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QUANTITATION WITH HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY AND PROGRAMMED MULTIPLE DEVELOPMENT WITH HIGH-PERFORMANCE MICRO-THIN-LAYER MATERIAL FOR DRUG ANALYSES IN BIOLOGICAL FLUIDS

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SUMMARY

The programmed multiple development thin-layer chromatography (PMD-TLC) technique was compared with the conventional chromatographic technique on both normal and HPTLC plates. The potency of this high-performance TLC is illustrated and discussed by means of the data obtained with the determination of digitoxin in human serum.

Improvement of the efficiency of TLC, resulting in a better resolution and sensitivity with PMD-TLC using HPTLC plates, makes this high-performance TLC technique comparable to high-performance liquid chromatography.

This TLC approach might lead to a sensitive, rapid, selective and simple assay for routine serial analyses and, because of its specificity and flexibility, it may facilitate drug interaction studies.

INTRODUCTION

In the development of potent drugs, the influence of substances in biological fluids that interfere in quantitative analysis is increasing, especially as drugs become more structurally related to naturally occurring substances. Because of the variations in drug treatment, metabolism and interactions in patients, there is a considerable demand for the development of improved separation methods together with optimal detection and data handling systems for increasing the sensitivity, specificity and precision. As detection at nanogram levels and below becomes even more important, there are a few separation and detection systems that will become the methods of choice, *e.g.*, radioimmunoassay, gas–liquid chromatography, high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry and fluorodensitometry[1,2], because of their sensitivity.

Programmed multiple development (PMD) has been used in order to improve the simplicity, sensitivity and precision in TLC. PMD is an automatic, cyclic method

for a new type of multiple development in TLC. To a certain extent the resolution of PMD chromatograms does not indicate either spot size or overloading, and auxiliary techniques are therefore used to enhance the sensitivity[3,4].

PMD in combination with high-performance thin-layer chromatography (HPTLC) material[5] further improves the quantitative TLC determination of drugs at nanogram levels, and at the microgram level the serum can be applied directly to the plate[6]. Some practical aspects of improving TLC densitometry have been discussed earlier[6]. As PMD-TLC results in the narrowing of spots perpendicular to direction of development, a better signal-to-noise ratio is obtained. This periodic re-concentration of sample components gives a greater resolution than can be obtained with a single elution and enhanced sensitivity in the subsequent fluorodensitometry[7,8].

To test the possibilities of the PMD-TLC method more extensively, we analysed digitoxin in human serum at the therapeutic level of 25 ng/ml.

EXPERIMENTAL

To improve the TLC method for digitoxin and to develop it for digoxin and possibly other structurally and non-structurally related glycosides[9] we studied the possibilities of PMD using micro-HPTLC plates.

Apparatus

Plate development with PMD was carried out with a Regis Model 2000 programmer and a Model 222 developer. The fluorescence of the spots was measured with a Vitatron TLD-100 densitometer or a Zeiss chromatogram spectrophotometer (KM_3) and Spectra-Physics autolab System I computing integrator.

The TLC plates were silica gel 60 plates on glass (dimensions 20 × 20 cm) and HPTLC-60 plates on glass and aluminium (sprayed black at the reverse side for better heat absorption) (dimensions 10 × 10 cm) (E. Merck, Darmstadt, G.F.R.). A 10- and a 25-μl Hamilton syringe (with a PTFE-coated plunger and a PTFE gasket tip) were used. A Desaga chromatography tank, a Vortex-Genie Vibromixer, a GLC-2 centrifuge with a maximum of 4955 *g* and a Bolex Lite 2M quartz–halogen lamp (1000 W) were used.

Preparation of hydrogen chloride vapour

Sulphuric acid (technical quality) was added slowly to sodium chloride (technical quality) in a 2-l flask. Subsequently the hydrogen chloride vapour obtained was led into a closed tank of dimensions 5 × 10 × 20 cm (Desaga) containing silica gel 60 plates, while a constant pressure was maintained with a connected sulphuric acid wash-bottle.

Reagents and chemicals

Chloroform (spectroscopic quality), acetonitrile (p.a.) and toluene (p.a.) were obtained from Merck. Methanol (minimum purity 99.5%), ethanol (99.5%) and acetone (99.5%) were obtained from J. T. Baker (Deventer, The Netherlands); digitoxin, Pharmacopoeia Neerlandica Ed. VII or from Merck; digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside, digitoxigenin and digitoxin from Boeh-

ringer (Mannheim, G.F.R.); digoxin, Ph. Ned. Ed. VII or from Merck; and digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside and digoxigenin from Boehringer. The stock solution of digitoxin was prepared by dissolving 5.000 mg of digitoxin in 50 ml of chloroform–methanol (1:5), and was stored in a refrigerator. Standard solutions of 1, 2, 10 and 100 ng/μl of digitoxin and its metabolites in chloroform–methanol (1:5) were used. Digoxin standard solution was prepared in the same way, but also in a concentration 10 times lower.

Extraction

A 2-ml volume of serum is shaken for 2 min with 8 ml of chloroform and 2 drops of 4 *N* sodium hydroxide solution in a 10-ml glass-stoppered centrifuge tube in a Vibromixer and the mixture is centrifuged for 15–20 min at 1417 *g*. Then 7 ml of the supernatant are transferred into a 10-ml centrifuge tube and washed with 3 ml of 1% ammonia solution and finally centrifuged. Six millilitres of the supernatant liquid are transferred to a 10-ml glass-stoppered tube with a tapered base of 0.2 ml volume. The chloroform extract is evaporated to dryness at 40° in the tube by the passage of dried compressed air for 20–30 min to prevent adsorption. The residue is dissolved in 200 μl of chloroform by mixing vigorously again for 2 min in order to maintain a high accuracy for digitoxin at the low concentrations involved.

Chromatography

With a 25-μl Hamilton syringe (right-angled tip), volumes of 10, 20 or 40 μl of two unknown solutions (X_1 and X_2) are applied on to a thin-layer plate, together with standards (S) of 5 and 10 ng of digitoxin using a 10-μl Hamilton syringe. The sequence of application is, *e.g.*, $X_2 = 40$ μl; $X_2 = 20$ μl; $S = 5$ ng; $S = 10$ ng; $X_1 = 20$ μl; $X_1 = 10$ μl. Two unknown samples can be examined because of the linearity and reproducibility of the method (see *Reproducibility of the method*).

In conventional TLC (silica gel 60, normal particle size[6] and micro-particle size), the centres of the spots are 1.5 cm apart and 1.8 cm from the edge of the plate. The eluent is chloroform–methanol–acetone–water (64:6:28:2), the development being carried out with a saturated tank using an elution time of about 20 min. The distance travelled is 10 cm for normal TLC plates and 6–8 cm for HPTLC plates; the plate is dried immediately with hot air in order to avoid sideways diffusion.

In PMD, the centres of the spots are 1.5 cm apart and 3 cm from the edge of the plate. The solvent selected for PMD with HPTLC material is acetonitrile–toluene (70:30) for aluminium plates and methanol–dichloromethane (7:93) for glass plates. Both solvent systems require the following programme:

mode 1: advance 1 — 0 cycle 8 removal 2 — 8
mode 3: advance 7 — 0 cycle 2 removal 2 — 8

After development, the TLC plate is dried for 2 min at 110° and placed in a closed tank, exposed to hydrogen chloride vapour for 60–75 min in the dark, and subsequently irradiated with artificial light (quartz halogen lamp at 150 V) for 12.5 min, still in the closed tank. This provides an acceptable and stable fluorescence while an increase in fluorescence intensity may be obtained by means of a non-volatile fluid film such as liquid paraffin[6].

Under UV light, a bluish fluorescence for digoxin and an orange-yellow fluorescence for digitoxin are observed. After heating for 5 min at 110° in order to remove the hydrogen chloride vapour, the digoxin remains bluish and the digitoxin becomes light yellow.

The spots are quantified by measuring the fluorescence immediately with a chromatogram spectrophotometer. The operating conditions with the Vitatron TLD-100 are as follows: light source, mercury lamp; mode, In II (+); level, f; coarse zero, 7; damping, 2; span, 10; excitation filter, 365 nm; emission filter, 536 nm; diaphragm, 2.5 × 0.5 mm; scanning speed, 1 cm/min; paper speed, 10 mm/min; integrator, 8. The operating conditions with the Zeiss KM_3 are as follows: light source, xenon lamp; stray-light filter (325–380 nm); mode, kompensation F; gain, automatic gain; damping, 1; photomultiplier voltage, 2; excitation wavelength, 365 nm; emission wavelength, 530 nm (optimal for digitoxin and metabolites), 455 nm (optimal for digoxin); slit, 6 × 0.5 mm; scanning speed, 10 mm/min; paper speed, 30 mm/min.

RESULTS AND DISCUSSION

Resolution and sensitivity

In order to obtain an acceptable resolution of digitoxin, its main metabolites and digoxin with PMD-TLC, a screening programme for eluent optimization as well as programme selection was necessary, and various solvent systems, mostly a combination of not more than two solvents, were tried. Sometimes a double solvent front appeared with PMD, which was caused by the different rates of evaporation of the two solvents during the removal of solvent with both infrared radiation and nitrogen gas.

If the resolution is affected by organic impurities on the silica gel plates or in the eluent itself, which become concentrated in bands with characteristic R_F values, pre-washing of the silica gel plates as well as distillation of solvents may be of additional help.

PMD-TLC with HPTLC plates on both glass and aluminium was performed with the same programme. To optimize the sensitivity and resolution of digitoxin from its metabolites and digoxin on a single plate, two programming modes (1 and 3) were examined. Mode 1 was used especially for narrowing the spots and mode 3 for efficient resolution.

The resolution shown in Fig. 1 appeared to be strongly dependent on both the TLC technique and the TLC material used. All measurements were performed with a Vitatron densitometer scanning in the direction of the solvent flow, but the fluorescence was not always of the same intensity; it was important to study the shape or dimensions of the spot with the application of the substance in microlitre or nanolitre amounts (Fig. 1a–e). In Fig. 1, an increased narrowing of spots is evident, resulting in a small base of the peaks in the densitogram obtained even at high recorder speeds, even when smaller amounts of substance were applied in the same or a larger volume. From this result, an increase in sensitivity (signal-to-noise ratio) can be concluded, resulting in a lower detection limit.

Figs. 2a and 2b show the separation of digitoxin from two extracts prepared from human serum at a concentration of 25 ng/ml and illustrates the narrow fluorescing bands of the unknown. The standards are situated in the middle of the

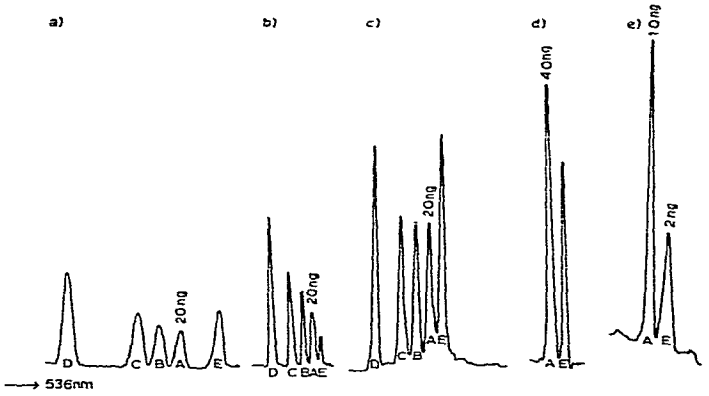


Fig. 1. Registration of the separation of digitoxin (A), its metabolites (B, C and D) and digoxin (E) with (a) conventional TLC, recorder 1 cm/min; (b) HPTLC, recorder 2 cm/min; (c) PMD on HPTLC material (Al plates), recorder 4 cm/min. Densitograms of the separation of digitoxin and digoxin with PMD on HPTLC material on both (d) aluminium plates (solvent removal with heat), recorder 2 cm/min, and (e) glass (solvent removal with nitrogen gas), recorder 4 cm/min. See text for details.

chromatogram. PMD-TLC on glass with a nitrogen cell (Fig. 2b) gives narrower bands and a better signal-to-noise ratio than PMD-TLC on aluminium with the use of infrared irradiation to remove the solvent front. In the latter instance, more circular spots were obtained and the resolution was even better, a horizontal line being observed. The recoveries were of the same order in both instances, with the best duplicate values being obtained when using the nitrogen flow cell. The data in Figs. 1 and 2 also illustrate two significant aspects of the PMD-HPTLC plate combination: with PMD high sample loadings can be used which give a performance that exceeds that of conventional development at much lower loadings. Hence PMD overcomes a major limitation associated with the routine use of HPTLC plates[6,8].

Linearity

It appeared that there was a linear relationship between the amount of digitoxin between 2 and 20 ng and digoxin between 0.2 and 2 ng and the corresponding fluorescence of the digitoxin derivative expressed as the peak area [height (mm) ×

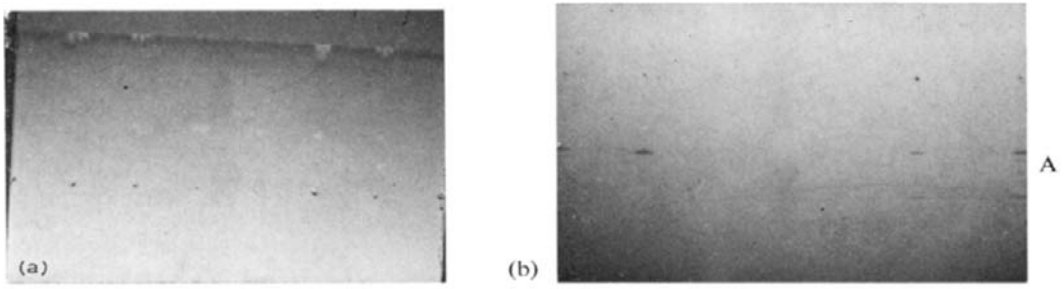


Fig. 2. Separation of digitoxin (A) after application of two different serum extracts from 25 ng/ml serum: (a) PMD with HPTLC material on aluminium; (b) PMD with HPTLC material on glass.

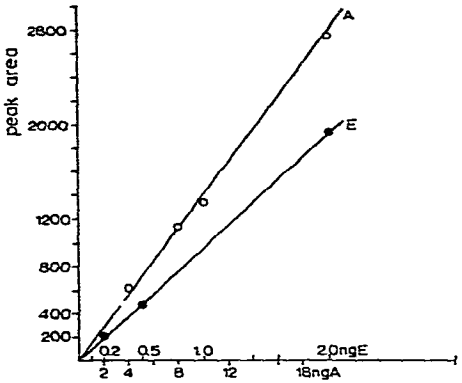


Fig. 3. Calibration graph for digitoxin (A) and digoxin (E).

width at half-height (mm)] (Fig. 3). Fig. 4a for digitoxin and Fig. 4b for digoxin show the densitograms from which the peak areas were calculated. Digitoxin was spotted singly in multiples of 2 ng/μl and the spots were scanned in the direction of the solvent flow, because of re-concentration of the spots in narrow bands.

Reproducibility of the method

The registration shown in Fig. 5 was obtained by spotting a standard of 8 ng of digitoxin six times ($n = 6$). The integrated values (Fig. 6) indicate the precision of the method and how it is influenced by the quantitative application, the chromatography and the fluorogenic reaction. The average value was 148 with a standard deviation of 5.09 and a coefficient of variation of 3.4%.

A volume of 20–100 μl of digitoxin was added to blank serum and the mixture was shaken for 2 min with a Vibromixer. In Fig. 7a and b the results of recovery ex-

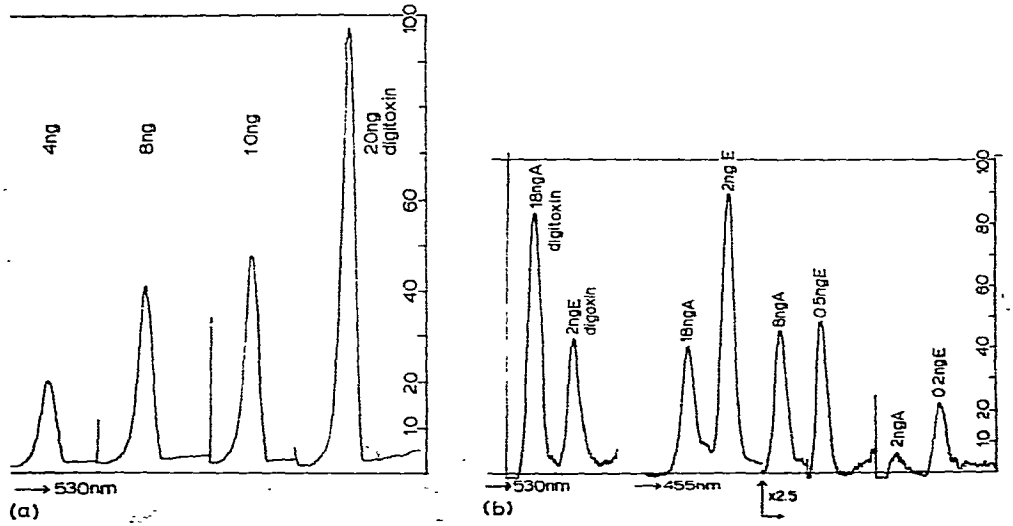
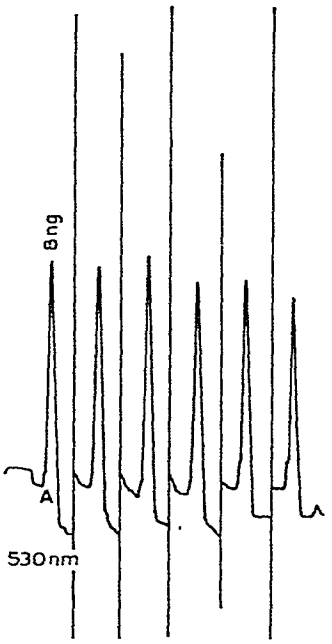


Fig. 4. (a) Registration of different amounts of pure digitoxin; (b) registration of different amounts of pure digoxin and digitoxin, measured at different wavelengths with a Zeiss KM_3 chromatogram spectrophotometer.

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  17       1510839 *A
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        20057 ID

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  15       1501111 *A
           1501111

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        20057 ID

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TIME          AREA
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Fig. 5. Registration of six replicates of the same amount of digitoxin (A) after development with PMD-TLC on glass with a nitrogen cell.

Fig. 6. Computer output of the integrated peak areas of the six replicates of the same amount of digitoxin (A) from Fig. 5.

periments for digitoxin are shown in a densitogram. The so-called "unknowns", X_1 and X_2, were spotted in volumes of 10, 20 or 40 μl from the 200 μl of extract. After calculation, we obtained recoveries of 85% at the 100 ng/ml level and 91% at the 50 ng/ml level, performed with PMD-TLC on glass, using the nitrogen cell and the Zeiss KM_3 at 530 nm (Fig. 7a). Fig. 8 gives the peak areas of standards and unknown from Fig. 7a. Fig. 7b indicates a recovery of 55% at the 50 ng/ml level and 64% at the 100 ng/ml level measured with the Vitatron TLD 100 at 536 nm. Duplicate results in both determinations were in excellent agreement. This much lower recovery, which was obtained in a series of survey experiments, is probably caused by the influence of the slit dimensions of the Vitatron.

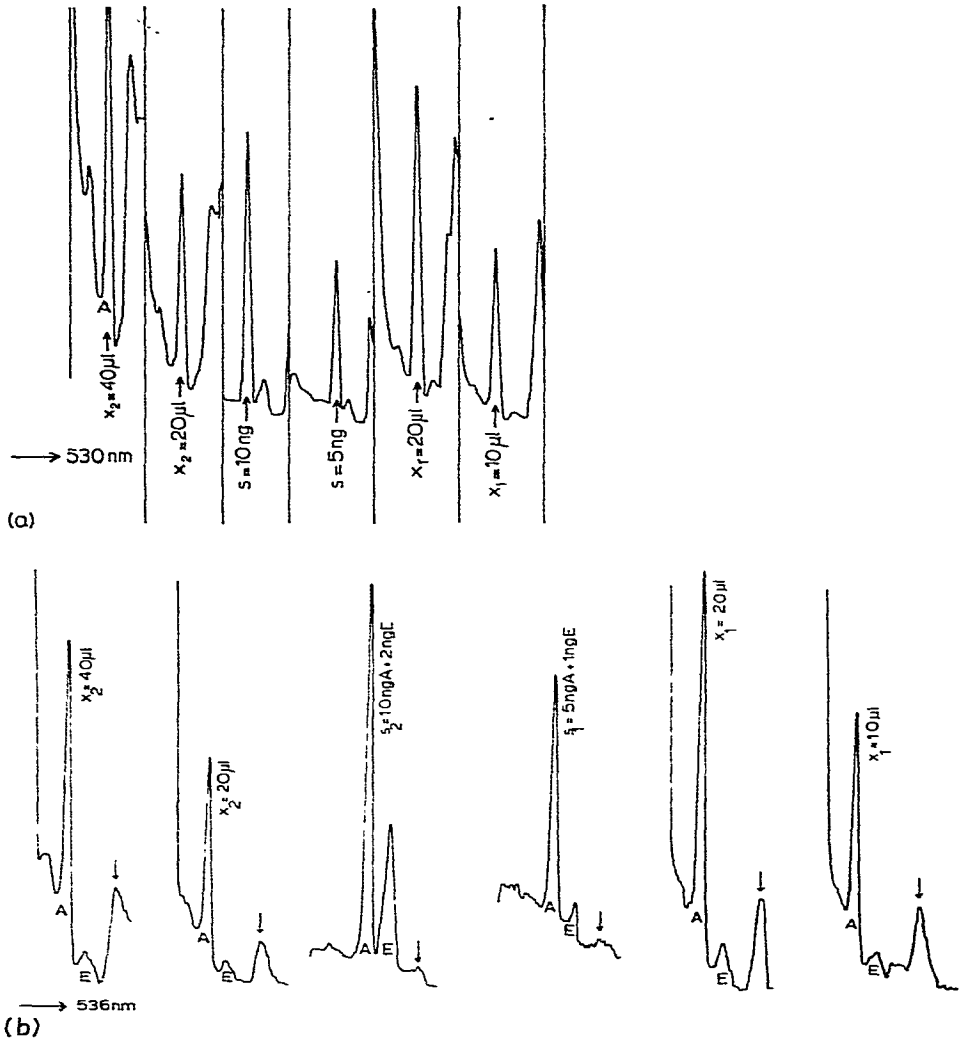

Fig. 7 (a) and (b). Registrations to show the determination of two known samples, X_1 and X_2, in recovery experiments (for details see text).

In the chromatogram in Fig. 9 separations at the 50 and 100 ng/ml levels are shown and illustrate the narrow bands for the two standards in the centre and the unknowns X_1 and X_2 at the sides (see *Resolution and sensitivity*).

As the quality of TLC plates is not always consistent, both the reproducibility and detection could be influenced to a considerable extent. The PMD-TLC technique has the capability of solving this problem.

However, it remains important to apply as small spots as possible. PMD-TLC has the advantage that rather large spots are reduced, resulting in even a better signal-to-noise ratio.

Using HPTLC material in conventional TLC it is possible to achieve a better

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        2742486
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Fig. 8. Computer output of the integrated peak area of the standards (S) and the unknowns X_1 and X_2.

resolution more quickly than with material of the normal particle size, but it should be noted that for an optimal effect spots have to be applied in nanolitre volumes, which is difficult to achieve with extracts from biological material.

The PMD technique on HPTLC material offers the advantage of larger application volumes coupled with the higher resolution given by the high-performance material. PMD-TLC, in comparison with conventional TLC, in separations from interfering substances, gives less streaking and a lower background, resulting in clearer results on the TLC plate. This improvement of the reproducibility and accuracy with this HPTLC method can be enhanced by scanning with the chromatogram spectrophotometer in the double-beam mode.

As the determination of digoxin has to be carried out in serum (1–2 ng/ml),

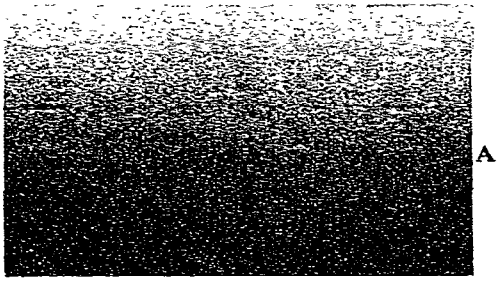

Fig. 9. Separation of digitoxin (A) after application of two different serum extracts of concentrations 50 and 100 ng/ml.

at least 2–4 ml of serum is required. Although the PMD technique permits enhancement of the signal-to-noise ratio, it will be necessary to purify the extract.

Using an external standard or, even better, an internal standard, serial analyses can be performed, while improvement of the fluorescence could simplify this HPTLC method even more. It is probably better to wash the TLC plate first and, from experience, it appeared that this is successful, especially with lower detection areas, as the first necessary "clean-up". For additional information, see the discussion regarding earlier investigations with quantitative TLC determinations[10].

Using the PMD-TLC method as a HPTLC technique, it depends on the physico-chemical properties of the substances concerned whether aluminium or glass plates should be used. In fact, the use of nitrogen gas for solvent removal is recommended as not heating the plate avoids decomposition. However, the combination of heat and nitrogen gas may lead to new possibilities for overcoming viscosity problems with solvent systems, especially when analysing extracts from biological material. This PMD-TLC method using HPTLC material on glass plates with a nitrogen flow cell appears to be a high-performance technique comparable to HPLC.

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