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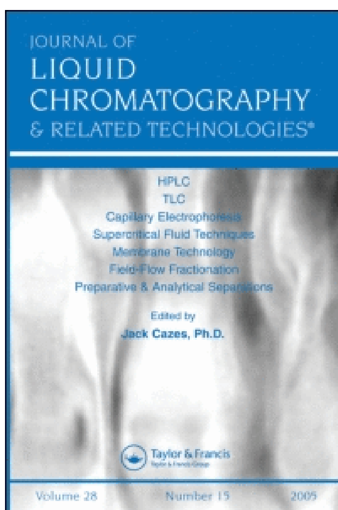

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THE APPLICATION OF CROSS-SCANNING TO CONVENTIONAL THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

The technique of cross-scanning, the scanning of thin-layer chromatographic (TLC) plates perpendicular to the direction of development, is applicable to conventional quantitative TLC where significant advantages over normal scanning are realized by its use. Several factors are discussed which influence the ability to cross-scan, such as application of the sample, solvent development and instrumentation.

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INTRODUCTION

Cross-scanning, the scanning of thin-layer chromatography (TLC) plates perpendicular to the direction of development, is a practice that has evolved from the development of high performance thin-layer chromatography (HPTLC) (1-4). HPTLC has gained wide-spread use because of improved resolution, shorter development distances, increased sensitivity and better reproducibility (1). However, conventional thin-layer chromatography continues to have important applications because of lower cost and greater capacity to accept large quantities of solute and solvent during the application of samples. This increased capacity is particularly important in the analysis of drugs and metabolites isolated from biological fluids and tissues since nanogram drug concentrations in the original samples necessitate that the extracts be concentrated and reconstituted with 50-100 µl of a solvent to facilitate the quantitative transfer of all the solute to a TLC plate. These large volume requirements exclude the use of not only the regular HPTLC plates but also those with the preadsorbent layer where 5 µl volumes are still considered maximal (5). The purpose of this report is to show that the technique of cross-scanning is applicable to conventional quantitative thin-layer chromatography and that significant advantages over scanning in the

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direction of development (normal scanning) are realized by its use. Absorbance and fluorescence methods employing cross-scanning have been applied to several experimental drugs in our laboratory. Data generated with two drugs under development are discussed to corroborate application of this approach.

MATERIALS AND METHODS

Compound I, cis-9-[3-(3,5-Dimethyl-1-piperazinyl)propyl]carbazole, was chromatographed in ethyl acetate-methanol-conc. ammonium hydroxide (85:10:5 v/v) and compound II, a substituted diphenylimidazole, was chromatographed in 100% acetone. The solvent systems were allowed to migrate 8 and 7 cm, respectively. All chemicals and solvents used in these analyses were reagent grade. Silica gel 60 plates (10 x 20 cm, EM Laboratories, Cinn., Ohio) were used as received with the addition of a parallel pencil line drawn 1.7 cm above the bottom edge of the plate. All samples were applied to this guideline with 100 µl gas/liquid syringes (Unimetrics, Anaheim, CA) using an 18-channel motor-driven multispotter (Analytical Instrument Specialties, Libertyville, Ill.). This unit aids with the application of large sample volumes (5-100 µls), since small incremental applications over several minutes minimize spot diameters (~2 mm). Rectangular chromatography tanks (23 x 29 cm) with saturation pads were used to develop the plates. Ultraviolet absorbance or fluorescence measurements were made by either normal scanning or cross-scanning the TLC plates with a single beam of a Schoeffel SD 3000 spectrodensitometer (Schoeffel Instruments, Westbury, NJ). The emission from the samples was determined with a reflectance mode assembly and a Schoeffel SD 300 density converter. Peak areas were automatically integrated and displayed with a 64K byte micro-computer (Digital Specialities, Carrboro, NC) equipped with a video display and hard-copy X-Y plotter (6). A less elaborate integrator, such as an Autolab Minigrator (Spectra Physics, Santa Clara, CA), works equally well.

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RESULTS AND DISCUSSION

Previously, cross-scanning in conventional TLC did not appear practical due to the non-uniform migration of solutes which necessitated optimization of the instrument's response by the centering of each spot prior to quantitation (7,8). Alleviation of this problem required instrument modification or alteratior of the chromatographic procedure, as done with HPTLC. Although the excitation beam of the TLC scanner used for this work illuminated an area (4x8 mm) that appeared to encompass any spot on the plate's surface, the photomultiplier did not respond uniformly when different regions of this beam were checked. To illustrate this point, figure 1 shows the sequential responses of four sections

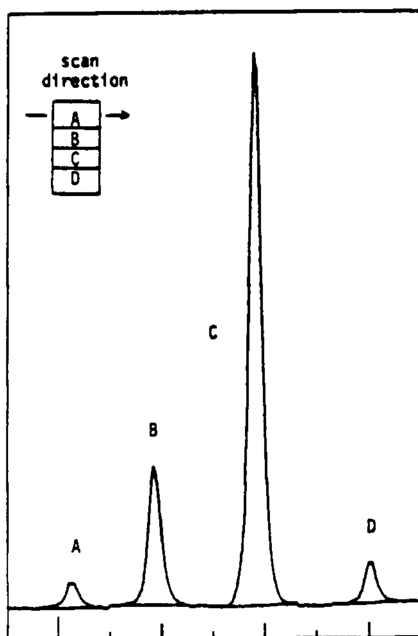

FIGURE 1

A chromatogram showing the markedly different responses of the detector when each of the regions A to D of a 4x8 mm excitation beam were scanned over a 1.5 mm dye spot.

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(A-D) of the excitation beam when each section was passed over a 1.5 mm diameter dye spot. The variation of these responses was due to the location and magnifying properties of a focusing device positioned between the plate and the photomultiplier tube. Partial or total removal of this lens system allowed the photomultiplier to respond uniformly over the entire illuminated area, but at a reduced sensitivity. Although this simple modification of the instrument did circumvent the requirement for manual optimization, altering the chromatographic procedure was found to be more desirable. Achieving reproducible solute migration (9) was accomplished by the precise application of samples parallel with the bottom edge of the TLC plate and decreasing development distances to minimize chromatographic abnormalities and spot diffusion.

Utilization of these variables to obtain reproducible R_f values was exemplified using conventional TLC plates. Fourteen aliquots (100 µl) of the chloroform extract from a plasma sample containing II were spotted, developed

and quantitated by measuring its natural fluorescence at an excitation wavelength of 280 nm. Peak areas obtained by normal scanning and cross-scanning had relative standard deviations of 3.4% and 2.8%, respectively. The accuracy and reproducibility of cross-scanning is illustrated in figure 2. Replicates of II were added to eight separate plasma samples which were extracted and quantitated. The three smaller peaks shown in this figure are 50 ng/spot standards while the larger peaks are the extracted samples, theoretically representing 80 ng/spot. The recoveries calculated following normal scanning and cross-scanning were 92.5 ± 4% and 93.5 ± 3.8%, respectively. A direct comparison of cross-scanning (peaks 1-5) and normal scanning (peaks 6-10) is shown in figure 3 for a series of plasma samples containing known amounts of I. The integrated peak areas at the top of this figure show that comparable results were obtained using the two procedures. Ripphahn and Halpaap also noted that scanning HPTLC plates in either direction gave concurring results (1). Finally, a practical example illustrating the speed of cross-scanning is shown in Figure 4. This chromatogram shows the results of a patient that received I in a recent clinical trial. Plasma was extracted with ethyl acetate and a portion of the organic solvent was concentrated prior to spotting. The acetic acid induced fluorescence of sample and reference spots was quantitated in 90 seconds by cross-scanning at an excitation wavelength of 270 nm. The above experiments establish that the advantages of cross-scanning suggested by Ripphahn and Halpaap (1) for HPTLC are applicable to conventional TLC also. These benefits include (a) wavelength optimization for each compound, (b) uncontaminated background reflectance in the vicinity of the sample giving greater specificity as illustrated in figure IV, and (c) substantially reduced time requirements for chromatographic measurements.

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The advantages of HPTLC plates are not totally derived from the 5 micron-sized particles used to prepare them, but also result from the shorter development distances and the minute amounts of material applied (10). As shown by Guiochon and Siouffi (10,11), the use of particles with a narrow size distribution is more important than particles of small size. Indeed, in terms of development time, resolution and cost, particles of 15 microns were considered superior to the 5 micron sized particles currently used in HPTLC plates. These same authors (12) further concluded that average plate heights ($\bar{H}$) comparable to HPTLC were obtained with 5-10 cm development of conventional silica gel plates prepared with 10-12 micron particles.

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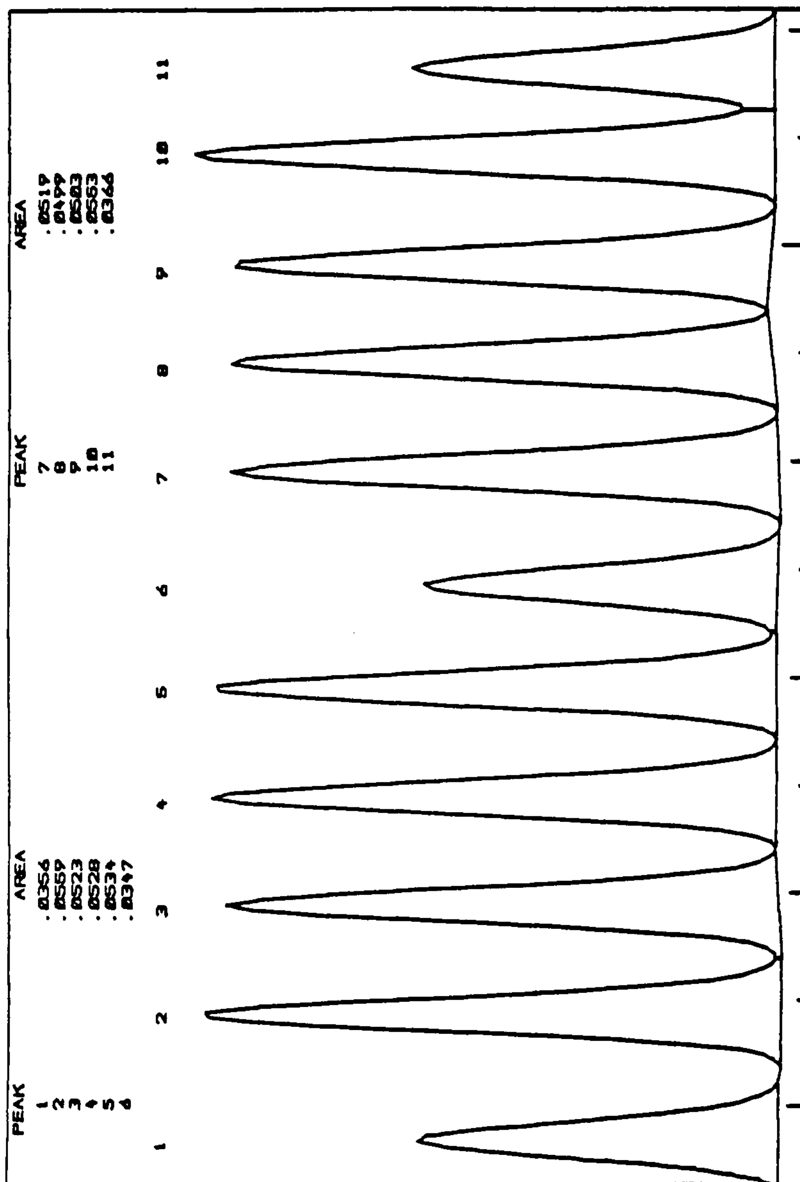

FIGURE 2. A chromatogram illustrating the reproducibility and accuracy of cross-scanning. The three smaller peaks (1, 6, & 11) are 50 ng/spot standards of II. The larger replicate peaks, which theoretically represent 80 ng/spot, had a 4.0% standard deviation in peak areas. Recoveries calculated from data obtained by normal scanning and cross-scanning were equivalent.

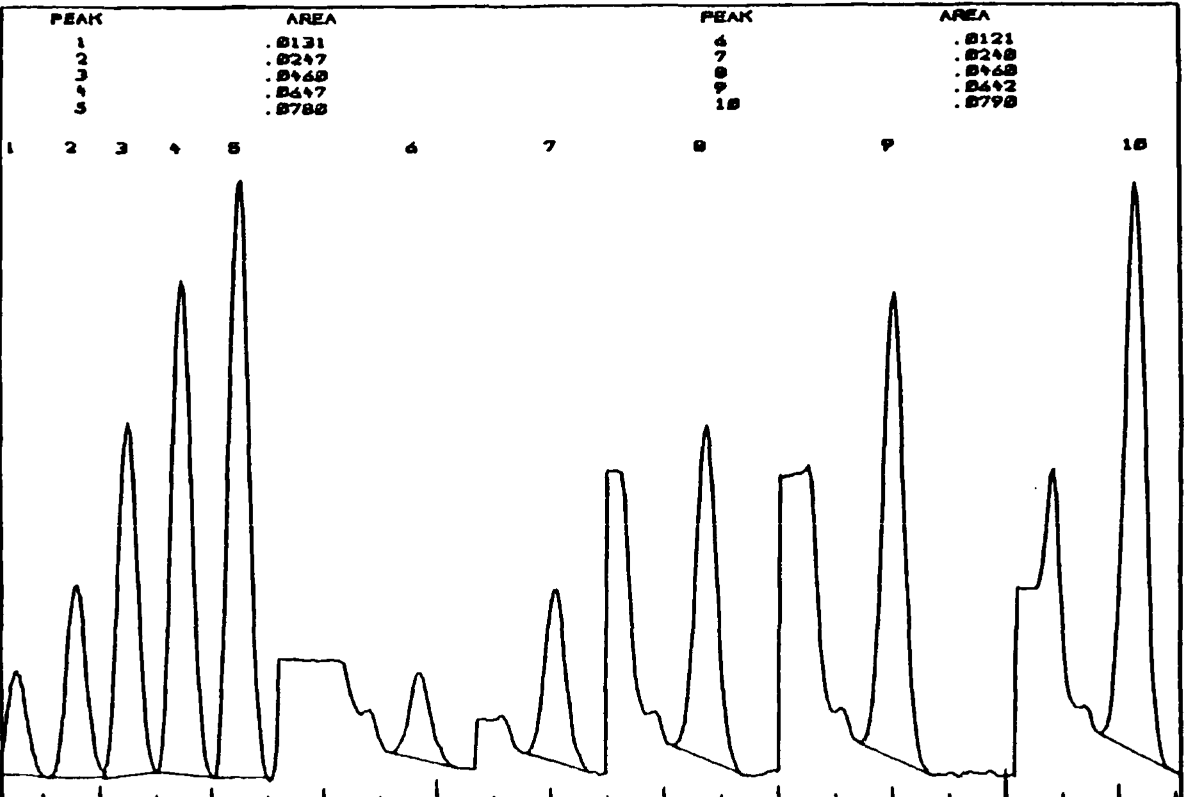

FIGURE 3. A chromatogram comparing cross-scanning (peaks 1-5) and normal scanning (peaks 6-10) for the same five plasma samples containing known amounts of I. The integrated peak areas listed at the top of this figure show that nearly identical results were obtained by either procedure.

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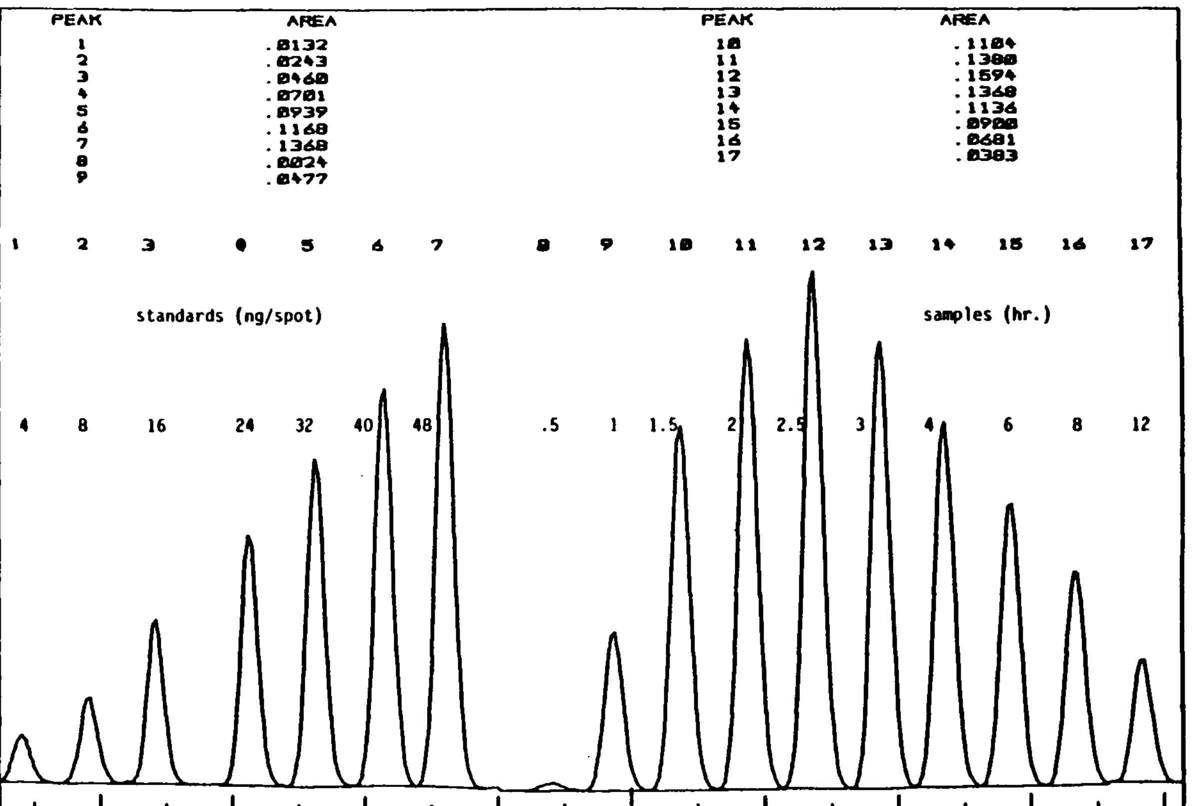

FIGURE 4. A chromatogram representing plasma data from a clinical trial and illustrating the speed of cross-scanning. The entire plate was scanned and quantitated in 90 seconds.

CONCLUSION

It is our contention that by developing conventional silica gel 60 (11-12 micron particle sized) plates 6-8 cm from the origin, their inherent benefits of economy and increased capacity are retained while the advantages of speed of development, reproducibility, increased sensitivity, and capability to cross-scan are added.

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