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V. QUANTITATIVE ANALYSIS OF CLADINOSE AND METHYLCLADINOSIDE BY DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS

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SUMMARY

A direct, quantitative, thin-layer chromatographic method is described for the determination of sugar cladinose and methylcladinoside in the presence of other acid-degradation products of the antibiotics erythromycin oxime and erythromycylamine. Cladinose and methylcladinoside are separated from compounds which cause interference on pre-coated silica gel F_{254} plates, and are measured directly on the thin-layer plate using a densitometer. Standard graphs obtained for cladinose and methylcladinoside show a linear relation between the square root of the peak area and the logarithm of the amount of substance present in the spot, as well as between the square of the area and the logarithm of the amount. This method is very successful in stability studies on the antibiotics erythromycin oxime and erythromycylamine in an acid medium. The technique seems to be particularly useful in instances in which the usual analytical methods either cannot be applied or can be applied only with difficulty.

INTRODUCTION

A number of publications have appeared on the quantitative determination of erythromycin and its derivatives[1–4]. Most of the methods employed, however, are non-specific and time-consuming, so they are not satisfactory for stability studies in the presence of degradation products and for the determination of very small quantities. Thin-layer chromatography (TLC)[5–7] is a practical method of solving these problems rapidly and satisfactorily. The substance to be analysed is separated from compounds which might cause interference by means of TLC, which permits a direct densitometric determination.

In our laboratory we have applied TLC to the identification of erythromycin oxime and erythromycylamine, of their derivatives and of their degradation products[8]. Continuing the work in this field, we studied the stability of the antibiotics erythromycin oxime and erythromycylamine[9] in an acid medium. Thus, reaction of these substances with 0.23% hydrogen chloride in methanol or in water at room temper-

ature removed the neutral sugar cladinose and provided products having low antibacterial activity. The structures of these antibiotics and their hydrolytic products are shown in Fig. 1.

In order to test the stability of the above antibiotics, the chromatography–densitometry method has been developed for rapid quantitation of the liberated sugar cladinose and methylcladinoside. Although a number of publications[10–16] give TLC methods for sugars, the quantitative determination of cladinose and methylcladinoside has not been described previously.

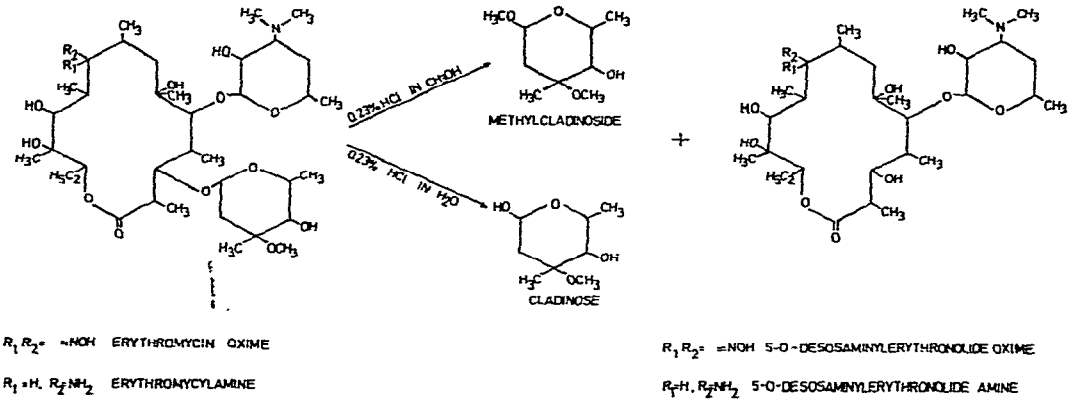

Fig. 1. Acid hydrolysis of erythromycin oxime and erythromycylamine at room temperature.

EXPERIMENTAL

Preparation of standards

The standards were prepared by the method for cladinose and methylcladinoside[17], and were kept in dark bottles at 17° as 1.60 and 1.45% solutions in chloroform, respectively.

Preparation of hydrolysates

Erythromycin oxime and erythromycylamine were hydrolysed in 0.23% HCl in methanol and water at room temperature for 48 h. Samples taken at intervals were neutralized with Na_2CO_3, the solvent was removed *in vacuo* and the residue was extracted with chloroform. The extract was dried and concentrated *in vacuo* to a solid. The sample solutions used for spotting were prepared in chloroform (10 mg/ml).

Chromatographic procedure

All of the solvents used were c.p. grade (E. Merck, Darmstadt, G.F.R.). Precoated silica gel F_{254} plates (20 × 20 cm, with a layer thickness of 0.25 mm) (Kemika, Zagreb, Yugoslavia) were used. The solutions were applied by means of 10-μl micropipettes and a semi-automatic Desaga micro-doser, in a 1-cm long lines. To set up the calibration graphs, amounts of standards of between 1 and 50 μg per spot were applied. In order to prevent the initial spots from spreading, the desired volume was added in 0.2-μl portions with drying between additions. After application of the

samples and standards, the plate was developed in chloroform–benzene (19:1) saturated with ammonia vapour, to a height of 18 cm, then heated in an oven at 110° for 10 min. The detection reagent was prepared by dissolving 3 g of phenol in 95 ml of absolute methanol and 5 ml of H_2SO_4. The plates were sprayed with this reagent and then heated for another 10 min at 110° to produce the dark brown spots.

Densitometry

Densitometry was carried out by use of a Photovolt 520 A densitometer, furnished with a drive unit, Model 42 B light source and Model 49 A integrator (Photovolt Corp,, New York, U.S.A.), through a blue filter of 465-nm wavelength. Plates were scanned parallel to the direction of development.

RESULTS AND DISCUSSION

Cladinose and methylcladinoside were separated satisfactorily from other components in hydrolysates. The R_F values for the antibiotics and their degradation products ranged between 0.08 and 0.70 (Table I).

TABLE I

R_F VALUES OBTAINED FROM TLC ON SILICA GEL WITH CHLOROFORM–BENZENE (19:1) IN A CHAMBER SATURATED WITH AMMONIA VAPOUR

Compound	R_F
Erythromycylamine	0.40
Erythromycin oxime	0.15
Cladinose	0.29
Methylcladinoside	0.70
5-O-Desosaminyl-erythronolide amine	0.35
5-O-Desosaminyl-erythronolide oxime	0.08

The spot areas of the analyses were compared with spot areas from standard solutions of the same compounds, the distribution of the substance within an individual spot being identical in the standard and the analysis sample. Standards were always applied near to the samples on the plates in order to avoid errors due to variations in the chromatographic conditions. In the range of 3–18 μg, the best linearity for cladinose and methylcladinoside was obtained when using the square root or the square of the area and the logarithm of the weight, as shown in Fig. 2. The expected variations in the areas observed during the scanning were determined by measuring the area of a cladinose standard. Three different amounts of cladinose, 20, 10 and 3 μg per spot, were each scanned 16 times. The coefficients of variation (1.4, 1.6 and 4.9% respectively) demonstrate the good reproducibility of the scanning.

After the areas of the standard and sample spots had been measured, the amounts of compound in the unknown spots were calculated by arithmetical and graphical methods. Arithmetically the results were calculated according to eqn. 1

$$\log C = \log C_M + \log d \frac{A^2 - A_M{}^2}{A_V{}^2 - A_M{}^2} \quad (1)$$

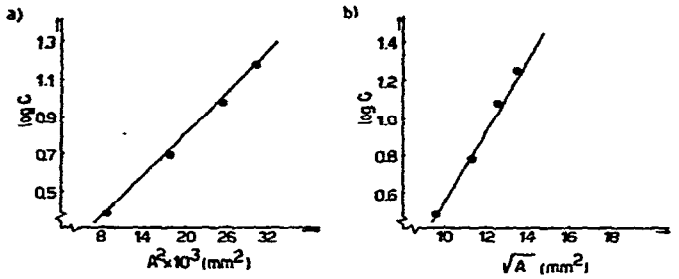

Fig. 2. Relation of the densitometric peak area (A) to the logarithm of the amount of sugar (log C) present on the chromatogram: (a) log C *versus* A^2; (b) log C *versus* $\sqrt{A}$.

TABLE II

QUANTITATIVE DETERMINATION OF CLADINOSE IN ACID HYDROLYSATES BY THE ARITHMETICAL METHOD

Amount of standard cladinose (μg)	*Dilution factor, d*	*Peak area of standard (mm²)*	*Peak area of cladinose in hydrolysates (mm²)*	*Amount of cladinose in hydrolysates* (μg)*
3	4	79	93	4.368
12		123	107	6.757
			108	6.990

* Calculated according to eqn. 1.

where C = the amount (μg) of the compound in the unknown spot, C_M = the amount (μg) of the smaller standard, d = the relation (dilution factor) between the two standards and A, A_M and A_V = the integral areas (mm²) of the unknown spot, of the smaller standard spot and of the larger standard spot, respectively. This equation is a modification of Nybom's equation[18], *i.e.*, instead of the area we used the square of the area. An example of the quantitative analysis by this method is given in Table II.

Beside the above arithmetical method we also applied a graphical method, where four or more standards were used. Straight lines were fitted to the calibration points by means of the least-squares method. The calibration graphs were individually calculated for each plate, and in this case we employed the linear relation between

TABLE III

QUANTITATIVE DETERMINATION OF CLADINOSE IN ACID HYDROLYSATES BY THE GRAPHICAL METHOD

Peak area of cladinose in hydrolysates, A (mm²)	$\sqrt{A} = x$	*Log* $C = y$*	*Amount of cladinose in hydrolysates, C (μg)*
93	9.64365	0.67632	4.746
107	10.34408	0.83671	6.866
108	10.39231	0.84776	7.043

* Calculated from the calibration graph: $y = 0.22899x - 1.53198$.

the logarithm of the amount of compound and the square root of the resulting spot area. An example of a quantitative analysis by this method is given in Table III.

Both the graphical and arithmetical methods of determination of cladinose and methylcladinoside gave similar results.

CONCLUSIONS

A quantitative analysis by direct densitometry of thin-layer chromatograms is described, which could be used for testing the stability of pharmaceutically active substances. In cases in which compounds causing interference are present and which necessitate the use of time-consuming separation techniques, this method is also suitable because of the accuracy of the results and the short period of time required. The results of the quantitative analysis presented here have successfully been employed for stability testing and defining the kinetic parameters of acid-catalysed hydrolysis of erythromycin oxime and erythromycylamine[19].

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