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DENSITOMETRIC DETERMINATION OF BILE PIGMENTS ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

Densitometric quantitation of bile pigments directly on the scored TLC silica gel and polyamide plates is described. Calibration curves are given for mesobiliviolin, stercobilin, mesobilirubin and also for dimethyl esters of stercobilin and biliverdin.

INTRODUCTION

The direct quantitation of compounds separated on thin-layer chromatographic (TLC) plates has the obvious advantage that in most cases the preliminary treatment required is minimal. The application of this method has already been quite extensive, including amino acids[1,2], fatty acids[3], proteins[4], pharmaceuticals[5], lipids[6–9], steroids[10,11], and other substances[12–15]. These uses have recently been divided into several general chemical groups[12–15]. For present purposes, however, a division into colored and colorless compounds is important. The latter have to be sprayed with a visualizing agent which introduces an additional possible source of error. Colored compounds (350–700 nm) are visible on the TLC plates without any treatment, and the bile pigments in particular absorb in the visible range, with exception of their chromogens which are readily oxidized to colored pigments. Hence they can be quantitated by use of visible light, and in the present study we have been mainly concerned with measurement of transmitted light[12] or of visible fluorescence excited by an appropriate source of light[12,14,15].

Extensive theoretical studies have been carried out as to the use of transmitted[12,22], reflected[12,22] and fluorescent light[13–15,22] for direct quantitation of compounds separated on TLC plates. These have revealed several factors which influence and limit the precision of the method, *e.g.* in particular a precision in spotting, the spot size on origin and spot size after development, the amount of substance in the spot, R_F values, visualization and quality of the thin-layer material[16–19]. It is extremely difficult to predict the total effect of the above-mentioned factors from the semi-theoretical studies when the influence of only one factor at a time is investigated. Thus we have chosen a different, purely pragmatic approach to the problem by experimentally standardizing the chromatography and spectrodensitometry for direct quantitation of bile pigments on TLC plates. Using the disc integrator for calculating the optical densities and peak areas, the calibration curves

have been plotted for each pigment. The most precise ranges of concentration and the limitations of the method have been determined.

EXPERIMENTAL

Materials

Silica Gel G was obtained from E. Merck, A. G. (Darmstadt, G.F.R.) for preparation of TLC plates, as described[20]. Polyamide ITLC plates 15 × 15 cm, coated on both sides, were from Gallard Schlesinger Chemical Manufacturing Corp. Chloroform, ethanol, benzene, methanol, ammonia were all analytical grade reagents; water was deionized and distilled. The Schoeffel double-beam densitometer was equipped with a monochromator and disc integrator[22].

Methods

Plates were scored into bands 10 mm wide: silica gel plates were scored with the Schoeffel scoring device and polyamide plates manually with a scalpel. Every second band was spotted, the unspotted band serving as a reference[22].

The chromatography systems were described previously[20], great care being taken to achieve reproducibility of R_F values[12]. Activation and storage of silica gel plates were rigorously followed. In an air conditioned laboratory the temperature was maintained at 21 ± 1°, and the relative humidity varied from 43 to 61%. These variations did not influence the quality of separation on TLC plates if the plates were not stored more than 48 h after activation. Silica Gel G plates are much more sensitive to humidity than polyamide plates, and the latter could be stored indefinitely without any visible change.

Special care was taken in spotting and developing the plates. Spotting was done with a micropipet, and approx. 0.3 μl were delivered at a time, followed by drying with a stream of nitrogen after which the next portion of the solution was delivered. In this way spot size could be controlled and did not exceed 30 sq. mm. The original size had some influence on quantitation, but the important point was the application of the pigment with formation of spots of the same sizes.

Development for each system must be performed in precisely the same way. The solvent front should always be the same distance from the origin. Whether the chosen distance was 10 or 12 cm was less important and could be selected according to R_F values of the separating pigments (the smaller the R_F value, the greater the distance from origin to front). Once the distance was chosen, it had to be adhered to in preparation of calibrating curves and for later analyses.

Spot area measurements were done with the Desaga–Brinkmann standard labeling template. The $\lambda_{max.}$ of each pigment was found by repeated optical density measurements of the same spot on the TLC plate by manual change of wavelength. Preceding each series of measurements, the instrument was checked with a set of five red filters. These were cut in pieces 2, 6, 7.5, 10 and 26 mm, positioned on the glass plate, and their optical densities and integrator readings were made. At least 32 measurements for each of the five filters provided sufficient data to calculate the standard deviation for the densitometer–recorder–integrator system. Coefficients of variation were ± 3.4, ± 3.9, ± 0.9, ± 5.5 and ± 4.0%, correspondingly.

Before standard curves could be prepared, it was necessary to determine the

error connected with spotting and chromatography. This was achieved by repeated spotting of 3 μl of the same solution, followed by chromatography and densitometric measurements. When integrator readings varied no more than $\pm$ 7%, the method was assumed to be acceptable for standard curve preparation. Such factors as chromatography, tank location, position of the TLC plate during the saturation, and uniform conditions of saturation had to be examined carefully as they might relate to significant variations. Background scintillations were measured by running a clean TLC plate in the spectrodensitometer, thus establishing values for Silica Gel G and polyamide plates as well as certain chromatographic papers.

The general method for preparation of standard curves was as follows. The pigment of known concentration was spotted on a plate with several spots containing various amounts. Preliminary runs of widely varying amounts gave a good indication of the desired range. On this basis more exact amounts were selected, *i.e.* 0.01, 0.05, 0.1, 0.3, 0.6, 1.0, 1.3, 1.6 and 2 μg. The plates were developed, and optical density was measured in the spectrodensitometer with the peaks integrated. The plots were then made with a quantity of pigment on the abscissa and the integrator reading on the ordinate. These plots permitted definition of the upper and lower limits of accuracy of densitometry (maximal sensitivity within the area in which the Lambert–Beer law was obeyed) (see Fig. 1).

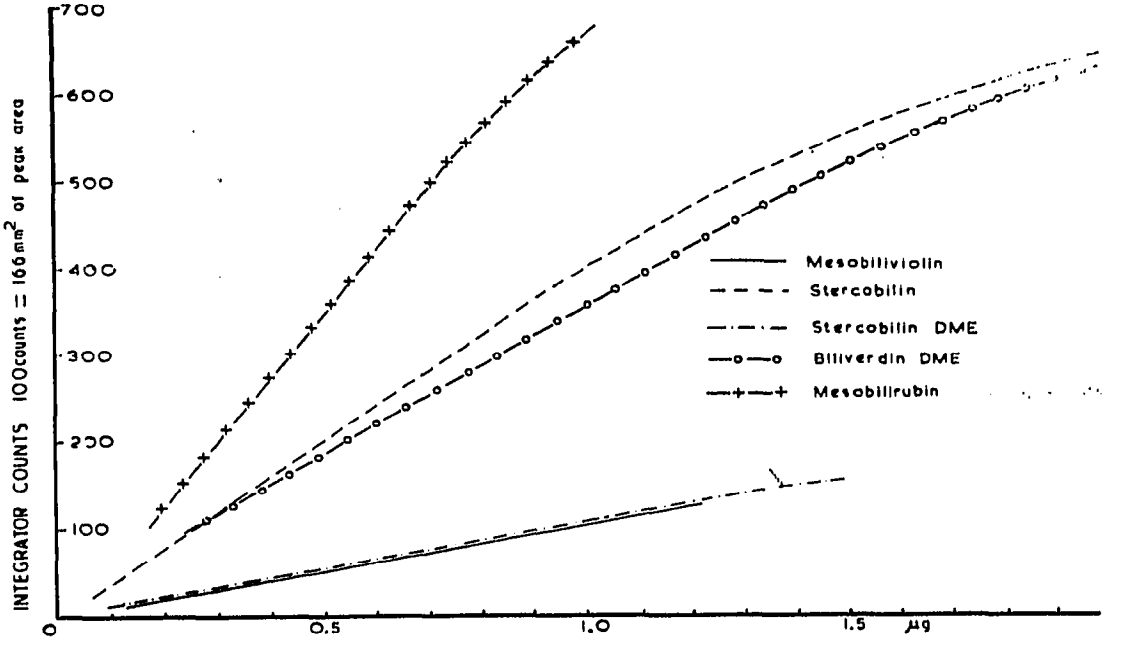

Fig. 1. Standard curves for bile pigment dimethyl esters on silica gel layers and standard curves for free bile pigments on polyamide. Chromatography systems as in ref. 20; calculated standard errors of estimate in Table II.

The influence of the R_F value on the results was determined by spotting the same amount of pigment at various distances from the bottom of the plate. The influence of spot size on the integrator reading was ascertained by spotting the same amount of pigment in varying dilution so that spots of various sizes were formed. The differences found were in agreement with published results[12].

TABLE I

ABSORPTION MAXIMA FOR BILE PIGMENTS ON TLC

Pigment	*Wavelength (nm)*	
	Free pigments on polyamide layer	*Pigment dimethyl esters on Silica Gel G*
Mesobilirubin	420	—
Mesobiliverdin	—	360
Stercobilin	480	480
Urobilin	480	480
Mesobiliviolin	580	—
Bilirubin	440	410
Biliverdin	370	320

RESULTS AND DISCUSSION

It is evident that the greatest sensitivity of this method is at the wavelength corresponding to the $\lambda_{max.}$ of each pigment, and these wavelengths were determined for each pigment on silica gel and polyamide thin-layer plates. Results are given in Table I.

Standard curves for bile pigment dimethyl esters on silica gel layers and standard curves for free bile pigments on polyamide are shown in Fig. 1. The sensitivity of direct quantitation on TLC is different for each bile pigment. Ranges of possible application (when integrator readings are directly proportional to pigment quantities, Beer's law and the ratios of integrator reading to μg pigment are constant)

TABLE II

STANDARD ERRORS AND RANGES FOR QUANTITATION OF BILE PIGMENTS

Pigment	*Standard error of estimate (ref. 23) (μg)*	*Range (μg)*	*Ratio* $\frac{\text{integrator counts}}{1\ \mu g\ \text{of pigment}}$
Mesobilirubin	± 0.015	0.02 → 0.7	831.9
Mesobiliviolin	± 0.021	0.1 → 0.7	104.7
Stercobilin	± 0.006	0.03 → 0.8	396.8
Biliver din DME[a]	± 0.041	0.082 → 1.2	362.3
Mesobilirubin DME	± 0.036	0.072 → 0.7	143.5
Stercobilin DME	± 0.055	0.11 → 1.0	90.9

[a] DME = Dimethyl ester.

are given in Table II. Examples of measurement of bile pigment mixtures at different wavelengths are shown in Fig. 2.

Absorption spectra of bile pigments are very dependent on media, and normally they are studied in solution. We have found that they differ when the pigment is dispersed on the absorbing medium of TLC plates. Since the maximum sensitivity

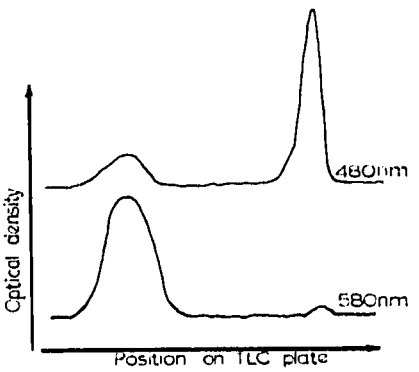

Fig. 2. Results of measurements of mesobiliviolin (left-hand peak) and stercobilin (right-hand peak) at 480 and 580 nm.

is at $\lambda_{max.}$ for a given pigment, the $\lambda_{max.}$ on the absorbing media was determined. The values are rounded up to 10 nm, corresponding to the smallest grating on the monochromator which permits duplication of the wavelength settings. True maxima are the recorded values $\pm$ 5 nm, but for purposes of quantitation this difference is insignificant.

Standard curves have different ratios of integrator reading to amount of pigment for each pigment. This is due to differences in the molar extinction coefficient, ε, and there was good correspondence between the ratio and ε as calculated for pigments in solution[21]. The greater the ratio, the greater the sensitivity of the densitometric method. The standard curves can be interpolated to 0; the lowest detectable quantity is considered to be an integrator value corresponding to doubled value for zero line variation of integrator reading or twice the standard error of estimate, whichever is greater.

The upper limits of standardization curves useful for quantitation are the points where the curve departs from the straight line, and those limits are given in Table II. The curves rise more rapidly at first and then level off as more pigment is added. This region might also be useful for quantitation; however there is no direct proportionality between integrator readings and the amount of pigment. All factors vital to reproducibility of the results are even more critical in this region. Leveling off of the curves is due to oversaturation with the pigment in the spot area[17], and then Beer's law is not obeyed (Fig. 1). If, however, too large an amount is applied for quantitation, simple dilution will permit a shift to a more favorable region on the calibration curve.

Dependence of calibration and quantitation on R_F values and spot size has been emphasized by others[12]. We have confirmed this and in addition have found an explanation for the dependence by means of using the scored TLC plates. Theoretically there is a maximal R_F value when no self-absorption and no scoring effect (error of quantitation due to scoring) are observed. The scoring effect changes the ratio of integrator counts to μg pigment and lowers the sensitivity. Scoring effect is related to a fraction of the pigment in a given spot on the very edge of the 10 mm band which is never placed in the light beam for measurement of absorption. The effect influences larger spots related to greater R_F values. It is closely related to the spot size on the origin, since the larger the spot, the sooner the scoring effect becomes noticeable—hence the necessity of rigorous attention to the conditions of spotting,

developing and measuring. When the pigment is spotted on the origin there is no scoring effect, since the whole spot is under the beam and then the oversaturation becomes prevalent. The straight part of the curve corresponds to the absence of both effects, scoring and oversaturation.

Mixtures of pigments are readily analyzed by means of the prepared calibration curves. Each pigment represented by a peak can be distinguished by density measurements at the appropriate wavelength (Table I). This technique can be used for quantitation of pigments separated on TLC. Also, it could be applied to qualitative identification of pigments by estimation of corresponding absorption maxima for each pigment. An example of this application limited to two pigments is shown in Fig. 2; however, it can be employed to multi-component mixtures.

Introduction of the densitometric method for direct determination of bile pigments on TLC necessitates some changes in the preliminary preparation of the solution. Previously it has been necessary to separate and partially purify pigments to be determined. With the present method a solution of a mixture of pigments can be subjected directly to TLC and densitometry.

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