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QUANTITATIVE ANALYSIS OF GLYCERYL NITRATES ON THIN-LAYER CHROMATOGRAMS

COMPARISON OF COLORIMETRY AND DENSITOMETRY

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SUMMARY

Densitometry and colorimetry were compared as methods for the quantitative determination of glyceryl nitrates on thin-layer chromatograms. For both methods a linear relationship exists between the amount of nitrate spotted and the results obtained. The reproducibility of densitometry seems acceptable at least for amounts of nitrate larger than 2 μg. As densitometry is much less time consuming than colorimetry after elution, the former method is preferable for our purposes. Application of a correction factor for baseline variation could be useful; correction for plate-to-plate variations is unrewarding, and internal standards have to be used on each plate.

INTRODUCTION

In our study of the metabolism of nitroglycerin, we previously used a quantitative analysis of different organic nitrates (tri-, di- and monoglyceryl nitrates) by separating them on thin-layer chromatograms followed by colorimetric determination of the individual nitrates on material eluted from the chromatograms[1,2]. Quantitative estimation of the different glyceryl nitrates had been made by CREW AND DICARLO[3] by radioscan on thin-layer plates. In our work with non-radioactive material, the elution and dosage procedure used hitherto is quite time- and effort-consuming. In the last years a number of instruments for direct quantitative spectrophotometry of thin-layer chromatograms were developed (for references see ZÜRCHER *et al.*[4]). In the present work we report our results with the *in situ* spectrophotometric determination of glyceryl nitrates on thin-layer plates, using a "flying spot" scanner, and we compared the results obtained with the results of colorimetric determination after elution from the plates.

MATERIALS AND METHODS

Materials

Glyceryl trinitrate (GTN, nitroglycerin or trinitrine) was available as a commercial 1% solution in ethyl alcohol. Glyceryl 1,3-dinitrate (GDN 1-3), glyceryl 1,2-

dinitrate (GDN 1-2) and glyceryl 1-mononitrate (GMN 1) were prepared from 1,3-dibromohydrine, 1,2-dibromohydrine and 1-chlorohydrine, respectively, according to the method of DUNSTAN *et al.*[5]. The substances obtained were purified and identified as described previously[6]. Appropriate dilutions of the various esters were prepared in ethyl alcohol. For practical purposes the amounts of the different glyceryl nitrates are always expressed in "μg nitrate", *i.e.* their content of nitrate.

Chromatography and detection

One-dimensional TLC of GTN, GDN 1-3, GDN 1-2 and GMN 1 was carried out on 0.25 mm Silica Gel F_{254} plates on glass (Merck). Using a microliter pipet (Haak 10:10), small samples (2–30 μl) of the nitrate solutions were carefully applied to the plates, and ascending chromatograms were run at room temperature. The solvent used for GTN, GDN 1-3 and GDN 1-2 was benzene–ethyl acetate, 4:1 (ref. 7). As solvent for GMN 1 we employed benzene–ethyl acetate, 2:5, with an R_F value for GMN 1 of 0.43. The solvent was allowed to travel 15 cm. The nitrates can be visualised on the thin-layer plate by spraying with a 1% diphenylamine–ethyl alcohol solution (15 ml) followed by irradiation with UV light for 10 min, the nitrate esters appearing as yellow–green spots on a light tan background[8].

Estimation with densitometry

This estimation is carried out after spraying the plates as described above, using an automatic "flying spot" scanner, the Universal Densitometer Vitatron, type TLD 100. The measuring system is an absorption measurement by means of transmitted light.

Experimental details were provided by the manufacturers (Vitatron N.V., Dieren, The Netherlands). The measurement of the peak area is done automatically by the integrator built into the recorder and is expressed in integration units. This peak area has a linear relation with the total concentration of the spot. Optimal scan conditions were experimentally determined: log (—); level b/5; span 9.60; damping 2; stroke 6 mm; filter 398 mμ.

One of the problems in densitometry is the variability in thickness of the silica gel layer; variations can be seen in one given plate, but even larger differences can be seen from one plate to another. Such variations undoubtedly influence the readings. The manufacturers of the densitometer suggest the use of a correction factor $e^{-2\Delta A_0}$ derived from GOLDMAN AND GOODALL's work on transmission of light through materials such as silica gel layers, with their inherent variability of thickness[9,10]. This correction factor can be applied for plate (or baseline) correction or for plate-to-plate correction. For plate correction, ΔA_0 is the difference of absorption between a "reference baseline point" and the baseline of each peak. For plate-to-plate correction, ΔA_0 is the difference of absorption between a reference baseline point on each plate and a fixed area of a standard plate.

Estimation with colorimetry after elution

The areas of the thin-layer plates corresponding to the various nitrate esters are scraped off individually; to allow exact localisation of these areas, guide spots of each of the nitrate esters used are applied on the plates, and after completion of the chromatogram these guide spots are colored by spraying with a 1% diphenylamine–

ethyl alcohol solution and by irradiating with UV light (see above). The material scraped off is transferred to glass-stoppered tubes; the nitrates are eluted by adding 3 ml of ethyl alcohol. The tubes are shaken for about 10 min and the silica gel is removed by centrifugation. A similar elution process is followed for a blank sample of the coating material alone. Colorimetric determination of the nitrates is carried out according to the method of LORENZETTI *et al.*[11] with an Universal Vitatron Colorimeter at 540 mμ, using 2-cm cells.

For both methods the relative standard deviations (s_{rel}) are calculated from

$$s = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

and used as an index of reproducibility.

RESULTS

Densitometry

Reproducibility of results in one given plate. Different amounts of GTN (2, 5, 10 or 20 μg of nitrate) were spotted on thin-layer plates; on each plate the same amount of the substance was spotted six times. The results are given in Table I, with the calculation of the reproducibility, as expressed by the relative standard deviation. The values for "corrected peak area" are obtained by multiplication of the peak area with $e^{-2\Delta A_0}$ for plate (or baseline) correction.

TABLE I

REPRODUCIBILITY OF SCANNING OF GTN SPOTTED ON ONE PLATE

Each amount of GTN was spotted 6 times and the mean of the six measurements calculated for each plate. The quantity of GTN spotted is expressed as μg nitrate. The peak area is given in integration units. Corrected peak area is obtained by multiplying with the plate correction factor (for details see text).

Quantity of GTN spotted	*Chromatogram No.*	*Peak area*		*Corrected peak area*	
		Mean	$s_{rel.}$	*Mean*	$s_{rel.}$
2	1	25.8	2.7	25.8	3.9
	2	50.4	11.1	50.7	11.1
	3	26.3	8.0	25.7	7.1
5	4	88.7	4.4	89.1	4.5
	5	118.2	3.7	116.3	3.2
10	6	198.6	2.9	196.6	2.2
	7	154.4	3.7	150.6	3.7
	8	139.3	3.2	137.1	2.1
20	9	251.2	4.1	249.6	2.9
	10	215.4	2.4	215.7	2.3

Reproducibility of results from different plates. Two concentrations of GTN, 10 and 20 μg nitrate, were spotted on fourteen different plates, and the peak areas, as measured by the densitometer, were compared. Corrected values are also given. They are obtained by multiplying the peak area by the factor $e^{-2\Delta A_0}$, for variations between plates (plate-to-plate correction) (Table II).

Calibration curves for the relationship between concentration of the different glyceryl

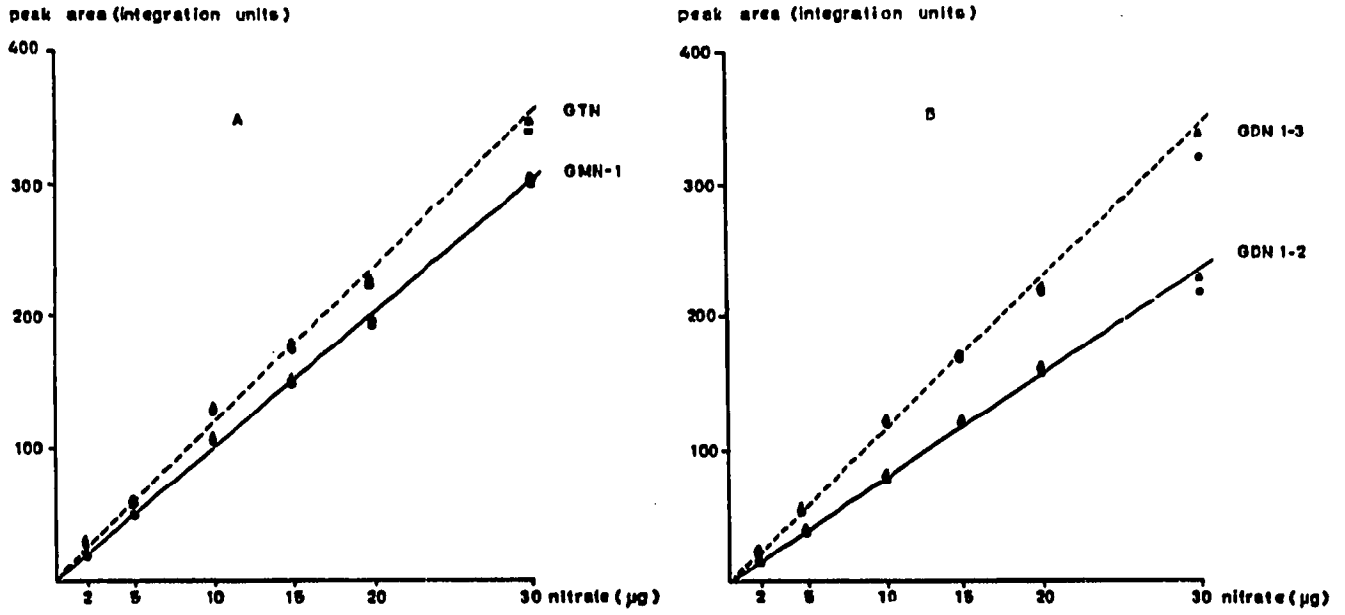

Fig. 1. Relationship between densitometer peak area (expressed in integration units) and amount of four different glyceryl nitrates (expressed as µg nitrate), spotted on thin-layer plates. (A) GTN: glyceryl trinitrate; GMN 1: glyceryl 1-mononitrate. (B) GDN 1-2: glyceryl 1-2-dinitrate; GDN 1-3: glyceryl 1-3-dinitrate. ● = uncorrected peak area; ▲ = peak area $\times e^{-2A_0}$ (plate or baseline correction).

nitrates and the peak area. These curves were obtained by spotting increasing amounts (2–30 µg of nitrate) of one of the glyceryl nitrates used on one plate and by plotting the peak area *versus* the amount spotted. Fig. 1 gives a representative example for each of the four glyceryl nitrates. As the plate-to-plate correction did not prove to be satisfactory (see DISCUSSION), no attempt was made to calculate mean calibration curves (*i.e.* curves calculated from results on different plates). The correction factor for variations in one plate was applied.

Colorimetry

If one measures colorimetrically increasing amounts of inorganic or organic nitrates, a linear relationship between absorbance and nitrate content can be seen, up to 30 µg of nitrate.

To test the reproducibility of the colorimetric determination, the absorbance for six 10-µl samples of GTN, equivalent to 10 µg nitrate each, was measured. For a mean absorbance of 0.289, a relative standard deviation of 4.1% was found. If one spots these small amounts of GTN on a thin-layer plate and elutes them without developing the plate, the material eluted gives an absorbance of 0.272 ($n = 6$), with a relative standard deviation of 7.0%. This absorbance equals 93.2% of the mean absorbance found with colorimetry without spotting (*i.e.* a recovery of 93.2%).

After spotting small quantities of GTN on a plate and developing the plate, the same elution and determination procedure can be followed. For 2 µg of GTN spotted, the mean absorbance ($n = 6$) is 0.054 with a relative standard deviation of 12.6%. The recovery of GTN is 90.0%. For 10 µg of GTN spotted, for six measurements on one plate the mean absorbance was 0.257, with a relative standard deviation of 7.5% and a recovery of 88.9%; for six measurements on another plate, the mean

TABLE II

REPRODUCIBILITY OF SCANNING OF GTN SPOTTED ON FOURTEEN DIFFERENT THIN-LAYER PLATES

10 and 20 μg GTN (expressed as nitrate content) were spotted on each plate. The peak area is given in integration units. Corrected peak area is obtained by multiplying with the plate-to-plate correction factor.

Chromatogram No.	*Peak area*		*Corrected peak area*	
	10 μg	*20 μg*	*10 μg*	*20 μg*
1	101.7	217.0	145.7	311.0
2	135.8	214.1	172.6	272.1
3	148.1	287.4	188.8	366.4
4	135.0	241.2	205.5	367.1
5	119.6	213.4	191.4	341.4
6	136.2	244.1	207.3	371.5
7	141.3	241.0	200.5	342.0
8	123.0	229.4	179.8	335.4
9	121.7	211.0	177.9	308.5
10	115.6	209.0	202.3	365.9
11	165.6	298.0	262.3	472.0
12	180.0	342.2	243.0	461.9
13	128.4	224.0	166.5	290.5
14	192.6	338.5	223.8	393.3
mean	138.9	250.7	197.8	357.1
s_{rel}	18.3	18.6	15.5	16.1

absorbance was 0.251 with a relative standard deviation of 7.9% and a recovery of 86.8%.

DISCUSSION

For quantitative analysis of glyceryl nitrates on thin-layer plates, both colorimetry and densitometry can be used. Indeed the calibration curves indicate a good linear relationship between peak area (densitometry) or absorbance (colorimetry) at one side, and the amount of glyceryl nitrates at the other side, and this up to a nitrate content of 30 μg. For both methods the organic nitrates used (and for that matter inorganic nitrates also) give, for the same content of nitrate, similar results.

Densitometry, with its obvious advantages of simplicity and speed, presents several difficulties. The unequal thickness of a plate provokes irregularities in the baseline. For a nitrate content of 5 to 20 μg, we found a reproducibility, for sets of six measurements, of 2.4 to 4.4% (uncorrected values). These values compare favourably to the results reported, *e.g.* for amino acid determination on thin-layer plates[4].

For small amounts of nitrates, *e.g.* 2 μg, the relative standard deviation can be as high as 11.1%. Such a variability can be ascribed to the irregular baseline course, making the definition of start and end of the peak area very difficult. Application of the plate correction yields better results in the case of large amounts of nitrate; for 2 μg nitrate this is not true. To us the results of densitometry for 2 μg nitrate look unacceptable.

A second problem in the densitometry is the use of a calibration curve obtained on a set of plates, for quantitation of unknown amounts of nitrates on other plates. For a set of measurements on 14 plates, a relative standard deviation of 18.3% (for

10 μg nitrate) and 18.6% (for 20 μg nitrate) is calculated. Application of the correction for plate-to-plate variation does not improve the results significantly. Although spraying and UV irradiation were rigidly standardised, they probably introduce a variability not correctable by the correction factor used; indeed in the case of dyes, where one can scan on a non-sprayed colourless background, the plate-to-plate correction yields very satisfactory results (non-published results). The lack of reproducibility from plate-to-plate precludes the use of a given calibration curve for measuring the amounts of nitrate on different thin-layer plates. This difficulty can be overcome by using internal standards, *i.e.* by spotting known quantities of the substances used on each plate.

Colorimetry is in itself a reproducible technique, for 10 μg of nitrate six measurements give a relative standard deviation of 4.1%; this variability is due in part to the manipulation of such small volumes (10 μl) and to the inherent variability of colorimetric procedures[12]. As expected, the relative standard deviations of measurements after spotting and elution increase. Here a calibration curve obtained once can be used for measurements from other plates. With this method one has to take into account the loss of material during the elution procedure; we usually obtain a recovery of around 90%. As we see no reason why recovery should be different from plate to plate, we think a sensible way of coping with this problem is to control the recovery from known amounts of material at given time intervals.

Concluding we can say that both colorimetry and densitometry can be used for quantitative estimation of glyceryl nitrates on thin-layer plates. An advantage of the colorimetry is that the spot size and uniformity of thickness of the silica gel layer are not important. Densitometry is obviously economical, considering time and effort. The reproducibility of the latter method seems to be at least as good as the reproducibility with colorimetry; it is, however, necessary to use internal standards because of the high plate-to-plate variability. Both methods seem less suitable for quantities of 2 μg of nitrate or less.

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