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THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF DIGITOXIN IN HUMAN SERUM

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SUMMARY

A specific assay for therapy control purposes and the toxicology of digitoxin is described. Digitoxin is isolated from serum (2 ml) by means of a single extraction with chloroform. Part of the organic phase is evaporated to dryness at 40° in a stream of dry air and the residue is dissolved in a small volume of chloroform. Conventional thin-layer chromatography (TLC) is used for the separation of digitoxin and its metabolites and digoxin. Fluorescence of the spots can be generated by treatment with hydrogen chloride vapour under the influence of a quartz-halogen lamp. Interpolation between two reference standards gives the concentration in the sample. Linearity is observed between 2 and 20 ng. Extensive recovery studies in the therapeutic range of 10—50 ng/ml have been performed. The results showed an overall recovery of 99.1% with a standard deviation of 11.2%. The sensitivity is 1—2 ng of digitoxin when standards are applied on a conventional TLC plate with a small diameter. The time needed for one analysis is about 4½ h, the real time of analysis being 2½ h; in serial studies, 20—24 determinations could be made daily. Whereas in the descriptions of most methods it is not mentioned whether digitoxin metabolites are co-determined, the present assay separates the digitoxin completely from the other compounds in serum, and thus enables the total fate of digitoxin in relation to the clinical effect to be studied more specifically than by radioimmunoassay.

INTRODUCTION

Digitoxin is prescribed in cases of cardiac failure and has a notably small therapeutic index. Although nowadays digoxin is used more frequently than digitoxin, it is still not certain which of these drugs is the most useful. In discussing this point [1, 2], the differences in the pharmacotherapeutic properties of digitoxin and digoxin become important in three main areas: protein binding [3], biological half-life and interactions with other drugs [4]. Digoxin seems to be preferred in cases of heart insufficiency alone, while digitoxin is suitable in cases of heart insufficiency combined with renal insufficiency [3]. To solve

such problems, it is essential that further research into the biological half-lives, interactions with other drugs and the protein binding of these two important drugs should be carried out.

A method is required that is very sensitive, accurate, specific, rapid, cheap and can be performed by the clinical pharmacist with a challenging analytical problem. Various procedures for the determination of digitoxin published during the past 10 years are mostly very time consuming or not specific enough [5—10]. Smith [11] developed a radioimmunoassay (RIA) method that is very sensitive, accurate, simple and requires only 1 h, but there are disadvantages related to digitoxin metabolism [12] and to the fact that patients with maintenance dosages of digoxin give false-positive values corresponding to levels of digitoxin of 1—2 ng/ml in serum. Another disadvantage is that laboratories which lack radiochemical equipment cannot use this procedure. Therefore, in order to eliminate these disadvantages, we decided to develop a new method [13—15], based on a fluoridensitometric approach as already applied to kinidine [16] and to amitriptyline and nortriptyline [17]. The conventional TLC method has been compared with the RIA method and the results are discussed.

EXPERIMENTAL

In establishing this method for the determination of digitoxin we tried to optimize important factors such as the thin-layer material, the sample application procedure, the use of one-dimensional or multi-dimensional development and the purity of the reagents. In this method, the ratio of digitoxin to interfering substances must be relatively high because of the low therapeutic blood level of digitoxin.

The method involves a single extraction of digitoxin from serum, concentration of the extract followed by thin-layer chromatography, a fluorigenic reaction with an acidic vapour and artificial light, quantitative measurement of the fluorescence of digitoxin with a densitometer and calculation of the amount in an unknown sample by interpolation from a calibration graph.

Apparatus and reagents

The TLC plates were Kieselgel 60 DC-Fertigplatten of dimensions 20 × 20 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) with a Hamilton repeating dispenser were used. A Desaga chromatography tank, a Vortex-Genie Vibromixer, a GLC-2 centrifuge with a maximum speed of 4955 *g*, a Bolex Lite 2M quartz-halogen lamp (1000 W) and a Vitatron TLD-100 densitometer were used.

Chloroform (spectroscopic quality) and hydrochloric acid (pro analysi; minimum concentration 37%) were obtained from Merck. Methanol (minimum 99.5%), ethanol (99.5%) and acetone (99.5%) were obtained from J.T. Baker Chemicals (Deventer, The Netherlands); digitoxin, Ph. Ned.* Ed. VII or Merck; digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside, digitoxigenin and digitoxin, Boehringer (Mannheim, G.F.R.); digoxin, Ph. Ned. Ed. VII or Merck; digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside and digoxigenin, Boehringer. The stock solution of digitoxin was prepared by dissolving 10.000 mg of digitoxin in 100 ml of chloroform, and was stored in a refrigerator. Standard solutions of 1 and 2 ng/μl of digitoxin in chloroform were used.

*Pharmacopoeia Neerlandica.

Extraction

A 2-ml volume of serum is shaken for 2 min with 8 ml of chloroform 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 glass-stoppered tube with a tapered base of volume 0.2 ml, passage of dried compressed air for 20—30 min to avoid adsorption. The residue is dissolved in 200 μl of chloroform by mixing vigorously again for 2 min to maintain high accuracy for digitoxin at the low concentrations involved.

Chromatography

With a 25-μl Hamilton syringe (right-angled), volumes of $x_1 = 20\,\mu l$, $x_2 = 40\,\mu l$ and $x_3 = 50\,\mu l$ of the unknown solution (x) and volumes of $b_1 = 20\,\mu l$, $b_2 = 40\,\mu l$ and $b_3 = 50\,\mu l$ of serum blanks are applied on a thin-layer plate, together with 4 ng (S_1), 9 ng (S_2), 7 ng (S_3), 14 ng (S_4), 5 ng (S_5) and 15 ng (S_6) of standard solutions of digitoxin using a 10-μl Hamilton syringe. The sequence of application is $S_1, b_1, x_1, S_2; S_3, b_2, x_2, S_4; S_5, b_3, x_3, S_6$. This is a large number of standards, which may be decreased by duplicate spotting of a few standards, so that on one plate more unknown samples can be examined because of the linearity and reproducibility of the method (see *Reproducibility of the method*). The centres of the spots are 1.5 cm apart and 1.8 cm from the edge of the plate. Before it was decided which solvent to use, at least 10 solvent systems were tested, because of the need to effect a separation from both metabolites and interfering substances in serum, and also to take account of the study of Zullich et al. [18] on the metabolism of digitoxin in rats. The eluent selected was chloroform—methanol—acetone—water (64:6:28:2), the development being carried out without a saturated tank with an elution time of about 20 min. The elution distance is 10 cm and the plate is dried directly with a warm hair drier to avoid sideways diffusion.

Fluorigenic reaction and detection

The determination of digitoxin and digoxin in biological material is not possible because of the lack of sensitivity of the fluorescent product being formed by a number of reagents. Only Seipel et al. [19] and Van Oostveen [20] have determined digitoxin in serum and digoxin in urine, respectively, with a TLC method using fluoridensitometry. The fluorescence was generated by a chloramine—trichloroacetic acid spray reagent. Other workers [21—26] have performed spectrofluorimetric determinations on pharmaceutical preparations. Some other spray reagents [27] and solvents for TLC mentioned in the literature lack stability and sensitivity, while Hoeke and Exler [28] and Frijns [23, 24] generated fluorescence by treatment with hydrogen chloride vapour under the influence of heat and artificial light, respectively. There was a low sensitivity or detection limit and the fluorescence colours were not reported.

As hydrogen chloride vapour gives a better sensitivity than other reagents and a reagent in the form of a vapour gives a homogeneous partition over the entire plate, we developed a more sensitive and less time-consuming modified assay. An increase in fluorescence intensity can be obtained by covering the chromatogram with a non-volatile fluid film such as liquid paraffin (Ph. Ned. Ed. VII).

To obtain optimal fluorescence, the TLC plate is placed in a tank saturated with hydrochloric acid for at least 1 h, in the absense of daylight, and is subsequently irradiated with artificial light (quartz-halogen lamp) for 12—13 min. This process converts digitoxin and digoxin and their metabolites into fluorescent compounds. Under UV light, an orange-yellow fluorescence is observed; after removing the acid from the plate by placing it in a drying oven for 1—2 min, the fluorescence appeared to be light yellow. This fluorescence is stable for days, provided that the plate is kept in the dark.

The spots are quantified by measuring the fluorescence directly with a Vitatron TLD-100 densitometer. The operating conditions are as follows: light source, mercury lamp; mode, ln II (+); level, f; coarse zero, 7; damping, 2; span, 10; excitation filter, 365 nm; emission filter, 536 nm (optimum for digitoxin and metabolites); diaphragm, 1.0; swing, 2; scanning speed, 1 cm/min; paper speed, 0.5 cm/min; integrator, 8.

RESULTS AND DISCUSSION

Linearity

It appeared that there was a linear relationship between the amount of digitoxin applied to the plate up to 20 ng and the corresponding fluorescence of the digitoxin derivative (integrated peak area) (Figs. 1 and 2). The numbers at the top of the peaks (integrated peak area) in Fig. 1 represent the fluorescence of the spots on the TLC plate. Digitoxin is spotted in duplicate in multiples of 2 ng. The spots are scanned on their R_F values, because a calibration graph is used,

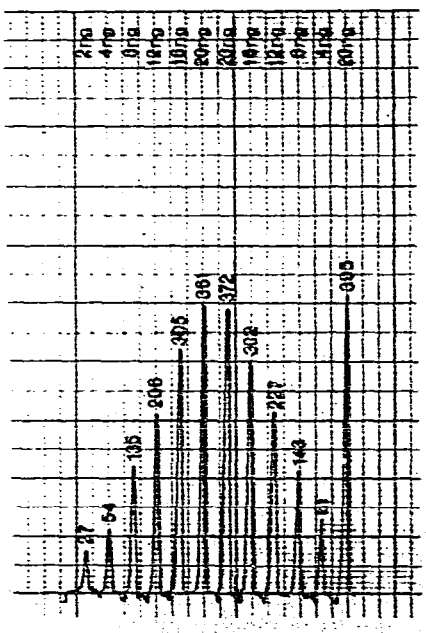


Fig. 1. Densitogram of different amounts of pure digitoxin.

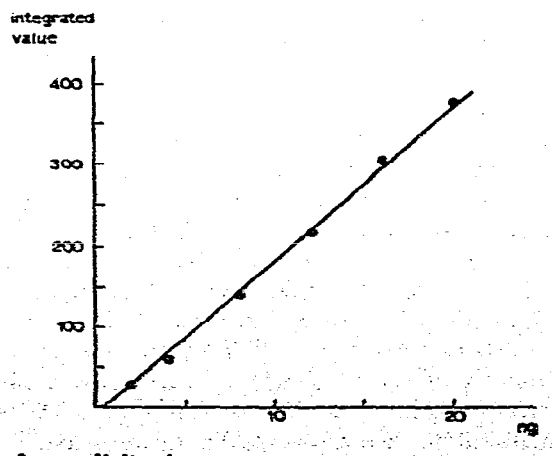


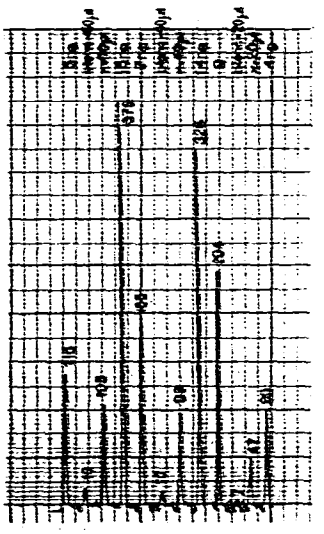
Fig. 2. Calibration graph for digitoxin (2—20 ng).

the shape of the spots (signal to noise ratio) and time saving. The calibration graph (Fig. 2) of digitoxin is constructed from the average value of the duplicate determinations from the densitogram of different amounts of pure digitoxin in Fig. 1.

To confirm these results, a number of duplicate determinations were carried out on solutions containing 2, 4, 8, 12 and 16 ng of the drug. After statistical analysis of the results [29], it appeared that the deviation from linearity was not statistically significant at a threshold value of 5%.

Calculation of digitoxin in an unknown sample

Fig. 3 shows a densitogram for the determination of digitoxin in an unknown sample. The unknown x is spotted in amounts of 20, 40 and 50 μl; blanks of the same amounts are run at the same time to give another impression of the sensitivity, accuracy and precision of the method. The calibration graph from which the digitoxin in the unknown sample is determined by interpolation (Fig. 4) shows again the good linear relationship. From the average value of the number of pulses of the unknown sample of digitoxin (x_1, x_2 and x_3) minus the avarage value (b_1, b_2 and b_3) of the serum blank, the amount in nanograms can be calculated directly from the calibration graph. It may be also sufficient to make the calculation from one or two reference standards (see *Reproducibility of the method*), depending on the purpose.



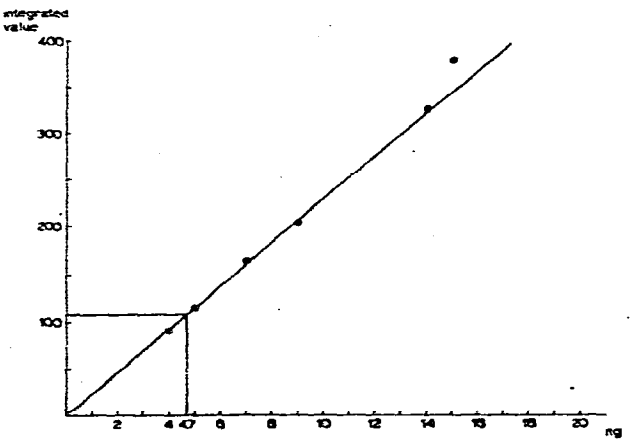


Fig. 3. Densitogram to show the determination of an unknown sample, x. The integrated value for each spot is given at the top of the curve.

Fig. 4. Calibration graph for the determination of the digitoxin in the unknown sample from Fig. 3.

Specificity of the method

The separation of digitoxin and its main metabolites and digoxin was carried out with the above solvent system. The dihydro products, with much lower fluorescence intensities and which rarely occur as metabolites, were not used.

TABLE I

hR_F VALUES OF DIGITOXIN, ITS MAIN METABOLITES AND DIGOXIN

Stationary phase: Kieselgel 60 (Merck, Fertigplatten). Solvent system: chloroform—methanol—acetone—water (64:6:28:2). Running distance, 10 cm; environment, unsaturated; fluorigenic reaction, 75 min under influence of HCl vapour in the dark and 12 min with both HCl vapour and artificial light.

Substance	hR_F value
Digitoxin	35
Digitoxigenin bisdigitoxoside	39
Digitoxigenin monodigitoxoside	44
Digitoxigenin	56
Digitonin	Origin
Digoxin	24

In Table I, it is shown that digitoxin is well separated from its main metabolite digitoxigenin bisdigitoxoside, other metabolites and digoxin.

Reproducibility of the method

A volume of 20—100 µl of digitoxin was added to blank serum the mixture was shaken for 2 min in a Vibromixer. The serum was incubated for at least ½ h. Table II summarizes the results of a series of recovery experiments in the therapeutic range of 10—50 ng/ml of digitoxin in serum and indicates the accuracy of the TLC method. In Fig. 5, the results of a recovery experiment for digitoxin are shown in a densitogram. The unknown x was spotted in a volume of 30 µl from the 200 µl of extract. The blank was also spotted in a volume of 30 µl. After calculation, we obtained a recovery of 114% at the 20 ng/ml level, which is above the average found value. In the experiment shown in Fig. 5, a standard of 8 ng was spotted six times ($n = 6$). These integrated values indicate the precision of the method and how it is influenced by the quantitative application, the chromatography and the fluorigenic reaction. The average value was 313 counts with a standard deviation of 8.3 and a relative standard deviation of 2.6%.

TABLE II

RECOVERY OF DIGITOXIN FROM SERUM ON CONSECUTIVE DAYS

Day	Digitoxin serum concentration (ng/ml)*				
	10	20	30	40	50
1	121	114	100	87	94
2		113	95	91	91
			96		
3	90	105	94	93	84
4	118		98		

*The overall recovery ($n = 17$) for digitoxin is 99.1% with a standard deviation of 11.2% with a range of 84—121 ng/ml.

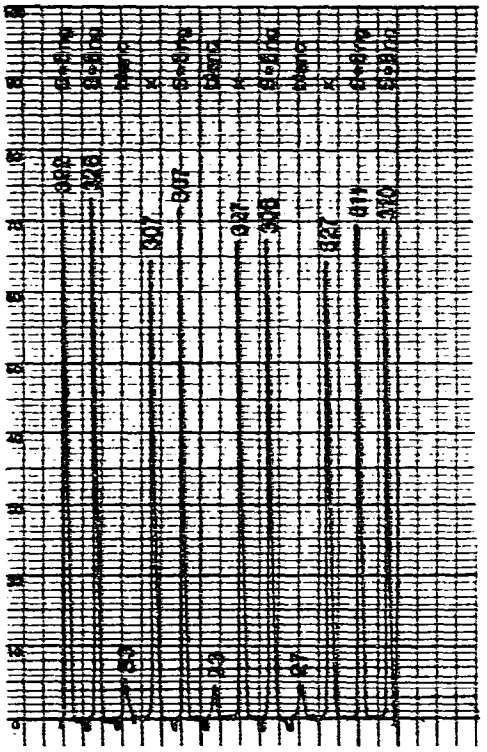


Fig. 5. Densitogram showing the results of a recovery experiment for digitoxin. For details, see text.

It appeared that the quality of the Merck plates was not consistent and both the reproducibility and the detection limit could be influenced to a large extent in different charges. Therefore, control of the charge is necessary. The relative standard deviation for 5 ng of digitoxin on one plate should not be more than 6%, when the detection limit is less than 0.5 ng.

All previous specific methods based upon chromatographic techniques are time consuming. All other methods for use with biological material are based on the affinity of digitoxin to biological receptors. Moreover, the specificity is dependent on the quality of the biological system, which may be uncertain. Our fluoridensitometric TLC method is principally suitable for determining both digitoxin and digoxin in any biological material, e.g., urine, faeces, heart tissue and muscle tissue.

Although the recoveries and the other features outlined above were acceptable, there were differences between the values obtained by fluoridensitometry and by RIA. The results of both methods applied to 11 patients are shown in Table III.

Since Vöhringer and Rietbrock [12] found that digitoxin is the main substance in the chloroform extract and all values obtained by the RIA method are

TABLE III

DETERMINATION OF SERUM LEVELS OF DIGITOXIN IN PATIENTS BY BOTH THE RIA AND TLC METHODS

Standard deviation 0.25 (n = 11); relative standard deviation of the mean ratio, S_{rel} = 53%.

Patient	RIA method [11]	TLC method]13]	Ratio serum levels, TLC/RIA
1	33	11.9	0.36
2	48	14.1	0.29
3	49	16.7	0.34
4	56	20.1	0.34
5	25	21.3	0.85
6	25	2.2	0.09
7	62	24.9	0.40
8	17	5.7	0.34
9	10	9.5	0.95
10	33	18.6	0.56
11	17	10.9	0.64
			Mean: 0.47

higher than those obtained by the TLC method (Table III), there must be a co-determination of other substances in the radioimmunoassay. Further, it can be concluded from the work of Vöhringer and Rietbrock [12] that it is important not to co-determine the main metabolites, because of changing of the ratio of chloroform-soluble and -insoluble metabolites in the first 48 h from 8.4 to 3.3, and because of the different half-lives of digoxin, digitoxin and their metabolites.

In the fluoridensitometric TLC method, there is no co-determination, and therefore this method is specific and is not influenced by structurally related compounds. The difference between the values obtained by the two methods (Table III) might be due to a lack of specificity in RIA or partly to a lack of attainment of equilibrium in the fluorimetric recovery experiments, although our recovery was almost 100% with reasonable accuracy. Hence the efficiency of recovery of the TLC method cannot be the reason for the difference in serum levels or for the difference in the precisions.

Any lack of specificity in the RIA method might be due to the co-determination of interfering substances such as metabolites of digitoxin, structurally related drugs and their metabolites and other structurally related substances. When the values obtained by the RIA and TLC methods do not bear a constant ratio to each other or there is an average ratio with a small standard deviation, while both methods have good precision, it can be concluded that the extent of the lack of specificity of the RIA method will be expressed by the standard deviation of the ratio of the serum levels obtained in the two methods.

CONCLUSION

A TLC method that is not too time consuming, not too expensive, with good accuracy and precision and specific and sensitive for the determination of digi-

toxin in the serum of patients in the therapeutic and toxic range of 10—60 ng/ml has been developed.

Improvements may come from investigating not only the sample preparation and the use of programmed multiple development [14, 15, 30, 31] and of high-performance TLC material [14, 15, 32], but also a better densitometric integration system. The result could be a fluoridensitometric TLC method that has advantages over RIA. As the fluorigenic reaction of digoxin and methyldigoxin surpasses even that of digitoxin, it seems possible that digoxin (lower therapeutic level 1—2 ng/ml) could likewise be assayed by fluoridensitometry.

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