QUANTITATIVE DETERMINATION OF GRISEOFULVIN AND GRISEOFULVIN-4'-ALCOHOL IN PLASMA BY FLUORIMETRY ON THIN LAYER CHROMATOGRAMS

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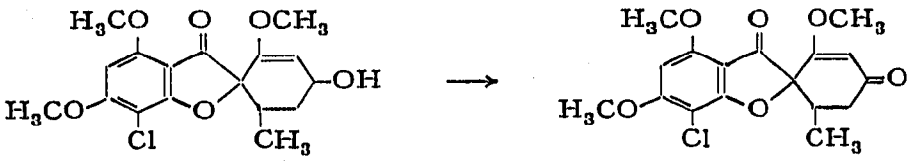
(Received July 26th, 1965)

In studying the *in vivo* conversion of griseofulvin-4'-alcohol (I) to griseofulvin (II), the need arose for a rapid, specific assay for these two compounds in plasma. The usual spectrophotofluorometric assay[1,2] for griseofulvin could not be



I II

applied due to the identical fluorescent spectrum of griseofulvin-4'-alcohol, and our inability to separate the two compounds by the usual extraction procedures. These compounds could be separated effectively, however, using thin layer chromatography (TLC). This paper reports a direct assay of the fluorescent spots on the chromatogram rather than utilizing an elution procedure.

A number of assays for biologically active substances have been developed employing quantitative thin layer chromatography[3–8]. The method, in most cases, has been to scrape the desired spot from the slide, elute, and analyze the compound in solution using an appropriate physical method. Densitometry has provided a means of quantitatively determining bile components[9], cholesteryl esters[10], and glycerides[11] directly on thin layer plates. SEILER *et al.*[12] have quantitatively determined some fluorescent substances directly on thin layer plates by scanning the chromatograms with a modified Zeiss Spectrophotometer. The Photovolt TLC Densitometer can be adapted to scan and record the relative fluorescence of spots on thin layer plates. This apparatus was used to determine quantitatively griseofulvin and griseofulvin-4'-alcohol in plasma, employing the method we describe as TLC fluorimetry.

EXPERIMENTAL

Apparatus

Modified Colab thin layer applicator; standard (Desaga) developing tank, Brinkmann No. 251020; Pyrex glass plates 200 × 50 mm, Brinkmann No. 251016;

Hamilton micro syringe 0.100 ml capacity; mechanical push button dispenser, Brinkmann No. 252035; Photovolt TLC Densitometer Model 530 (Photovolt Corp., N.Y.), and Varian G-10 recorder (Varian Assoc., Palo Alto, Calif.).

Materials

Silica gel, less than 0.08 mm (E. Merck, Darmstadt); Baymal (technical colloidal boehmite alumina), Dupont; ethanol, 95 %; acetone; griseofulvin (McNeil Laboratories Lot No. 0524), and griseofulvin-4'-alcohol[13] [m.p. 162–164° (decomposes)].

Preparation of plates

A stock suspension of 4 % technical colloidal boehmite alumina (Baymal) was prepared with high shear agitation using a Waring blender. A 50-ml quantity of this solution was diluted with an equal part of 95 % ethanol to make a 2 % suspension of Baymal in 50 % ethanol. The coating slurry was prepared by mixing 100 ml of the freshly prepared 2 % Baymal suspension with 30 g of silica gel (Merck) in a mortar. The final mixture which had a consistency similar to glycerin was poured into the modified Colab applicator. Heat resistant glass plates, 200 × 50 mm, were coated with a 250 micron layer and allowed to set for 15 min at room temperature. The plates were activated by heating in an oven at 110° for one hour and stored in a desiccator over anhydrous calcium chloride.

It should be emphasized that the inclusion of colloidal boehmite alumina in the silica gel provides an excellent binding with the glass surface. The layers produced by this procedure are less subject to cracking, result in less powdering, and are generally far more stable to rough handling. Further, the silica gel slurry is sufficiently stable so that there is no sharp time limit in manipulation. Although not important to this study, it should be noted that the Baymal contains small amounts of acetic acid.

Extraction of plasma samples

Plasma was obtained by centrifuging, at 3,500 r.p.m., heparinized blood taken from the marginal ear vein of rabbits. One ml of plasma was placed in a 15 ml glass stoppered tube and 8 ml of anhydrous ether added. The plasma was extracted by shaking on a mechanical shaker for 10 min. After separation, 6 ml of the ether layer were placed in a 20 ml glass vial and the solvent evaporated to dryness on a steam bath. The residue was subsequently spotted on thin layer plates. Lesser amounts of plasma were proportionally extracted or a smaller portion of the ether layer was taken for evaporation when the griseofulvin plasma concentration was higher than 2.0 μg/ml. In this manner readings were kept within the limits of the assay.

Spot application and development of plates

To obtain quantitative results, it was essential that the size of the spot applied to the thin layer plate be reproducible. Excellent quantitative spot application was obtained using a simple apparatus consisting of a push button dispenser (Brinkmann) fitted with a 0.1 ml Hamilton syringe which delivered 0.002 ml of solution in graduated steps. The plate was placed on the platform of a laboratory jack and raised until the silica gel coating was approximately 1 mm below the needle of the Hamilton

syringe. The solution to be applied was dispensed in 0.002 ml steps allowing the solvent to evaporate from the spot without the use of heat before dispensing the next aliquot. In this manner, the dosing needle did not touch the silica gel layer and the size of the spot could be controlled to provide the necessary uniformity.

Samples were dissolved in ether for spot application by adding 0.2 ml of ether to the glass vial containing the residue of the plasma extraction. The vial was capped and rotated to insure solution of the residue. The ether was then taken up in the dispensing syringe and applied to the plate using the technique described. An additional 0.2 ml of ether was added to the residue and again taken up with the syringe and reapplied to the original spot. The syringe and needle were adequately rinsed with solvent before application of another sample. Two plasma extract spots could be placed on one 200 × 50 mm plate.

The spotted plates were placed in a standard developing tank which was completely lined with filter paper to insure saturation of the chamber. The solvent system used for development was a mixture of 3 parts of anhydrous ether and 2 parts of acetone. Development time was approximately 45 min and was terminated when the solvent reached the end of the plate coating. Griseofulvin had an R_F value of 0.62, while that of griseofulvin-4'-alcohol was 0.50. It was found that fluorescence of the spots increased to a small extent upon allowing the plates to stand at room temperature overnight; therefore, the chromatograms were scanned for fluorescence 12–15 h after development. Duplicate determinations were carried out on all unknown plasma samples.

Adaptation and operation of the Photovolt TLC Densitometer

The Photovolt TLC Densitometer Model 530, equipped with a mechanical stage for automatic scanning, was adapted for fluorimetry by using the U.V. light source assembly (Cat. No. 5139), primary filter (Cat. No. 5267) and photomultiplier search unit (Cat. No. 5221) as described in the instruction manual supplied with the instrument. A combination of Wratten filters 42A and 49 were installed in the aperture of the search unit to provide a transmission range of 412 mμ to 490 mμ with a peak at 450 mμ. The addition of filters was advantageous in reducing stray light as the compounds to be determined had fluorescent spectra with a maximum at 450 mμ. It was found that the influence of background light could be further eliminated by reducing the source aperture and search unit aperture to 1 mm slit using black electrical tape. With this arrangement, the photometer was operated at maximum sensitivity without significant interference from stray light. Recordings of relative fluorescence of the scanned spots were made using a Varian Model G-10 recorder having a chart speed of 2 in. per min.

The developed thin layer plate was placed on the stage of the densitometer with the coated side of the place facing the U.V. light source. By manually adjusting the stage, the fluorescent spot to be scanned was located and the plate positioned on the stage to give a maximum fluorescent reading. This positioning procedure insured that the spot would be scanned through its center axis. The zero or blank reading was taken on a blank portion of the chromatogram between the position of the original spot before developing and the spot to be scanned. At this point the photometer needle was brought to zero with the zero control knob and the spot was mechanically scanned in the direction of solvent flow.

RESULTS AND DISCUSSION

Typical curves produced from scanning spots of known concentrations of griseofulvin on a developed chromatogram are shown in Fig. 1. The indicated amounts of griseofulvin were applied to the plate in 50 λ of chloroform. Uniformity of spot application was found to be most critical, and round spots of 7 to 8 mm in diameter

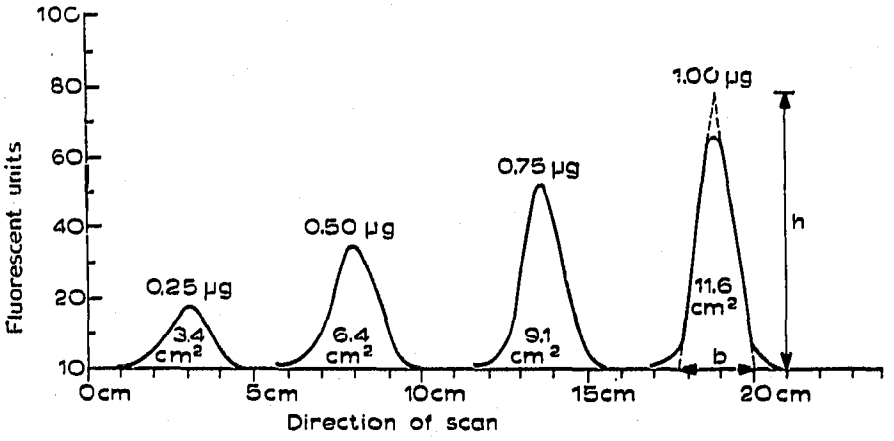


Fig. 1. Typical curves produced by scanning the fluorescent spots of known amounts of griseofulvin on thin layer chromatograms. The amount of griseofulvin applied to the plate in 50 λ of chloroform appears above each peak. The respective areas shown under each curve were calculated using the triangulation procedure shown on the 1.0 μg curve.

were applied with the application device described. A consistently low, background reading was obtained from the thin layer plates employed in the assay. Purposely selected plates with imperfections were shown to have little influence on this reading.

The area under the symmetrical peaks was calculated using the triangulation technique shown on the 1.0 μg peak in Fig. 1 with area (cm^2) = $^1/_2\ bh$. The areas obtained by this method were in excellent agreement with the areas calculated using the method of BARTLETT AND SMITH[14] for gas chromatographic peaks. Linearity between the area under the peaks produced by the scan and the amount of material applied to the spot existed between 0.25 and 1.5 μg for both griseofulvin and griseofulvin-4'-alcohol. Higher amounts of either compound on a spot appeared to cause quenching of fluorescence. The slope of such a standard curve of pure griseofulvin varied slightly from day to day, however, such variation was insignificant in terms of the whole assay.

It should be noted that during spot application, the drug concentrates at the periphery of the spot, forming a fluorescent ring. However, after development the spots were uniformly homogeneous as evidenced by the symmetrical peaks reproduced in Fig. 1. These peaks are similar in shape to normal or Gaussian distribution curves and conform to the test for such curves reported by BARTLET AND SMITH[14]. Recently KLAUS[15] has reported on possible errors caused by deformation of spots using similar techniques. Errors from spot defects or peak skewness were not observed in our method.

Known amounts of griseofulvin and griseofulvin-4'-alcohol were added to rabbit plasma and extracted with ether. An aliquot of the ether extract was evapo-

rated to dryness, taken up in ether and applied to the plate. Heating the plate during spot application caused plasma contaminants to hold back the fluorescent drugs upon development and was avoided. Fig. 2 shows typical recordings produced by scanning plasma extracts containing known amounts of griseofulvin and griseofulvin-4'-alcohol. The background of a blank plasma extract was found to be free of fluorescent material which would interfere with analysis of the drugs. From the method

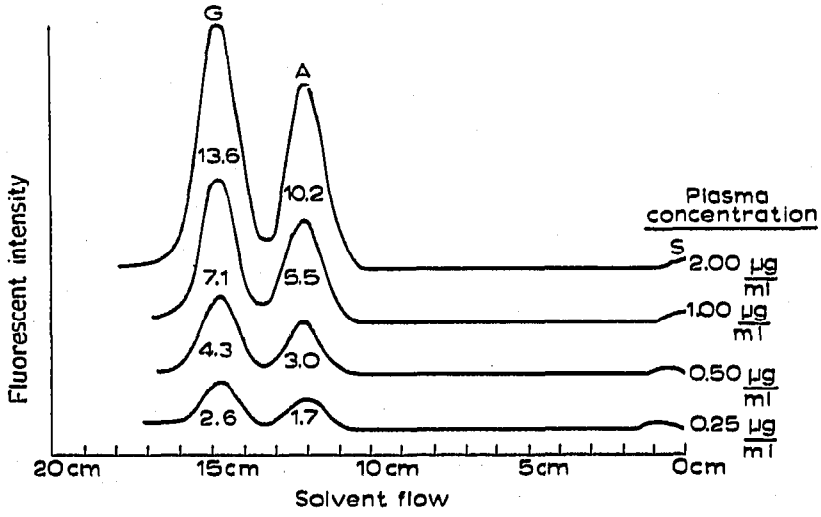


Fig. 2. TLC fluorimetric recordings of chromatograms containing griseofulvin (G) and griseofulvin-4'-alcohol (A) extracted from plasma. An ether extract of 1 ml of plasma to which had been added the indicated amounts of each compound was applied to the plate at point S and developed in an ascending manner in an ether–acetone (3:2) solvent system.

reported by BARTLET AND SMITH[14], it is possible to correct for peak overlap as a result of inadequate separation; however, such a correction was not necessary in our system, as the separation was more than adequate according to the criteria set forth by these authors.

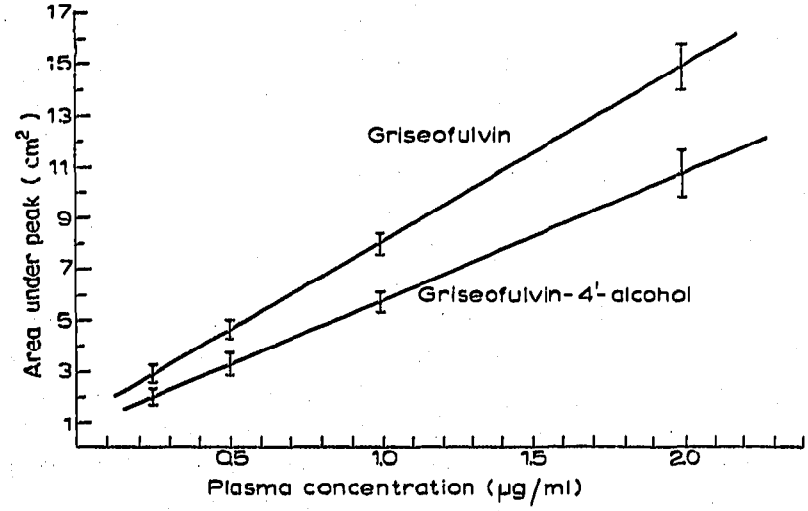


Fig. 3. Extraction curves of griseofulvin and griseofulvin-4'-alcohol from rabbit plasma obtained from the TLC fluorimetric assay. The 95% confidence intervals for each set of experimental points are shown.

The standard extraction curves for griseofulvin and griseofulvin-4'-alcohol and the 95 % confidence intervals are shown in Fig. 3. The curves were constructed using the method of least squares and the standard errors of the estimates were 3.2 for griseofulvin and 4.7 for griseofulvin-4'-alcohol. It appears to be a characteristic of the system that the standard curve for griseofulvin and the extraction curves do not extrapolate through zero. Some densitometric measurements reported also produce curves which do not intersect the origin[11].

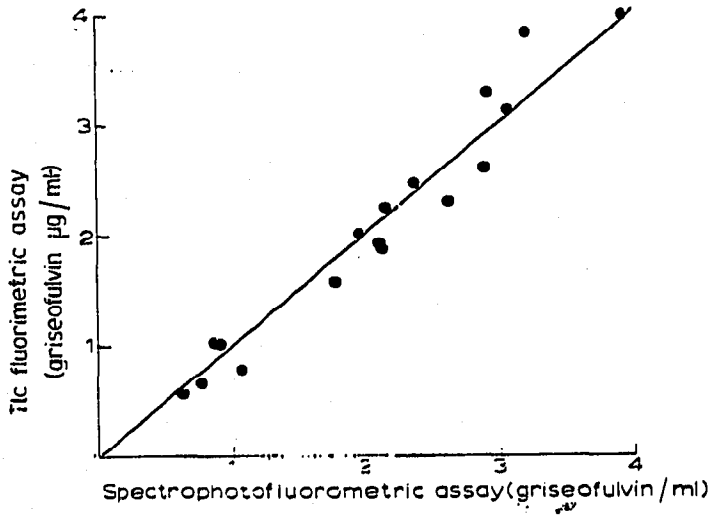


Fig. 4. Comparison of the spectrophotofluorometric assay and the TLC fluorimetric assay for griseofulvin in plasma showing experimental points grouped around the 100 % correlation line.

Both the spectrophotofluorometric[16] and thin layer fluorimetric assays were used to determine griseofulvin concentration in plasma samples following oral administration of 200 mg/kg of griseofulvin to rabbits. The results of the two different assays on each plasma sample are compared on the graph in Fig. 4. There is some scattering of values around the 100 % correlation line as a result of the errors in-

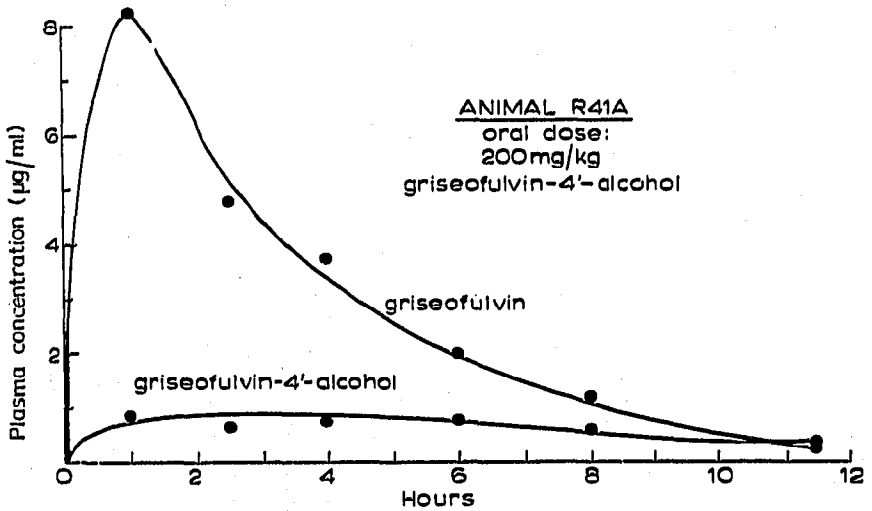


Fig. 5. Rabbit blood levels of griseofulvin and griseofulvin-4'-alcohol following an oral dose of the alcohol derivative. Both compounds were determined quantitatively using the TLC fluorimetric assay.

curred in each assay. Good agreement between the assays was shown by the calculated coefficient of correlation (r) of 0.974 which is significant beyond P (0.001) by the t test.

The plasma levels of griseofulvin and griseofulvin-4'-alcohol resulting from oral administration of 200 mg/kg of griseofulvin-4'-alcohol is seen in Fig. 5. The low plasma concentration of the alcohol and the higher level of griseofulvin indicated rapid *in vivo* conversion of the inactive (*in vitro*) precursor to griseofulvin. This rapid conversion was also seen following intravenous administration of griseofulvin-4'-alcohol. The significance of these results and the results of testing other griseofulvin derivatives will be published in the near future.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service Research Grants AI 05241 and 5 TI GM 728 from the National Institutes of Health. Pure griseofulvin was furnished by McNeil Laboratories, Fort Washington, Pennsylvania (U.S.A.). The authors gratefully acknowledge Miss DARLENE TANNEBERG for her technical assistance.

SUMMARY

A method for quantitatively determining griseofulvin and griseofulvin-4'-alcohol in plasma was developed employing measurement of fluorescence directly on thin layer chromatograms. Chromatograms of plasma extracts were mechanically scanned with a modified Photovolt TLC Densitometer. The areas under the peaks produced by the scan were found to be linear with the amount of fluorescent material on the spots within a useful range. The errors incurred in the assay were 3.2 for the determination of griseofulvin and 4.7 for griseofulvin-4'-alcohol. The results of the TLC fluorimetric assay and the spectrophotofluorometric assay on plasma samples following oral doses of griseofulvin to rabbits were in good agreement.

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