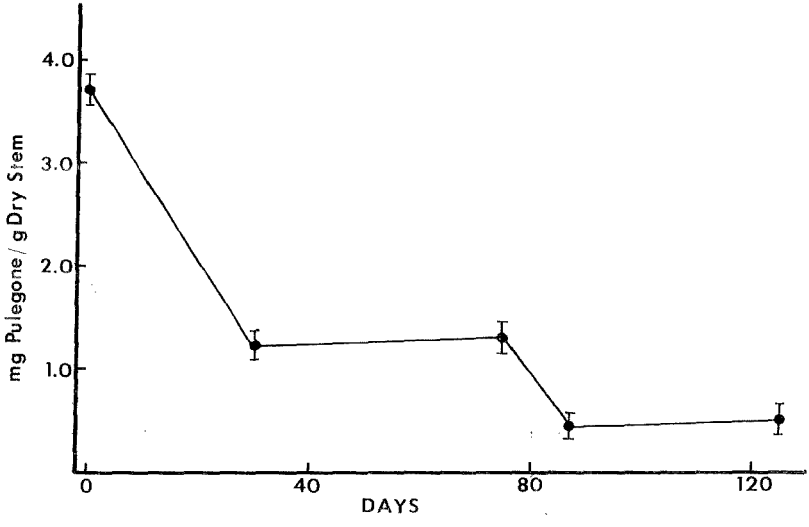

FIGURE 2. Graph of mean pulegone concentration in H. pulegioides stem as a function of date of collection.

of the season. The concentration of pulegone was also highest in the stem at the beginning of the season. This concentration decreased sharply until late June and then gradually decreased through the rest of the growing season, as shown in Figure 2. The time relationship generally corresponds to the study of Farley and Howland (5), which found a decrease of pulegone content from August to September.

To verify complete pulegone extraction, a number of the initial foliage samples was extracted for a second 24 hour period, and this extract was spotted with the first for TLC analysis. Since results showed that at least 95% of the total pulegone was recovered in the first extract, a single extraction was routinely used in all analyses.

The anisaldehyde reagent detected pulegone as a purple zone against a white background at an R_F of 0.43. The reagent is relatively selective for terpenes, and only two faint zones other than pulegone were detected on chromatograms of extracts spotted at the levels described above. Typically, one brown zone was located near the origin and a faint green zone, probably due to chloroplast pigments, close to the solvent front. The densitometric calibration curve [peak area (mm^2) vs. ng pulegone spotted] was fit to the linear equation $Y = A + (B \cdot X)$ with the following typical values: index of determination (r^2) = 0.999, A = -42.0, B = 3.74. Because of slight differences in these values from layer to layer, bracketing standards were always developed in parallel with samples.

To confirm identity of pulegone, foliage extract was streaked onto a preparative TLC plate, the pulegone zone was scraped and eluted with ethanol, and the eluate was analyzed with a directly coupled gas chromatograph quadrupole mass spectrometer. The mass spectrum had a molecular ion peak of m/z = 152, a base peak of 81, and other prominent peaks were at 137, 109, 95, 91, 67, 53, 41. The close match (r = 0.998) between the extract spectrum, the spectrum of an injected pulegone standard, and the pulegone spectrum in the instrument library, and the correspondence of GC retention times (2.8 minutes) between the sample and standard identified the TLC zone as pulegone.

The concentration of pulegone in a "concentrated peppermint oil" purchased in a local health food store was found to be 5.8 mg/ml by the densitometric method, which is within the range of pulegone content found for a series of commercial peppermint oils by a much more complex GC method (5). To verify the accuracy of quantification by densitometry, 2.0 ml of this sample was spiked with 20.0 mg of pulegone, and reanalyzed. The resultant value of 14.9 mg/ml represented 91% recovery of the added pulegone, which is adequate for trace analysis at the ng level. Several other purple and brown zones appeared on the chromatogram of the oil (R_F values of 0.28, 0.59, 0.65, 0.70), but none interfered with scanning the pulegone zone.

The quantitative TLC method described provides a simple, accurate means for determination of pulegone during botanical phytotoxicity studies or for assay of this toxic compound in peppermint oil to be added to consumer products. It would also be useful for analysis of finished products, for which the International Organization of the Flavor Industry Committee of Experts (4) has set a pulegone limit of 20-250 ppm, after extraction and concentration steps. Because of the resolving power of TLC and the use of a sensitive and relatively selective terpene detection reagent, pulegone appeared as a separate zone that was not overlapped by interferences, as is often the case when analyzing similar samples by gas chromatography. Samples other than those used in these studies might require cleanup prior to spotting.

ACKNOWLEDGMENTS

We acknowledge the help of Dr. R. J. Newland, Department of Chemistry, Lafayette College, and Dr. Bill Anderson, Department of Chemistry, Lehigh University, in performing the GC/MS analysis and interpreting the results.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1183-1192 (1983)

DENSITOMETRIC QUANTITATION OF UREA, CARBAMATE, AND ANILIDE HERBICIDES ON C_{18} REVERSED PHASE THIN LAYERS USING BRATTON-MARSHALL DETECTION REAGENT

Joseph Sherma and Joel L. Boymel
Department of Chemistry
Lafayette College
Easton, PA 18042

ABSTRACT

Twelve urea, carbamate, and anilide herbicides were detected on KC_{18} reversed phase thin layers with the Bratton-Marshall reagent after hydrolysis to produce aromatic amines. Spots were quantitated at the nanogram level by scanning with a densitometer. Analyses of spiked water and soil samples were carried out to demonstrate the practicality of the method.

INTRODUCTION

Thin layer chromatography with densitometry has been widely used for the accurate and precise quantitation of residues of many classes of pesticides (1). The Bratton-Marshall detection reagent has been shown to be especially effective for determination of pesticides with an aniline moiety, such as asulam (2) and chloramben (3). The Bratton-Marshall reagent has also been used for the qualitative detection and semiquantitative (visual) determination of some substituted urea herbicides that are hydrolyzed to their anilines on thin layer plates (4). In this paper this approach has been extended to the detection of N-phenylcarbamate and anilide pesticides. Common hydrolysis conditions for these pesti-

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cides and quantitation of the resulting anilines by scanning densitometry are reported. The method is demonstrated with standard pesticides from these three classes and with soil and water samples fortified with representative pesticides.

EXPERIMENTAL

The following pesticide standards were obtained from the U.S.EPA pesticide repository, Research Triangle Park, NC: Urea herbicides-diuron (bromacil; 5-bromo-3-sec-butyl-6-methyluracil); linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea); monuron [3-(p-chlorophenyl)-1,1-dimethylurea]; neburon [1-n-butyl-3-(3,4-dichlorophenyl)-1-methylurea]; monolinuron [N-(4-chlorophenyl)-N'-methoxy-N-methylurea]; metrobromuron [3-(4-bromophenyl)-1-methoxy-1-methylurea]. Carbamates-barban (4-chloro-2-butynyl m-chlorocarbanilate); carbetamide [D-N-ethyllactamide carbanilate (ester)]; chlorpropham (isopropyl m-chlorocarbanilate); propham (isopropyl carbanilate). Anilide-propanil (3', 4'-dichlorophenylpropionanilide).

Stock solutions of these pesticides were prepared at the 10 mg/ml level in ethyl acetate, and these were volumetrically diluted to prepare other standard solutions, as required. Sample solutions were applied to layers with 1 µl Drummond microcap micropipets or a 25 µl Drummond Dialamatic microdispenser (1-25 µl variable delivery volume). Spiking solutions of metrobromuron and chlorpropham were prepared at a concentration of 0.50 mg/ml in ethyl acetate.

Whatman K5 silica gel and $KC_{18}D$ and $KC_{18}DF$ chemically bonded reversed phase thin layer plates (20x20 cm) were predeveloped with methanol-chloroform (or methylene chloride) (1:1 v/v) prior to spotting. Plates were developed in rectangular glass TLC tanks for a distance of 10-15 cm beyond the origin line, which was located

2.5 cm above the bottom of the plate. A pool of mobile phase 2-3 mm deep and a paper liner were contained in the tank, which was equiliberated for 10-15 minutes prior to inserting the spotted plate.

For detection of zones, the chromatogram was air dried and sprayed lightly and evenly, using a Kontes sprayer, with 6N ethanolic hydrochloric acid (60 ml of conc. HCl + 50 ml of absolute ethanol). The plate must not be oversprayed, or it will pucker; the layer should be wet but not visibly soaked. The sprayed layer was covered with a clean glass plate and placed in a 180°C oven for 10 minutes (5). The plate was removed from the oven and placed on an asbestos pad, and the layer was uncovered and allowed to cool. The layer was then sprayed with a 1% sodium nitrite solution until visibly saturated. This solution was prepared by dissolving 1.0 g of $NaNO_2$ in 20 ml of distilled water and diluting to 100 ml with 2N ethanolic HCl (17 ml conc. HCl + 83 ml ethanol). The layer was air dried (ca. 5 min) and then sprayed with a fresh 1% N-(1-napthyl)ethylenediamine dihydrochloride solution (1.0 g of reagent dissolved in 10 ml of H_2O, and diluted to 100 ml with ethanol) until visibly saturated.

Zones were scanned with a Kontes Model 800 fiber optics densitometer using the white phosphor (440 nm peak, 300 nm band width) and the single beam, transmission mode (BC setting) with an 8 mm light source length to match the 8 mm wide lanes of the KC_{18} divided plates. The scan rate was 5 cm/min. Recorder peaks were measured using the formula height x width at half-height.

Control pond water and soil (Keyport silt loam), which were pre-analyzed to prove they contained none of the herbicides of interest, were fortified by adding an appropriate volume of pesticide standard solution to the samples, which were shaken to distribute the spike.

Samples were prepared for TLC by procedures described by Ambrus *et al.* (6). Soil (100g) was extracted by shaking in turn with 100 ml and 70 ml of acetone containing 2 ml of 2N ammonium acetate. The extracts were filtered, combined, evaporated with nitrogen gas, and taken up in 4 ml of benzene. The benzene solution was cleaned up on a 1 cm id glass chromatography column containing 8 g of deactivated Woelm 200 neutral alumina (81 g Super I alumina + 19 g H_2O, mixed, and equiliberated for 2 hr). One ml of benzene solution was added to the column, which was pre-wet with 10 ml of hexane. The column was eluted with 30 ml of *n*-hexane (Fraction I) and 30 ml of hexane-ethyl ether (7:3 v/v) (Fraction II). Lake water (1 liter) was extracted with 100, 50, and 50 ml portions of methylene chloride after adding 50 ml of saturated NaCl solution. The combined extracts were then treated as above for the soil extract.

Adsorbent was pretested with standard solutions of pesticides to assure proper elution characteristics of columns prior to the analyses. Column eluates were evaporated and the residue dissolved in ethyl acetate in a small tube to prepare the TLC spotting solution.

RESULTS AND DISCUSSION

Numerous mobile phases from the literature were tested for separation of the 12 pesticides of interest on silica gel thin layers. These included 2-, 3-, and 4-component solvents composed of various proportions of hexane, acetone, chloroform, and methanol; chloroform-nitromethane (1:3 v/v); and ethyl ether-toluene (1:3 v/v). Both saturated and unsaturated development conditions were evaluated. No system could be found that provided adequate resolution of the pesticides by silica gel TLC.

KC_{18} reversed phase plates with and without fluorescent indicator were then tested. Only the layers with

TABLE 1

R_F Values and Spot Colors of Pesticides on $KC_{18}F$ Layers Developed with Methanol-Acetonitrile-Tetrahydrofuran-Water (50:15:8:27 v/v)

Pesticide	R_F	Color
Diuron	0.47	red-purple
Fluometuron	0.55	red
Linuron	0.38	red-purple
Metobromuron	0.50	purple
Monolinuron	0.53	purple
Monuron	0.62	purple
Neburon	0.27	red-purple
Barban	0.33	red-purple
Carbetamide	0.60	red-purple
Chlorpropham	0.32	red-purple
Propham	0.49	purple
Propanil	0.40	red-purple

fluorescent indicator allowed detection of the zones; plates without fluorescent indicator did not show any spots. The presence of the phosphor apparently was necessary for successful completion of either the hydrolysis reaction or the detection reaction, or both.

Mobile phases for KC_{18} TLC were systematically designed by the procedure described by Sherma and Charvat (7) based on the paper of Lehrer (6). A solvent strength of 2.1 gave the optimum R_F range for the pesticides, and the mixture methanol-acetonitrile-tetrahydrofuran-water (50:15:8:27 v/v) was the best mobile phase with this strength. R_F values and spot colors in this system are shown in Table 1. Differences among R_F values would allow this method to detect and quantitate multiresidues of certain pesticides which are adequately re-

solved. This would be true for the three phenylcarbamates and for neburon, diuron, and monuron, for example. Developed zones were typically narrow and round in this system, generally covering an R_F range of ± 0.03 on either side of the R_F values in Table 1. All R_F values were within the optimum R_F range of 0.2-0.8 for accurate and precise densitometry (9).

As seen in Table 1, urea, phenylcarbamate, and anilide pesticides were detected as red, purple, or purple-red spots after in situ hydrolysis with ethanolic HCl, diazotization with $NaNO_2$, and coupling with Bratton-Marshall reagent. The reagent solution should be prepared fresh just before use and degrades within four hours. Spots appear immediately, and reach maximum color intensity within about 15 minutes after drying. The plate background becomes irreversibly purple after about 30 minutes, which allows more than enough time for scanning of the zones.

Representative compounds were chosen from each class of pesticides to demonstrate quantitation. These were metobromuron, monuron, and neburon (ureas), chlorpropham (carbamate), and propanil (anilide). Stock solutions of these compounds were diluted with ethyl acetate to prepare a series of standard solutions ranging in concentration from 10 ng/µl to 5 µg/µl. Increasing weights of each compound were spotted across individual plates, which were developed and the spots detected. For each of the five compounds, 50 ng was the lowest amount that was visually detected and 100 ng was the lowest amount that consistently gave a densitometer peak that was at least 10% of full scale deflection (10 times the noise level).

Peak areas (cm^2) were plotted vs ng spotted to construct calibration curves for each compound. Figure 1 shows the plot for 100-2000 ng of metobromuron. It is

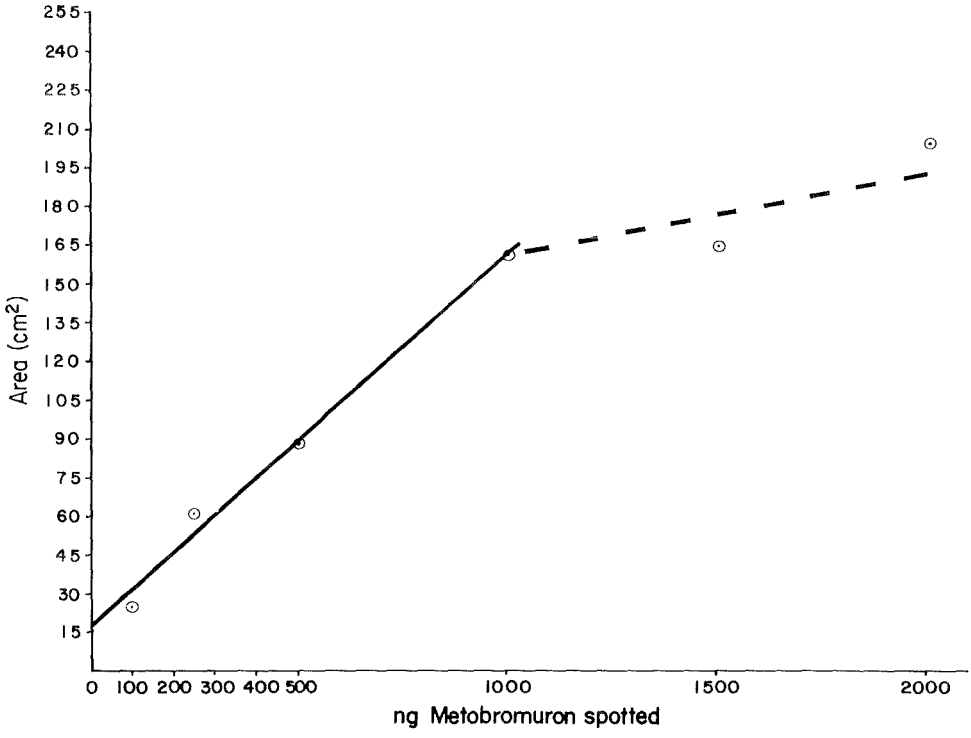

Figure 1. Calibration curve for the urea herbicide metobromuron from 100-2000 ng. Attenuation was X4 for the 100 ng peak and X8 for the others.

seen that the plot was linear from 100-1000 ng, and then curved downward toward the X-axis. Figure 2 shows the corresponding scanner peaks up to 1000 ng. The calibration plot for propanil was linear up to 1500 ng, monuron and neburon to 2000 ng, and chlorpropham to 3000 ng.

Calibration curves were quite reproducible in terms of slope, intercept, and linearity from plate to plate, but standards should always be run on the same plate with samples to obviate the effects of any variations when using the method.

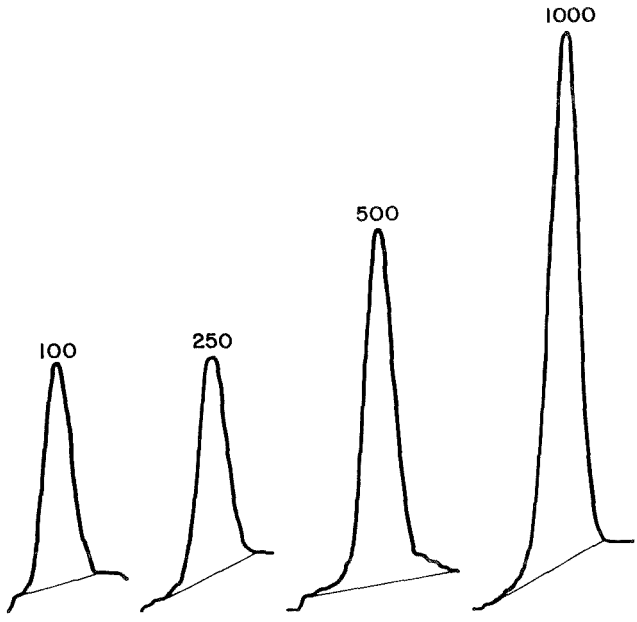

Figure 2. Scans of 100-1000 ng of metobromuron using the Kontes Model 800 scanner with attenuation 32. Constructed baselines are shown.

To check reproducibility, four 700 ng spots of each of the five compounds were spotted across separate KC_{18} plates, the plates were developed, and the zones were detected and scanned. The relative standard deviations of the areas ranged from 4.9 to 14.6%, with a mean of 9.8%. This is satisfactory precision considering the three spraying operations required for detection, and is more than adequate for pesticide residue determinations at concentrations below 1 ppm.

Lake water was spiked with chlorpropham and soil with metobromuron at 0.50 ppm by adding 500 µg of chlorpropham (10 ml of spiking solution) to 1.00 liter of control lake water and 50 µg of metobromuron (1 ml of spiking solution) to 100 g of air dried soil. Samples

were extracted and cleaned up by chromatography on a neutral alumina column as described in the Experimental section. Assuming complete recovery, the 1 ml of benzene solution added to the column represented 125 µg of chlorpropham (250 ml of water sample) and 12.5 µg of metobromuron (25 g of soil). The column eluate residues were dissolved in 200 µl of ethyl acetate, and 1 µl of the chlorpropham and 10 µl of the metobromuron sample solutions were spotted along with standards for TLC. If recovery was complete, these volumes would contain 625 ng of the respective pesticides. The 10 µl sample was spotted in small increments, with intermediate drying with a stream of nitrogen gas, to keep the initial zones compact and comparable in size to the standard zones. Duplicate samples and bracketing standards were applied to each plate, e.g., 200, 400, 600, 800, and 1000 ng.

The first (n-hexane) column eluate contained all of the chlorpropham, as reported by Ambrus et al (6). This was checked by spotting both eluates separately, which proved the second fraction was negative. Metobromuron split between the two fractions, with most of the pesticide being eluted in the first (6).

The recoveries reported by Ambrus et al. (6) for the column chromatography were greater than 80% for chlorpropham and 95% for metobromuron. Our overall recoveries from the spiked samples, obtained by interpolating the amount of pesticide in sample spots from the standard curve and comparing to the theoretical 650 µg level, averaged 78.6% (4 trials, range 74.0-83.3%) for chlorpropham in water and 81.8 (5 trials, range 76.5-84.8%) for metobromuron in water. Chromatograms had no zones interfering with the scanning of the pesticide zones. The chromatogram of the water sample was especially clean, and this sample could probably have been analyzed without column cleanup. The soil chromatogram had detected

zones at the origin plus one or two other zones well resolved from the pesticide.

SUMMARY

Advantages of TLC for pesticide analysis include simplicity, high sample throughput, and the ability to analyze multiple samples at the same time under identical conditions and to process standards in parallel. The method described in this paper is the first multiresidue procedure reported for determining urea, carbamate, and anilide type herbicides after detection as colored zones. Precision and accuracy (recovery) are shown to be adequate for analyses below 1 ppm, and two typical applications are demonstrated. Extension of the method to other sample matrices and additional pesticides of these types should be possible if the required extraction and cleanup steps are carried out.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1193-1211 (1983)

COMPARISON OF REAGENTS FOR LIPID AND PHOSPHOLIPID DETECTION AND DENSITOMETRIC QUANTITATION ON SILICA GEL AND C_{18} REVERSED PHASE THIN LAYERS

Joseph Sherma and Susan Bennett
Department of Chemistry
Lafayette College
Easton, PA 18042

ABSTRACT

A variety of chromogenic and fluorogenic detection reagents were evaluated for detection and densitometric quantification of lipids and phospholipids on silica gel and chemically bonded octadecylsilane reversed phase thin layer plates. Phosphosphomolybdic acid (PMA) was found to be the most generally favorable reagent, followed by cupric acetate and ethanolic sulfuric acid. The quantification of lipids in blood serum by chemically bonded RP-TLC was demonstrated using PMA with direct sample application to preadsorbent plates.

INTRODUCTION

The determination of neutral lipids and phospholipids in various biological samples is one of the areas of greatest application for qualitative and quantitative thin layer chromatography (1). A great number of detection reagents have been used for these determinations, but only a few limited comparisons have been reported (e.g., 2,3). This paper reports a comparative study of the suitability of common chromogenic and fluorogenic reagents for detection and quantification by scanning of lipids and phospholipids on silica gel and C_{18} chemically bonded reversed phase silica gel thin

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layer plates. Reagents were evaluated on the basis of convenience, visual detection sensitivity, stability of zones, densitometric sensitivity, reproducibility of scanning, and the nature of the calibration curve. Detection and quantification on silica gel and reversed phase layers were also compared.

Phosphomolybdic acid (PMA) and cupric acetate proved to be the most advantageous reagents, and PMA was especially useful for quantifications on C_{18} layers. This was demonstrated by determination of cholesterol, triolein, and cholesteryl oleate in human blood serum by reversed phase TLC with densitometry.

EXPERIMENTAL

Materials

Lipid standards were purchased from Nu-Chek-Prep, Inc. and phospholipids from Avanti Polar Lipids, Inc. The lipids studied were methyl oleate, cholesteryl oleate, triolein, and cholesterol. The phospholipids were lecithin (dipalmitoyl); lecithin (egg); sphingomyelin (bovine); and phosphatidyl ethanolamine (egg), inositol (bovine), serine (bovine), and glycerol (egg). Stock solutions of each compound were prepared at a concentration of 1.00 µg/µl, and volumetric dilutions were made to give solutions containing 100, 50.0, 10.0, and 1.00 ng/µl. Lipids were dissolved in chloroform, and phospholipids in chloroform-methanol (1:1) except for phosphatidyl inositol (hexane-ethanol, 9:1) and phosphatidyl serine (benzene).

Whatman 20 x 20 cm scored, preadsorbent silica gel plates (LK5DF), 10 x 20 cm scored, preadsorbent high performance silica gel plates (LHPKDF), and 20 x 20 cm scored, preadsorbent C_{18} chemically bonded reversed

phase plates ($LKC_{18}DF$) were used. Comparable plates without fluorescent indicator were used for reagents producing spots fluorescing at 254 nm. To remove impurities and increase sensitivity of detection reagents, plates were prewashed by development with chloroform-methanol (1:1) (silica gel) or chloroform-acetonitrile (80:20)(C_{18}) and dried in a fume hood prior to spotting. The properties of these plates and general techniques for their proper utilization have been described (4).

Procedures

Solutions were applied to the preadsorbent spotting area by streaking with a 25 µl Drummond Dialamatic microdispenser, followed by drying with warm air from a hair drier. Plates were developed in filter paper-lined glass chambers that were equilibrated with the mobile phase for approximately 15 minutes. One hundred ml of solvent gave a pool in the chamber that was about 3 mm deep. Silica gel TLC and C_{18} layers were developed for 10-12 cm and HP silica gel 6 cm.

Developed plates were air dried in a fume hood, and detection reagents were applied either by spraying from a Kontes Chromaflex sprayer or by dipping in a Thomas-Mitchell metal dip tank as described in the individual sections below. Heating steps were carried out in a temperature-regulated Precision Model 15 or Blue M Electric Co. Model OV-8A oven.

Zones were scanned with a Kontes Model 800 fiber optics densitometer equipped with a Hewlett Packard Model 3390A calculating integrator/recorder. Scanning was done in the double beam mode using the source phosphor found to be most appropriate, based on maximum signal-to-noise ratio, for each detection reagent. A shroud was used to concentrate the light source. Cali-

bration curves were plots of integrator areas vs nanograms spotted for a series of standard spots.

Fingertip blood serum was analyzed on C_{18} plates by the following procedure: the first few drops from a pricked finger were rejected, and the sample (about 5 µl) was then allowed to flow freely into tubes designed for use with a Fisher Model 59 centrifuge. Blood was centrifuged at 7000 rpm for 5 minutes, and the serum was diluted 1:10 or 1:20 with distilled water to produce a spot with an area falling on the linear portion of the calibration curve for the compound of interest when 5 µl was applied for TLC. The initial zones of the sample (spotted in duplicate) and bracketing standards were dried for 5 min with a hair drier, and the plate was developed with acetonitrile-methyl ethyl ketone-chloroform-acetic acid (50:30:15:5) for a distance of 13 cm (about 25 minutes). Chromatograms were oven dried (5 min, 100°C), cooled, dipped in Whatman 5% PMA reagent, and heated for 8 minutes at 110-120°C. The plates were cooled and scanned, and the average sample spot area was interpolated from the calibration curve constructed from the standards in the same plate to give the ng of lipid present. Conversion was made to mg/100 ml present in blood based on the amount of sample spotted and the dilution factor.

RESULTS AND DISCUSSION

Lipid and phospholipid visualization reagents were evaluated on silica gel TLC and HPTLC layers and on C_{18} RP layers in terms of ability to detect 2-3 µg amounts statically (without development); visual and scanning sensitivity after development; densitometric calibration curve (slope, linearity); stability of detection (by scanning a single zone repeatedly over a

period of time); and quantification reproducibility (relative standard deviation of the scan areas of six standard zones after spotting, development, and detection). Mobile phases used on silica gel for the calibration curves, reproducibility, and time studies were chloroform-ethyl acetate (94:6) for cholesterol (R_F 0.40), hexane-ethyl ether-acetic acid (80:20:1) for methyl oleate (R_F 0.60), and chloroform-ethyl acetate-n-propanol-methanol-0.25% aq. KCl (25:25:25:13:9) for sphingomyelin (R_F 0.10) and lecithin (R_F 0.20). On HP silica gel, chloroform-methanol-ethanol-acetic acid (40:30:20:10) or chloroform-methanol-ethanol-0.25% KCl (40:20:20:20) were used to provide increased R_F values for lecithin and sphingomyelin. On C_{18} RP layers, ethanol-acetic acid-formic acid (80:10:10) was used for cholesterol (R_F 0.35), acetonitrile-chloroform (60:40) for cholesteryl oleate (R_F 0.30) and triolein (R_F 0.30), and acetonitrile-methyl ethyl ketone-chloroform (50:35:15) for methyl oleate (R_F 0.55). These mobile phases were designed to yield R_F values that were usually within the optimal range of 0.3-0.7 for densitometric analysis (5). On HP plates, the short (6 cm) development distance required an R_F value of at least 0.25 so that the spot could be scanned without interference from the preadsorbent-sorbent junction.

The Nu-Chek Prep 18-4A neutral lipid standard mixture was completely separated on a reversed phase layer using the mobile phase acetonetrile-methyl ethyl ketone-chloroform-acetic acid (50:35:15:5). R_F range values were cholesteryl oleate 0.19-0.22, triolein 0.25-0.30, cholesterol 0.31-0.36, methyl oleate 0.58-0.60, and oleic acid 0.65-0.67 after a 13 cm development (25 minutes). The R_F values for this mixture on silica gel developed with hexane-diethyl ether-acetic acid (80:20:1) were

cholesterol 0.08, oleic acid 0.24, triolein 0.37, methyl oleate 0.49, and cholesteryl oleate 0.62. Comparisons of these values indicate some sequence changes but not the exact reversal that would be expected if the mechanism on the C_{18} layer was totally reversed phase partition. Phospholipids were difficult to quantify on C_{18} layers because four of the six natural standards gave two zones upon development with mobile phases such as those listed above and detection with PMA. For example, development of phospholipid standards on C_{18} with ethanol-acetic acid-H_2O (80:10:10) gave the following R_F values: (lecithin 0.21-0.27 Ostreak), phosphatidyl serine 0.39 + 0.45, sphingomyelin 0.17 + 0.26, and phosphatidyl glycerol 0.47 + 0.51. These multiple compounds apparently were not resolved on silica gel in the mobile phases employed in this research. Phosphatidyl ethanolamine and phosphatidyl inositol gave single spots with respective R_F values of 0.46 and 0.49 in this mobile phase.

The following subsections describe procedures and results for the various detection reagens. In each case all four lipids and seven phospholipids were tested for static detection, and selected compounds were developed and evaluated in terms of sensitivity, stability, and densitometry.

Phosphomolybdic Acid (PMA)

PMA was prepared as a 5% solution in absolute ethanol, followed by vacuum filtration through a Buchner funnel. The solution is bright yellow when fresh but darkens to yellow-green on storage. Whatman PMA reagent (Catalog No. 4911-119) was found to be equivalent to fresh laboratory-prepared solution and not to require storage in a refrigerator. Chromatograms were dipped

into the reagent and then heated at 110-120°C for 8-12 minutes to produce blue spots on a yellow background. Overheating caused darkening of the background. PMA can be incorporated into the layer by dipping prior to development if the mobile phase will not wash out the reagent (6). Pre-dipping was possible with the phases used for lipids but not those for the phospholipids. Since there were no significant advantages found for pre-dipping, post-dipping was routinely used. The dip solution was reused as long as it retained its light yellow color. The red Kontes phosphor was employed to scan spots produced by PMA.

All of the lipids and phospholipids except dipalmitoyl lecithin (containing only saturated phospholipid) were strongly detected at 2 μg level statically on both silica gel TLC and HPTLC layers. The visual and scanning detection limits were 100 and 200 ng, respectively, for silica gel TLC and 50 ng and 100 ng for HPTLC. Scan areas were stable for at least 60 minutes. On silica gel, calibration curves were typically linear from 200-1200 ng with correlation (linearity) coefficients (r) of 0.98. Reproducibility of scanning six spots ranged from 3.1-4.9% for both lipids and phospholipids. On HP silica gel, linearity was obtained from 100-1000 ng, r was approximately 0.96, and reproducibility was 1.4-4.8%. Figure 1 shows a calibration curve for sphingomyelin on HP silica gel.

On C_{18} RP layers, all compounds were detected with visual limits of 25-100 ng and scanning limits of 50-200 ng. Calibration curves were linear to about 1000 ng and r values were at least 0.98. Reproducibility of quantification for cholesterol was 4.9%. A calibration curve for cholesteryl oleate is illustrated in Figure 2.

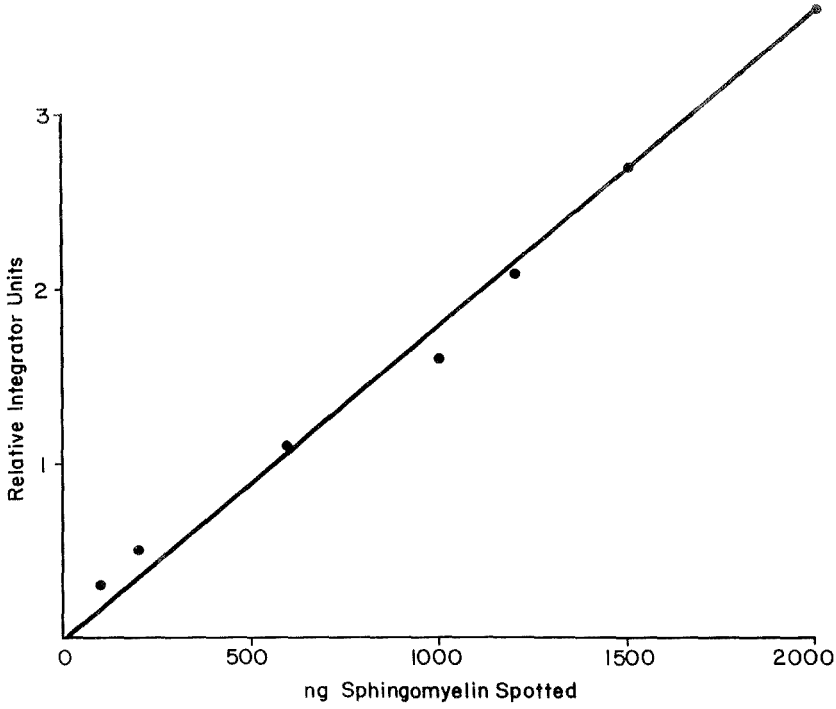

Figure 1. Calibration curve for 100-2000 ng of sphingomyelin developed on a high performance silica gel plate with chloroform-methanol-ethanol-0.25% KCl (40:30:20:10) and detected with PMA.

Scans of the peaks used to construct this curve are shown in Figure 3.

Cleaning of plates by predevelopment was very important in obtaining a light yellow background with PMA, and use of an oven with even heat distribution was important for good precision. Freshness of the PMA solution was more important on RP plates than on silica gel. Laboratory-made solution could be used only within 24

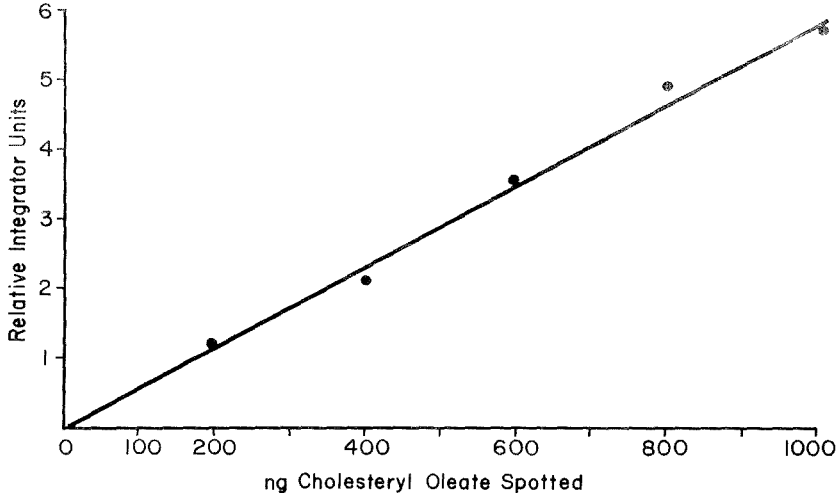

Figure 2. Calibration curve for 200 to 1000 ng of cholesteryl oleate developed on a C_{18} reversed phase plate with acetonitrile-chloroform (60:40) and detected with PMA.

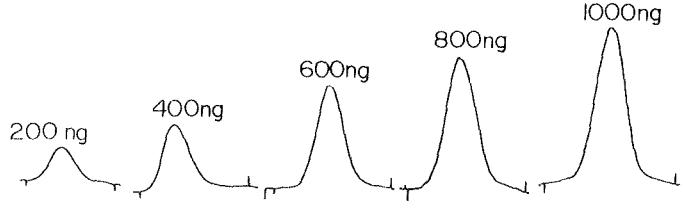

Figure 3. Scans of 200-1000 ng of cholesteryl oleate on a C_{18} layer using the Kontes Model 800 scanner and Hewlett Packard Model 3390A integrator with attenuation X8.

hours of preparation. However, Whatman PMA reagent solution was useful over a period of at least one week.

Cupric Acetate

The reagent was prepared by mixing for 1 hour with a magnetic stirrer 30 g of cupric acetate monohydrate and 80 ml of phosphoric acid with sufficient distilled water to make one liter of solution. Plates were dipped into the reagent and then heated (160°C for 8-15 min for silica gel and 120°C for 4-5 minutes for C_{18}) to produce charred spots on a white background. The white phosphor was inserted when scanning the zones (7).

All compounds were statically detected at the 2 µg level on silica gel except dipalmitoyl lecithin (saturated) (8). Visual and scanning sensitivity were both 100 ng for cholesterol and sphingomyelin and 200 ng/400 ng for lecithin. The linear range of calibration curves was typically 100-1000 ng and correlation coefficients were 0.96-0.99. The reproducibility for cholesterol was 4.4% and for lecithin 7.1%. A time study indicated stability within 2.5% over a period of 30 minutes upon scanning of a 400 ng cholesterol zone.

Visual detection limits on HP silica gel were 25 ng for cholesterol and lecithin, and 50 and 100 ng for these compounds, respectively, for scanning. Calibration curves were linear up to 1000-1200 ng, with r values of 0.91-0.98. Reproducibility for cholesterol and lecithin were 2.9 and 4.3%, respectively.

Cupric acetate was not successfully applied to C_{18} layers. The heating time was very critical, and it was virtually impossible to produce dark spots with good contrast before the layer itself began to char. Even on silica gel, the time and temperature of heating were

more critical for reproducible zone color and a light, consistent background than with PMA. Zone scans, baselines, calibration curves, and r values also tended to be less erratic for PMA compared to cupric acetate. The best results that could be achieved on RP layers were a 200 ng visible detection limit for cholesterol and a 16% RSD reproducibility for scanning replicate zones.

Cupric Sulfate

The reagent was prepared by dissolving 100 g of anhydrous cupric sulfate plus 80 ml of phosphoric acid in enough water to make one liter and magnetically stirring for 1 hour(8). Plates were dipped and heated for 10-15 minutes at 160°C to produce charred zones on a white background. The white phosphor was used to scan these zones.

Static detection of all compounds, including dipalmitoyl lecithin (saturated), was obtained at the 2 µg level. The detection of saturated phospholipid with $CuSO_4$ reagent but not cupric acetate has already been reported (8).

Detection limits of lipids and phospholipids were 50-100 ng visually and 100-200 ng for scanning on silica gel. Limits for dipalmitoyl lecithin were 200 and 400 ng, respectively. Calibration curves were linear to 1200 ng, with r values of 0.90-0.98. RSD values were 2.5% for cholesterol and 1.5% for egg lecithin. On HP silica gel, detection levels were similar, but calibration curves had considerably lower r values due to zone scans with uneven baselines that were difficult to measure accurately. Backgrounds were generally darker and less even than with cupric acetate reagent. Two spots were detected for some of the natural phospholipids which

had given only one spot with cupric acetate or PMA, indicating the presence of saturated phospholipid in these standards (8). One 800 ng zone of cholesterol was scanned over a 30 min period and areas were constant within 5.5%. Cupric sulfate was not applicable with C_{18} layers; the plate turned dark before spots appeared with all heating conditions attempted.

Dittmer-Lester Reagent

The reagent was prepared from molybdic anhydride and molybdenum in acid solution as described earlier (4,10). The plate was sprayed uniformly until lightly damp to produce blue zones for phospholipids, but lipids were not detected. Visual detection limits were about 1 µg for dipalmitoyl and egg lecithin and phosphatidyl ethanolamine, and approximately 2 µg for the others after development. The blue spots appeared on a lighter blue background and were blotchy and grainy; after 4 hours, the spots became white. The blue zones were not suitable for scanning on either silica gel TLC or HPTLC plates. On C_{18} layers, only phosphatidyl serine and glycerol were detected at the 2 µg level during static tests, and these spots were blotchy and unstable.

Phospray

The prepared reagent from Supelco was applied by using a procedure recommended by the manufacturer during a phone conversation. The chromatogram was thoroughly air dried, heated at 100°C for 10 sec, sprayed moderately, and then re-heated for 30-60 seconds. Green spots were produced against a dark blue background for all phospholipids at the 2 µg level in static tests, with the egg lecithin and phosphatidyl serine and inositol zones being most strongly colored. Lipids were not detected. The plate background was always uneven except after the lightest spraying, which did not allow the phospholipids

to be detected. Whatman K4 gypsum-bound silica gel plates gave much better results than on the originally used K5 silica gel TLC and HPTLC plates, but silica gel G was not included as part of this research project. No detection was obtained for any of the phospholipids or lipids in static tests at the 2-3 µg level on C_{18} RP layers.

Bromothymol Blue

Commercial reagent solution (Supelco No. 3-4656) was sprayed on the plate until wet. All lipids and phospholipids, including depalmitoyl lecithin, were detected on silica gel as aquamarine spots on a green background. Scanning was attempted using the red phosphor, but uneven backgrounds and white shadows near the spots precluded satisfactory results. For example, a reproducibility study with egg lecithin resulted in a 21% relative standard deviation. Sensitivity of visual detection was approximately 50-100 ng. Detection was not successful for lipids or phospholipids on C_{18} RP layers because the layer background was blue.

1,2-Naphthoquinone-4-Sulfonic Acid

The reagent solution (100 mg of 1,2-naphthoquinone-4-sulfonic acid dissolved in 100 ml of a mixture of ethanol-60% $HClO_4$-H_2O-40% formaldehyde, 20:10:9:1) was applied by dipping the plates, which were then heated for 7-8 minutes at 80°C to produce blue spots against a tan background. The red phosphor was used for densitometry. All compounds except dipalmitoyl lecithin were detected on silica gel at minimum levels of 200 ng (visual) to 400 ng (scanning). The calibration curve for cholesterol was linear from 400-1000 ng, the correlation coefficient was 0.99, and reproducibility was 13% (RSD). The major problems with the reagent were the sensitive nature of the heating step required to achieve the maxi-

mum contrast between the spots and the background, as well as the generally blotchy and uneven background produced. These problems were even worse on HP silica gel than on K5 silica gel. On C_{18} RP layers, all compounds were detected at the 200-400 ng level, but dipping the reagent caused the layer to loosen and sometimes come off the glass, precluding use of the reagent for quantification.

Acid Fuchsin-Uranyl Acetate

Attempts to apply this reagent, previously described by Michalec and Reinisova (10) and Michalec and Kolman (11), were unsuccessful. All lipids and phospholipids were detected as faint red spots against a light red, uneven background at the 500-1000 ng level on silica gel. Attempts to dip C_{18} layers caused the layer to be removed from the glass, and spraying the reagents gave poor detection sensitivity and an uneven, colored background. No conditions could be found using these two solutions to obtain red spots on a white background as reported earlier.

6-*p*-Toluidino-2-Naphthalenesulfonic Acid, Potassium Salt

A 1 mM TNS solution was prepared in 50 mM Tris-HCl, pH 7.4 and lightly sprayed onto the layer (12). All lipids and phospholipids were detected visually on silica gel at 100-1000 ng levels under shortwave UV light, but the spots faded quickly and could not be reproducibly scanned. The reagent could not be used on C_{18} plates because the layer became fluorescent under shortwave UV light after application.

Primuline

The reagent was prepared by diluting 1 ml of stock solution (0.1 g primuline in 100 ml H_2O) to 100 ml with acetone-H_2O)(4:1) (13). Plates were sprayed until wet

and viewed immediately. All lipids and phospholipids were detected visually at the 1 µg level after development as purple spots against a green background on silica gel when viewed under shortwave UV light. As the plate dried after spraying, the spots faded and sensitivity decreased so that reproducible scanning was not possible. No spots were visible under either longwave or shortwave UV light on C_{18} layers.

Sulfuric Acid in Ethanol

Plates were sprayed lightly but uniformly with absolute ethanol-conc. H_2SO_4 (9:1) and heated at 180°C for 5-10 minutes (14). Tan spots on a purple background were visible under longwave UV light on silica gel for all compounds except dipalmitoyl lecithin, but visual sensitivity was not as good as for PMA. Therefore, quantification by scanning was not studied.

The same compounds were visually detected on C_{18} layers at minimum levels ranging from 400 ng-9 µg. Scanning in the longwave UV region was done by placing the 254 nm filter on top of the 351 nm phosphor and taping a plastic "sunscreen" over the densitometer head to block 360 nm radiation from reaching the detector. The calibration curve for cholesterol was linear from 1-3 µg with a correlation coefficient of 0.99. The curve for methyl oleate was linear from 5-9 µg with a linearity of 0.99; 5 µg was the lowest level that gave a peak for this compound, although the visual detection limit was 400 ng. Peak areas were constant over a period of at least 30 minutes, and reproducibility of scanning was 4.7% (RSD). It is important not to overheat the plate or the spots (and eventually the background) will char instead of becoming fluorescent.

Nitric Acid Vapors

Plates were placed in a closed chamber saturated (for 1 hour) with vapors from conc. HNO_3 contained in a 30 ml beaker (15). After 10 minutes exposure, the plates were heated for 10 minutes at 180°C to produce orange-green spots against a purple background when viewed under longwave UV light. Cholesterol, cholesteryl oleate, sphingomyelin, and phosphatidyl serine, inositol, and ethanolamine were visually detected at levels of 2-5 µg after development on silica gel, but background noise precluded successful quantification by scanning. On a C_{18} layer, only phosphatidyl serine was well detected at approximately 2 µg, but again an uneven background did not allow successful scanning.

2',7'-Dichlorofluorescein

Plates were dipped for 10 seconds in the reagent solution (1.5 mg/100 ml isopropanol), air dried for 15 minutes, and viewed under longwave UV light (16). Spots with sufficient contrast to the background to allow sensitive visual detection or quantification were not produced on any of the layers tested.

Conclusions

Based on the above results, in our hands PMA proved to be the overall best reagent for detection and *in situ* quantification of lipids and phospholipids on all three layers considering the aforementioned criteria. Cupric acetate was also excellent for silica gel but not for C_{18} bonded layers. Ethanolic sulfuric acid was the second best reagent found for the RP layers, but the sensitivity of quantification was not equal to PMA.

It must be stressed that our study was made using closely controlled, standardized procedures, but only with three types of polymer-bound (hard) layers that

are the most widely used today. Results with any given reagent might be different on another type of layer, e.g., silica gel G, which may have been used when the reagent was originally reported in the literature, or if a different scanner was used.

Analysis of Blood Serum

To demonstrate the application of RP layers and PMA for the analysis of lipids in biological fluids, blood serum from a single subject, an apparently healthy adult male, was analyzed by direct spotting on C_{18} preadsorbent plates, as described in the Experimental section, followed by development with acetonitrile-methyl ethyl ketone-chloroform-acetic acid (50:30:15:5). The average values of morning blood were 50.0 mg of cholesterol and 32.0 mg of cholesteryl oleate per 100 ml. Figure 4 shows the scans of duplicate 5 µl of this serum after dilution (1 + 9) with water. The peak areas for cholesterol agreed within 7.7% and for cholesteryl oleate within 1.3%. The triolein content was not quantified. Blood taken from the same subject in the afternoon of the same day had 67.5 mg/100 ml of cholesterol and 36.0 mg/100 ml of cholesteryl oleate.

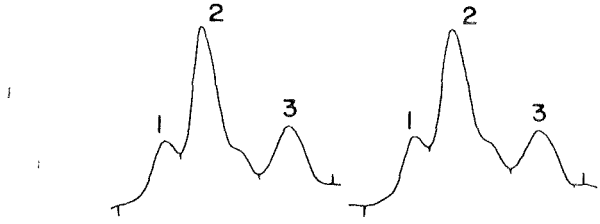

Figure 4. Scans of chromatograms of duplicate 5 µl blood serum samples; peak 1 is cholesterol, 2 is triolein, and 3 is cholesteryl oleate.

On day 2 of the study, morning blood contained 28.0 mg/100 ml of cholesteryl oleate and 69.0 mg of cholesterol. Triolein was again identified but not quantified. On day 3, morning and afternoon blood samples were collected, diluted 20:1, and the triolein content was determined to be 240 mg/100 ml in both samples. In all analyses, duplicate samples agreed within 10% and usually within 5%.

These limited analyses demonstrate the practicality of preadsorbent RP-TLC for lipid determination using PMA detection reagent.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1213-1220 (1983)

A COMBINED THIN-LAYER CHROMATOGRAPHY/MICRO INFRARED METHOD

Haleem J. Issaq
Chemical Synthesis and Analysis Laboratory
NCI-Frederick Cancer Research Facility
P.O. Box B
Frederick, Maryland 21701

ABSTRACT

A method is described for the automatic elution of chromatographed compounds on thin-layer chromatography plates and their subsequent identification by micro infrared spectroscopy.

The method is simple, easy to perform in a few minutes, and requires 5 μg of material, 3 mg of KBr powder, and 150 μl of solvent.

INTRODUCTION

The use of combined techniques for chemical analysis is not only useful but in most cases time saving. The combination of chromatography, which gives a pure sample, and spectrometry, which provides structural information, supply the analyst with valuable data.

Numerous gas chromatography/mass spectrometry (GC/MS) (1-5), liquid chromatography/mass spectrometry (LC/MS) (6-10), and recently thin-layer chromatography/mass (TLC/MS) (11) coupling systems have been reported. The combination of TLC with flameless atomic absorption spectrometry for the identification of inorganic ions and organometallic complexes has

Research sponsored by the National Cancer Institute under Contract No. NO1-CO-23910 with Program Resources, Inc.

0148-3919/83/0607-1213$3.50/0

been described, and that of TLC with infrared spectroscopy (TLC/IR) was summarized by Szekely (13). However, most of the methods described require scraping and subsequent elution of the sample from the adsorbent which leaves adsorbent particles in the eluate. These particles cannot be removed by filtration and, as a result, interference signals are observed. These aforementioned methods require a large amount of solvent, compared with the sample, for spot elution. They are time consuming and in certain cases, the recoveries are not quantitative.

This paper presents a combined TLC/IR method which, unlike the other methods (13), uses a novel elution technique requiring approximately 150 µl of solvent for quantitative elution. The sample obtained is free from the adsorbent and ready for analysis. Six samples can be eluted from the plate simultaneously. The amount of sample required is approximately 5 ug.

EXPERIMENTAL

Reagents and Materials

Silica gel 60 precoated TLC plates (EM Labs, Inc., Elmsford, NY) were used after activation at 110°C for 2 hrs. All solvents were previously distilled in glass (Burdick and Jackson, Muskegon, MI). Benzo(a)pyrene (B(a)P), and dimethylbenzanthracene (DMBA) (Aldrich Chemical Co., Milwaukee, WI) and pyrene Chem Service, Inc., West Chester, PA) were used without purification. Sample solutions were freshly prepared in chloroform. Benzene: hexane (10:90) was used as a developing solvent, and methanol as an eluant.

Apparatus

IR. Perkin-Elmer, (Norwalk, Conn.) Model 180 IR spectrometer with a 6X beam condenser attachment was used. KBr pellets were prepared using a Perkin-Elmer ultra-micro die kit and the AgCl mini-cell windows (Wilks Scientific, Norwalk, Conn.) were supported on a Perkin-Elmer demountable micro cell mount.

TLC. The Eluchrom Automatic Elution System by Camag, Inc. was used to elude the spots from the plate. Standard TLC developing tanks were used. Samples weere spotted and eluted under subdued light in an inert atmosphere (nitrogen) to prevent sample decomposition.

Method

10 µl of freshly prepared solution (1 µg/µl) were spotted on silica gel plates which were then developed in benzene:hexane (10:90). Following developement the plates were dried under nitrogen at room temperature. The plate was then covered with a clean glass plate and spots were located under UV radiation. A circle perimeter of 2.5 cm was scored through the plate adsorbent coating and centrally about each spot to be eluted, using a special milling device which is provided as an accessory to the Eluchrom System. After a plate was scored, it was placed on the Eluchrom unit and an elution head was placed over each scored circle. The elution heads were tightly clamped on the plate, forming a teflon (elution head) - glass (TLC plate) seal. Eultion was carried out at the slowest flow setting (0.1 ml/min) and the spot eluant was collected into a clean test tube. 150 µl of methanol was required to quantitatively elute each spot. The sample was eluted into a 0.5 ml reaction tube (Supelco, Inc., Bellefonte, PA), containing approximately 3 mg of KBr powder (Harshaw Chemical Co., Solon, OH). After elution the reaction tube containing the sample was placed overnight in a vacuum oven at 35°C to assure complete dryness (2 hours are an adequate time). The dry KBr powder with the sample was formed into a 1.5-mm pellet of which the spectra was recorded. A background spectrum was obtained for a blank which was similarly prepared from part of the plate where no samples were spotted.

Alternatively the eluant was collected in a clean 1 ml screw cap septum vial in which the solvent was evaporated to 10 µl. The sample was then deposited in the center of a silver chloride (AgCl) mini-cell

window using a 10 μl syringe. The sample deposit was built up as a circle with a diameter of about 2-mm, by carefully transferring the solution to the AgCl window in 2 μl portions and evaporating the solvent with nitrogen before further addition. A demountable micro cell mount was used to support the AgCl window. A 2-mm metal disc pellet holder was sandwiched between the AgCl window and the base-plate in order to mask off an area of the crystal so that only radiation passing through the sample was allowed to reach the detector. The spectra were then recorded using a Perkin Elmer X6 beam condensor and a reference beam attenuator. Using a blank AgCl window, a transmittance reading of 48% was obtained at 1,000 cm^{-1} while under similar conditions a blank 1.5-mm KBr pellet transmitted ~ 10% of incident radiation. The better energy transmittance offers advantages in terms of spectral recording accuracy and scan time saving.

DISCUSSION

The use of combined TLC/IR is not new (13), however, almost all of these methods require (a) eluting the sample from the adsorbent after scraping the spot and (b) redissolving the concentrated extract in a minimum amount of solvent or forming a micro-KBr pellet. The disadvantages of scraping and subsequent elution were recently discussed (14). The most prominent disadvantage and the one that affects the results most is that the extremely fine adsorbent particles cannot be removed from the eluate by filtration or centrifugation and leads to interference signals. For example, silica gel produced strong signal at about 9 μ.

Much better results are obtained by those methods in which the substance is transferred with a little solvent from the spot into the KBr powder. Some of the techniques used for obtaining a sample free from the adsorbent are (a) the wick-stick method (15) which utilizes a KBr triangle (25 x 8 x 2 mm) (Harshaw Chemical Co.). The triangle is placed

in a small beaker. The adsorbent, which contains the separated substance, is transferred into the beaker, a small amount of solvent is added, the substance is extracted from the sorbent, the solution rising in the wick-stick, and the solvent evaporates in the upper region where the compound accumulates at the tip of the triangle. The tip is cut, dried and used to obtain the spectrum. The advantage of this method is that it enables the spectra to be obtained free from background interferences. Good IR spectra are obtained for 10 μg of component, however, the recovery is 50-80%; (b) the direct transfer method (16) requires 20 μg of KBr and works as follows: after the spot is located on the plate, the adsorbent around the spot is scraped to form a "tear-drop" shape. The KBr powder is then placed on the plate in the form of a rod 6-8 mm long and 2 mm wide. The KBr is in contact with the adsorbent. The substrate is then eluted from the adsorbent to the KBr by adding solvent from a syringe to the adsorbent. The nearer half of KBr which is in contact with the adsorbent is discarded. Spectra is obtained by using the other half of the KBr. The disadvantages of the method are (i) 20 mg of KBr are used, this is too large an amount for micro sample; (ii) too rapid an addition of solvent can lead to a loss of substance; and (iii) not all the sample is eluted. In a modification of the above method De Klein (17) formed a half-moon arrangement from the KBr. A good spectra was obtained but severe contamination effects were obvserved due to the presence of adsorbent material. (c) Another microtransfer procedure was developed (18) in which the TLC spot is scraped off, collected and placed on top of a small amount of KBr powder, tamped down the cone joint of an 18-gauge hypodermic needle. A 1-ml glass syringe is filled with pure acetone, connected to the needle and solute is eluted drop by drop into 10 mg KBr. Each drop is allowed to evaporate completely before the addition of the next drop. About twenty drops are needed. A 1.5 mm disc is formed. 5-30 μg of sample is needed. (d) The plate transfer method (19) uses

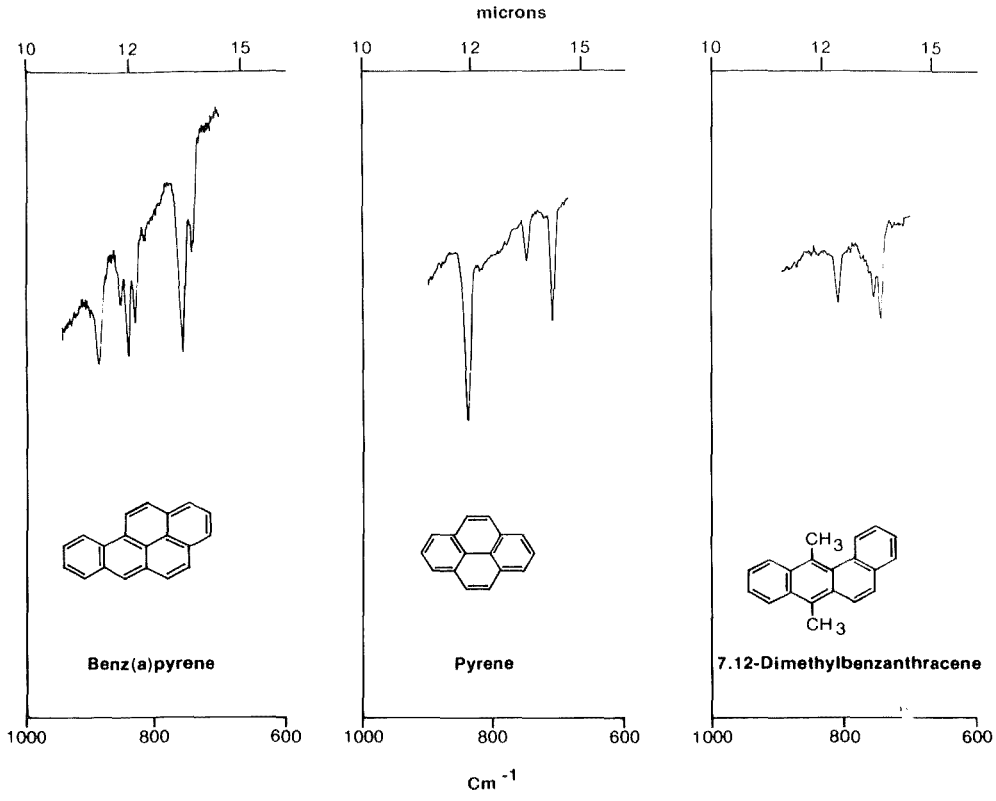

FIGURE 1

two plates, one coated with an adsorbent, the other with KBr, connected layer against layer the sample is spotted on the adsorbent layer after separation spots are eluted from the separating layer into the KBr layer. The size of sample required for identification is 50 µg.

All the above transfer methods have a common weakness, namely quantitative transfer of the sample from the plate into the KBr free of adsorbent. This was easily achieved in our laboratory by employing a unique elution system, the Eluchrom automatic elution system. This unit is capable of eluting six samples simultaneously. The amount of solvent

required for elution of each sample is approximate 150 µl. The sample is eluted directly into 3 mg KBr, placed in a 0.5 ml reaction vessel, after which the sample is evaporated and a 1.5 mm KBr pellet is prepared from which the spectra (shown in Figure 1) was recorded. The advantages of this method are:

1. A sample free of adsorbent is obtained (no silica gel bands were observed).

2. Only 150 µl of methanol was required to elute each of pyrene, benzo(a)pyrene, and dimethylbenzanthracene quantitatively with better than 98% recovery.

3. Six samples can be eluted simultaneously in approximately two minutes.

4. No loss of sample due to scraping and transferring, since these steps are eliminated by using the Eluchrom.

The amount of sample needed to produce good quality IR spectra ranges between 1-10 µg, depending on the sample. 5 µg were needed to produce the spectra shown in Figure 1.

Compared to the other TLC-IR techniques, the method presented here is superior, it is quantitative, sensitive, and time saving.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1221-1226 (1983)

STRUCTURE AND TLC MOBILITY RELATIONSHIPS FOR TEBUTHIURON AND RELATED THIADIAZOLES

Rex W. Souter and Rafik H. Bishara
The Lilly Research Laboratories
Eli Lilly and Company, M-769
307 E. McCarty Street
Indianapolis, IN 46285

ABSTRACT

Tebuthiuron and structurally-related thiadiazoles are separated by thin-layer chromatography on silica gel plates in two developing solvent systems. Relationships between chemical constitution and chromatographic mobility are discussed.

INTRODUCTION

The 5-substituted 1,3,4-thiadiazole ureas are of interest as possible herbicides (1). Tebuthiuron, 1-(5-t-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea, is an herbicide developed in these laboratories. Also known as GRASLAN®, SPIKE®, or PERFLAN®, the compound is effective for broad spectrum weed control. It has been found useful in forests and rangeland (2), in crops (3, 4) and is effective for control of woody plants (5, 6).

An organic synthesis of tebuthiuron has been described (1) as has been an improved synthesis of 1,3,4-thiadiazoles in general (7). However, little chromatographic work with tebuthiuron and related substances has been reported. HPLC of selected herbicides including tebuthiuron has been discussed (8) as well as GC analysis of the latter with its metabolites in grass and sugarcane (9). In a toxicology study of tebuthiuron metabolism

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in some animals, TLC of the herbicide and metabolites was reported (10).

In this work, separation of tebuthiuron from some structurally related thiadiazoles by TLC is reported. Mobilities of the spots are discussed with reference to structural features. With the exception of this work and (10), no reports of TLC behavior of thiadiazoles are known to exist.

EXPERIMENTAL

Apparatus

Thin-layer plates were precoated silica gel F_{254}, 20 cm x 20 cm with 0.25 mm adsorbent layer (E. Merck, available from Brinkmann Instruments, Inc., Westbury, N. Y.). Spotting pipets were disposable (Drummond type, distr. by Ace Glass Co., Louisville, Ky.). Rectangular, heavy-wall glass tanks 30 cm x 10 cm x 28 cm (Brinkmann) were used as developing chambers. The plates were viewed (after development and drying) under short-wave UV (254 nm) light using a Chromato-Vu (Ultra-Violet Products, Inc., San Gabriel, Ca.).

Reagents

Reagent grade solvents were used in these experiments. Methyl alcohol was used to dissolve the samples while 30 ml/75 ml acetone-chloroform (System A) or 70 ml/30 ml diethyl ether-acetonitrile (System B) were used to charge the developing tanks at each use. All test samples were from in-house sources.

Thin-Layer Chromatography

The adsorbent layer of each TLC plate was scored 15 cm above the starting line which was 2.5 cm from the bottom of the plate. One hundred micrograms of each compound was spotted in

adjacent lanes across the plates. Developing solvents were charged into the glass chambers lined on the sides and back with Whatman No. 1 filter paper, and 30 minutes equilibration time was allowed before use. Plates were developed at room temperature until the solvent reached the scored line. Plates were removed, allowed to dry at room temperature, and were viewed under 254 nm light.

RESULTS AND DISCUSSION

In Table 1, all compounds with the exception of VI have the general formula shown. Compound IV and the triazoline VI (structure shown complete in Table 1) are isomers.

While IV and V (tebuthiuron) contain t-butyl substituents as R_1, R_2 in IV is a secondary amine and in V is more tertiary-amine-like. The greater mobility of V may be explained by the low polarity (11) and adsorption capacity (12) of tertiary vs secondary amines. Mobilities of amines are also increased as chain length on the amino group increases (12) and with aliphatic substitution on the chain (13). Similar results were reported for some thiadiazole ureas and their metabolites (10).

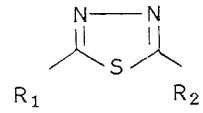
Note that R_f values are very low for compounds I - III in System A while System B starts to differentiate III from the others. Low R_f's may be due to solubility considerations, but III is more mobile as expected for reducing the amino character of the molecule (14).

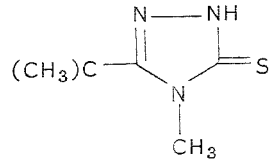
In Compounds III and V, substituent R_2 is constant but R_1 is varied. The aliphatic t-butyl group has the effect of lowering the polarity and thus increasing the mobility of the spot (14). The same argument applies to II and V. V is much more mobile than II because V is more aliphatic.

Triazoline Compound VI has a much greater mobility than its isomer IV which may be accounted for by two facts. VI lacks a conjugated double-bond system (15) and a secondary amine group at the 5-position on the ring.

TABLE I.

STRUCTURES AND TLC R_f VALUES FOR TEBUTHIURON AND RELATED THIADIAZOLES



Cmpd.	Substituent R_1	Substituent R_2	Rf in System A	Rf in System B
I	$-NHCH_3$	$-NHCH_3$	0.03	0.04
II	$-NHCH_3$	$-(NCH_3)CO(NHCH_3)$	0.03	0.07
III	$-(NCH_3)CO(NHCH_3)$	$-(NCH_3)CO(NHCH_3)$	0.07	0.15
IV	$-C(CH_3)_3$	$-NHCH_3$	0.18	0.35
V	$-C(CH_3)_3$	$-(NCH_3)CO(NHCH_3)$	0.30	0.55
VI			0.51	0.85

In addition to the related materials described in Table I, all starting materials and intermediates from the synthesis of tebuthiuron may be separated from the latter in either of the TLC systems.

ACKNOWLEDGEMENTS

The technical assistance of Mr. K. W. Taylor for completing the experimental TLC procedures is appreciated.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1227-1238 (1983)

ROUTINE HPTLC IN INDUSTRIAL ANALYSIS: PROCESS CONTROL OF FERMENTATIONS

F. Kreuzig
Research and Development, Biochemie Ges.m.b.H.
A-6250 Kundl/Austria

ABSTRACT

Quantitative HPTLC is a cost saving and reliable chromatographic routine method for the control of many fermentation processes, as for example the fermentation of penicillin V. The following substances are analyzed: lactose or sucrose, soya oil (against foaming), phenoxyacetic acid (precursor), penicillin V and p-hydroxypenicillin V, as final products. The derivatization after chromatography is performed with an automated spraying device, the measurement - perpendicularly to the direction of chromatography - and evaluation are computerized. The time requirements per sample range from 6 to 15 minutes, the pure analysis time per sample from 5 to 12 minutes. The break-down time of the complete HPTLC apparatus system is about 0,6 % of the working time, all substances to be determined can be measured with one scanner, in every interval and sequence desired. The accuracy, expressed by the coefficients of variation (N = 8 - 9, on one plate), ranges from 1,6 to 3,0 %,for very low concentrations up to 6,6 %.

INTRODUCTION

Reducing costs is a must in all fields of economy; it is also advisable to look, where analytical costs in research, development and production can be decreased. Especially for process control, additional methodic criteria,besides costs,are of importance, namely: speed, simplicity, reliability and accuracy.

Lecture, presented at the "Second International Symposium on Instrumental HPTLC" (2 - 5 May, 1982, in Interlaken)

0148-3919/83/0607-1227$3.50/0

A chromatographic method fulfilling these requirements is quantitative HPTLC, a method, which has already been used for 6 years in many laboratories. This method allows the routine process control of many substances in every sequence with only one measuring instrument.

Industrial fermentations for the production of antibiotics are characterized by the need of information about the concentration of different substances in the fermentation liquid during fermentation in certain intervals: carbohydrates and precursors for the biosynthesis of the antibiotic, oils against foaming and the antibiotic to be produced as well, so that adding different substances, batch by batch or continuously, can be performed in the fermenter if necessary.

An on-line coupling of an HPLC device directly to a fermenter for a continuous and automated analysis would be - from the point of view of automation - an optimum, but contradictions to this concept are the uncertainty of representative sampling, the extraction and dilution steps (1), and the break-down time of HPLC devices. Although quantitative HPTLC can be only particularly automated, this method offers an extremely high reliability. This reliability is a "conditio sine qua non", because the liquid in a fermenter represents an enormous financial value; an incorrect or delayed process control would result in high financial loss. In this paper, the process control of a penicillin V fermentation is presented.

The interesting parameters of a penicillin fermentation are the C-sources lactose or sucrose, vegetable oils, as soya oil for example, the precursor phenoxyacetic acid, and the final product penicillin V and p-hydroxyphenicillin V, a by-product, in low concentration, not desired.

EXPERIMENTAL

The plates used were commercially available HPTLC-plates; samples and standards, dissolved in chloroform, were applied with a Pt-Ir-capillary (250 nm), whilst solutions of water or water/methanol were applied with a CAMAG-Nano Applicator or CAMAG-Nanomat. For chromatography, TLC chambers (from TLC kit Merck no 11622) or twin-through chambers (CAMAG), lined with filter paper, were used.

The cleaning up of the samples from the penicillin fermenters is done in different ways, according to the biological matrix and the substances to be determined. The separations are performed conventionally in the linear manner over 3 to 5 cm, the derivatizations for several reasons preferably with a special spraying device (2, 3).

The measurement of the spots with a Zeiss-chromatogram-spectrophotometer is performed perpendicular to the direction of chromatography, to and from, the technique of automated evaluation is the same as shown earlier (2), with two exceptions: a balance (for weighing the fermentation broth samples) is coupled to the computer, and a fast integrator (HP 3390 A) is used, so that the speed of scanning can be raised to 100 mm/min. As function for the calibration line that of area^2/concentration, sometimes log a/log c have proved; the computation of the calibration line was not done by the least-square method, but by the percentual method, as described earlier (3).

Determination of lactose/sucrose

Materials:	HPTLC plates silicagel 60 F_{254}, 20 x 10 cm, Merck no 5642 CAMAG-Nano Applicator, 230 nl
Standards:	lactose and/or sucrose 1 1 mg/ml CH_3OH/H_2O = 1/1 2 2 mg/ml - " - 3 3 mg/ml - " -
Solvent system:	1-butanol/acetic acid/water = 40/50/7,5
Spray reagent:	2 ml aniline + 2 g diphenylamine + 10 ml H_3PO_4 + 88 ml CH_3OH
Samples:	The fermentation liquid is diluted with CH_3OH/water = 1/1 and filtered or centrifugated.
Procedure:	Samples A - I and standards 1 - 3 are applied as follows: A B 1 C D 2 E F 3 G H I A B 1 C D 2 E F 3 G H I The separation (distance 5 cm) takes about 30 minutes. The plate is dried for 30 minutes at 120°C, sprayed automatically with the reagent and placed in a drying

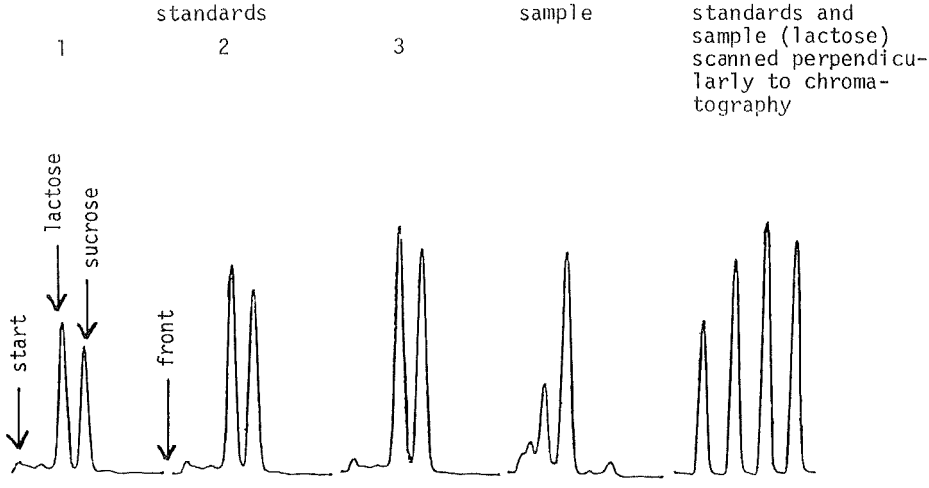

FIGURE 1
Chromatogram of Lactose/Sucrose Separation

oven for 15 minutes (110°C). Lactose (R_f = 0,16) shows grey-blue spots, sucrose (R_f = 0,26) brown-green spots.
Conditions: M-Pr, A/I/7, λ = 523 nm, remission, slit length 3,5 mm, slit width 0,5 mm, calibration line: a^2/c

Determination of soya oil

Materials:	HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642 Pt-Ir-capillary 250 nl
Standards:	soya oil 1 0,6 mg/ml $CHCl_3$ 2 1,2 mg/ml - " - 3 1,8 mg/ml - " -
Solvent system:	petrol ether/diethyl ether/acetic acid = 135/15/1,5
Samples:	The broth is extracted and diluted with $CHCl_3$.

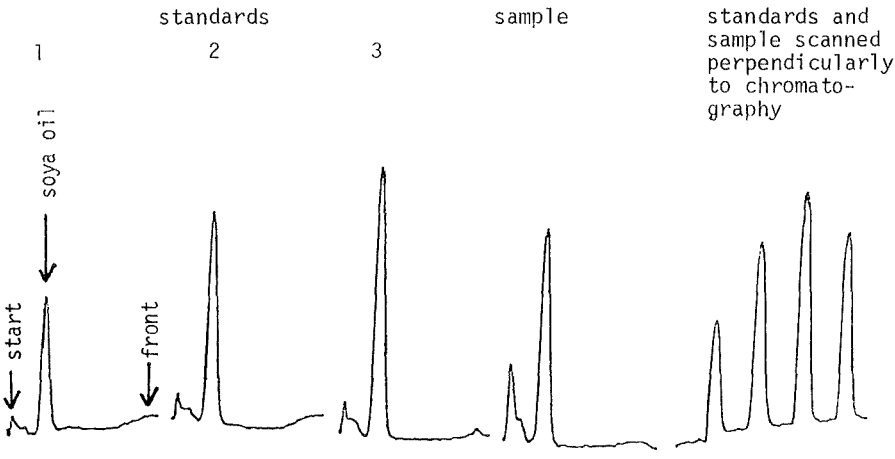

FIGURE 2
Chromatogram of Soya Oil Separation

Spray reagent: 3 g NH_4 molybdate + 25 ml water + 30 ml 0,1 N HCl + 15 ml $HClO_4$ (60 %) + 70 ml CH_3OH

After spraying the plate automatically, it is heated for 2 minutes at 115°C in a drying oven. R_f = 0,16, the spots are blue.

Conditions: M-Pr, A/I/7, λ = 630 nm, simultaneously in transmission and reflectance mode, $f_R : f_T$ = 100 : 20, slit length 6 mm, slit width 1,0 mm, calibration line: a^2/c

Determination of phenoxyacetic acid

Materials: HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642
CAMAG-Nano Applicator, 230 nl

Standard: phenoxyacetic acid
1 2 mg/ml water
2 4 mg/ml water
3 6 mg/ml water

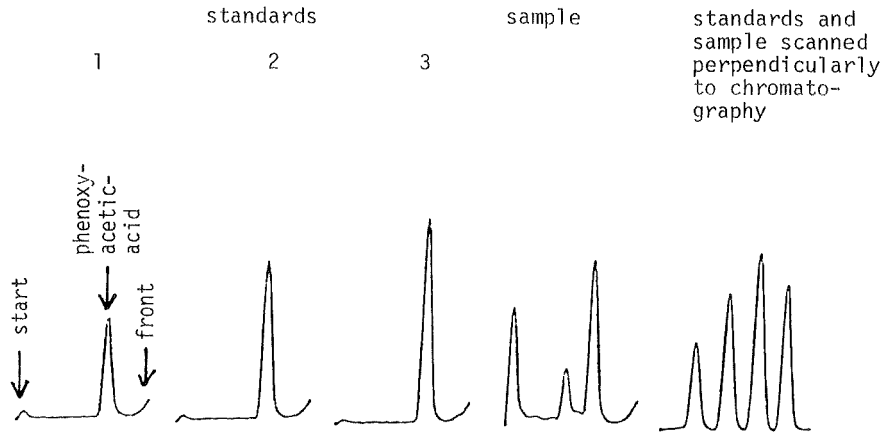

FIGURE 3
Chromatogram of Phenoxyacetic Acid Separation

To raise the solubility of the standard, the water must contain 1 mg $NaHCO_3$/ml.

Solvent system: butylacetate/methylenechloride/acetic acid = 80/10/)

Sample: The fermentation broth is filtered and the filtrate diluted with water.

Procedure: Samples A - I and standards 1 - 3 are applied as follows:
A B 1 C D 2 E F 3 G H I A B 1 C D 2 E F 3 G H I
The separation (distance 5 cm) takes about 7 minutes.
The plate is dried for 30 minutes at 120°C. R_f = 0,51.
Conditions: M-Pr, F/II/1, λ= 268 nm, remission, slit length 3,5 mm, slit width 1,5 mm, calibration line: a^2/c

Determination of penicillin V and p-OH-penicillin V

Materials: HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642, CAMAG-Nano Applicator, 230 nl

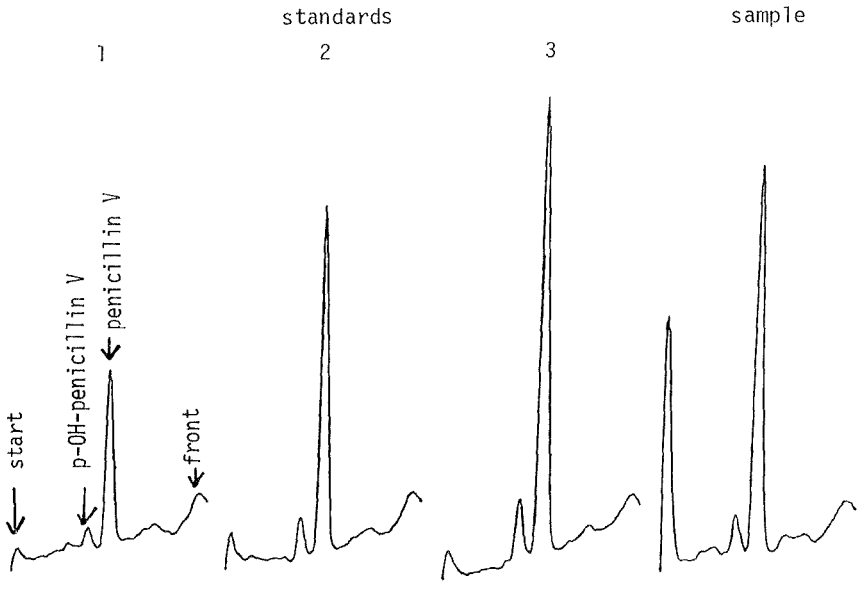

FIGURE 4
Chromatogram of Penicillin V and p-OH-Penicillin V Separation

Standard: penicillin V, K-salt; p-OH-penicillin V, Na-salt
1 2 mg penicillin V + 0,2 mg p-OH-penicillin V/ml water
2 4 mg - " - + 0,4 mg - " -
3 6 mg - " - + 0,6 mg - " -

Solvent system: toluene/ethylacetate/acetic acid = 40/40/20

Sample: 1 volume of fermentation liquid + 1 volume of CH_3CN, the precipitate is filtered and the filtrate diluted with water.

Procedure: The samples A - H and the standards 1 - 3 are applied in the following way:
A B 1 C D 2 E F 3 G H A B 1 C D 2 E F 3 G H
The separation distance of 5 cm is reached within 9 minutes, the plate is dried for 20 minutes at 120°C.

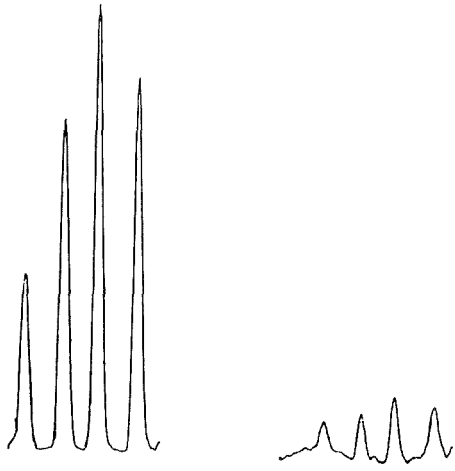

FIGURE 5

Chromatogram of Penicillin V and p-OH-Penicillin V Separation Standards and Sample, scanned perpendicularly to Chromatography

The R_f-value of penicillin V is 0,44, for p-OH-penicillin V 0,34.

Conditions: M-Pr, F/II/8, λ = 270 nm, remission, slit length 3,5 mm, slit width 1,0 mm, calibration line: log a/log c (for penicillin V)
a^2/c (for p-OH-penicillin V)

RESULTS AND DISCUSSION

The reason for the preference of quantitative HPTLC for routine analyses in antibiotic industry are, of course, the usual, wellknown benefits, as for instance speed (4, 5, 6, 7). But these benefits can be utilized to its fullest extent if chromatography and detection are optimized in such a way that measurement can be done perpendicularly to the direction of chromatography (3, 8), the saving of time is remarkable and besides that Gaussian peaks will result. With this method of mea-

surement a complete analytical system is available which is so fast that it is not displaced by HPLC. This kind of scanning HPTLC plates is practicable if the spot to be measured is separated sufficiently from neighboured spots who will often orginate from the matrix of the samples. The analyst has to select

- an appropriate method to clean up the sample (which should be a simple one-step method)
- and/or a special solvent
- and/or a selective derivatization reagent.

The optimal combination of these possibilities will always offer a proper solution of problems. It it is necessary to scan in the direction of chromatography, an automated system should be used (9).

The following table illustrates the speed of analyses:

TABLE 1

Time Requirement (min) for preparing and analyzing 8 - 9 Samples.

substance	preparation (A)	analysis (B)	(A)+(B)	$\frac{(A)+(B)}{sample}$	$\frac{(B)}{sample}$
lactose or sucrose	25	96	121	15	12
soya oil	10	38	48	6	5
phenoxy-acetic acid	10	55	65	7	6
penicillin V + p-OH-penicillin V	30	53	83	10	7

The slowest method is that for the determination of lactose/sucrose, because the solvent is rather viscous. On the other hand, separation with HPLC will take about 25 minutes per sample.

Simplicity and reliability depend on each other. Quantitative HPTLC is, in comparison to other chromatographic methods, not a sophisticated one and can be adapted quickly to various scanning conditions, the results can be given without delay at a distinct time.

In the last two years we could reduce the break-down time (for instruments for sample application, scanner, integrator, computer, printer) to about 0,6 % of the working time. For scanning, the presence of smoke, dust and vapor of aromatic solvents must be strictly avoided.

For evaluation of the accuracy of the quantitative determination of different substances one sample was analyzed 8 to 9 times on one plate and the coefficients of variation computed as follows:

TABLE 2
Accuracy of quantitative Determination

substance	coefficient of variation (%)	N
lactose/sucrose	1,6	9
soya oil	3,0	8
phenoxyacetic acid	2,1	9
penicillin V	1,9	8
p-OH-penicillin V	6,6	8

The value of 6,6 % is due to the low concentration of p-OH-penicillin V.

Typical quality coefficients of calibration lines are shown in table 3.

TABLE 3
Quality Coefficients of Calibration Lines

substance	quality coefficient (%)	calibration-line
lactose/sucrose	3,8	a^2/c
soya oil	4,4	a^2/c
phenoxyacetic acid	1,9	a^2/c
penicillin V	1,8	log a/log c
p-OH-penicillin V	7,5	a^2/c

These coefficients are stored on the disc of the computer and printed out on request, arranged according to substance. This information is of importance in controlling the quality of analytical work.

If one spot, standard 2, is scanned ten times, the following coefficients of variation were found:

TABLE 4
Accuracy for Scanning one Spot

substance	coefficient of variation (%)
lactose/sucrose	0,4
soya oil	0,6
phenoxyacetic acid	1,0
penicillin V	0,9
p-OH-penicillin V	2,7

These measurements may signal the analyst, if for example the spot shape should be further optimized.

Quantitative HPTLC is the cheapest chromatographic method. The more different substances have to be analyzed with the same priority - and this is typical for process control of fermentation - the more the superiority of this method is obvious. Furthermore the scanner can be used beneath routine analyses for working out and optimizing HPTLC-methods.

If these process control analyses for the determination of penicillin V would be performed with HPLC, there would be three complete liquid chromatographs necessary.

ACKNOWLEDGEMENTS

I am indebted to Mrs. Anna Greil, Miss Veronika Bichler and Miss Edith Trainer for their skilful technical assistance and to Mr. Helmut Wolff for writing the computer programs.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1239-1247 (1983)

A TLC-HPLC METHOD FOR DETERMINATION OF TIAZOFURIN (NSC 286193) IN SERUM

Cynthia C. Ackerly, Robert A. Newman* and John J. McCormack

Vermont Regional Cancer Center and *Departments of Pharmacology and Medicine, College of Medicine, Given Building, University of Vermont, Burlington, VT 05405 (U.S.A.)

INTRODUCTION

The nucleoside compound 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC 286193; tiazofurin; see Fig.1) has recently been shown to possess significant antitumor activity against L1210 and P388 murine leukemias (1,2). Its demonstrated antitumor efficacy in treating mice with Lewis lung carcinoma (2), a tumor refractory to many chemotherapeutic drugs, is of considerable potential significance. Anabolism of the compound is required for its antitumor activity and involves the production of a state of guanine nucleotide depletion secondary to inhibition

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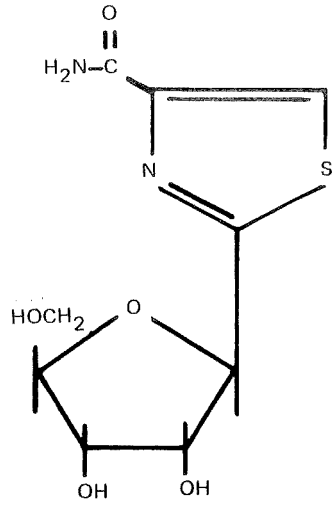

FIGURE 1

Structure of 2-β-D ribofuranosylthiazide-4-carboxamide (tiazofurin).

of inosine monophosphate (IMP) dehydrogenase (3). Thiazolecarboxamide adenine dinucleotide is formed in the metabolism of tiazofurin and appears to be responsible for the inhibition of IMP dehydrogenase (4,5).

The National Cancer Institute will soon commence with clinical Phase I trials of tiazofurin. The present study describes a useful and sensitive procedure for the determination of tiazofurin concentrations in biological fluids through a combination of thin layer (TLC) and high pressure liquid chromatographic (HPLC) techniques. The method has been applied for the measurement of serum

tiazofurin levels in mice and demonstrates the utility of this method in anticipated pharmacokinetic studies soon to begin in humans.

MATERIALS AND METHODS

Standards and Reagents

Tiazofurin (NSC 286193) was obtained from the Investigational Drug Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD, U.S.A.). 2-propanol, sodium acetate and HPLC grade water and methanol were purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.). Porcine serum was obtained from Pel-Freeze (Rogers, AR, U.S.A.) and was routinely used in the development of analytical methodology.

Chromatographic Apparatus and Conditions

All analyses were performed with a Spectra-Physics Model 8000 liquid chromatograph equipped with a Schoeffel Model 770 variable-wavelength U.V. detector set at 235 nm. The mobile phase consisted of 96% 0.02M sodium acetate pH 4.5 and 4% methanol which was used with an analytical reverse-phase C_{18} μ-Bondapak column (Waters Assoc. 3mm ID x 30cm, 10μ particle). A flow rate of 0 ml/min was used. A guard column (Brownlee Labs, 1C RP-18 LiChrosorb, 4.6mm ID x 3cm) was installed between the injector valve and the

main column for extended column life. Avicel-F (250μ particle size) TLC plates were purchased from Analtech (Newark, DE, U.S.A.).

Sample Preparation and Drug Analyses

For the determination of drug recovery, serum aliquots were spiked with known amounts of tiazofurin. Protein was precipitated from the spiked serum samples by addition of 5 volumes (V/V) of acetonitrile. Samples were centrifuged (600g, 10 min., room temp.) and the supernatant solution decanted and evaporated to dryness at 60°C with air purging in a Fisher IMD sample concentrator (Fisher Scientific, Pittsburg, PA, U.S.A.). The residue was reconstituted with 0.4ml HPLC grade water and an aliquot (50ul) of this suspension was spotted manually along with appropriate tiazofurin standards onto TLC plates.

Initial studies of the serum elimination of tiazofurin were performed using male CD_1 mice (Charles Rivers Canada, St. Constant, Quebec, Canada). Serum was obtained by retro-orbital puncture at specified times (0, 5, 10, 15, 30, 45, 60, 90 min.; 2, 3, 4, 5, 6, 7, 8 and 24 hrs.) after the intravenous administration of 250 mg/kg (750 mg/m^2 body surface area) of tiazofurin. There was sufficient tiazofurin concentration in the murine blood to permit direct spotting of small aliquots of plasma (10 ul) onto the TLC plate thereby eliminating the need for the deproteinization and concentration steps.

All TLC plates were developed over a six-hour period in a solvent system of 2-propanol:water (70:30, V/V) in a closed humidified chamber at room temperature. In this system tiazofurin migrated with an RF of 0.61. The area of the chromatogram corresponding to tiazofurin (identified under U.V. light at 254 nm) was excised and eluted into 0.3 to 0.5 ml of HPLC grade water. This suspension was sonicated, centrifuged (600g., 3 min.) and 0.1 ml of the supernatant solution was injected onto the HPLC column. The resulting peak was integrated by the preset program in the Spectra-Physics data system using peak area to determine drug concentration.

Results and Discussion

The analytical methodology for the determination of the thiazole derivative, tiazofurin, described in this paper is closely analogous to the combined TLC-HPLC approach recently reported by our group for the determination of a related thiadiazole derivative (6). A typical standard curve of tiazofurin extracted from spiked serum samples is shown in Fig. 2. Drug levels from HPLC injected samples are linear over at least one hundredfold concentration (10 ng to 1000 ng) with an average correlation coefficient of 0.995 as calculated by the least squares regression method. The average inter-day recovery of tiazofurin from serum is 88.4±2.4% despite the various chromatographic procedures to which the sample is subjected.

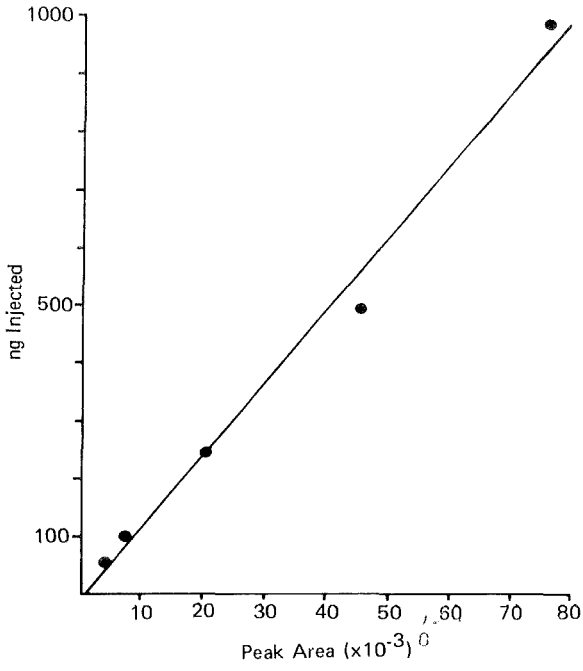

FIGURE 2

Standard curve of tiazofurin extracted from serum.

A representative chromatograph of the TLC-processed serum sample is shown in Fig. 3. This method, to the best of our knowledge, is the first reported for quantitation of tiazofurin in serum samples. Serum levels of tiazofurin as low as 3.2 ug/ml can be accurately and reproducibly determined.

In an effort to determine the applicability of this method for the measurement of murine serum drug pharmaco-

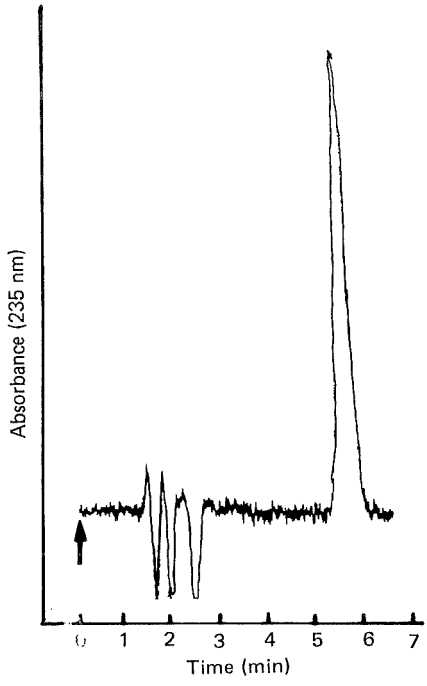

FIGURE 3

Representative chromatograph of TLC-processed mouse serum sample obtained 30 minutes following I.V. injection with tiazofurin. Arrow indicates point of injection.

kinetics, mice were administered 250 mg/kg tiazofurin which is one-tenth the LD_{10} (dose producing 10% mortality) for mice as reported by Southern Research Institute (7). The mouse LD_{10} dose ($MELD_{10}$) is typically ten times the initial drug dose administered to humans in a Phase I clinical trial of experimental cancer chemotherapeutic agents. Since serum drug levels were easily detected as late as 7 hr. after drug

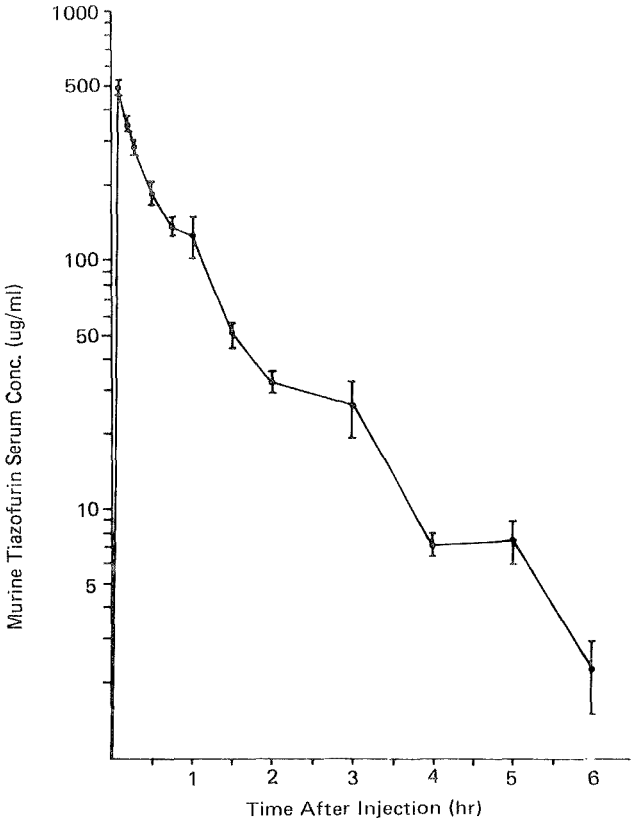

FIGURE 4

Serum drug concentrations from mice administered 250 mg/kg (I.V.) of tiazofurin. Data are presented as mean ± S.E. of 8 mice per time point.

administration, it is clear that this method will be suitable for establishing pharmacokinetic parameters in humans. As seen in Fig. 4, a terminal drug elimination phase of 60 to 72 min. can be calculated from the murine serum drug levels; more

extensive studies will obviously be required for complete kinetic analysis in mice and in anticipated Phase I studies in man.

ACKNOWLEDGEMENTS

This work was supported by grants PHS P30 CA 22435 and contracts No. NO1 CM 97278, and No. NO1 CM 27547 awarded by the National Cancer Institute.

The authors wish to thank Jeanette Hartshorn and Linda Mathews for skillful technical assistance, also Joan Barney for her assistance in the preparation of this manuscript.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1249-1252 (1983)

QUANTITATIVE SEPARATION OF Zr^{4+} from La^{3+} and Ce^{3+}; W^{6+} FROM Cr^{3+}, Mo^{6+} and VO^{2+} BY THIN LAYER CHROMATOGRAPHY.

M. QURESHI
CHEMISTRY SECTION
Z.H. ENGINEERING COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH, INDIA

B.M. SETHI and S.D. SHARMA
CHEMISTRY DEPARTMENT
HINDU COLLEGE
MORADABAD, INDIA

ABSTRACT

Methods have been developed for the quantitative separation of Zr^{4+} from La^{3+} and Ce^{3+} using 0.1M ammonium oxalate solution as eluant. W^{6+} can also be separated quantitatively from Cr^{3+}, Mo^{6+} and VO^{2+} using DMSO-1MHCl (1:9). Both the methods are fast and quantitative. The separation takes about 40-50 min. The average error is about 3%.

INTRODUCTION

The thin layer chromatography of metal ions on silica gel layers has been studied during the last ten years (1-11). Very few references are available for the quantitative separation of metal ions using TLC (4,5) which offers a simple, rapid and versatile method for the separation of metal ions. However, it has been used more in organic analysis than in inorganic one.

In this article, we present a method for the separation of Zr^{4+} from La^{3+} and Ce^{3+}. This separation has been achieved using a simple solvent i.e., 0.1M ammonium oxalate solution. W^{6+} has been separated from Cr^{3+}, Mo^{6+} and VO^{2+} using DMSO-1M HCl (1:9) as the developing system.

MATERIALS AND METHODS

Apparatus

Thin layer chromatography apparatus (Toshniwal, India) for the preparation of silica gel-G layers on 20x3 cm. glass

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plates was used. These plates were developed in glass jars (20x5cm). Spectrophotometric studies were performed on a Spekol colorimeter (made in Germany).

Reagents

Silica gel-G, dimethyl sulphoxide (DMSO), sodium tungstate and zirconium oxychloride were all from B.D.H., England. Other chemicals were of AnalaR grade.

Test solutions and Detectors

The test solutions were generally 0.1M in metal chloride, nitrate or sulphate. 0.1M solutions of sodium tungstate and zirconium oxychloride were used. All the cation solutions were made in 5% citric acid solution. Conventional spot test reagents were used for detection purposes (12).

Preparation of Silica gel-G layers

The slurry used was prepared by mixing silica gel-G with demineralised water in the ratio of 1:3 with constant shaking for 5 min. The slurry was coated on clean glass plates with the help of an applicator to give a layer of ~ 0.15 mm thickness for qualitative studies and ~ 0.50 mm thickness for quantitative studies. The plates were first dried at room temperature and then in an electric oven at 100 ± 5°C for about 2 hr. These plates were then stored in an oven at room temperature until used.

Procedure

The metal ion solution was spotted with the help of a lambda pipette in the form of a streak. The development was performed in the chosen solvent systems. The solvent was allowed to ascend 13 cm from the starting line on the plates in all cases. A pilot plate was run simultaneously in order to locate the exact position of the spot on the plate. The area of Zr^{4+} and W^{6+} was scratched and was eluted with 1:1 HCl and 0.5M NaOH solutions respectively. The final volume of the filtrate in case of Zr^{4+} was reduced to about 1 ml. by evaporation and then was determined spectrophotometrically using alizarin at 560 mμ (13). In case of W^{6+} the final volume of the filtrate was reduced to about 10 ml and was determined spectrophotometrically by thiocyanate method (14).

RESULTS

The quantitative separation of Zr^{4+} from La^{3+} and Ce^{3+} in 0.1M ammonium oxalate is summarized in table 1. Table 2 shows the quantitative separation of W^{6+} from Cr^{3+}, Mo^{6+} and VO^{2+} in DMSO-1M HCl (1:9).

TABLE 1
Quantitative Separation Of Zr^{4+} From La^{3+} and Ce^{3+}

Zr^{4+} taken (µg)	Interfering ions added (µg)	Zr^{4+} found (µg)	Error (%)
20	La^{3+} (52.9)	19.25	-3.75
80	La^{3+} (26.4)	78.75	-1.56
20	Ce^{3+} (52.5)	20.00	-
80	Ce^{3+} (26.2)	77.50	-3.12
20	(La^{3+}+Ce^{3+}) (26.4+26.2)	19.25	-3.75
80	(La^{3+}+Ce^{3+}) (26.4+26.2)	76.00	-5.00

DISCUSSION

0.1M ammonium oxalate solution is an interesting solvent. It separates Zr^{4+} (R_f = 0.10) from La^{3+} (R_f = 0.83) and Ce^{3+} (R_f = 0.84). Zr^{4+} is probably precipitated as zirconium oxalate while La^{3+} moves almost to the solvent front. When 0.1M ammonium oxalate solution is added to the La^{3+} solution in test tube, a white precipitate is obtained. This points to the fact that the reaction in the test tube may be quite different from the reaction on the plate. The results in table 1 show that Zr^{4+} can be separated quantitatively from La^{3+} in the ratio of 1:2.5 and 2.5:1 and from Ce^{3+} in varying ratios. Zr^{4+} can also be separated from a mixture of La^{3+} and Ce^{3+} by using this solvent.

W^{6+} is quantitatively separated from Mo^{6+}, Cr^{3+} and VO^{2+} using DMSO-1M HCl (1:9). In excess HCl, W^{6+} is probably precipitated

TABLE 2
Quantitative Separation Of W^{6+} From Cr^{3+}, Mo^{6+} And VO^{2+}

W^{6+} taken (µg)	Interfering ions added (µg)	W^{6+} found (µg)	Error (%)
480	Cr^{3+} (80)	460	-4.16
200	Cr^{3+} (80)	190	-5.00
480	Mo^{6+} (123)	468	-2.56
200	Mo^{6+} (123)	182	-4.03
480	VO^{2+} (175)	471	-1.87
200	VO^{2+} (175)	182	-4.03

as tungstic acid while Mo^{6+}, Cr^{3+} and VO^{2+} move almost to the solvent front. The results are given in table 2. The methods are precise and accurate and it is possible to separate small amounts of Cr^{3+}, Mo^{6+} and VO^{2+} from a very large amount of W^{6+}.

ACKNOWLEDGEMENT

The authors thank the University Grants Commission, New Delhi (India) for financial assistance to B.M.S.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1253-1264 (1983)

PREDICTION OF K_{sp} FROM Rf VALUES: THIN LAYER CHROMATOGRAPHY OF 47 METAL IONS ON STANNIC ARSENATE IN AQUEOUS HYDROCHLORIC ACID SYSTEMS.

S.D.SHARMA*, T.R.SHARMA and B.M.SETHI
Chemistry Department, Hindu College, Moradabad, 244001, India.

Abstract

The adsorption behaviour of 47 metal ions has been studied in aqueous HCl systems using stannic arsenate layers. The effect of solvent pH on R_f alongwith the complexation effect of HCl has been investigated. On the basis of R_f values the K_{sp} of some metal arsenates have been predicted. HCl has been utilized to resolve some binary and ternary mixtures such as Be^{2+} - Mg^{2+}, Zr^{4+} - La^{3+}, Th^{4+} - Ce^{4+}, Th^{4+} - UO_2^{2+}, Cr^{3+} - Mo^{6+} or W^{6+}, Sb^{3+} - Bi^{3+}, Cd^{2+} and Ag^{+} - Cu^{2+} - Cd^{2+}.

INTRODUCTION

The use of inorganic ion-exchangers in thin layer chromatography of metal ions has received some attention in recent years (1-5). In these studies a binder such as silica gel, starch or cellulose, which clouds the interpretaion of the mechanism, is not used. However, the following limitations are noticed:

(a) Numerous ions have not been systematically studied.
(b) The effect of solvent pH on the Rf value has not been investigated and hence the separations can not be put to much practical use.

Stannic arsenate is known to possess some unusual and promising ion exchange properties (6,7). Its analytical utility has been demonstrated in paper chromatography (8-10) and electrochromatography (11) of inorganic ions. The present study summarises our efforts to use thin layers of this material without any binder in aqueous HCl systems. As a result some very important and difficult separations were achieved. HCl has been chosen owing to its complex forming ability. An effort has been made to predict on the basis of Rf values, the Ksp of some metal arsenates.

*Address for correspondence:
Dr. S.D.Sharma, A-82, Gandhi Nagar, Moradabad, 244001, INDIA.

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EXPERIMENTAL

Apparatus:

Thin layer chromatography applicator of Toshniwal (India) was used to prepare the layers on 20 x 3.5 cm glass plates. Chromatography was performed in 24 x 6 cm glass jars.

Reagents:

Stannic chloride pentahydrate (PPH, Poland) and sodium arsenate heptahydrate (Riedel, Germany) were used. All other chemicals were of AnalaR grade.

Test solutions and Detectors:

Test solutions were generally 0.1 M in the metal nitrate of chloride and were prepared as described earlier (8). Conventional spot test reagents were used for detection purposes (8).

Preparation of Thin Layer Plates:

Stannic arsenate in the H^+ form, prepared according to the procedure described earlier (6), was powdered and slurried with a little demineralized water in a mortar. Vigorous grinding for a long time proved to be very important for complete adhesion. The slurry was then spread over the clean glass plates with the help of an applicator, and uniform thin layers (~ 0.1 mm. thick) were obtained. The plates were ready for use after drying at room temperature.

Procedure:

One or two drops of the test solution were placed on the plates with thin glass capillaries. After drying the spots, development was made in different solvent systems and the ascent was fixed as 11 cm in all cases. After development, the plates were dried and the cation spots were detected using the appropriate spraying agent.

RESULTS

The chromatographic behaviour of 47 ions on stannic arsenate layers has been studied using HCl as solvent in the concentration range 10^{-5} M to 5 M. The binary and ternary separations achieved experimentally are given in Tables 1 and 2. The ions investigated were :
Ag^+, Tl^+, Hg_2^{2+}, K^+, Rb^+, Cs^+, Hg^{2+}, Pd^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, UO_2^{2+}, VO^{2+}, Ca^{2+}, Be^{2+}, Zn^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Sb^{3+}, Bi^{3+}, Fe^{3+}, Cr^{3+}, Ir^{3+}, Al^{3+}, Ga^{3+}, Au^{3+}, Y^{3+}, La^{3+}, Pr^{3+}, Nd^{3+}, Sm^{3+}, Ce^{3+}, In^{3+}, Pt^{4+}, Se^{4+}, Te^{4+}, Ce^{4+}, Ti^{4+}, Th^{4+}, Zr^{4+}, Mo^{6+} and W^{6+}.

In order to check the reproducibility of the R_f values, some ions were chromatographed in 1.0 M HCl, 0.1 M HCl and 0.01 M HCl. It was observed that the variations does not exceed 10% of the average R_f value.

DISCUSSION

It is evident from Tables 1 and 2 that HCl in a wide concentration range offers numerous possibilities for analytically difficult separations, which

TABLE 1

Some Binary Separations Achieved On Stannic Arsenate Layers

Solvent	Separations Achieved (R_T-R_L)
0.00001 M HCl	Cu^{2+} (0.00 - 0.16) - Ni^{2+} (0.58 - 0.80)
	Cr^{3+} (0.86 - 0.96) - VO^{2+} (0.00 - 0.08)
	Mn^{2+} (0.70 - 0.80) - VO^{2+} (0.00 - 0.06)
	Ni^{2+} (0.56 - 0.74) - VO^{2+} (0.00 - 0.10)
	Y^{3+} (0.00 - 0.10) - Mg^{2+} (0.72 - 0.98)
	Al^{3+} (0.00 - 0.04) - Mg^{2+} (0.70 - 0.95)
	Be^{2+} (0.00 - 0.05) - Ba^{2+} (0.74 - 0.84)
	Al^{3+} (0.00 - 0.08) - Ba^{2+} (0.75 - 0.86)
	Ti^{4+} (0.00 - 0.05) - Cr^{3+} (0.85 - 0.96)
0.001 M HCl	Cu^{2+}(0.00 - 0.18) - Mn^{2+} (0.73 - 0.91)
	Fe^{3+}(0.00 - 0.20) - Mn^{2+} (0.75 - 0.95)
	Pb^{2+}(0.00 - 0.06) - Mn^{2+} (0.70 - 0.95)
	Ti^{4+}(0.00 - 0.00) - Mn^{2+} (0.75 - 0.92)
	Al^{3+}(0.00 - 0.15) - VO^{2+} (0.51 - 0.60)
	Be^{2+}(0.00 - 0.06) - Ga^{3+} (0.30 - 0.58)
0.1 M HCl	UO_2^{2+} (0.00 - 0.10) - VO^{2+} (0.55 - 0.63)
	MoO_2^{2+} (0.00 - 0.03) - VO^{2+} (0.53 - 0.65)
	Cu^{2+} (0.00 - 0.16) - Cd^{2+} (0.64 - 0.76)
	Cu^{2+} (0.00 - 0.22) - Cr^{3+} (0.67 - 0.78)
	Cu^{2+} (0.00 - 0.21) - Mg^{2+} (0.70 - 0.98)
	Pb^{2+} (0.00 - 0.08) - Hg^{2+} (0.70 - 0.80)
	Pb^{2+} (0.00 - 0.10) - Cd^{2+} (0.65 - 0.76)
	Bi^{3+} (0.00 - 0.08) - Cd^{2+} (0.64 - 0.77)
	Bi^{3+} (0.00 - 0.05) - Pd^{2+} (0.62 - 0.80)
	Ti^{4+} (0.00 - 0.00) - Au^{3+} (0.82)- 1.00)
	Ti^{4+} (0.00 - 0.00) - VO^{2+} (0.55 - 0.64)
	Mo^{6+} (0.00 - 0.04) - Au^{3+} (0.80 - 1.00)
0.5 M HCl	Fe^{2+} (0.59 - 0.80) - Fe^{3+} (0.00 - 0.20)
	Tl^{+} (0.00 - 0.00) - Tl^{3+} (0.89 - 1.00)
	Fe^{3+} (0.00 - 0.20) - Ni^{2+} (0.72 - 0.87)
	Fe^{3+} (0.00 - 0.21) - VO^{2+} (0.68 - 0.78)
	Bi^{3+} (0.24 - 0.44) - Sb^{3+} (0.00 - 0.00)
	UO_2^{2+} (0.59 - 0.80) - Pb^{2+} (0.00 - 0.16)
	Te^{4+} (0.00 - 0.00) - Pd^{2+} (0.80 - 1.00)

Table 1 (Contd.)

Solvent	Separations Achieved (R_T-R_L)
1.0 M HCl	Fe^{3+} (0.00 - 0.16) - Cr^{3+} (0.90 - 1.00)
	Cr^{3+} (0.87 - 1.00) - Mo^{6+} (0.00 - 0.12)
	Cr^{3+} (0.90 - 1.00) - W^{6+} (0.00 - 0.08)
	Th^{4+} (0.00 - 0.10) - Ce^{3+} (0.71 - 0.92)
	Th^{4+} (0.00 - 0.12) - Ce^{4+} (0.60 - 0.94)
	Zr^{4+} (0.00 - 0.06) - La^{3+} (0.70 - 0.92)
2.0 M HCl	Zn^{2+} (0.71 - 0.95) - Hg^{2+} (0.48 - 0.60)
	Th^{4+} (0.00 - 0.08) - UO_2^{2+} (0.35 - 0.55)
	Th^{4+} (0.00 - 0.05) - Y^{3+} (0.82 - 0.97)
3.0 M HCl	Hg_2^{2+} (0.00 - 0.00) - Hg^{2+} (0.62 - 0.82)
	Th^{4+} (0.00 - 0.00) - VO^{2+} (0.66 - 0.74)
	Th^{4+} (0.00 - 0.04) - Mg^{2+} (0.90 - 1.00)
0.1 M HNO_3	Cr^{3+} (0.00 - 0.00) - Ni^{2+} (0.55 - 0.71)
	Be^{2+} (0.00 - 0.12) - Mg^{2+} (0.77 - 0.95)

have been actually realised. Some of the more important are Be^{2+} - Mg^{2+}, Zr^{4+} - La^{3+}, Th^{4+} - Ce^{4+}, Th^{4+} - UO_2^{2+}, UO_2^{2+} - VO^{2+}, Cr^{3+} - Mo^{6+} or W^{6+} and Sb^{3+} - Bi^{3+} - Cd^{2+}, Ag^{+} - Cu^{2+} - Cd^{2+} etc.

These separation possibilities arise from two effects:
(a) Formation of Chloro-complexes due to the presence of HCl,
(b) Selective adsorption of certain cations by stannic arsenate layers.

In order to bring out the more interesting features of these studies R_f values were plotted against pH (Figs. 1a and 1b). The following trends were noticeable:

(i) For most cations there is no significant change in R_f values with the change in pH. Ag^{+}, Hg_2^{2+}, Sb^{3+}, Tl^{+}, Fe^{3+}, Mo^{6+}, W^{6+}, Se^{4+}, Te^{4+}, Th^{4+} and Zr^{4+} have almost zero R_f value at all pH. Ag^{+} is strongly adsorbed on stannic arsenate. Tl^{+} is probably precipitated as TlCl or Thallous arsenate. Sn^{4+} from stannic arsenate precipitates Se^{4+}, Te^{4+}, Mo^{6+} and W^{6+}. Excessive hydrolysis of Sb^{3+} in acidic solution may be attributed to its very low R_f value. Stannic arsenate is highly selective for Fe^{3+} and even at low pH its R_f is not significant. Zr^{4+} and Th^{4+} form insoluble arsenates

TABLE 2

Some Ternary Separations Achieved On Stannic Arsenate Layers

Solvent	Separations Achieved (R_T - R_L)
0.00001 M HCl	Ag^+ or Bi^{3+} or Pb^{2+} (0.00 - 0.06) - Cd^{2+} (0.48 - 0.60) - Pd^{2+} (0.80 - 0.98)
0.1 M HCl	Ag^+ or Se^{4+} or Te^{4+} (0.00 - 0.00) - Au^{3+} (0.80 - 0.96) - Ni^{2+} (0.50 - 0.68)
0.5 M HCl	Ag^+ (0.00 - 0.00) - Cu^{2+} (0.75 - 1.00) - Cd^{2+} (0.48 - 0.58)
1.0 M HCl	Fe^{3+} or Mo^{6+} (0.00 - 0.16) - UO_2^{2+} (0.30 - 0.49) - VO^{2+} (0.68 - 0.78)
3.0 M HCl	Ag^+ (0.00 - 0.04) - Cu^{2+} (0.50 - 0.68) - Au^{3+} (0.80 - 1.00)
5.0 M HCl	Ag^+ (0.00 - 0.05) - Pb^{2+} (0.33 - 0.45) - Cu^{2+} (0.70 - 0.75)
	Sb^{3+} (0.00 - 0.00) - Bi^{3+} (0.53 - 0.61) - Cd^{2+} (0.80 - 0.98)

resulting in almost zero R_f value. Hg_2^{2+} precipitates as its chloride and thus there is no movement. Hg^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Ir^{3+}, Au^{3+}, Pt^{4+}, Fe^{2+}, Mg^{2+}, Ca^{2+} and Pr^{3+} have high and constant R_f values. This is due to the solvation of the cations or their chloro complexes. Au^{3+} and Pt^{4+} exist as their anionic complexes which are not sorbed significantly. Hg^{2+} exists almost exclusively as $HgCl_2$ which is largely covalent in character and hence it has R_f value of approximately 1. Zn^{2+} and Mn^{2+} form anionic complexes like $[ZnCl_4^{--}]^{2-}$ and $[MnCl_4^{--}]^{2-}$ resulting into a higher R_f.

(ii) For K^+, Rb^+ and Cs^+, the R_f value slightly decreases from pH 0 to 1. Further increase in pH has no effect on the R_f values of these cations. These metals are not known to form complexes and the decrease in R_f is due to strong adsorption on the stannic arsenate layers.

(iii) Cations such as Bi^{3+}, Pb^{2+}, Cu^{2+}, UO_2^{2+}, Al^{3+}, Be^{3+}, In^{3+}, Ce^{4+} and Ti^{4+} show a sharp decrease in R_f values between pH 0 and 1. At pH $>$ 1 Bi^{3+} has zero R_f value owing to hydrolysis but at lower pH there is an increase in R_f value due to the formation of anionic complexes such as $BiCl_4^-$. Similarly in the case of Pb^{2+}, at lower pH i.e. at higher Cl^-

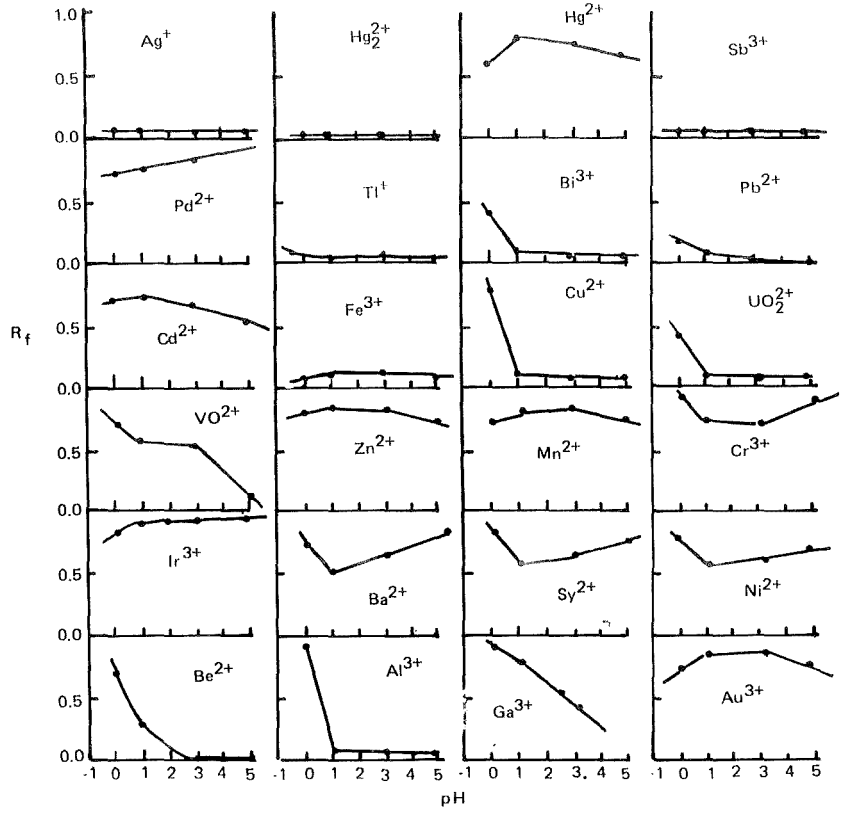

Fig. 1a & b: Plots of R_f vs. pH.

concentration, it forms anionic complexes such as $[PbCl_4^{--}]^{2-}$ which results in an increase in the R_f value of the cation. The same explanation holds good for other cations as well. Ions such as Ba^{2+}, Sr^{2+}, Ni^{2+}, Co^{2+} and Cr^{3+} have minimum R_f at pH 1. This is probably due to the maximum ion-exchange which occurs at this pH.

(iv) Ga^{3+}, VO^{2+}, Y^{3+}, La^{3+}, Nd^{3+}, Sm^{3+} and Ce^{3+} show exceptional behaviour. At pH $\rangle$ 2 Nd^{3+} and Sm^{3+} are almost completely adsorbed on stannic arsenate layers resulting in a very low R_f. The R_f values of VO^{2+}, Ga^{3+} and Ce^{3+} gradually decrease with an increase in pH and become almost zero at pH 5. This is probably due to the formation of stable complexes at higher Cl^{-} concentration as evidenced by Figure 2.

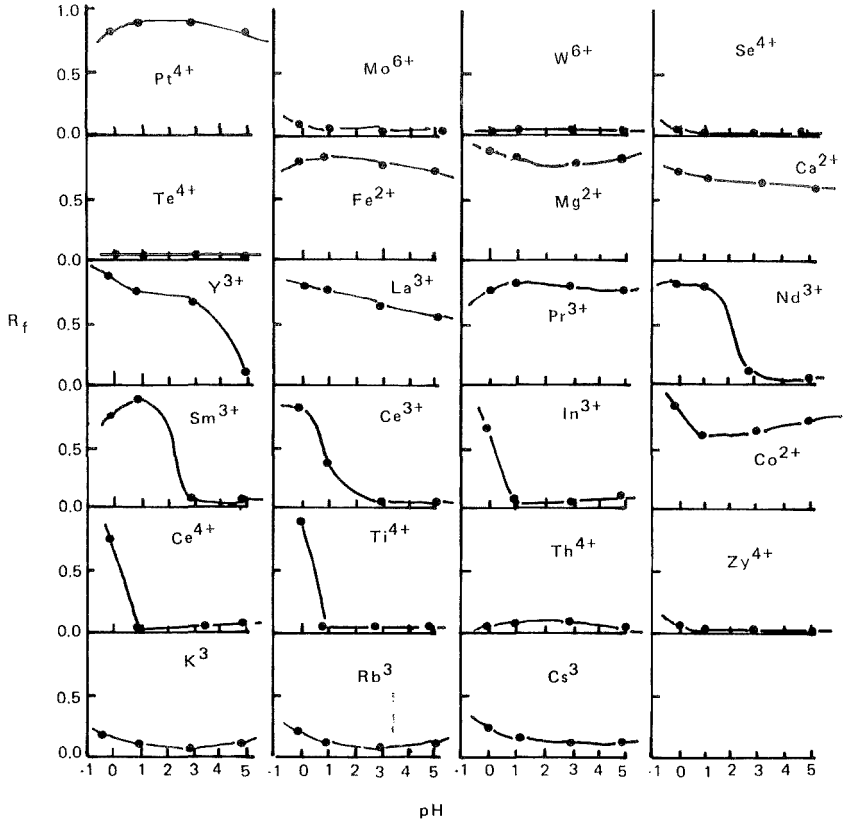

Fig. 1a & b (Contd.)

A survey of the plots (Figs 1a and 1b) reveals that the R_f generally decreases with an increase in pH, the steep fall being between pH 0 and 1. The value further decreases upto pH 2 and then becomes almost constant. This shows that pH 1 to 2 is the most favourable acidity for ion-exchange. This observation is in conformity to our earlier experience on stannic arsenate papers (9).

A comparison of R_f values in 0.1 M HNO_3 on stannic arsenate layers and stannic arsenate impregnated papers (9) prepared under similar conditions suggests that there is a decrease in the R_f value on layers for almost all cations probably due to the higher adsorption. Greater total ion-exchange capacity of the thin layer plates than that of the papers may be responsible

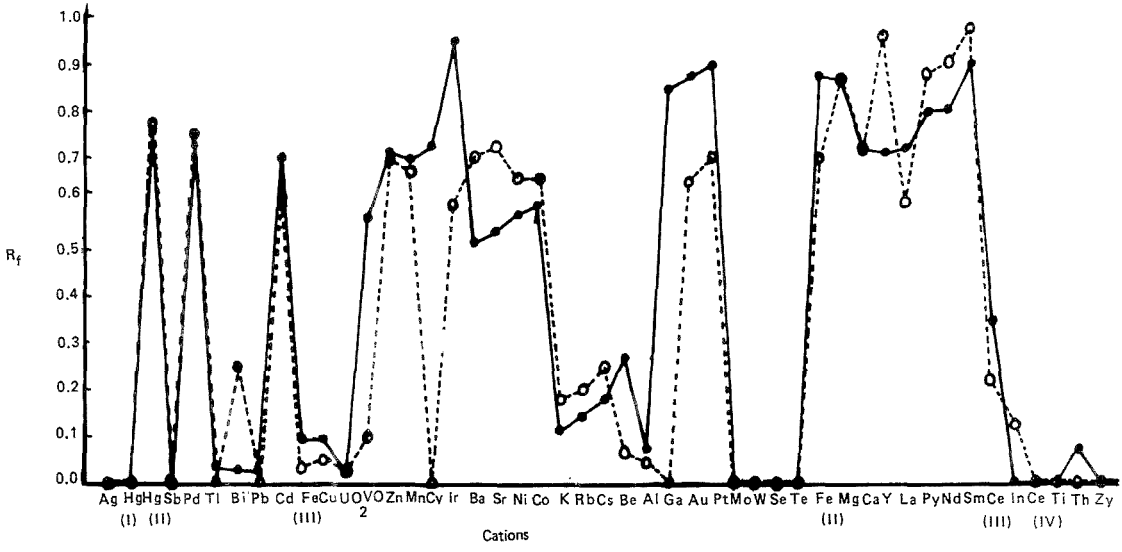

Fig. 2 : Comparison of R_f values in 0.1 M HCl and 0.1 M HNO_3 on stannic arsenate layers.

TABLE 3

Precipitation of Cations in the Mixture of Solvent and Impregnating Material

Solvent	Cation + Sodium arsenate + Solvent	
	Cations which precipitate	Cations which do not precipitate
1.0 M HCl	Hg_2^{2+}, Te^{4+}, Tl^{+}, Ag^{+}, Zr^{4+}	Fe^{3+}, Se^{4+}, Mo^{6+}, W^{6+}, Th^{4+}, Sb^{3+}

for this decrease. The decrease is more pronounced in case of Tl^{+}, Cu^{2+}, VO^{2+}, Cr^{3+}, Y^{3+} and La^{3+}. This is due to the fact that stannic arsenate is more selective for these cations and thus there is a greater exchange resulting in much lower R_f value.

Ions which have zero R_f values may do so owing to (a) precipitation, (b) ion-exchange and (c) strong adsorption due to high charge. In order to similate conditions on thin layers, sodium arsenate was added to the cation solution followed by the solvent. A number of ions precipitate under these conditions (Table 3). In these cases the precipitation mechanism hol-ds good. For other ions, Fe^{3+} is selectively adsorb-ed by stannic arsenate, Sb^{3+} hydrolyses and Se^{4+}, Th^{4+}, Mo^{6+} nd W^{6+} are strongly adsorbted owing to their high charge.

To study the effect of complexation, R_f values were determined in 0.1 M HNO_3. A plot of R_f versus cations for 0.1 M HCl and 0.1 M HNO_3 as solvent (Fig.2) indicates the increase in the R_f value of certain cations such as VO^{2+}, Cr^{3+}, Ga^{3+}, Ir^{3+}, Be^{2+} and Th^{4+} due to the formation of anionic chloride complexes. A higher R_f value of Au^{3+} and Pt^{4+} in HCl media can be explained on the same basis.

It is known that R_f value amongst other factors, depend-s upon the solubility product of the metal salt. On co-relating the R_f values of the metal ions with the K_{sp} of their arsenates, the following linear relationship is obtained:

$$R_f = 0.325 + 0.011 \quad \frac{10^3}{-\log K_{sp}}$$

Figure 3 shows this linear relationship. With the help of this equation it is possible to predict the K_{sp} values of different metal arsenates (Table 4). The K_{sp} values of the various arsenates which have been used in plotting figure 3 are given in Table 5. In our earlier communication (8), we have predicted the K_{sp} of metal arsenates from the R_f value of metal ions on stannic arsenate papers. The only difference in the two

TABLE 4

Predicted Values of K_{sp} for Different Metal Arsenates

Metal arsenate	K_{sp}
Hg^{2+}	2.5×10^{-39}
Pd^{2+}	4.6×10^{-30}
Ir^{3+}	2.8×10^{-21}
Be^{2+}	6.9×10^{-28}
Ga^{3+}	2.2×10^{-17}
Fe^{2+}	5.2×10^{-26}
Y^{3+}	1.7×10^{-22}
La^{3+}	2.1×10^{-23}
Ce^{3+}	2.1×10^{-23}
Ce^{4+}	1.9×10^{-25}

TABLE 5

Experimental Values of K_{sp} for Some Metal Arsenates

Metal arsenate	K_{sp}
Pb^{2+}	4.1×10^{-36}
Cu^{2+}	7.6×10^{-36}
Cd^{2+}	2.2×10^{-33}
Mn^{2+}	$(1.9) \times 10^{-29}$
Cr^{3+}	$(7.8) \times 10^{-21}$
Zn^{2+}	1.3×10^{-28}
Co^{2+}	$(7.6) \times 10^{-29}$
Sr^{2+}	8.1×10^{-19}
Ca^{2+}	6.8×10^{-19}
Al^{3+}	$(1.6) \times 10^{-16}$
Ni^{2+}	$(3.1) \times 10^{-26}$
Mg^{2+}	2.1×10^{-20}

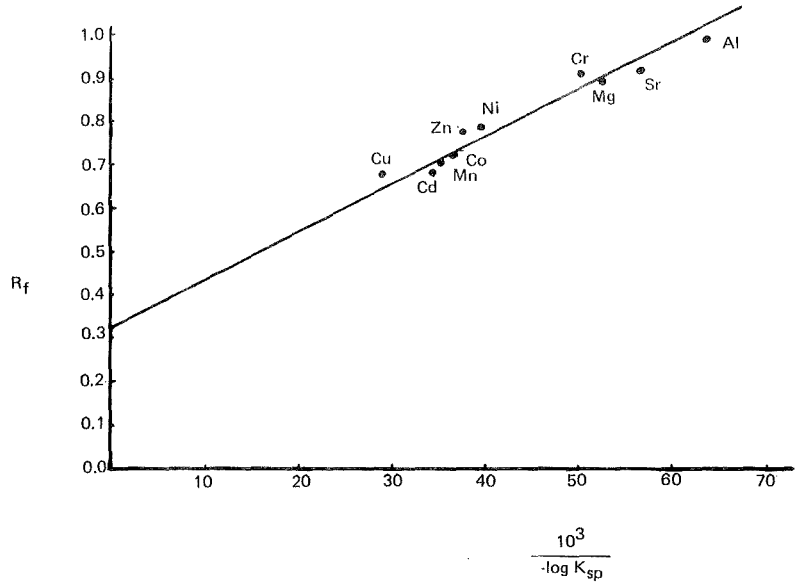

Fig. 3 : Plot of R_f vs. $\frac{10^3}{-\log K_{sp}}$ for metal ions in 1M HCl.

linear relationships obtained for impregnated papers and thin layer plates is that in the latter case, the charge on the ion and the bare ion radii has little effect on the R_f value and only the K_{sp} of metal arsenate is the deciding factor.

Acknowledgement

The authors thank Dr. R.N.Gupta for providing research facilities. Financial assistance from U.G.C. (India) to one of us (S.D.S.) is also gratefully acknowledged.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1265-1272 (1983)

RAPID SEPARATION OF PROSTAGLANDINS BY LINEAR HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

B. J. Smith[1], R. M. Ross[1], C. R. Ayers[2],
M. R. Wills[1,2] and J. Savory[1,3]
Departments of Pathology[1], Internal Medicine[2] and Biochemistry[3],
University of Virginia Medical Center
Charlottesville, Virginia 22908

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

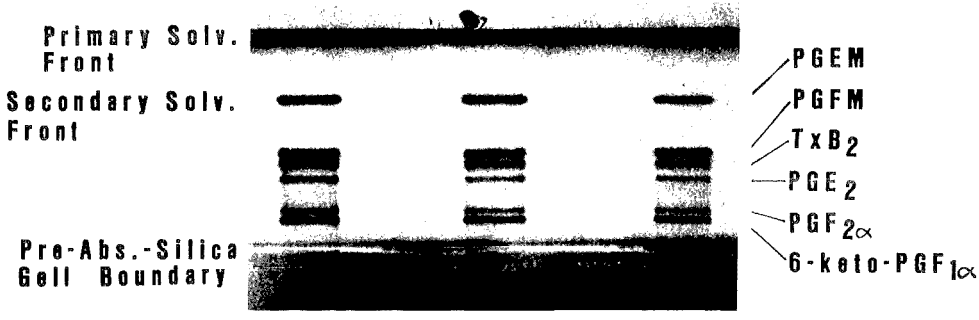

Figure 1. Prostaglandin standard chromatogram. The compounds were applied as a mixture in 20 µl aliquots. From left to right, the total volume applied to each lane was 60 µl, 40 µl, and 20 µl. Each 20 µl aliquot contained 1 µg each of 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and TxB_2, and 5 µg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

ACKNOWLEDGEMENTS

We wish to express our appreciation to Dr. John E. Pike (The Upjohn Company, Kalamazoo, MI) for his generous gift of the 13,14-dihydro-15-keto metabolites of PGE_2 and $PGF_{2\alpha}$. This work was supported in part by NIH Training Grant #5 T32 HL07355-05.

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REGULAR CONTRIBUTED PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1275-1289 (1983)

A CRYSTALLINE QUARTZ, LASER-EXCITED FLUORESCENCE, PHOTOACOUSTIC, AND TWO-PHOTON PHOTOIONIZATION DETECTOR FOR HPLC

E. Voigtman and J.D. Winefordner
Chemistry Department
University of Florida
Gainesville, Florida 32611

ABSTRACT

A crystalline quartz flow cell has been developed for simultaneous fluorescence, photoacoustic effect, and two-photon photoionization detection of aromatic compounds in HPLC eluents. Excimer laser-excited three mode detection of naphthalene, 7,8-benzoflavone, N-ethylcarbazole, and anthracene in 70/30 v/v acetonitrile/water is exhibited. The flow cell is suitable for use with deoxygenated solvent systems and is orientation independent.

INTRODUCTION

Recently, we demonstrated the feasibility of a laser-excited windowless flow cell which allows the simultaneous and independent detection of HPLC eluents by their fluorescence (FL), photoacoustic effect (PA), and two-photon photoionization (PI) [1]. The analytical figures of merit (detection limits) previously reported, though good, are far from optimal since any one of the three techniques may be greatly improved by re-designing the flow cell to eliminate the other two techniques. See, for example, Yamada et al. [2] for a windowless flow cell design which results in greatly improved photoionization detection power at the expense of elimin-

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ating the FL and PA detection modes. We have deliberately chosen to emphasize the development and optimization of three mode detectors since they afford the possibility of monitoring the three most important photophysical energy loss pathways available to excited molecular species. This should be especially advantageous in HPLC applications, where the mixture to be separated is complex and/or the number of analyte species is only a fuzzy estimate, since more information is available per analyte. This also means that the laser excitation system is better utilized, i.e., the bother and expense of a laser-based technique is traded off for greater detection power and selectivity per laser pulse.

The single greatest disadvantage of our windowless three mode flow cell is the relative insensitivity of the PA mode: it is about 100× worse than a cuvette cell (static) PA detection system [3,4]. This is presumably due to inefficient acoustic coupling between the transducer and the region where the acoustic waves originate. Other (minor) disadvantages include the need to operate the flow cell in an upright position and the inability, without inert gas sheathing modifications, to handle de-oxygenated solvent systems.

Since the cuvette cell was much better, in the PA detection mode, than the windowless flow cell, it was used as the starting point in a new three mode flow cell design intended to eliminate the afore-mentioned disadvantages. The cuvette cell detector consisted of a lead zirconate-titanate (PZT) ceramic piezoelectric transducer glued to a thick fused quartz disc (1.25 cm diameter and thickness). A Suprasil fluorescence cuvette (1 cm × 1 cm) is

placed on the disc with a drop of glycerol (an acoustic coupling fluid) between the cuvette bottom and disc surface. The preamplifier enclosure shields the transducer since the quartz disc passes through a rubber grommet in the enclosure wall.

Elimination of the fused quartz disc and glycerol by gluing the PZT transducer directly to the cuvette bottom improves the acoustic coupling efficiency, but complicates the shielding of the transducer and cleaning of the cuvette. It also increases the likelihood of false signal production by the PZT transducer via the pyroelectric effect. This effect is strong in barium titanate and very strong in PZT [5]. It can be eliminated by using a crystalline quartz piezoelectric transducer since quartz has neither a primary pyroelectric effect (transient polarization change due to uniform temperature change at constant strain) nor a secondary pyroelectric effect (transient polarization change due to uniform-temperature-induced strains and consequent direct piezoelectric effect)[6]. The acoustic coupling efficiency also improves in going from a fused quartz/PZT interface to a fused quartz/crystalline quartz interface. It takes no great leap of the imagination to simplify the fused quartz cuvette and attached crystalline quartz transducer combination to a simple hollowed out quartz crystal which serves as both the solution container and transducer. For an HPLC flow cell, it is convenient to use a drilled quartz crystal as the integrated detector. Our design, which also incorporates the FL and PI detection modes, is shown in Fig. 1. Note that this cell may also be considered as being related to the cylindrical

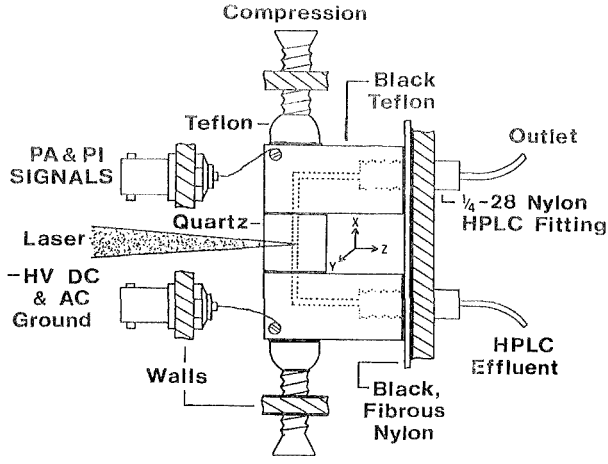

Figure 1: Schematic of the crystalline quartz cube HPLC flow cell.

piezoelectric ceramic transducer designs of Lahmann et al. [7] and Oda et al. [8] because they also integrate the transducer and vessel functions. The major difference with the present design is that laser excitation occurs through the transducer itself. It is therefore unnecessary to employ adhesives to attach windows to the cylindrical PZT transducer. It is also easier to contact the signal electrode in the present design.

Crystalline quartz was selected as the cell material because it is doubtful whether any other material would be suitable in such a design. Since the laser beam passes through the transducer, it is imperative that the transducer material not exhibit primary or secondary pyroelectric behavior. It must also have extremely low absorbance in the ultraviolet (UV) and visible spectral regions to avoid solarization, filtering of FL emission, and the tertiary pyro-

electric effect (transient polarization change due to non-uniform-temperature-induced strains and direct piezoelectric effect)[6,9]. Crystalline quartz is well-suited in this regard since it is the material of choice for the intracavity end windows of high power argon ion laser tubes characterized for UV operation. It is also chemically inert, is relatively inexpensive and easily fabricated, and offers high sensitivity, i.e., the piezoelectric g coefficient (generated electric field per unit pressure input) is 5.8×10^{-2} V $m^{-1}Pa^{-1}$ for quartz and only $\sim 10^{-2}$ V $m^{-1}Pa^{-1}$ for barium titanate and PZT [5]. It also has low thermal expansion coefficient and high volume resistivity ($\sim 10^{12}$ Ω m) at ordinary temperatures [5]. The latter property is essential for PI mode operation with non-polar, i.e., non-shunting, solvent systems in order to avoid 'shorting' the bias and signal electrodes together.

Disadvantages include low dielectric constant (which necessitates placement of the preamplifier as close to the transducer as possible) and lack of a volume expander piezoelectric mode. The latter causes quartz to be useless in 'hydrostatic' pressure sensing applications, i.e., it cannot respond to isotropic pressure changes. Since volume expander piezoelectric behavior may be exhibited only by members of the polar crystal classes, and the same applies for primary and secondary pyroelectric behavior [6], the trade-off of volume expander mode for freedom from pyroelectric effect is generally unavoidable.

To overcome lack of sensitivity to isotropic pressure changes, the hole drilled in the quartz cube of Fig. 1 is through the cen-

ters of the X faces, i.e., the cube faces normal to the X piezoelectric axis (electric, compression, or dihedral axis). The laser beam is incident along the Z axis (optic, crystallographic 'c' axis) for two reasons. First, the very small absorbances of the solvent and eluent species cause a negligible transient pressure gradient along the piezoelectrically inactive Z axis. Second, the unpolarized incident laser beam is uneffected by the relatively high UV optical activity (circular birefringence) of quartz along the optic axis [10]. Thus, the 'cylindrical' acoustic waves spread out radially, from the illuminated fluid region, in the X,Y plane, but effectively only stimulate (the transducer) along the Y axis.

Estimation of the PA preamplifier output voltage due to weak absorption by eluting analyte species is difficult for several reasons. First, both the excitation and detection geometries are quite different from the usual cases of coaxial laser beam excitation and fluid flow considered by others [11-17]. These geometric effects cannot properly be ignored since, for example, the generated pressure is strongly dependent on laser spot size (w) when the laser pulse duration (τ_p) is much less than the acoustic transit time ($\equiv w/v_a$ where v_a is the speed of sound in the liquid) of the pressure disturbance across the illuminated region [16]. This condition is satisfied in the present case since $\tau_p \simeq 10$ ns, $w \simeq 100$ μm, and $v_a \simeq 10^3$ m s^{-1}. A second difficulty arises because of the critical dependence of the output signal on the relative frequency responses of the generated pressure pulse, semi-clamped transducer, and preamplifier, and on the acoustic impedance match between the eluent liquid and the transducer [16].

Since we have previously shown that 10 ns laser pulses are only slightly more efficient in PA signal generation than 1 μs pulses of the same pulse energy [3], the expected pressure may be roughly estimated as ∿ 1 Pa (which is the calculated value given by Patel and Tam [15] for typical conditions such as 10^{-3} cm^{-1} absorptivity and 1 mJ pulse energy). Since the molar absorptivity of anthracene at 308 nm is ∿3200 M^{-1} cm^{-1}, the anthracene concentration corresponding to 10^{-3} cm^{-1} is ∿60 ppb. Thus, the transducer output can be no larger than ∿580 μV for a 1 cm cube cell. This value will be greatly reduced, despite the preamplifier gain, due to various coupling losses (acoustic and electrical), bandwidth restrictions in the preamplifier, and wiring and preamplifier input capacitance.

EXPERIMENTAL

A 1 inch cube of oriented, synthetic quartz crystal (Sawyer Research Products, Eastlake, OH) was fabricated into several 1 cm oriented, polished cubes (B. Collins, Hyde Park, NY) with the cube faces normal to the X, Y, and Z piezoelectric axes. A hole of 0.027 inch diameter was drilled through the center of the cube normal to the X faces (Wilmad Glass, Buena, NJ). The drilled hole was successively polished with diamond powder paste (15, 6, 1, & ¼ μm particle sizes, Abrasive Sales, Rindge, NH) and waxed dental floss. For clarity, the quartz cube in Fig. 1 is shown rotated 90° about the X axis (so that the unabsorbed portion of the excitation beam is shown impinging on the black background rather than passing out of the electrically shielding cell housing).

The bias and signal electrodes were fabricated fron conductive black PTFE (30% by weight graphite-filled PTFE, Fluorocarbon, Anaheim, CA). This material was convenient because it is easily machined, chemically inert, has low reflectivity, and deforms slightly under pressure, thereby conforming to the polished quartz cube surface (for efficient charge collection) and providing a fluid-tight seal.

Solutions were prepared by four successive five-fold dilutions in ethanol of a freshly prepared stock solution of naphthalene (200. μg/mL), 7,8-benzoflavone (200. μg/mL), N-ethylcarbazole (200. μg/mL), and anthracene (82.0 μg/mL). Solvents were de-oxygenated by bubbling with He for ∿1 hr prior to the HPLC injections of 20 μL samples. The HPLC system, column, and flow rate (1.5 mL/min) were the same as before [1]. Solvent programming was used to prepare the 70/30 v/v acetonitrile/water mobile phase.

The cube cell was operated simultaneously with and downstream from a conventional 254 nm UV absorbance detector with connections to the HPLC as shown in Fig. 1. Excitation pulse energy was kept low (∿0.1 mJ, see below) and 308 nm scatter was removed on the FL emission side by a 400 nm long pass filter. No artifact signals were observed, in any mode, upon illumination of neat solvent flowing through the cell. However, damage to the drilled hole was observed when focussed beams of ∿1 mJ per pulse were incident at the quartz/liquid interface. Microscopic examination revealed conchoidal fracturing, but no evidence of melting. No damage occurred when the cube cell was empty.

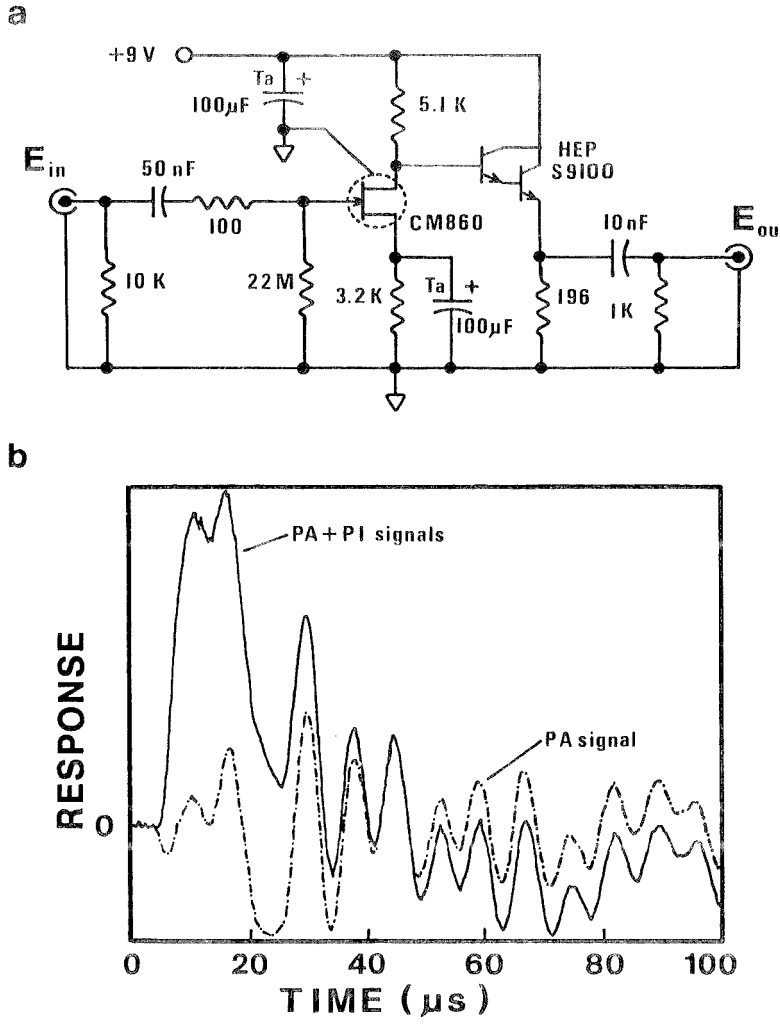

Figure 2: (a) Low noise preamplifier with R-C input coupling for polar mobile phases. The CM860 JFET is the four lead version of the 2N6550 JFET (Teledyne Crystalonics, Cambridge, MA).
(b) Typical preamplifier output waveforms for an (unseparated) five-fold stock solution dilution. Full scale is ∿13 μV.

The detection scheme is almost identical to that previously employed [1] except in one regard. Note in Fig. 1 that the DC bias voltage (V_b) of -1 kV was applied to the bottom PTFE electrode. This electrode was capacitively bypassed to ground (50 nF, 1600 WVDC, silver-mica). The other electrode is both the piezoelectric and photoionization signal pick-up. The joint signals were amplified with the preamplifier shown in Fig. 2a and typical waveforms with V_b = 0 V (PA effect only) and V_b = -1 kV (PA and PI effects) are shown in Fig. 2b. Photoionization chromatograms may be obtained with negligible PA signal addition by gating the boxcar averager so that it is near the peak of the PI waveform and overlapping a zero-crossing of the PA waveform. It is also possible to utilize the sum of the two non-radiative effects as a single analytical measure. Both effects are proportional to analyte concentration, but it should be remembered that the PA technique readily measures small solvent absorbances [18] while the PI technique is more nearly 'zero-baseline' with respect to solvent signals.

RESULTS AND DISCUSSION

Three mode chromatograms of naphthalene, 7,8-benzoflavone, N-ethylcarbazole, and anthracene in 70/30 v/v acetonitrile/water are shown in Figs. 3 and 4 together with a companion UV absorbance chromatogram. Detection limits, calculated as before [1], are presented in Table 1. The UV absorbance and PI results are essentially unchanged from the windowless flow cell values. The FL results are ∿2× worse while the PA results are ∿4× worse.

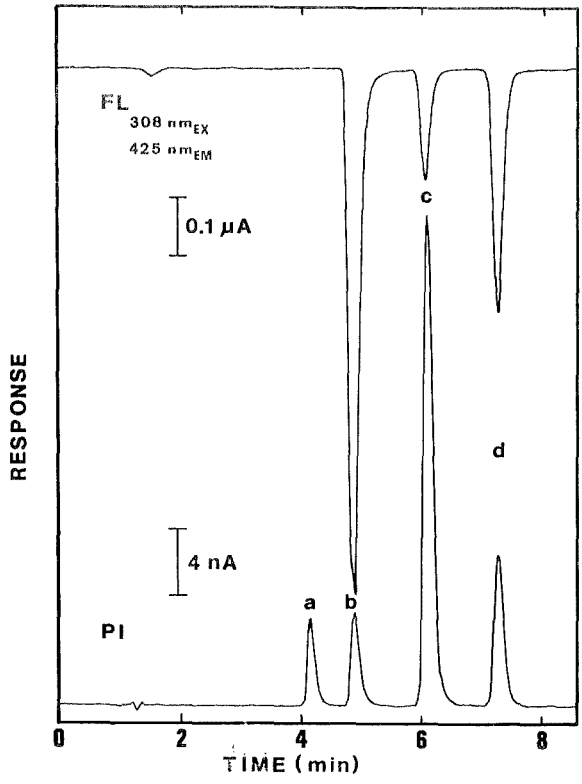

Figure 3: Fluorescence (FL) and Photoionization (PI) chromatograms for a five-fold dilution of stock solution of (a) naphthalene, (b) 7,8-benzoflavone, (c) N-ethylcarbazole, and (d) anthracene.

As stated earlier, none of the three flow cell modes is optimized. Even so, both the FL and PI modes exhibit the high detection power needed for HPLC applications. Both modes can be improved greatly (>100×) with relatively minor changes in the construction and operation of the cube cell. For example, the PI mode can be

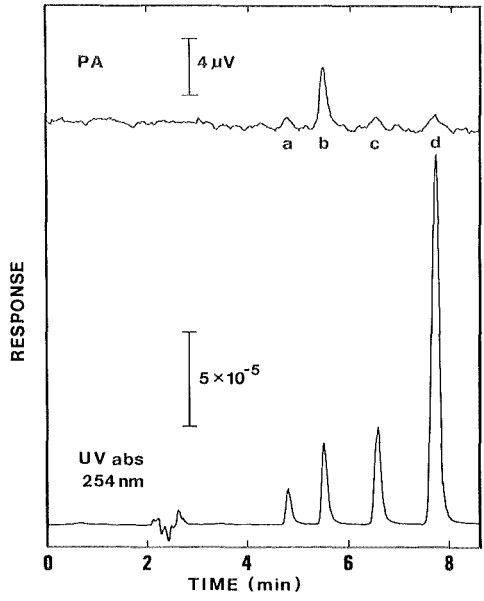

Figure 4: Photoacoustic (PA) and UV absorbance chromatograms for the same solution as in Fig. 3.

TABLE 1

Chromatographic LODs (S/N=3) for Four Aromatic Compounds in 70/30 v/v CH_3CN/H_2O. Units are μg/mL.

	Naphtha-lene	7,8-benzo-flavone	N-ethyl carbazole	Anthra-cene
UV absorbance	0.4	0.2	0.2	0.02
FL emission	---	0.1	0.5	0.09
PA	30.	5.	30.	10.
PI	0.5	0.5	0.08	0.1

improved by using higher bias voltage (-5 kV), higher pulse energy (∿1 mJ), and closer electrode spacing (∿2 mm) [2,19,20]. This means using MHV connectors rather than BNC connectors and using a 2 mm quartz cube.

Unfortunately, the PA mode performance is not so readily improved. Although the cell meets the goal of gas-tight operation in any orientation, it is ∿100× worse than a conventional (254 nm UV absorbance) detector. Even taking into account the disparity in excitation wavelengths, the low laser pulse energy, and the use of polar solvents (which 'short' the transducer faces), it probably cannot be improved more than a hundred-fold at best. This is not the case with PA mode detection using PZT detectors since, for example, the first PA mode HPLC detector (Oda and Sawada [21]) achieved detection limits for several azobenzene isomers which were ∿25× better than UV absorbance results (7.9×10^{-6} cm^{-1} at S/N=2). They used an acousto-optically modulated Ar ion laser, PZT disc, and lock-in amplifier detection and expected to greatly improve their PA mode results by using pulsed laser excitation. Lai et al. [22], using both nitrogen and XeCl excimer laser (pulsed) excitation, a non-contacting PZT cylindrical transducer, and boxcar averager signal processing, achieved LODs which were ∿10× worse than the conventional detector. The disparity in PA mode HPLC flow cell detection powers for the pulsed versus CW laser excitation cases is curious and precisely the opposite from the observed behavior in static cells [15]. Consequently, future efforts will be concentrated on resolving the anomaly and in developing a three mode

cell which is at least 10× better than UV absorbance detectors in all modes.

ACKNOWLEDGMENTS

This work was supported by NIH-GM-11373-19 and by DOE-DE-AS05-780R06022.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1291-1317 (1983)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DITHIOLTHIONES

Asaad N. Masoud and Ernest Bueding

Departments of Environmental Health Sciences and Immunology and Infectious Diseases, The Johns Hopkins University, Baltimore, MD.

ABSTRACT

Sixteen dithiolthiones including oltipraz (5-(2-Pyrazinyl)-4-methyl-1-1,2 dithiol-3-thione) and anethol dithiolthione (P-methoxyphenyl-1,2-dithiol-3-thione) have been studied using an isocratic high performance liquid chromatographic system. The retention characteristics of these compounds were determined using a conventional C 18 reverse phase column and a μ Bondapack phenyl column. The mobile phases used consisted of several concentrations of methanol and water. All compounds studied were adequately detected in the nanogram range (30-100 ng on column) using

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ultra violet detection (UV) set at 300 nm. Several analogs and isomers were separated. The retention characteristics of the 16 compounds studied are reported for both columns using 4 mobile phases and chromatograms of reference standards are presented.

INTRODUCTION

Oral administration to mice of dithiolthiones results in chemoprotective and radioprotective effects (1). This is associated with significant elevations in tissue glutathione levels and in the activity of a number of enzymes, such as glutathione-S-transferases, catalyzing the inactivation of carcinogens and other toxic products (1). One dithiolthione, oltipraz, (5-(2-pyrazinyl)-4 methyl-1,2 dithiol-3-thione) has antischistosomal activity (2,3,4,) and anethol dithiolthione (ADT) (3-(p-methyoxyphenyl)-1,2-dithiol-3 thione) has been reported to stimulate salivary secretion and to antagonize the dryness of the mouth, produced by certain antidepressant drugs (5).

Because of the numerous biological activities, and because of their presence in several edible plants (Brassica species) (6), this class of compounds is assuming considerable interest.

No methods have been reported in the literature for the separation of dithiolthiones by high performance liquid chromatography (HPLC). In this paper we are describing conditions for the separation and detection of 16 dithiolthiones using two reverse phase columns and four mobile phases. Absorption maxima and the extinction coefficients (α) of these compounds in the UV and visible regions are reported also.

MATERIALS AND METHODS

Instrumentation

An isocratic HPLC system assembled in our laboratory consisted of a Milton Roy

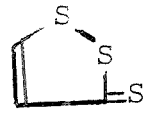
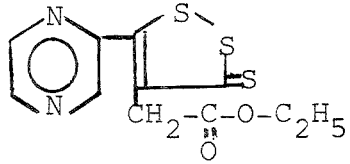
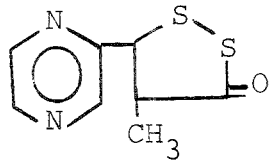
reciprocating minipump, model 396; a stainless steel tube, 1/4 inch (0.64 cm) outer diameter by 1 meter as a pulsation damper; a 5,000 pounds per square inch (34.5 megapascal) pressure gauge; a Rheodyne injector model 7125 and a variable wavelength ultraviolet (UV) detector, model Spectro-Monitor III. All parts were obtained from Laboratory Data Control (Riviera Beach, Florida). A Hewlett Packard computing integrator model 3390A (Avondale, Pennsylvania) was connected to the detector. A Brownlee RP 18, Lichrosorb 3 cm guard column (Santa Clara, California) was connected to the analytical column. Two analytical columns were used in this study; a Whatman, Partisil PXS 10/25, ODS-2 microparticulate, reverse phase column (Clifton, New Jersey) which will be referred to as column A, and a Waters, μ Bondapack phenyl (Milford, Massachusetts), which will be referred to as

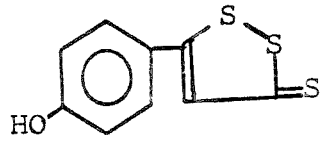
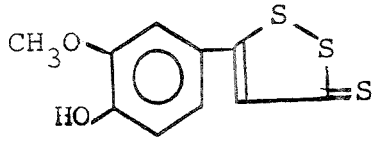
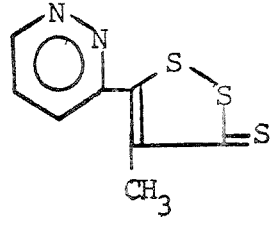
Column B. Columns were kept inside a Bioanalytical Systems heater model LC-23A (West Lafayette, Indiana) and column temperature was maintained at 35°C throughout this study.

Materials:

The compounds investigated were obtained courtesy of Dr. Baronnet of Laboratoires Therapeutique Moderne, Suresnes, France (Latema), Dr. Benazet of Rhone Poulenc, (RP), Research Division Vitry Sur-Seine, France and Dr. R. Gyurick of Smith Kleine and French, USA. They were used without further purification. The sources of these compounds, their structures, absorbance maxima, excitation coefficients and their names or their company identification numbers are listed in Table 1. These compounds were arranged in order of their elution on column A with 70% methanol as a mobile phase

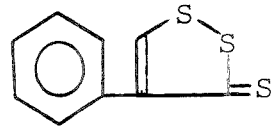
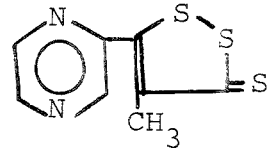
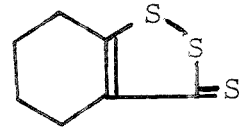
TABLE 1
Compounds Studied, Their Structures, Sources, Names or Code Numbers, Absorbance Maxima and Extinction Coefficients.

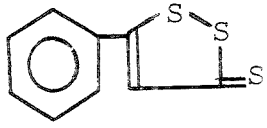
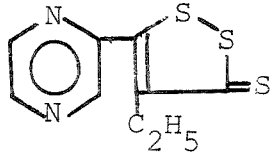
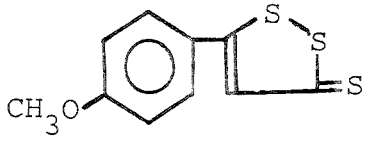
Roman Numeral of Comp.[1]	Structure	Name or Code Number[2]	Source[3]	Abs. Max. (nm)	Extinc. Coef. (α)
I		Dithiocylopen-ten.ethione	Latema	225	46.0
				245	29.0
				320	25.0
				405	60.0
II		38656	RP	230	52
				270	45.2
				420	39.5
III		36642	RP	230	26.5
				288	64.5
				350	20.5

IV		Des-methyl ADT	Latema	230	31.0
				350	50.0
				425	35.0
V		120L	Latema	230	68.0
				255	65.5
				370	92.0
				430	79.0
VI		36733	RP	220	43.0
				265	40.0
				435	29.0

(continued)

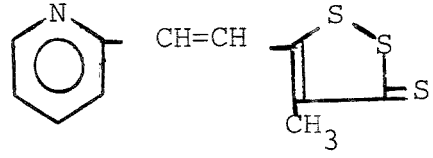
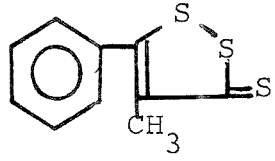
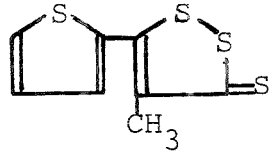
Table 1 Cont.

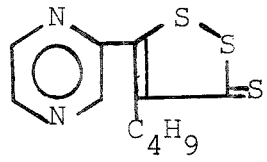
VII		1129L	Latema	210	65.3
				230	45.9
				320	18.8
				420	33.5
VIII		Oltipraz (OLT)	RP	220	50.5
				295	63.5
				430	39.0
IX		82013	SKF	220	58.0
				275	40.5
				315	32.5
				404	66.5

X		116L	Latema	220	57.0
				265	49.0
				310	94.5
				425	52.0
XI		36334 (Ethyl OLT)	RP	225	45.5
				290	56.0
				435	36.5
XII		Anethol-dithiolthione (ADT)	Latema	225	61.5
				265	35.5
				342	96.0
				425	57.0

(continued)

Table 1 Cont.

XIII		40863	RP	238	29.0
				350	49.5
				460	19.5
XIV		37528	RP	215	63.5
				238	46.5
				295	56.0
				415	51.0
XV		36731	RP	225	56.5
				265	37.5
				335	65.5
				430	51.0

XVI		35919	RP	222	53.0
		(Butyl OLT)		290	62.0
				440	41.5

1. These compounds eluted in that order on a Whatman partisil, PXS, 10/25, ODS-2, Column and when mobile phases containing 70% or 75% methanol were used.
2. These numbers are the numbers assigned to these compounds by the manufacturers.
3. Latema - Laboratoires de Therapeutique Moderne; RP - Rhone Poulenc; SKF- Smith Klein and French.

and assigned a Roman numeral based on that order for the purpose of referring to these compounds throughout this paper.

Methanol, (Glass-distilled) was purchased from Burdick and Jackson laboratories (Muskegan, Michigan). Water was distilled, deionized and demineralized.

Individual reference standards were dissolved in methanol and further dilutions and mixtures were also prepared in methanol. Solutions were stored in vials provided with teflon-lined caps and the vials were wrapped with aluminum foil to protect the samples from light. Samples were gassed with nitrogen and kept refrigerated when not in use. No decomposition was observed under these conditions for at least two months.

Mobile Phases

Four mobile phases containing 75, 70, 65 and 60% methanol in water were used. These

mobile phases will be referred to throughout this paper as 75%, 70%, 65% and 60% methanol respectively. The mobile phases were degassed under vacuum immediately prior to use and kept at approximately 40°C during chromatography to prevent the introduction of air bubbles into the system. The flow rate was kept at 1.1 ml/min throughout this study.

Detector and Integrator Settings:

The absorption characteristics of the compounds were studied using a Varian scanning spectrophotometer model Cary 219 in the UV and visible range (Table 1). All the compounds were found to possess a maximum at a wavelength close to 300 nm. Thus when the UV detector was set at that wave length, all the compounds were adequately detected when nanogram amounts (30-100 ng on column) were chromatographed.

The UV detector provided a constant signal to the computing integrator of 1 absorbance unit (AU)/volt. Thus height counts provided by the integrator were constant for a given peak, regardless of the attenuation setting of the integrator. The actual size of the peaks as they appear on the chromatograms however are determined by the attenuation of the integrator. Throughout this study, the attenuation was set at 3 which equals to 8 mV full scale. Since the detector provides 1 AU/V, the sensitivity of the system was 0.008 absorbance units full scale, (AUFS).

RESULTS AND DISCUSSION

Working standards of individual compounds were chromatographed to determine their retention time (t_R) and their detector response. The amount introduced onto the column was in the range of 3 to 9 µl

of solutions containing 10 μg/ml. The capacity factor (k') for each compound was determined using the 4 mobile phases on each column according to the equation K' = t_R-t_0/t_0 where t_0 represents the solvent front which was 2.70 min. on column A and 3.35 min. on column B. Table 2 lists the K' values of these compounds. Figures 1 and 2 provide a graphic representation of these data.

As expected the compounds were generally retained more on column A than on column B. Also their retention was increased as the percentage of methanol was decreased in the mobile phase. The order of elution was consistent for most of the compounds with all mobile phases used. Only few compounds reversed order as the amount of methanol was decreased and the degree of reversal was not very significant.

TABLE 2
K' Values and Detection Responses of the Compounds Studied

	COLUMN A				COLUMN B				
	PERCENT METHANOL IN MOBILE PHASES								
Compound	75	70	65	60	75	70	65	60	mAU[1] Per µg on column
I	0.66	0.74	1.00	1.39	0.38	0.32	0.46	0.61	77.17
II	1.21	1.45	2.15	3.37	0.80	0.90	1.44	2.03	63.33
III	1.47	1.62	2.21	3.17	0.70	0.71	1.04	1.45	156.67
IV	1.51	1.98	3.02	4.94	0.76	0.91	1.54	2.33	34.67
V	1.67	2.21	3.46	5.52	0.83	0.98	1.67	2.56	47.11
VI	1.72	2.25	3.18	4.70	0.74	0.80	1.23	1.70	71.56

VII	2.08	2.57	3.94	6.31	1.02	1.24	2.13	2.96	44.00
VIII	2.92	3.64	5.08	7.70	1.18	1.18	2.13	2.95	85.67
IX	3.11	3.81	5.87	8.39	1.25	1.55	2.39	3.37	50.17
X	3.66	4.83	7.63	12.68	1.49	1.93	3.28	4.91	111.33
XI	3.72	4.99	7.60	12.24	1.42	1.78	2.90	4.19	76.67
XII	4.30	5.91	9.61	16.52	1.67	2.24	3.93	6.06	33.50
XIII	4.80	7.00	10.23	17.02	1.38	1.95	3.23	4.77	10.77
XIV	4.92	7.32	11.84	20.58	2.03	2.84	5.07	7.80	30.17
XV	5.66	8.05	13.13	22.50	2.07	2.84	5.007	7.64	31.77
XVI	7.37	10.67	18.09	32.31	2.35	3.35	6.06	9.63	52.44

1. These responses were calculated for individual compounds when chromatographed on column A and eluted with 75% methanol in water.

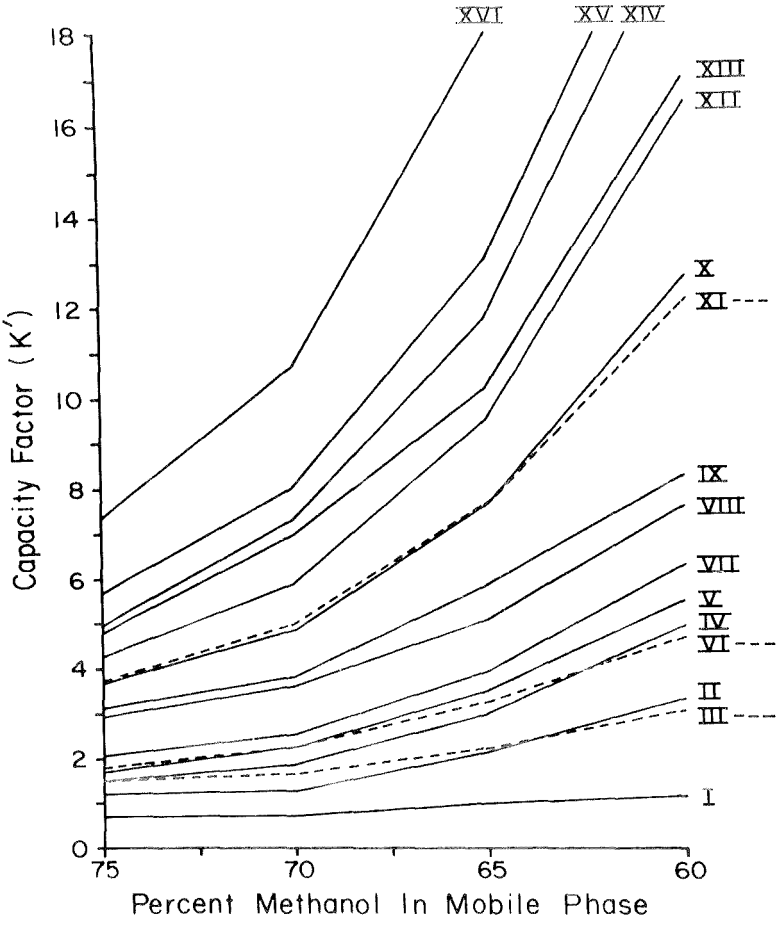


FIGURE 1

Capacity factors (k') of the compounds studied when chromatographed on column A.

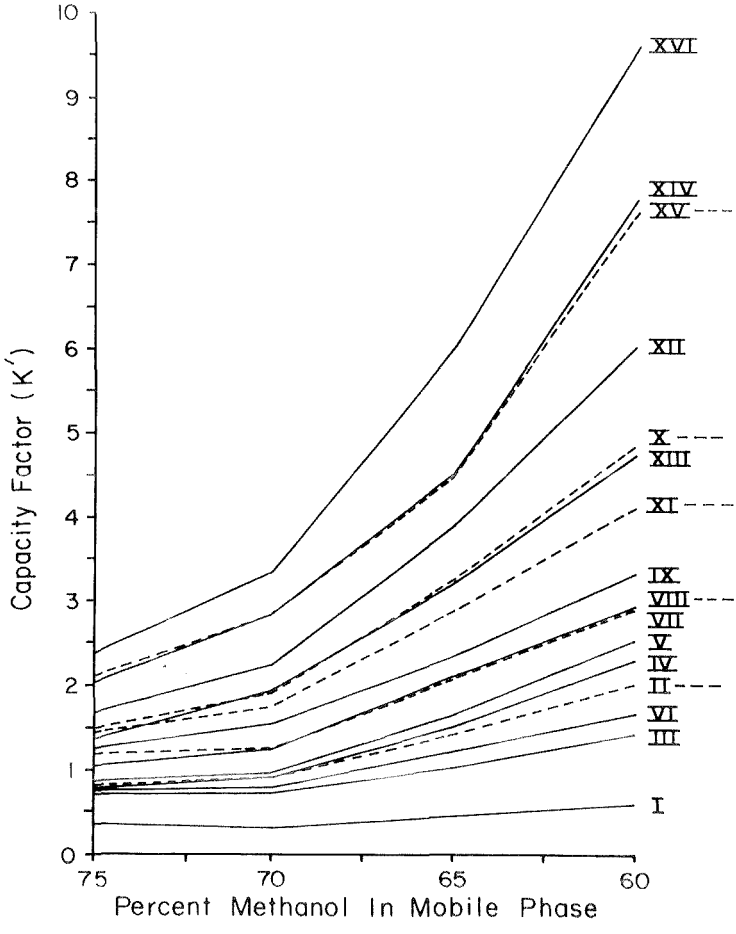


FIGURE 2

Capacity factors (K') of the compounds studied when chromatographed on column B.

For column A, compound III was eluted slightly earlier than compound II when 60% methanol was used. Compound VI was eluted earlier than V with 65% methanol and earlier than IV with 60% methanol. Compounds X and XI also reversed order in a similar fashion when the concentration of methanol in the mobile phase was reduced to 60%. It is difficult to interpret with certainty this difference in response to a lower methanol concentration by compounds III, VI and XI. However, the only structural characteristic common to the three compounds is the presence of two nitrogens in the ring of the side chain of the dithiocyclopetene thione. This effect could not be observed in compounds II and XV possibly due to the presence of a reasonably large side chain which could have overcome that effect. The order of elution from column A could be used as an index of the lipophilicity of these compounds.

The order of elution from column B was significantly different from that of column A. Retention on column B was no longer dependent on the lipophilicity of the compounds and the affinity of the compounds to the phenyl function of the stationary phase resulted in a different pattern of separation. As with column A, few compounds reversed the order of elution as the concentration of methanol in the mobile phase was reduced, however this phenomenon was less pronounced than with column A.

The choice of the column and of the mobile phase to be used for the separation of a given mixture is clearly dependent on the components of the mixture and the particular compound(s) of interest. Figures 3 and 4 illustrate the separation of the same mixture containing the 16 compounds studied using two different chromatographic systems.

Figure 3 reproduces the separation obtained on column A using a mobile phase

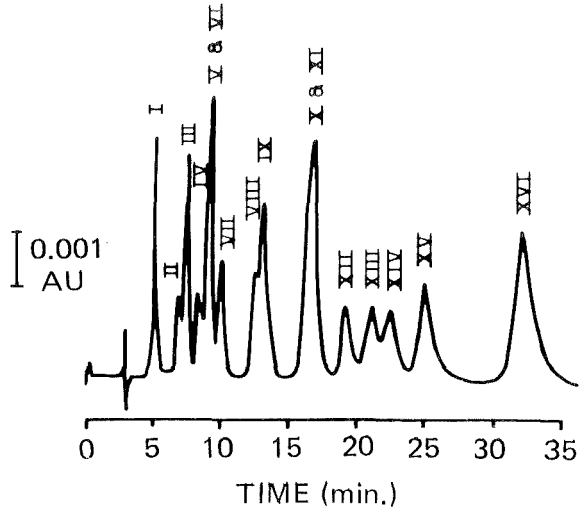


FIGURE 3

A chromatogram representing the separation of the compounds studied on a reverse phase column. The chromatographic conditions are as follows: column, Whatman PXS, 10/25 ODS-2 (column A); mobile phase, 70% methanol in water; detection, UV, 300 nm at 0.008 AUFS; flow rate, 1.1 ml/min; chart speed 0.2 cm/min.

The compounds chromatographed and the amounts on column are as follows: I = 60 ng; II = 30 ng; III = 30 ng; IV = 60 ng; V = 60 ng; VI = 60 ng; VII = 60 ng; VIII - 30 ng; IX = 60 ng; X = 30 ng; XI = 60 ng; XII = 60 ng; XIII = 120 ng; XIV = 60 ng; XV = 90 ng; XVI = 90 ng.

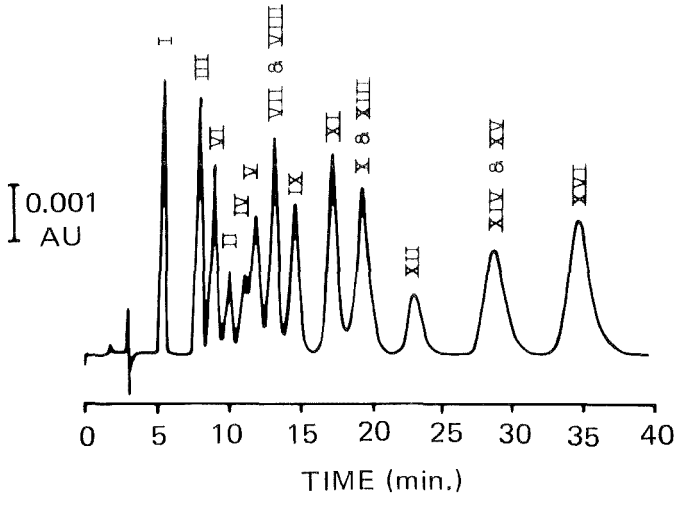


FIGURE 4

A Chromatogram representing the separation of the compounds studied on a phenyl column. The chromatographic conditions are as in Figure 3 except the column used was Waters μ Bondapack Phenyl and the mobile phase containing 60% methanol in water. The components and amounts were the same as in Figure 3.

containing 70% methanol. Under these conditions compounds V and VI; VIII and IX and X and XI co-eluted. Also, most of early eluting compounds, II through VII, did not produce adequate separations.

Figure 4 represents the separation of the same mixture obtained on column B using a mobile phase containing 60% methanol. Under these conditions, compound V (which co-eluted with VI under the previous conditions) was clearly separated from VI but not from IV. Compound VIII, similarly was adequately separated from IX but co-eluted with VII. Finally compound X was adequately resolved from XI but co-eluted with XIII.

In summary, the use of the combination of two columns and four mobile phases allowed most of the compounds studied to be separated adequately.

Detection Responses

Peak height counts computed for the compounds chromatographed on column A using 75% methanol as a mobile phase by the computing integrator, were converted to MV's using the conversion factor provided by the

Hewlett Packard Instruction Manual for the instrument. These values were used to calculate the milli-absorbance units (mAU) per μg on the column which is reproduced in column 10 of Table 2. This value is a useful indication of the signal produced by each compound and can provide an approximation of the detection limits of the method for each compound. Assuming that a sensitivity setting of 0.01 absorbance units full scale (AUFS) is a reasonable setting for a routine operation of most instruments, thus a signal of 1 mAU would represent 10% of the full scale which would certainly be considered a detectable signal (the chromatograms reproduced in Figures 3 & 4 a sensitivity setting of 0.008 AUFS was used). Based on these data and those recorded in column 10 of Table 2, the lowest detectable amounts on column can be calculated by dividing 1000 by the mAU produced by 1 μg on column to result in the

amount in nanograms necessary to produce a signal of 1 mAU. The range of sensitivity of the compounds studied will then be approximately 100 ng on column for compound XIII to 5 ng on column for compound III.

ACKNOWLEDGEMENTS

The authors thank Drs. R. Baronnet of Latema Laboratories, Dr. F. Benazet of Rhone Poulenc Laboratories and Dr. R. Gyurik of SKF laboratories for generously providing the reference standards used in this study. They also acknowledge their appreciation for the constructive suggestions of Dr. Andrew Monjan and Ms. Angela James for typing the manuscript. This work was partly supported by NIH Grant ES- 00093

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1319-1332 (1983)

DETERMINATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN BLOOD PLASMA SAMPLES

John K. Baker*, John Kapeghian, and
Anthony Verlangieri
School of Pharmacy
University of Mississippi
University, MS 38677

ABSTRACT

Following the stabilization of the plasma samples with $HClO_4$ and EDTA, the samples could be directly analyzed by HPLC using electrochemical detection and reversed-phase columns. The accuracy and precision of the method was evaluated using plasma samples spiked with ascorbic acid (10 μg/ml) and the results were also compared to the classical colorimetric procedure. Dehydroascorbic (5 μg/ml) was determined in plasma samples using UV detection following derivatization at room temperature for 45 minutes with o-phenylenediamine.

INTRODUCTION

In normal human adults the plasma concentration of ascorbic acid ranges from 4 to 14 μg/ml (1) while the reported values for dehydroascorbic acid range from 0.6 to 2.0 μg/ml (2,3). The most widely used method for the analysis of ascorbic acid in plasma is based on the colorimetric reaction with 2,6-dichlorophenolindophenol (1). "Total ascorbic acid" in plasma has been measured by dinitrophenylhydrazine derivatization of the dehydroascorbic acid initially present and the ascorbic acid oxidized to dehydroascorbic acid (1,2).

Ascorbic acid has been determined in animal tissue samples in the 200 to 3,000 μg/gm range using HPLC analysis with UV detection at 254 min (4). In the present study, it was also confirmed that HPLC analysis

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with UV detection was satisfactory for the high ascorbic acid concentrations found in tissue samples, however, it was not satisfactory for the 4 to 14 μg/ml range found in plasma samples. HPLC analysis with electrochemical detection has been used for the analysis ascorbic acid in marine invertebrates in the 4 to 30 μg/g range (5) and this detection technique was utilized in the present study.

Dehydroascorbic acid could theoretically be detected using electrochemical methods in the reductive mode but the diffusion current is only a small fraction of the expected value (6). While this manuscript was in preparation, a HPLC method for dehydroascorbic acid in fruit juice (100 μg/ml range) using derivatization with o-phenylenediamine was published (7). This highly conjugated derivative formed in aqueous solution at room temperature within 30 minutes and it was moderately stable (8). In the present study, the o-phenylenediamine derivatization procedure has been modified for the analysis of dehydroascorbic acid in plasma in the 0 to 5 μg/ml range.

EXPERIMENTAL

Determination of Ascorbic Acid by HPLC:

Approximately 3 ml of whole blood was collected in a tube containing 10 mg EDTA and immediately centrifuged for 5 minutes. Then 500 μl of the plasma was transferred to a fresh sample tube containing 25 μl of concentrated (70%) perchloric acid, agitated using a vortex mixer, then centrifuged to yield a clear, nearly colorless layer. The total time from sample collection to stabilization with perchloric acid was generally less than 10 minutes.

A calibration sample of 10 μg/ml ascorbic acid was prepared fresh daily using ascorbic acid (reagent grade, Fisher Scientific Co.) with 0.1 mg/ml Na_2EDTA and 0.1 N $HClO_4$ as the diluent. Unstablized solutions

of ascorbic acid in this concentration have a half-life of only 1-2 hours and were not satisfactory for calibration purposes. A calibration solution of 10 μg/ml of uric acid was also freshly prepared.

A 5.0 μl sample of the stabilized plasma sample or calibration sample was injected into the HPLC system using a U6K injector (Water's Assoc.). A C-18 guard column and a 3.9 mm x 30 mm μ-Bondapak C-18 analytical column was used with a mobile phase (1.5 ml/min) prepared with 0.2 mg/ml Na_2EDTA, 0.001 M N-octylamine, and 0.1M sodium acetate. The pH of the mobile phase was adjusted to 5.0 with glacial acetic acid before use.

A glassy carbon electrode at +0.60 volts (100 nA range) was used with an electrometer (Model LC-3A, Bioanalytical Systems, Inc.) and a strip chart recorder or a recording integrator (Model 3390A, Hewlett Packard). If the calibration sample was less than 25% of a full scale response, the electrode surface was polished (every 3-4 wks. typical).

Determination of Dehydroascorbic Acid:

Stock solution of 1.0 mg/ml of dehydroascorbic acid (ICN nutritional Biochemicals) were prepared using distilled water and these standard solutions were discarded if not utilized within 4 hours. Calibration samples of 5 μg/ml dehydroascorbic acid were prepared immediately before use because of the poor stability of the dilute solutions.

In a typical determination, 3 ml of whole blood was collected in a tube containing 10 mg EDTA, centrifuged, and then 500 μl of the plasma (or 5 μg/ml standard) was transferred to a fresh tube containing 50 μl of 0.5 mg/ml o-phenylenediamine. The total time between collection to the blood sample and the addition of the o-phenylenediamine was typically less than 7 minutes.

Following a mininum 45 min. incubation period, a 10 µl sample was subjected to HPLC analysis. A µ-Bondapak C-18 column was used with a mobile phase (1.5 ml/min) comprised of 6.6 g K_2HPO_4, 8.4 g of KH_2PO_4, 400 ml CH_3OH and 3.6 liters of water. Dual UV detectors set at 254 nm and 280 nm using the 0.02 AUFS range (Model 440, Waters Assoc.) were used for the detection of the dehydroascorbic acid derivative. The identity of the peak was verified by a comparison of retention time and the absorbance ratio observed with the two UV detectors.

Colorimetric Determination of Ascorbic Acid:

As an additional tool in establishing the validity of the HPLC method, the most commonly used colorimetric procedure (1) was also utilized for the analysis of some of the plasma samples. In this procedure, the blood was collected and the plasma was stablized using exactly the same procedure as used for the HPLC determination. A 500 µl sample of the stabilized plasma, 300 µl of a citrate buffer (0.22 g/ml trisodium citrate adj. to pH 4.15 with glacial acetic acid), and 1.0 ml of 2,6-dichlorophenolindophenol (0.1 mg/ml in water) were mixed directly in a spectrometer sample cell. After exactly 30 sec., the absorbance (A_1) of the sample at 520 nm was measured. Approximately 2 mg of ascorbic acid crystals were added to sample and the absorbance reading (A_2) of the mixture was measured again. The difference in the reading (A_1-A_2) was taken to be proportional to the ascorbic acid content and the procedure was calibrated using the same procedure.

RESULTS AND DISCUSSION

Using the ion-pair HPLC system with electrochemical detection, the normal physiological levels of ascorbic and uric acids present in human plasma could be detected easily (Fig. 1). Using the 100 nA range (the least sensitive range on the unit) the ascorbic acid peak appeared as a

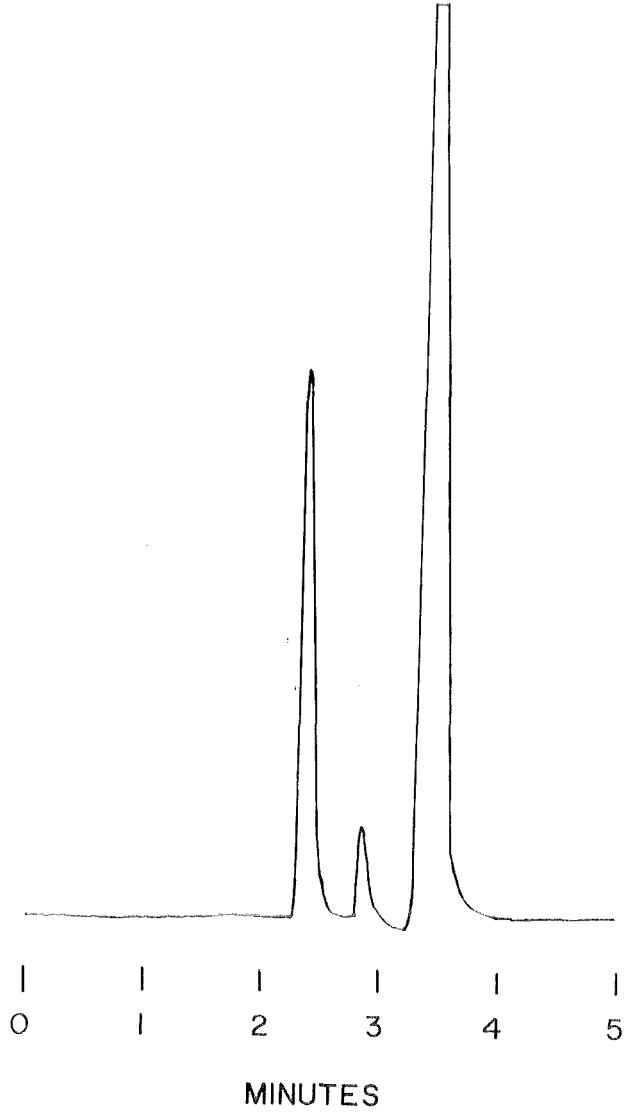

FIGURE 1: Chromatogram of Human Plasma Containing Uric Acid (t_R=3.4 min) and 10 μg/ml Ascorbic Acid (t_R=2.3 min.)

nearly full scale deflection at 2.30 min while the solvent peak at 1.3 minutes was just barely discernible. In the examination of fresh plasma samples from man, guinea pig, and monkey; it was found that the size of the ascorbic acid peak was of the same order of magnitude for the three species but there were marked variations in the size of the uric acid peak between species (man > guinea pig >> monkey).

To determine the accuracy and precision of the HPLC method, human plasma that had been depleted of ascorbic acid was spiked with 10.0 μg/ml of ascorbic acid, then the pooled specimen was divided into five samples for HPLC analysis and five samples for the classical colorimetric method. The results of this comparison (Tab. 1) showed that the HPLC method gave accurate results, however, the standard deviation of the series of measurements was higher than the standard deviation observed for the colorimetric method. If greater precision were needed, a fixed-loop injector could be substituted for the open-loop style injector to improve the precision of the volume of sample delivered to the column.

TABLE 1

Comparison of the Standard Colorimetric and HPLC Methods for the Determination of Ascorbic Acid (Spiked at 10.0 μg/ml) in Human Plasma

Plasma Sample	Colorimetric Method	HPLC Method
1	11.53 μg/ml	11.38 μg/ml
2	11.59 μg/ml	10.47 μg/ml
3	10.92 μg/ml	9.77 μg/ml
4	10.97 μg/ml	9.17 μg/ml
5	11.08 μg/ml	9.29 μg/ml
average	11.22 μg/ml	10.02 μg/ml
standard dev.	±0.32 μg/ml	±0.92 μg/ml
plasma blank	1.79 μg/ml	0.26 μg/ml

The analysis of the spiked plasma samples with the colorimetric method gave results that were 12% higher than the true value. The plasma blank also gave a fairly high false background value (1.79 μg/ml) which probably arose from redox reactions with other plasma constitutents with the indophenol dye. Though the absorbance reading for the plasma blank was taken 30 sec after the addition of the indophenol dye, the absorbance at 520 nm by the dye continued to fall at a fairly rapid rate.

In the early stages of this investigation, a number of different methods for stabilizing ascorbic acid were investigated. Metaphosphoric acid worked well for simple solutions or tissue samples of ascorbic acid, but it was found not to be useful for the stabilization of plasma samples. Trichloroacetic acid was also investigated, but it did not give a clear solution after precipitation of the plasma proteins. The combinations of EDTA and $HClO_4$ was found to be the most useful for the stabilization of ascorbic acid and precipitation of plasma proteins. Using this method, samples could be stored at room temperature for 6 hr. with no loss, 24 hr. at 5° with 14% loss, 7 days at -70° with no loss.

There are frequent references to rapid decomposition of ascorbic acid in plasma samples (1,9), but there are few kinetic studies using the more selective analytical procedures. A large sample of monkey plasma was collected and stored at room temperature. At various time periods, a portion of the plasma was stabilized and the ascorbic acid content was determined using the HPLC procedure. The results of this experiment (Fig. 2) showed the ascorbic acid was lost at a high rate ($t_{\frac{1}{2}}$ = 0.55 hr.) and the loss was much faster than would occur in a simple solution. The same monkey plasma sample was stored at 5° for 7 days, returned to room temperature, spiked with ascorbic acid, then

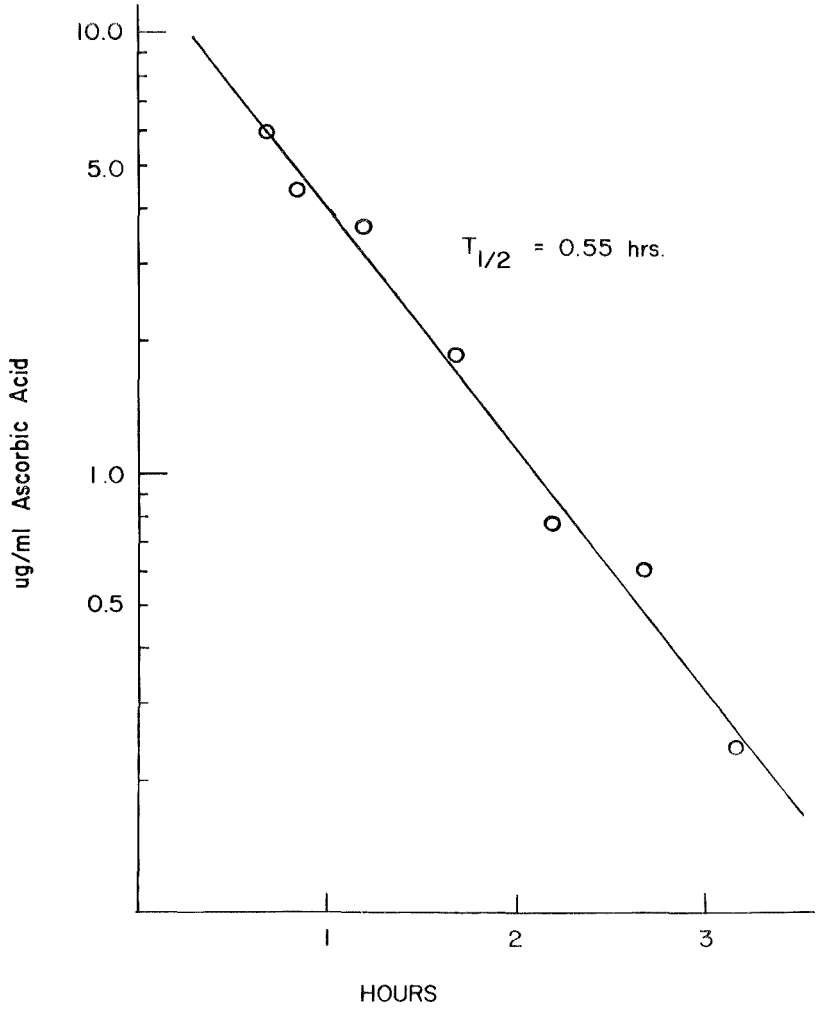

FIGURE 2: Degradation of Ascorbic Acid in a Fresh, Unstabilized Monkey Plasma Sample Held at Room Temperature.

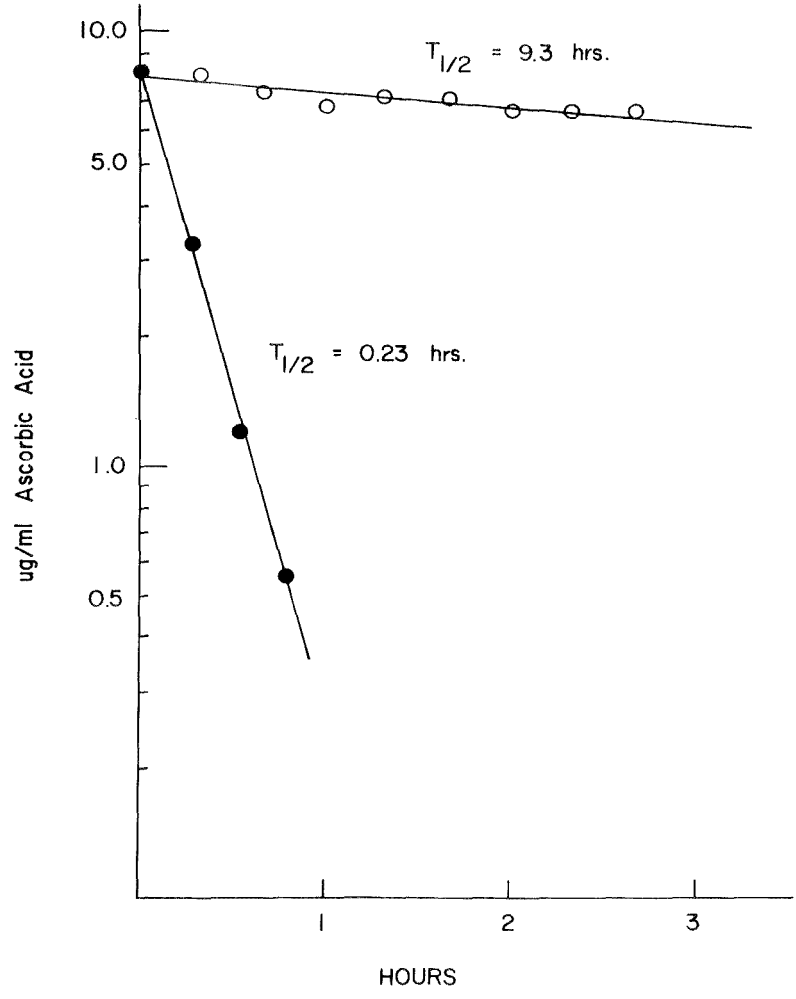

FIGURE 3: Degradation of Ascorbic Acid in Unstabilized Human Plasma Samples Held at Room Temperature. Filled circles-fresh plasma. Open circles-aged plasma.

assayed at various time periods. In this aged plasma sample, the ascorbic acid appeared to be much more stable ($t_{\frac{1}{2}}$ = 0.95 hr) compared to the fresh sample.

Similar results were also obtained using human plasma (Fig. 3). In freshly collected human plasma, the shortest half-life observed was 0.23 hours. In human plasma that had been stored at 5° for several weeks, then returned to room temperature, the half-life of ascorbic acid ($t_{\frac{1}{2}}$ = 9.3 hr) was considerably longer. Previous literature reports (1,9) have indicated that there was a 5-8% loss per hour, but the age of the plasma samples used for the evaluation was not indicated. The present study indicated that the stability of ascorbic acid in freshly drawn, hemolysis-free plasma samples was much poorer than previously estimated. Thus if one desires to have an accurate estimate of the ascorbic acid plasma levels of a given subject, it is essential that the total time between collection of the whole blood and the addition of the $HClO_4$ stabilizer be less than 10 minutes.

The method that was developed for the HPLC analysis of dehydroascorbic acid utilized a reaction with o-phenylenediamine (Fig. 4). Though this reaction has been studied for over 50 years, there are some indications that the structure shown for the product in Figure 4 may not be correct (10). However, under the conditions used for the derivatization of dehydroascorbic acid in the present study, only one product was detected in the model systems or with the plasma samples. The reaction occurs rapidly at room temperature in neutral aqueous solutions or in plasma samples. The derivative appeared as a peak at 8.7 min, was well separated from the excess o-phenylenediamine reagent at 5.5 min, and could be detected at the 5 μg/ml level in human plasma (Fig. 5). A derivatized human plasma blank did not show any other peak at that retention time. As an additional tool in the identification of

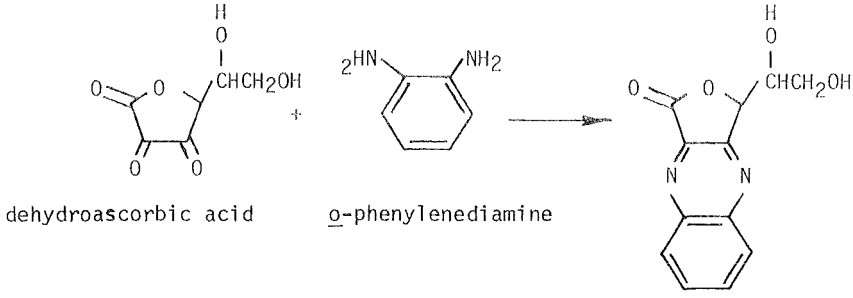

FIGURE 4: Derivatization Reaction for Dehydroascorbic Acid Showing Tentative Structure of Reaction Product.

the dehydroascorbic acid derivative peak, dual 254 nm and 280 nm detectors were used. With this system, the ratio of the detector response (A_{254}/A_{280} = 1.33) for the peak was very reproducible from week to week and the ratio was markedly different from other peaks naturally occurring in the plasma samples.

To establish the accuracy and precision of the method, human plasma was spiked with 5.0 µg/ml dehydroascorbic acid and divided into 5 specimens for analysis. The results of this analysis (Tab. 2) indicated that the precision of the method was satisfactory with a ±4.0% relative standard deviation. However, the average value for the five determinations (4.19 µg/ml) was found to be 16% lower than the expected

TABLE 2

Determination of Dehydroascorbic Acid (Spiked at 5.0 µg/ml) in Human Plasma

Plasma Sample	Dehydroascorbic Acid
1	4.34 µg/ml
2	4.40 µg/ml
3	4.07 µg/ml
4	4.11 µg/ml
5	4.07 µg/ml
average	4.19 µg/ml
standard dev.	±0.17 µg/ml (± 4.0%)

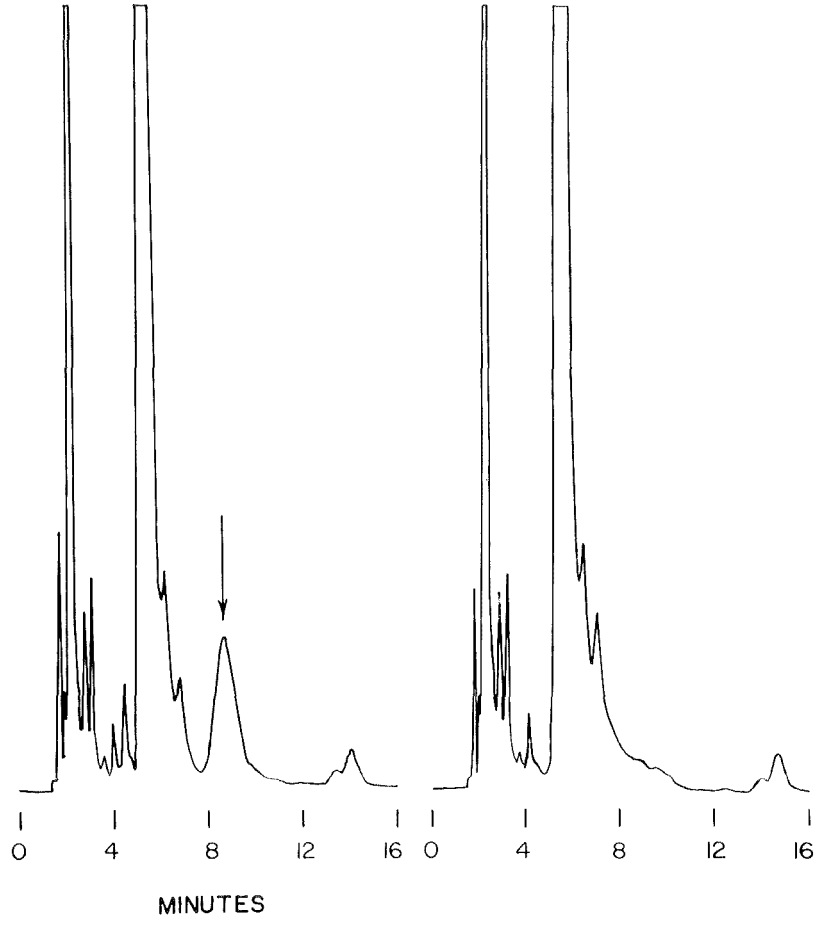

FIGURE 5: Chromatograms of Human Plasma containing Dehydroascorbic Acid Derivatized with o-Phenylenediamine. Left-sample spiked with 5.0 μg/ml dehydroascorbic acid, arrow indicated position of derivative. Right-derivatized plasma blank. Excess o-phenylenediamine appears at 5.5 min. in both chromatograms.

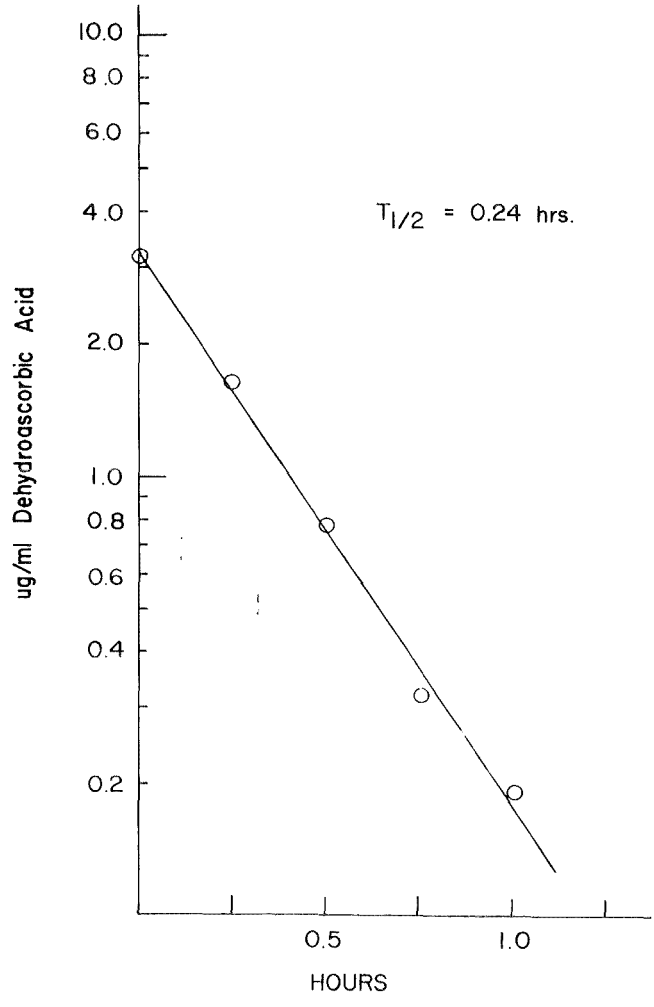

FIGURE 6: Degradation of Dehydroascorbic Acid in Unstabilized Human Plasma Held at Room Temperature.

value. Though the samples were handled quickly, it would appear that a significant portion of the added dehydroascorbic acid decomposed before the derivatization reagent was added.

To determine the stability of dehydroascorbic acid in the sample, human plasma was spiked with the material at room temperature, and samples were assayed over a one hour period. The results of this experiment (Fig. 6) showed that dehydroascorbic acid had a very short half-life ($t_{\frac{1}{2}}$ = 0.24 hr) under these conditions. With a half-life of only 15 minutes, it was essential to keep the time between the collection of the sample and the assay to a minimum.

ACKNOWLEDGEMENT

This work was supported in part by the Coronary Heart Disease Research Project of the American Health Assistance Foundation, Washington, D.C., and in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(7), 1333-1344 (1983)

HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROMETHAZINE HYDROCHLORIDE IN THE PRESENCE OF ITS THERMAL AND PHOTOLYTIC DEGRADATION PRODUCTS: A STABILITY INDICATING ASSAY

S. Stavchansky, *J. Wallace, M. Chue, and J. Newburger

College of Pharmacy
Drug Dynamics Institute
The University of Texas
Austin, Texas 78712

*Department of Pathology
The University of Texas Health Science Center
San Antonio, Texas 78284

ABSTRACT

A stability indicating method has been developed for the quantitation of promethazine hydrochloride in the presence of its photolytic and thermal degradation products. Following a basic extraction with acetonitrile, promethazine is separated from its internal standard, promazine, and vehicle components by direct high performance liquid chromatography using ultraviolet detection (249 nm) and a stainless steel column 25 cm in length, 0.46 cm i.d. packed with octa-decyl silica 5μ in diameter. A linear relationship was obtained between peak height ratio (promethazine/promazine) and promethazine hydrochloride in water over the range 30-600 g/ml. The percent coefficient of variation of the assay is 0.8% and the recovery of promethazine hydrochloride from aqueous solutions is 99.7%. The photolytic degradation of promethazine hydrochloride does not follow simple first order kinetics. Potassium iodide and p-benzoquinone had a significant effect on the degradation rate of promethazine during the first 30 minutes of the photolytic degradation reaction.

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However, after one hour there is no apparent quenching effect on the photolytic degradation rate of promethazine hydrochloride in the presence of these quenchers.

INTRODUCTION

Promethazine hydrochloride is known to undergo thermal and photolytic degradation which is oxidative in character, yielding a wide variety of degradation products including some which are colored. Promethazine hydrochloride has been determined by forming a colored product in various acidic mediuims (1,2) or by forming a colored complex with palladium chloride (3-5). It has been analyzed by the formation of photo-oxidation products which are free radicals characterized by their strong absorption in the visible region (6). Separation of promethazine hydrochloride prior to spectrophotometric determination by partition column chromatography (7,8), and ion exchange chromatography (9,10) has been reported. The USP XX assay for promethazine hydrochloride in injectable solutions is based on the determination of salts or organic nitrogenous bases (11). The method described for syrups and tablets uses ultraviolet spectrophotometry after separation from the inert ingredients by a biphasic extraction or a partition column. These methods are time consuming and may not have the specificity required for the quantitation of promethazine in the presence of its degradation products. In addition, the USP does not describe an assay method for promethazine hydrochloride in suppository dosage forms containing polyethylene glycol. The separation of drugs in proprietary preparations containing phenothiazine by high pressure liquid chromatography (HPLC) has been reported (12-14); however, these methods are not stability indicating. Other HPLC methods have been developed to quantitate promethazine hydrochloride in biological fluids (15-17). In the present investigation, an HPLC method has been developed to

specifically determine promethazine hydrochloride in polyethylene glycol suppositories undergoing stability testing. In addition, the influence of potassium iodide and p-benzoquinone on the photolytic degradation rate of promethazine hydrochloride is reported.

EXPERIMENTAL

Promethazine Hydrochloride (Napp Chemicals, Inc., Ladi, New Jersey) and Promazine Hydrochloride (Wyeth Labs, Inc., Philadelphia, Pennsylvania) were used as received. All solvents and chemicals were commercial analytical grade (Fisher Scientific Co., Fair Lawn, New York).

Chromatographic Conditions

A dual pump high performance liquid chromatographic system with a microprocessor control was used (Beckman Model 100 A Pump, Beckman Instruments, Inc., Berkeley, California). A stainless steel column, 25 cm in length, 4.6 mm i.d., was packed with Octadecyl silica, 5 μ in diamter (Ultrasphere ODS 5 μ . Altex Scientific Inc., Berkeley, California). The mobile phase consisted of 15 mM monobasic potassium phosphate pH 5 and a mixture of 9×10^{-3} mM triethanolamine and acetonitrile in a ratio of 15:85, respectively. The column temperature was maintained at 45° C by means of a water circulator. The flow rate of the mobile phase was 1.6 ml/hr and the variable wavelength detector was set at 249 nm (Hitachi Model 200-40 Spectrophotometer., Altex Scientific Inc., Berkeley, California).

Internal Standard Solution

50 mg of Promazine hydrochloride was accurately weighted and placed in a 200 ml volumetric flask and appropriately diluted with deionized water.

Polyethylene Glycol Vehicle Solutions

A stock solution was prepared using the excipients and preservatives employed in the manufacture of the suppositories. Appropriate dilutions were made with deionized water. Ten ml of this solution is approximately equivalent to the vehicle content of a 2.5 g PEG suppository.

Standard Solution

60 mg of promethazine hydrochloride was accurately weighted and placed in a 100 ml volumetric flask and brought to volume with deionized water. Two, 4, 6, 8, and 10 ml of the above solution were pipetted into five separate 10 ml volumetric flasks, and each flask brought to volume with deionized water. The concentration of the resultant promethazine hydrochloride solutions were 120, 240, 360, 480, and 600 µg/ml, respectively.

Standard Curve of Promethazine Hydrochloride in Water

One ml of each standard solution was placed in a 12 ml centrifuge tube and to each tube was added 0.5 ml of internal standard solution and 0.2 ml of 5 N NaOH. The centrifuge tube was thoroughly mixed for 30 seconds. Then, 4.0 ml of acetonitrile was added to each tube, mixed thoroughly, and centrifuged at 2000 rpm for 5 minutes. Ten µl of supernatant were directly injected into the HPLC.

Photolytic Degradation of Promethazine Hydrochloride

One hundred mls of a 1.56×10^{-3} M solution of promethazine hydrochloride in a Sörensen citrate buffer (pH 5) were placed in a quartz reaction tube (The South New England Ultraviolet Co., Connecticut), length 43 cm, o.d. 2.6 cm and i.d. 2.4 cm, equipped with a water cooling tube, o.d., 1.2 cm and exposed to ultraviolet light (350 nm) by means of a photolytic reactor (The South New England Ultraviolet Co., Connecticut). The reaction temperature was maintained at 22°± 0.2°C.

One ml samples were withdrawn at zero time and at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, 48.0, and 60.0 hours. One half ml of each sample was analyzed as described in the section under the standard curve of promethazine hydrochloride in water. The influence of radical quenchers on the photolytic degradation rate of promethazine hydrochloride was studied by adding 2.34×10^{-4} moles of potassium iodide, or 3.12×10^{-4} moles of p-benzoquinone. The promethazine concentrations were determined as previously described.

Thermal Degradation of Promethazine Hydrochloride

Promethazine hydrochloride, 0.3 g, was dissolved in 10 ml of water. Forty-nine mls of oxygen saturated Sorensen citrate buffer solution (pH 5) was placed in a separate 100 ml light proof amber volumetric flask wrapped with aluminum foil and kept at a temperature of 70°C ± 0.2°C, in a thermostatically controlled oil bath. One ml of the stock solution was added and the flask agitated for 5 seconds. Samples of 1.5 ml were taken at zero time and at appropriate time intervals up to 60 hours. One ml of each sample was analyzed as described previously. After each sample was withdrawn from the volumetric flask, the flask was immediately recharged with a stream of oxygen in order to maintain an oxygen atmosphere.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram for the analysis of promethazine hydrochloride in water. Using the appropriate mobile phase, as described previously, the retention times for promethazine and promazine were 5.9 and 9.0 minutes respectively. Quantitation of promethazine hydrochloride in water was obtained from a standard curve in which the peak height ratio (promethazine/promazine) was plotted against the promethazine

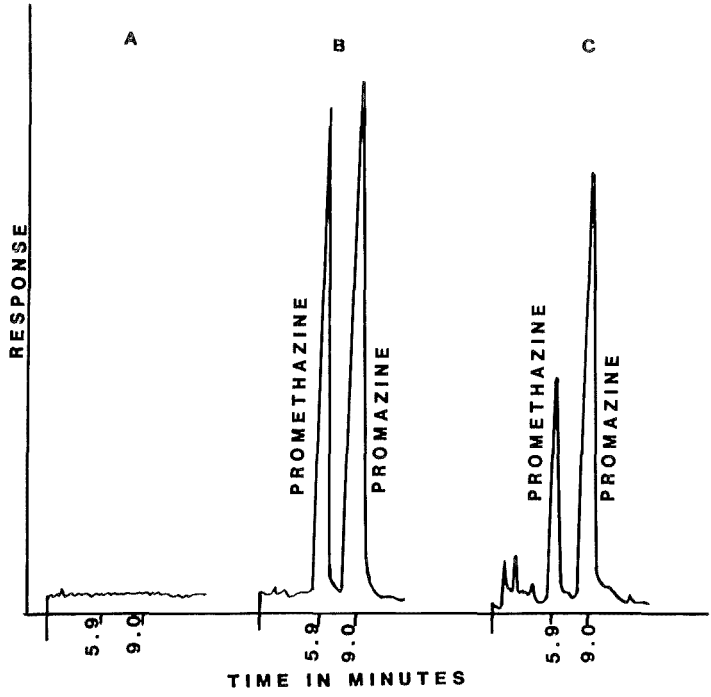

Figure 1. Chromatogram of promethazine hydrochloride blank vehicle extract, (A); 360 mcg/ml of promethazine hydrochloride extracted from an aqueous solution, (B); and chromatogram obtained after 35 hours of photolytic degradation at 340 nm of a 0.5% solution of promethazine hydrochloride aqueous solution.

hydrochloride concentration. There is a linear relationship between peak height ratios of promethazine to promazine and the concentration of promethazine hydrochloride in water over the range of 30-600 µg/ml. The least-square regression equation for the curve is $y = 0.00179X + 0.0262$, and the correlation coefficient is 0.999. The results of ten replicate assays carried out over several days indicate that the assay method has adequate precision. The percent coefficient of variation of the assays is 0.8%. The presence of the polyethylene glycol vehicle did not

have an effect on the assay. The recovery of promethazine hydrochloride from aqueous solution is 99.7%.

The method described by Underberg was used to extract the thermodegradation products (21,22). Nine spots were visualized under ultraviolet light after thin layer chromatography on silica gel G.F. and using acetone:6N NH_3 (100:2) as the mobile phase. The spots were extracted and reconstituted in methanol and injected into the chromatograph. The thermal degradation products did not interfere with the determination of promethazine hydrochloride using the HPLC procedure. In our laboratories, gas chromatographic methods (GC) using electrolytic conductivity or flame ionization detectors have been used to quantitate promethazine hydrochloride in the presence of its thermal degrada-

TABLE 1

Comparison of the Analysis of Promethazine Hydrochloride in Aqueous Solution by a Gas Chromatographic Method and a High Performance Liquid Chromatographic Method

Parameter	Method	
	GC[a]	HPLC[b]
Sensitivity in Nanograms	7.5	1.2
Correlation Coefficient of Standard Curves in Water	0.999	0.999
Promethazine Retention Time in Minutes	10.8	5.9
Percent Coefficient of Variation	0.7	0.8
Percent Recovery	100.0	99.7

a. Gas chromatography with electroconductivity detection.

b. High pressure liquid chromatography with U.V. detection at 249 nm.

tion products (18). Thus, a comparison between the HPLC method and the GC method with electrolytic conductivity detection was performed for the purpose of determining the efficiency of the methods. Table 1 illustrates a comparison of sensitivity, coefficient of correlation of promethazine hydrochloride standard curves in water, retention times, percent coefficient of variation, and percent recovery. The retention time by the HPLC method is estimated to be 1.83 times less than by the GC method. In addition, the HPLC method is more sensitive.

TABLE 2

Thermal Degradation of 600 µg/ml of Promethazine Hydrochloride in pH 5.0 Sörensen Citrate Buffer Solution at 70°C

Time in Hours	Percent Promethazine Hydrochloride Remaining in Solution	
	GC[a]	HPLC[b]
0	100.0	100.0
1	88.2	90.0
2	84.3	85.7
3	82.8	--
4	80.2	78.2
6	75.5	71.5
8	69.5	65.8
12	59.3	56.4
24	35.7	35.2
36	19.2	21.3
48	13.7	13.2
60	7.8	9.8

a. Gas chromatography with electroconductivity detection.

b. High pressure liquid chromatography with U.V. detection at 249 nm.

Table 2 illustrates the results of a thermal degradation study at pH 5 and at 70°C. Three replicate assays using the GC and HPLC methods were performed at each time interval. In order to determine if a statistical significant difference existed between the results obtained by these methods an F-test was performed ($p = 0.05$). The results indicate that there is no significant difference between the degradation kinetics of promethazine hydrochloride as determined by GC or HPLC. The results of this study suggest that both the GC and HPLC methods are suitable for the analysis of promethazine hydrochloride in the presence of its thermal degradation products. The HPLC method is more sensitive and faster than the GC method, therefore, it is recommended as the method of choice for the quantitation of promethazine hydrochloride in polyethylene glycol solid delivery systems. This method may also be extended to cocoa butter-wax solid delivery systems.

The photolytic degradation products were separated by thin layer chromatography using acetone:methanol:6N NH_3 (140:60:1) as mobile phase. Five spots were visualized under ultraviolet light. The spots were extracted with methanol and injected into the chromatograph. No interferences were found in the chromatographic region of promethazine. Figure 2 shows the results obtained from the photolytic degradation studies. In addition, it illustrates the effect of potassium iodide and p-benzoquinone on the degradation rate of promethazine hydrochloride in aqueous solution. Each point in the graph represents the mean of three determinations. It was interesting to note that during the first 30 minutes the promethazine hydrochloride concentration decreased rapidly. No linear relationship could be found from the logarithmic percent of promethazine versus time plots. This result is consistent with the report by Cox (19). However, as shown in figure 2, the degradation rates of promethazine hydrochloride from 1 hour to 60 hours followed a pseudo first-order process. The observed mag-

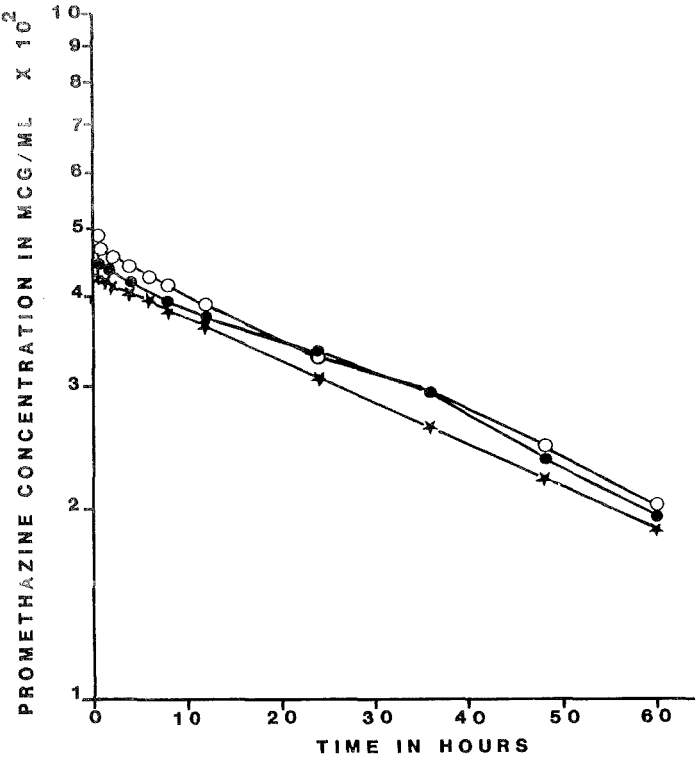

Figure 2. Influence of radical quenchers on the photolytic degradation rate of promethazine hydrochloride in aqueous solution. ★, 1.56×10^{-3} M promethazine hydrochloride aqueous solution; in the presence of: ○, 3.12×10^{-3} M of p-benzoquinone; ●, 2.34×10^{-3} N if potassium iodide.

nitude of the rate constants (Kobs ± standard deviation) were found to be $1.33 \times 10^{-2} \pm 0.18$ hrs^{-1}, $1.53 \times 10^{-2} \pm 0.25$ hrs^{-1}, and $1.65 \times 10^{-2} \pm 0.2$ hrs^{-1} in the absence and presence of potassium iodide and p-benzoquinone, respectively. In order to determine if a significant difference existed among the photolytic degradation rate constants obtained in the presence and absence of the radical quenchers (potassium iodide and p-benzoquinone) a t-test was performed ($p=0.05$).

The results indicate that there was no statistical difference between the photolytic degradation rates. There was 12.7 and 5.6 percent more promethazine hydrochloride remaining in solution in the presence of p-benzoquinone and potassium iodide during the initial 30 minutes of the reaction, respectively. After one hours, there is no apparent quenching effect on the photolytic degradation rate of promethazine hydrochloride in the presence of these quenchers. The results of this study suggest that the mechanism of decomposition of promethazine is not just a simple free radical mechanism. Potassium iodide is known to be a quencher of triplet state of photo sensitive drugs (20). However, the results of this study suggest that the mechanism of photolytic oxidation of promethazine after 30 minutes may not proceed through a triplet state step or that potassium iodide may not act as a quencher of choice for the promethazine triplet state.

In summary, a sensitive and specific high pressure liquid chromatographic method with a simple organic phase extraction was developed with the purpose of determining promethazine hydrochloride in polyethylene glycol suppositories.

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LC NEWS

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LC CALENDAR

1983

APRIL 25-27: Fifth International Symposium on Coal Slurry Combustion and Technology, Hyatt-Regency Hotel, Tampa, Florida. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Rd., Pittsburgh, PA, 15236.

APRIL 26-28: Capillary Chromatography: 5th Int'l Symposium, Riva del Garda, Italy. Contact: Dr. P. Sandra, Laboratory of Organic Chem., University of Ghent, Krijgslaan 281(S4), B-9000 Ghent, Belgium.

MAY 2-6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 6-11: Engineering Foundation Conf. on Fundamentals of Adsorption, Schloss Elmau, Bavaria, W. Germany. Contact: A. L. Myers, Chem. Eng. Dept., University of Pennsylvania, 220 S. 33rd St., Philadelphia, PA, 10104, USA.

MAY 10-13: 123rd ACS Rubber Div. Meeting, Toronto, Ont., Canada. Contact: Mrs. M. E. Bauer, Rubber Div., Inc., Univ. of Akron, Akron, OH, 44325, USA

MAY 15-17: International Symposium on LCEC and Voltammetry, Hyatt - Regency Hotel, Indianapolis, Indiana, USA. Contact: 1983 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

MAY 16-18: Symp. on Anal. Chem. of Pollutants, Jekyll Island, GA. Contact: E. McGarity, USEPA, Envir. Res. Lab., Athens, GA, 30613, USA

MAY 22-27: 8th American Peptide Symposium, Tucson, AZ. Contact: V. J. Hruby, Chem. Dept., Univ. of Arizona, Tucson, AZ, 85721, USA

MAY 23-25: ACS Regional Meeting joint with Ohio Valley

Chromatography Symposium, Miami Univ., Oxford, OH. Contact: J. R. Grunwell, Chem. Dept., Miami Univ., Oxford, OH, 45056, USA.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1-3: The Budapest Chromatography Conference, Budapest, Hungary. Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JUNE 5-8: 66th Canadian Chem. Conf. & Exhibition, Calgary, Alta., Canada. Contact: A. Rauk, Chem. Dept., Univ. of Calgary, Calgary, Alta., Canada, T2N 1N4.

JUNE 5-10: 29th IUPAC Congress, Cologne, W. Germany. Contact: W. Fritsche, c/o Ges. Deutscher Chemiker, P. O. Box 90 04 40, D-6000 Frankfurt Main 90, West Germany.

JUNE 6-9: Symp. on Milestones & Trends in Polymer Sci, Midland, Mich. Contact: J. K. Rieke, 1702 Bldg., Dow Chem Co., Midland, MI, 48640, USA.

JUNE 14-15: 2nd Annual AOAC Midwest Regional Section Meeting, Ames, IA. Contact: H. M. Stahr, Veterinary Lab., Iowa State Univ., Ames, IA, 50011, USA.

JUNE 15-16: DECHEMA Annual Meeting, Frankfurt. Contact: Secretariat, DECHEMA, P. O. Box 97 01 46, D-6000 Frankfurt 97, West Germany.

JUNE 20-22: Intl. Symp. on Chrom. & Mass Spectrom. in Nutrition Sci. & Food Safety, Montreaux, Switzerland. Contact: Secretariat, Via Eritrea 62, 20157 Milano, Italy.

JUNE 21-23: ACS Anal. Div. Summer Symp., Lincoln, Nebr. Contact: [illegible] Carr, Univ. of Nebraska, Chem. Dept., Lincoln, NB, 68588, USA.

JUNE 22: ASTM Symposium: Computers in Chemical Analysis, Kansas City, MO, Contact: K. Greene, ASTM Publications Div., 1916 Race Street, Philadelphia, PA, 19103, USA.

JUNE 26-29: ACS 13th Northeast Regional Meeting, Univ. of Hartford, Hartford, Conn. Contact: J. Burlow, P. O. Box 418, Glastonbury, CT, 06033, USA.

JUNE 27 - JULY 1: 3rd Symp. on Separation Sci. & Technol. for Energy Applications, Gatlinburg, Tenn. Contact: A. P.

Malinauskas, Oak Ridge National Lab., P. O. Box X, Oak Ridge, TN, 37830, USA.

JULY 11-14: IUPAC Prague Meeting on Macromolecules, Prague. Contact: PMM Secretariat, Inst. of Macromolec. Chem., 16206 Prague 6, Czechoslovakia.

JULY 17-23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V OBV, United Kingdom.

JULY 27-30: 3rd Int'l. Flavor Conf., ACS, The Corfu Hilton, Corfu, Greece. Contact: S. S. Kazeniac, Campbell Inst. for Food Res., Campbell Place, Camden, NJ, 08101, USA.

AUGUST 10-12: 22nd Cnadian High Polymer Forum, Univ. of Waterloo, Canada. Contact: A. Garton, NRC of Canada, Div. of Chem., Ottawa, Ont., Canada, K1A OR6.

AUGUST 14-19: 25th Rocky Mountain Conference, Denver Convention Complex, Denver, Colorado. Contact: E. A. Brovsky, Rockwell International, P. O. Box 464, Golden, CO, 80401, USA.

AUGUST 15-19: Coal Science: 1983 Int'l Conference, Pittsburgh, PA. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Road, Pittsburgh, PA, 15236, USA.

AUGUST 22-26: 7th Australian Symposium on Analytical Chemistry, Adelaide, Australia. Contact: D. Patterson, AMDEL, P.O.Box 114, Eastwood S.A. 5063, Australia.

AUGUST 26 - SEPTEMBER 2: Int'l. Symp. on Solvent Extraction, Denver, CO. Contact: D. Nowak, AIChE, 345 E. 47th St., New York, NY, 10017, USA.

AUGUST 28 - SEPTEMBER 2: 11th World Petroleum Congress, London. Contact: Amer. Petrol. Inst., 2101 L St., N.W., Washington, DC 20037, USA.

AUGUST 28 - SEPTEMBER 2: ACS 186th Nat'l Meeting, Washington, DC. Contact: A. T. Winstead, ACS, 1155 16th St., NW, Washington, DC, 20036, USA.

AUGUST 29 - SEPTEMBER 2: 4th Danubé Symposium on Chromatography & 7th Int'l. Sympos. on Advances & Applications of Chromatography in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal. Sect., Czech. Scientific & Techn. Soc., Slovnaft, 823 00 Bratislava, Czechoslovakia.

SEPTEMBER 25-30: Federation of Anal. Chem. & Spectroscopy

Societies (FACSS) Conf., Philadelphia. Contact: M. O'Brien, Merck, Sharp & Dohme Res. Labs., West Point, PA, 19486, USA.

OCTOBER 2-6: 97th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact: K. Fominaya, AOAC, 1111 N. 19th St., Suite 210, Arlington, VA, 22209, USA.

OCTOBER 3-5: Chemexpo '83, Harbor Castle Hilton Hotel, Toronto, Ont., Canada. Contact: ITS Canada, 20 Butterick Rd., Toronto, Ont., Canada, M8W 3Z8.

OCTOBER 12-13: 8th Annual Baton Rouge Anal. Instrum. Disc. Grp. Sympos., Baton Rouge, LA. Contact: G. Lash, P. O. Box 14233, Baton Rouge, LA, 70898, USA.

OCTOBER 12-14: Analyticon'83 - Conference for Analytical Science, sponsored by the Royal Society of Chemistry and the Scientific Instrument Manufacturers' Ass'n of Great Britain, Barbican Centre, London. Contact: G. C. Young, SIMA, Leicester House, 8 Leicester Street, London WC2H 7BN, England.

NOVEMBER 3-4 ACS 18th Midwest Regional Meeting, Lawrence, Kansas. Contact: W. Grindstaff, SW Missouri State Univ., Springfield, MO, 65802, USA.

NOVEMBER 9-11: ACS 34th SE Regional Meeting, Charlotte, NC. Contact: J. M. Fredericksen, Chem. Dept., Davidson College, Davidson, NC, 28036, USA.

NOVEBER 14-16: 3rd Int'l. Sympos. on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, Monaco. Contact: S. E. Schlessinger, 400 East Randolph, Chicago, IL, 60601, USA.

NOVEMBER 16-18: Eastern Analytical Symposium, New York Statler Hotel, New York City. Contact: S. David Klein, Merck & Co., P. O. Box 2000, Rahway, NJ, 07065, USA.

1984

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

1985

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. Winstead, ACS, 155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 8 - 13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1986

APRIL 6 - 11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7 - 12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1987

APRIL 5 - 10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

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ISBN: 0-8247-6935-X

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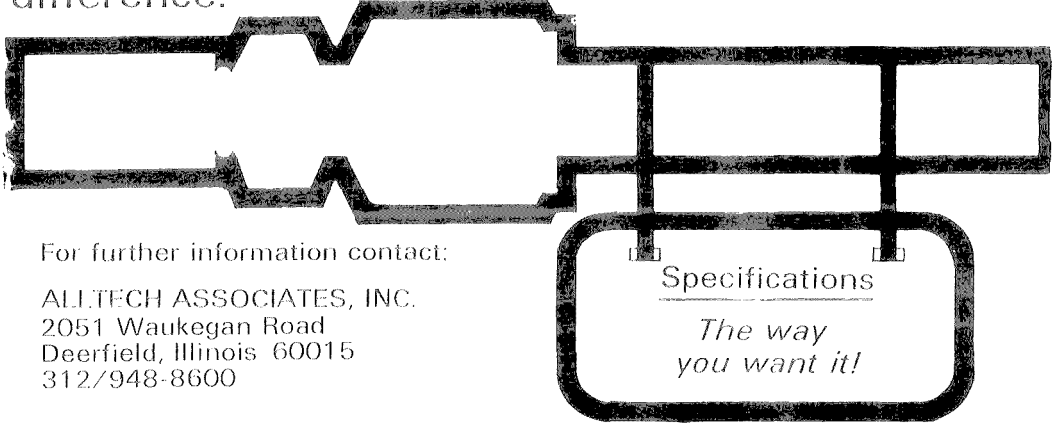

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