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Determination of thevetin B in serum by fluorescence polarization immunoassay

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Abstract: Thevetin B, a cardiac glycoside of *Thevetia neriifolia* Juss. seeds, was determined in serum by fluorescence polarization immunoassay. Anti-digitoxin antibody was used, thevetin B genin being structurally identical to digitoxigenin. Cross-reactivity of 94% was found by this method, for concentrations from 5 to 80 ng ml^{-1}.

Keywords: *Thevetin B; digitoxin; cross-reactivity; fluorescence polarization immunoassay.*

Introduction

The seeds of *Thevetia neriifolia* Juss. (Apocynaceae) contain several cardiac glycosides, the two main ones being thevetin A and B. The latter glycoside is the easiest to obtain as the pure form [E. Uber-Bucek, unpublished results]. Moreover it shows a significant chemical relationship with digitoxin and digoxin glycosides, especially with digitoxin.

The genins of thevetin B and digitoxin (Fig. 1) are identical, and the difference between these two compounds resides only in the glycosidic structure. The carbohydrate part of thevetin B (Fig. 2) is composed of one thevetose (desoxy-6-*O*-methyl-3-glucose) and two glucose units (gentiobiose). Those of digoxin and digitoxin are composed of three digitoxose units (didesoxy ribohexose).

The aim of this work is to apply the fluorescence polarization immunoassay (FPIA) to the determination of thevetin B in serum, with use of an anti-digitoxin antibody.

Because of the lack of available specific anti-thevetin B antibody and of the structural analogy of thevetin B with digoxin and digitoxin compounds, two available anti-digitoxin and anti-digoxin antibodies have been used for this assay. The cross-reactivity study of these two antibodies against thevetin B was realized by FPIA in sera spiked with thevetin B. The anti-digitoxin antibody was finally chosen because of its high levels of cross-reactivity (94%).

FPIA is an homogeneous fluoroimmunologic quantification based on the competition of a drug tested and the same drug labelled by fluorescein called the 'tracer' against its specific antibodies. Only labelled drugs bound to antibodies are detected by the fluorescence polarization system [1, 2].

This method is often used in hospitals, either for drug therapeutic monitoring or for toxicological assays in biological fluids [1–4].

Fluorescence polarization immunoassay is a very rapid method because its system is entirely automated. The total time for the analysis of 20 samples is about 10 min. Its sensitivity for digitoxin and digoxin determination in serum is in the nanogram per millilitre range, and its reproducibility is good with a relative standard deviation (RSD) lower than 10% with the apparatus used (TDx–Abbott).

Jolley *et al.* [3], have compared FPIA with high-performance liquid chromatography (HPLC), enzyme multiplied immunotechnique (EMIT) and radioimmunological assays for the determination of diverse drugs. They found a correlation of 0.935–0.992.

The main advantages of FPIA [5] over physical techniques (GC, HPLC) are absence

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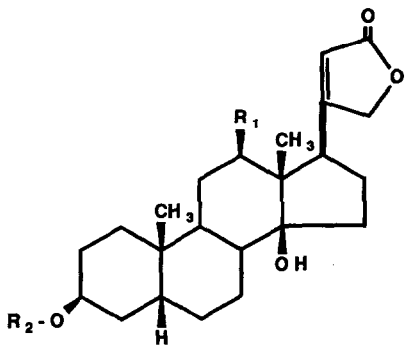

Thevetin B : R_1 = H; R_2 = one thevetose and one gentiobiose; Mwt = 858.95

Digoxin : R_1 = OH; R_2 = three digitoxoses; Mwt = 780.92

Digitoxin : R_1 = H; R_2 = three digitoxoses; Mwt = 764.92

Figure 1
Chemical structures of cardiac glycosides.

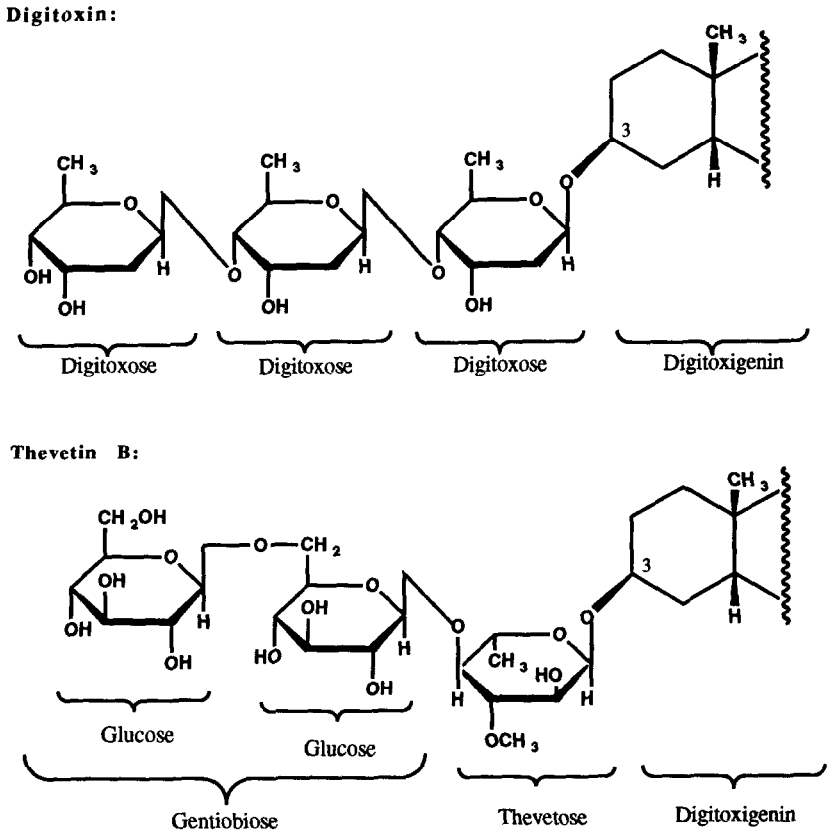

Figure 2
Chemical structures of glycosidic-chain in thevetin B and digitoxin.

of the need for extraction, the use of a small sample volume (about 100 µl) and the rapidity of the analysis.

Moreover, the physical techniques involving extraction and spectrophotometric detection are impractical for cardiac glycoside therapeutic monitoring, because they are not sensitive enough to detect the nanogram per millilitre range. For these reasons, FPIA was chosen for determination of thevetin B in serum with use of an anti-digitoxin antibody.

Materials and Methods

Instrumentation

The FPIA analysis was performed on a TDx assay system (Abbott Diagnostics Division) including a polarization spectrofluorimeter with auto sampler, auto diluter and centrifuger.

Reagents

A. TDx–Abbott kit of digitoxin and digoxin. This contains: (1) pre-treatment reagent solution tensioactive buffer; (2) solution of rabbit anti-digoxin or anti-digitoxin antiserum in buffer; (3) digoxin or digitoxin marked with fluorescein; (4) reference solutions in human serum at concentrations from 0 to 5 ng ml^{-1} for digoxin and from 0 to 80 ng ml^{-1} for digitoxin; (5) three control solutions in human serum for digitoxin (7.5, 15 and 35 ng ml^{-1}) and digoxin (0.75, 1.5 and 3.5 ng ml^{-1}).

B. Thevetin B. This was isolated from the seeds of *Thevetia neriifolia* Juss. (Apocynaceae) with a 99.6% purity for the first time [E. Uber-Bucek, unpublished results]. The stock solution of thevetin B at 1 mg ml^{-1} was prepared by dissolving thevetin in methanol. The aqueous solution of thevetin B at 1 µg ml^{-1} was prepared by diluting the 1 mg ml^{-1} stock solution of thevetin B 1000-fold.

Sensitivity of the apparatus

The detection limit is defined as the lowest concentration of the cardiac glycosides that can be resolved from the zero calibration standard, with 95% confidence. For the digoxin assay it was 0.2 ng ml^{-1} (0.26 nmol l^{-1}) and for the digitoxin assay 1 ng ml^{-1} (1.31 nmol l^{-1}).

The RSD was 3–7% for the digitoxin assay, under the best working conditions. Control solutions of digitoxin at 7.5, 15 and 35 ng ml^{-1} were used to validate the measurements: deviations from the standard curve must not exceed 15% for control solution at 7.5 ng ml^{-1} and 10% for the two others.

Preparation of standard sera

These were prepared by dilution of an aqueous solution of thevetin B (1 µg ml^{-1}) in drug free lyophylized ox serum (Biotrol 3, France), after dissolution in water. The concentrations of thevetin B in spiked sera varied from 5 to 80 ng ml^{-1} as did also the concentrations of reference solutions of digitoxin.

For a daily set of tests (intraday) the same solutions at different concentrations of thevetin B (5–80 ng ml^{-1}) were used. These solutions were prepared fresh each day for the interday tests.

Cross-reactivity analysis

(1) With anti-digitoxin antibodies. A 100 µl volume of thevetin B standard sera were deproteinized with 300 µl of methanol in 1.5 ml polypropylene conical tubes (Eppendorf tubes). The mixture was vortexed, then centrifuged for 5 min. The supernatant was transferred to an Abbott cup which was placed on a carrousel of the automatic sampler. The kit of digitoxin reagents including digitoxin rabbit antiserum, fluoresceine-labeled digitoxin ('tracer') and pretreatment reagent was placed in the Abbott system. The analysis was performed automatically according to Abbott instructions.

The calibration standard curve of digitoxin was realized in duplicate in the same manner and recorded in the computer of the TDx system. The concentrations of thevetin B (ng ml^{-1}) in known standards were given from the digitoxin calibration standard curve and calculated by the computer. The calculation was based on polarization intensity (NET P) of sample with a precision of 0.001 polarization unit for concentrations of fluorescein less than 10^{-10} M [6–8].

(2) With anti-digoxin antibodies. A 300 µl volume of thevetin B standard sera was deproteinized with 300 µl of precipitating reagent of digoxin containing 5% sulphosalicylic acid in methanol. Subsequent operations were the same as above. A digoxin reagent kit was utilized in this study. The concentrations of thevetin B (ng ml^{-1}) in known standards were given from a digoxin calibration curve and calculated as previously.

Results and Discussion

Affinity of the antibodies

Affinity is defined as the strength of bond between antigen and antibody and usually represented by the equilibrium constant K [9].

The assay of a molecule by a specific antibody of another molecule is realizable when the level of cross-reactivity is high and the response is constant under any concentration used. In this study the responses of thevetin B to anti-digitoxin and anti-digoxin antibodies have been determined.

The results shown in Table 1 indicate that anti-digitoxin antibody had the greater response for thevetin B and that this response is roughly proportional to thevetin B concentration. The amount found, expressed as digitoxin, corresponds approximately to 82% of the actual concentration of thevetin B.

The results of the thevetin B assays with the anti-digoxin antibody (Table 1) showed a much lower response (20–43%) than those obtained with the anti-digitoxin antibody. Moreover the response was not proportional to thevetin B concentration.

As results showed a higher sensitivity of thevetin B to anti-digitoxin antibody, a higher affinity of thevetin B with this antibody is inferred.

Intra- and inter-assay precision and accuracy of the method

Intraday test was realized with the same kit and with a single standard curve. Six samples ($n = 6$) where measured for each concentration. Interday test corresponds to a reproducibility study. It was carried out during 2 months, with 13 samples (prepared each day), tested with four different kits and analysed from different standard curves.

Statistical calculations are based on Shapiro–Wilk and Dixon tests, then on F test and t test. The values found are normally distributed at risk $a = 5\%$. The RSD is less than 10%, except for interday test at low concentrations. The results for thevetin B and digitoxin are shown in Tables 2 and 3, for concentrations from 5 to 80 ng ml^{-1}.

For the interday test, the RSD for the lowest concentration increased up to 22% for thevetin B and to 15% for digitoxin. These variations are coherent with the validation procedure of the apparatus for digitoxin essay at low concentrations (a variation of 15% is allowed for control solution at 7.5 ng ml^{-1}). Large RSDs (15%) at low concentration were also observed in the assay of other drugs with the same apparatus, under similar conditions, and is explained by the use of different kits [10].

The comparison of averages for t test shows a significant difference between the averages for all studied concentrations. This difference of averages confirms the lack of behavioural similarity between the samples.

Drug-free control ox and human serum (blank) give a positive value in the digitoxin assay system. This 'false positive' response can be explained according to the presence in the serum of endogenous substances 'digitoxin-like', which interact with anti-digitoxin antibodies [11] and/or the presence of fluorescent substances (hemoproteins, bilirubin, . . .) [1].

Ox serum was used because it contains less 'digitoxin-like' than human serum. The value found, expressed as digitoxin, for a deproteinized drug-free ox serum was about 0.2 ng ml^{-1} and it was less than 0.8 ng ml^{-1} for

Table 1
Thevetin B assay using anti-digitoxin and anti-digoxin antibodies

Sample	Theoretical conc. (ng ml^{-1})	Anti-digoxin antibody		Anti-digitoxin antibody	
		Conc. read (ng ml^{-1})	% of response	Conc. read (ng ml^{-1})	% of response
Thevetin B	20	3.96	19.8	15.27	76.4
	15	3.60	24.0	—	—
	10	2.95	29.5	8.22	82.2
	5	2.14	42.8	4.32	86.4
Digoxin	3.5	3.85		—	
	1.5	1.61		—	
Digitoxin	35	—		37.38	
	15	—		14.30	
	7.5	—		6.58	

Table 2
Statistical analysis of measured concentrations for digitoxin and thevetin B (intraday test)

	Thevetin B			Digitoxin			
Theoretical conc. (ng ml^{-1})	Conc. read (ng ml^{-1}) (n = 6)	SD	RSD	Conc. read (ng ml^{-1}) (n = 6)	SD	RSD	t test (5%)
0	0.20	—	—	0.88	—	—	—
5	3.93	0.4	9.4	5.54	0.5	8.9	S*
10	8.08	0.1	1.8	10.36	0.7	7.2	S
20	17.01	0.6	3.5	20.51	0.6	2.8	S
40	32.96	0.7	2.1	40.41	1.4	3.5	S
80	67.83	2.3	3.5	80.59	2.3	2.9	S

*S: Significant difference.

Table 3
Statistical analysis of measured concentrations for digitoxin and thevetin B (interday test)

	Thevetin B			Digitoxin			
Theoretical conc. (ng ml^{-1})	Conc. read (ng ml^{-1}) (n = 13)	SD	RSD	Conc. read (ng ml^{-1}) (n = 13)	SD	RSD	t test (5%)
0	0.26	—	—	0.29	—	—	—
5	3.61	0.8	21.9	4.99	0.8	15.1	S*
10	7.22	1.1	15.6	9.91	0.8	7.7	S
20	14.50	1.3	8.8	19.85	1.7	8.4	S
40	31.96	2.7	8.5	39.30	2.0	5.1	S
80	65.72	6.4	9.8	75.66	3.5	4.6	S

*S: Significant difference.

deproteinized human serum (Tables 2 and 3). It was considered as zero because it was inferior to the theoretical detection limit (1 ng ml^{-1}) of the apparatus for digitoxin assay.

Intraday test results expressed in nanogram per millilitre (Fig. 3), show a good correlation (r = 0.998) between the y values obtained from different concentrations of thevetin B, expressed as digitoxin, and those x values from the same concentrations of digitoxin. The response is linear as a function of the concentrations, where $y = 0.846x - 0.66$.

The curve equation realized from the interday tests gives a similar result, where $y = 0.847x - 0.96$, with a correlation coefficient, r = 0.994.

The values obtained with thevetin B, expressed as digitoxin, are transformed to true thevetin B concentrations by multiplying by a correction factor (CF) which is 1/0.84. The uncertainty, evaluated as ±9% from intraday test measurements, is included in the ±10% precision range of the method. Thus, the value of the correction factor is 1.19 ± 0.11.

Cross-reactivity specificity

Specificity is the degree of freedom from interference caused by substances other than the intended compound. Interference can be caused by, heterologous antibody populations, cross-reactivity with structurally related compounds, and nonspecific interference due to low molecular-weight compounds that alter the reaction conditions [9].

The values of molar concentrations of thevetin B, expressed as digitoxin, have been transformed into true concentrations of thevetin B by using the correction factor.

The cross-reactivity was then determined by comparing the standard curve of digitoxin with that of true thevetin B (Fig. 4). The two curves have the same origin, and the first point of each curve corresponds to zero concentration (blank). The same curves were obtained for the intra and interday tests.

The cross-reactivity at 50% [9, 12] of the curve is 94%. This percentage is constant throughout the curve, from 5 ng ml^{-1} (5.82 nmol l^{-1} of thevetin B and 6.54 nmol l^{-1} of digitoxin) to 80 ng ml^{-1} (93.14 nmol l^{-1} of thevetin B and 104.59 nmol l^{-1} of digitoxin). The uncertainty of the value of the cross-reactivity is ±10%. It derives from the uncertainty of the correction factor.

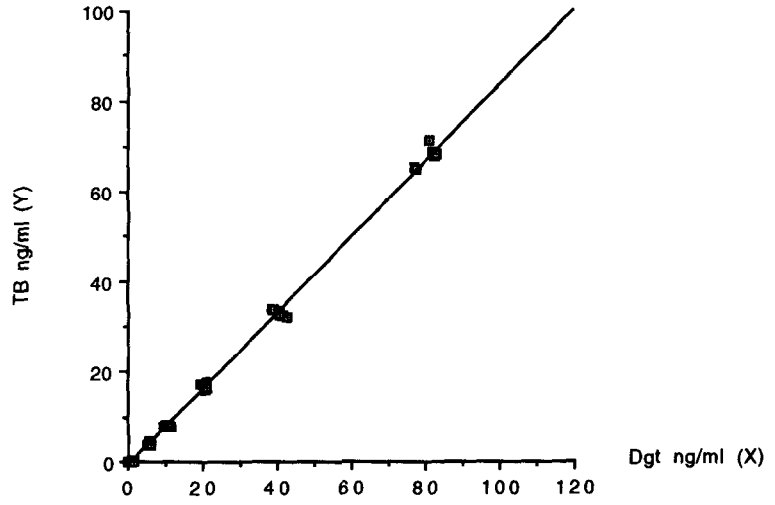

Figure 3
The linear response of anti-digitoxin antibody against different concentrations of thevetin B (TB) and digitoxin (Dgt) with $y = 0.84x - 0.66$. The coefficient of correlation is $r = 0.998$.

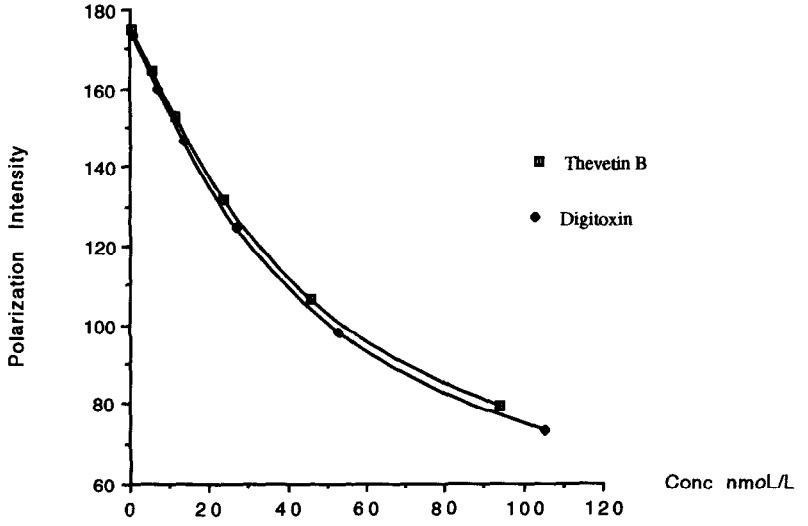

Figure 4
Standard curves of digitoxin and thevetin B.

These results allow accurate determination of the concentration of thevetin B in sera by fluorescence polarization immunoassay (FPIA) using anti-digitoxin antibodies and the TDx–Abbott methodology.

The high cross-reactivity found between thevetin B and anti-digitoxin antibody is explained by the identical genin structure of the two molecules [5, 13].

Cross-reactivity of thevetin B with anti-digoxin antibody was not studied because the preliminary tests (Table 1) showed a low sensitivity of the response.

Conclusion

The determination of thevetin B in sera can be carried out accurately and easily by fluorescence polarization immunoassay with use of an anti-digitoxin antibody. Further work is necessary to verify the affinity of anti-digitoxin antibody with thevetin B at concentration below 5 ng ml^{-1}. The different glycosidic structures of thevetin B and digitoxin interfere little in the affinity of anti-digitoxin antibody. The development of this method will be the object of a subsequent pharmacokinetic study of thevetin B.

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