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High-performance thin-layer chromatographic determination of digoxin and related compounds, digoxigenin bisdigitoxoside and gitoxin, in digoxin drug substance and tablets

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ABSTRACT

A high-performance thin-layer chromatographic (HPTLC) method for the determination of digoxin and its related compounds digoxigenin bisdigitoxoside (DBD) and gitoxin in digoxin drug substance and tablets was developed. Separation of the three compounds was accomplished on a C_{18} wettable reversed-phase plate using water–methanol–ethyl acetate (50:48:2, v/v/v) as the mobile phase. The analytes were determined by densitometry using absorbance for digoxin and fluorescence for the two related compounds. All peaks were quantified by peak-height analysis. Linear regression analysis of the data was performed for all three compounds. The calibration range for digoxin was set at 320–480 ng per 5-mm band, equivalent to 80–120% (w/w) of a 400-ng band load, that for DBD was set at 4–12 ng per 5-mm band, equivalent to 1–3% (w/w) of the digoxin load, and that for gitoxin was set at 0.4–1.6 ng per 5-mm band, equivalent to 0.1–0.4% (w/w) of the digoxin load. The limit of quantification (LOQ) for digoxin was 64 ng per 5-mm band with a limit of detection (LOD) of 8 ng per 5-mm band. The LOQs for both DBD and gitoxin were 0.12 ng per 5-mm band with LODs of 0.4 ng per 5-mm band. The linearity range for the digoxin peak height in the absorbance mode was 0–5000 ng per 5-mm band. The linearity range for DBD and gitoxin peak heights in the fluorescence mode was 0–2000 ng per 5-mm band.

INTRODUCTION

Digoxin is an extremely potent cardiotonic glycoside of the digitalis family that is widely used in modern medicine for its effect on the heart's force and speed of contraction [1]. Gitoxin and digoxigenin bisdigitoxoside (DBD) may be present in Burroughs-Wellcome digoxin. Material made by other processes may have different impurity profiles [2]. Suitable analytical techniques are necessary to detect and determine these compounds in both digoxin drug substance and tablets. Previous methods to assay for these compounds have included GC, HPLC and TLC techniques. The GC methods involve the hydrolysis of the sugar moieties to form the digoxigenin aglycone, which is subsequently trimethylsilylated. However, digoxin, DBD, digoxigenin monodigitoxoside and digoxigenin present in digoxin drug substance are all converted into the same aglycone as digoxin and will artificially inflate the digoxin response due to the formation of identical trimethylsilyl derivatives [3,4]. Methods have been developed using both reversed and normal phases to separate and determine various cardiotonic glycosides from the digitalis family. Analyses to separate drug substances from either metabolites or degradation products have used both isocratic or gradient elution HPLC systems. However, none of these methods can separate and determine the compounds of

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interest in this paper at the desired levels [5–9]. The TLC procedures vary in their capability to assay these compounds. In general, they do not provide determination at the desired levels and resolution can be a problem. Typically, normal-phase TLC methods give fast development times, but show irreproducible quantification owing to poor resolution. Reversed-phase TLC methods give better resolution of the three compounds, but have long development times and poor precision. Overpressurized reversed-phase TLC methods take only a few minutes, but the technique is not as widely accepted as classical TLC [10]. For none of the TLC methods has the quantitative recovery of these three compounds from commercial digoxin tablets been reported.

A perusal of the current literature indicates that there is no single method for the determination of digoxin and the two related compounds DBD and gitoxin. The USP XXII method uses HPLC to determine digoxin in digoxin tablets and drug substance [11]. The inclusion of DBD in the HPLC assay method for digoxin drug substance and tablets is used as a system suitability test only. A compendial limit test for digoxin drug substance uses a TLC response to gitoxin to provide information about related glycoside levels (≤3%, w/w). Neither of the related compounds is determined at the levels described here. In this paper, a high-performance TLC (HPTLC) method for the determination of the three compounds is reported. The substances are separated on a 100% wettable octadecylsilane HPTLC plate using water–methanol–ethyl acetate (50:48:2, v/v/v) as the mobile phase. The plate is scanned in the absorption mode for digoxin followed by fluorophore development and scanning in the fluorescence mode for DBD and gitoxin.

EXPERIMENTAL

Reagents and chemicals

Digoxin (99% purity) and digoxigenin bis-digitoxoside (92% purity) were supplied by Burroughs-Wellcome (Greenville, NC, USA) and gitoxin (95% purity) by Sigma (St. Louis, MO, USA). Chloroform, absolute methanol and ethyl acetate (J.T. Baker, Phillipsburg, NJ, USA) were of HPLC grade. Ethanol (USP) and concentrated hydrochloric acid (analytical-reagent grade) were obtained from the Central Research Store of the University of Georgia.

Instrumentation

The HPTLC system consisted of a Camag Linomat IV band applicator (Camag Scientific, Wilmington, NC, USA) equipped with a 100-μl syringe and a Camag Densitometric Scanner II operated by a Camag software package (System HPL 2.1 Rev. 7.01) on a Hewlett-Packard Model 9121 microcomputer. The developing chamber and conditioning tray were purchased from Camag. A Model C-31 microbalance (Cahn Instruments, Cerritos, CA, USA) and a Model AE50 balance (Mettler Instruments, Greifensee, Switzerland) were used for weighing.

Preparation of stock solutions

A digoxin stock solution was prepared by weighing 10.0 ± 0.1 mg of the digoxin powder and transferring it into a 50-ml acid-washed light-resistant volumetric flask. Chloroform–methanol (50:50, v/v) was added to volume and mixed by shaking to obtain a 0.2 mg/ml solution of digoxin.

A DBD stock solution was prepared by weighing 1.08 ± 0.05 mg of the powder and placing it in a 100-ml acid-washed light-resistant volumetric flask. Chloroform–methanol (50:50, v/v) was added to volume and mixed by shaking to obtain a 0.1 mg/ml solution of DBD.

A gitoxin stock solution was prepared by weighing 1.05 ± 0.05 mg of the powder and placing it in a 10-ml acid-washed light-resistant volumetric flask. Chloroform–methanol (50:50, v/v) was added to volume and mixed by shaking. A 1-ml volume was pipetted into a 100-ml acid-washed light-resistant volumetric flask. Chloroform–methanol (50:50, v/v) was added to volume to obtain a final concentration of 0.001 mg/ml of gitoxin.

Preparation of calibration solutions

Aliquots of 4.0, 5.0 and 6.0 ml of the digoxin stock solution were accurately pipetted into each of three individual acid-washed 100-ml volumet-

ric flasks, then 1.0, 2.0 and 3.0 ml of the DBD and 1.0, 2.5 and 4.0 ml of the gitoxin stock solutions were added to the flasks, respectively. A 40-ml volume of ethanol–water (50:50, v/v) was added to each flask and the flasks were shaken in a horizontal shaker (Eberbach, Ann Arbor, MI, USA) for 10 min. The flasks were allowed to stand at ambient temperature for an additional 10 min and chloroform–methanol (50:50, v/v) was added to volume.

Preparation of spiked tablet samples

Into each of three 100-ml acid-washed light-resistant volumetric flasks were accurately pipetted 4, 5 and 6 ml of digoxin stock solution, 1, 2 and 3 ml of DBD stock solution and 1, 2.5 and 4 ml of gitoxin stock solution. Each mixture was evaporated to dryness with the aid of a nitrogen flow at ambient temperature. Then, 800 mg placebo 0.125 mg tablet matrix or 500 mg placebo 0.25 mg tablet matrix was accurately weighed and transferred into each flask [12] and 10 ml of chloroform–methanol (50:50, v/v) and 40 ml of ethanol–water (50:50, v/v) were added to each flask. Each flask was shaken in a horizontal mixer for 30 min, then allowed to stand at ambient temperature for a minimum of 10 min. Next, chloroform–methanol (50:50, v/v) was added to volume. Each solution was filtered through a medium-porosity sintered-glass funnel (15 ml) into a clean Büchner flask and 40 μl were spotted on to the HPTLC plate.

Preparation of assay sample from commercial tablets

Either twenty digoxin 0.125-mg tablets or twenty digoxin 0.25-mg tablets were accurately weighed and powdered. An accurately weighed portion of the powder equivalent to 1.0 mg of digoxin was transferred into a 100-ml acid-washed light-resistant volumetric flask and the procedure described above for spiked tablet samples was followed.

Chromatography

A 100% wettable HPTLC plate (10 × 10 cm octadecylsilane) (Merck) was prewashed with absolute methanol and dried with forced air. A 40-μl aliquot of each calibration solution and tablet sample were applied in duplicate as 5-mm bands at the rate of 10 s/μl.

The vertical twin-trough chamber was lined on two sides with saturation pads (Universal Scientific, Atlanta, GA, USA) and equilibrated for no more than 6 min with mobile phase. The plate was developed for 30–35 min, removed and dried with forced heated air for 5–10 min. Each lane was scanned for digoxin peak height using 218 nm in the absorbance mode.

Fluorophore development

After scanning for digoxin, the plate was exposed to HCl vapor for 60 min in a conditioning tray. The plate was then heated in an oven for 30 min at 120°C, allowed to cool for 10 min at ambient temperature and each lane was scanned in the fluorescence mode for DBD and gitoxin peak heights using 365 nm with a K400 cut-off filter.

RESULTS AND DISCUSSION

The structures of the analytes are shown in Fig. 1. Many TLC systems were initially investigated in these studies. Normal-phase HPTLC systems using silica plates and mobile phases containing various proportions of chloroform–methanol, methyl ethyl ketone–toluene, methanol–formamide, cyclohexane-acetone–glacial acetic acid and chloroform–methanol–formamide provided fast development times, but gave inadequate resolution of the three compounds. Reversed-phase HPTLC methods showed more promise, but gave unique problems. Mobile phases consisting of methanol–water and acetonitrile–water were investigated. It was found that methanol–water (70:30, v/v) gave a good resolution of the three compounds, but the development time was 2.5 h with a relative standard deviation (R.S.D.) of peak heights of *ca.* 5–15%.

With the commercial availability of a 100% wettable reversed-phase C_{18} plate, adequate resolution of the three compounds was obtained using a mobile phase of water–methanol–ethyl acetate (50:48:2, v/v/v). The development time

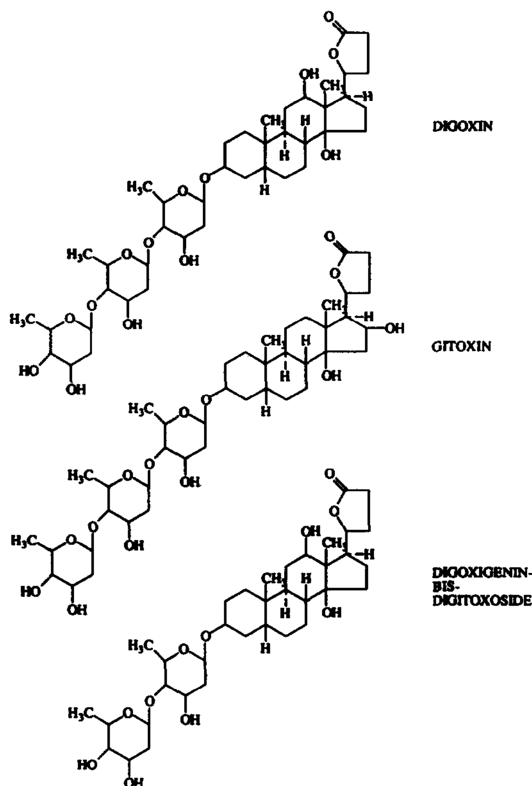

Fig. 1. Structures of analytes.

was improved to 30 min with an R.S.D. of peak heights of ≤5%.

Single-step densitometric scanning and quantification by peak-height analysis of all three analytes on the HPTLC plate was investigated in both the absorbance and fluorescence scanning modes. It soon became apparent that a single-scanning mode was unsuitable for the compounds. There was a linear response for digoxin in the absorbance mode at 218 nm, but DBD and gitoxin were not detectable at the required levels. Therefore, it was decided to investigate fluorescence detection for the three analytes. None of the compounds possessed native fluorescence, so methods to induce fluorescence were examined, including dips such as zirconium tetrachloride, sprays such as chloramine-T–trichloroacetic acid, absolute methanol–sulfuric acid, *p*-toluenesulphonic acid, inorganic acids, hydrogen peroxide, potassium bromide–potassium bromate–hydrochloric acid and vapors such as inorganic acids. Different fluorophores were created which emitted blue to yellow light. The various sprays were not reproducible enough for this study, and the zirconium tetrachloride dip removed the coated phase from the HPTLC plate. The best fluorophore development system that gave repeatable scanning results with sufficient fluorescence to detect the DBD and gitoxin at the required levels was exposure of the developed plate to hydrogen chloride vapor for 60 min followed by heating for 30 min in an oven at 120°C. However, plots of digoxin concentration *versus* fluorescence emission were not linear in its respective calibration range. Therefore, it was necessary to scan digoxin in the absorbance mode (see Fig. 2), develop the fluorophores for DBD and gitoxin and determinate them using the fluorescence mode (see Fig. 3). In this manner, all three compounds were detected and determined at levels within their specified linear ranges.

The coefficient of determination (r^2) in the absorbance mode for digoxin in the range 0–5000 ng per 5-mm band was 0.9912 ($n = 6$), DBD in the fluorescence mode gave $r^2 = 0.9904$ ($n = 7$) in the range 0–2000 ng per 5-mm band and gitoxin in the fluorescence mode gave $r^2 = 0.9986$ ($n = 7$) in the range 0–2000 ng per 5-mm band.

The calibration range for digoxin was set at 320–480 ng per 5-mm band, equivalent to 80–120% (w/w) of a 400-ng band load, that for DBD was set at 4–12 ng per 5-mm band, equivalent to 1–3% (w/w) of the digoxin load, and that for gitoxin was set at 0.4–1.6 ng per 5-mm band, equivalent to 0.1–0.4% (w/w) of the digoxin load. The limit of quantification (LOQ)

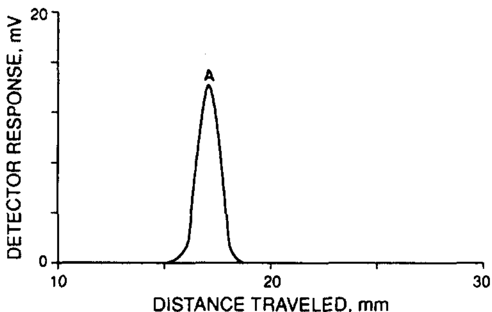

Fig. 2. Typical chromatogram of 400 ng per 5-mm band of digoxin (A) scanned in the absorbance mode at 218 nm.

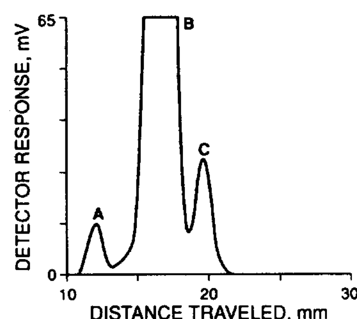

Fig. 3. Typical chromatogram of (A) 1.6 ng per 5-mm band of gitoxin, (B) 400 ng per 5-mm band of digoxin and (C) 12 ng per 5-mm band of DBD, after induced fluorescence and scanned in the fluorescence mode at 365 nm with a K400 filter.

for digoxin was 64 ng per 5-mm band with a limit of detection (LOD) of 8 ng per 5-mm band. The LOQs for both DBD and gitoxin were 0.12 ng per 5-mm band with LODs of 0.04 ng per 5-mm band.

Recovery data for spiked samples of the analytes at the three concentrations of each analyte employed for the calibration graphs are given in Table I for both the 0.125- and 0.25-mg placebo digoxin tablet matrices. Analysis of variance (ANOVA) tests run on the recovery data from spiked and commercial tablet samples showed that there was no significant difference between the data across plate lots and on analysis days at the 95% confidence level.

Actual commercial digoxin tablets were assayed for digoxin, DBD and gitoxin content using the HPTLC method described here. The results are given in Table II. The digoxin levels were within 90–105% (w/w) of the labeled amount established following the USP XXII digoxin monograph [11]. The DBD and gitoxin levels were also within the allowable ranges except for tablets C and D, which were beyond the stated date of expiration and the DBD levels were elevated compared with in-date digoxin tablets (A, B, E and F). However, the DBD levels in the out-of-date tablets are still below the 3.0% (w/w) upper limit specified in the USP XXII monograph.

The assay was unaffected by exposure of the plate to light, longer plate development times and a lined *versus* unlined developing chamber. It is important that the mobile phase be prepared fresh daily. A temperature of 22 ± 1°C was necessary as higher temperatures gave faster

TABLE I

RECOVERY DATA FOR DIGOXIN, DIGOXIGENIN BISDIGITOXOSIDE (DBD) AND GITOXIN FROM SPIKED PLACEBO DIGOXIN 0.125- AND 0.25-mg TABLET MATRICES

Analyte	Amount loaded (ng per 5-mm band)	Mean recovery (%)			
		0.125-mg matrix[a]	R.S.D. (%)	0.25-mg matrix[b]	R.S.D. (%)
Digoxin	320	99.84 ± 2.30	2.30	99.64 ± 0.12	0.12
	400	100.20 ± 0.62	0.62	100.33 ± 1.22	1.21
	480	100.27 ± 2.20	2.19	99.35 ± 0.16	0.16
DBD	4[c]	98.99 ± 1.53	1.55	96.25 ± 0.13	0.14
	8[c]	98.91 ± 1.92	1.94	96.45 ± 0.22	0.23
	12[c]	97.78 ± 3.05	3.12	97.42 ± 0.54	0.55
Gitoxin	0.4[d]	96.46 ± 2.92	3.03	94.37 ± 0.19	0.20
	1.0[d]	96.65 ± 2.10	2.17	94.35 ± 0.07	0.07
	1.6[d]	97.16 ± 3.04	3.13	97.35 ± 3.31	3.40

[a] Placebo tablet matrix used in 0.125-mg digoxin formulation. Results are means ± S.D. ($n = 10$).
[b] Placebo tablet matrix used in 0.25-mg digoxin formulation. Results are means ± S.D. ($n = 18$).
[c] Equivalent to 1, 2 and 3% (w/w) of digoxin level at 400 ng per 5-mm band.
[d] Equivalent to 0.1, 0.25 and 0.4% (w/w) of digoxin level at 400 ng per 5-mm band.

TABLE II

HPTLC ASSAY OF COMMERCIAL DIGOXIN TABLETS FOR DIGOXIN AND RELATED COMPOUNDS

Tablet	Labeled amount of digoxin (mg)	Analyte	Amount found (mg)	Recovery (%)
A	0.125	Digoxin	0.127 ± 0.003[a]	101.84 ± 2.27[b]
		DBD	<0.001	<1.0[c]
		Gitoxin	<0.0001	<0.1[d]
B	0.125	Digoxin	0.123 ± 0.005[e]	99.02 ± 3.01[e]
		DBD	<0.001	<1.0
		Gitoxin	<0.0001	<0.1
C	0.125	Digoxin	0.126 ± 0.002[e]	100.65 ± 2.03[e]
		DBD	0.0014 ± 0.0001	1.13 ± 0.09[f]
		Gitoxin	<0.0001	<0.1
D	0.25	Digoxin	0.249 ± 0.006[e]	99.4 ± 2.5[e]
		DBD	0.003 ± 0.0004	1.24 ± 0.17
		Gitoxin	<0.0002	<0.1
E	0.25	Digoxin	0.246 ± 0.004[g]	98.6 ± 1.5[g]
		DBD	<0.002	<1.0
		Gitoxin	<0.0002	<0.1
F	0.25	Digoxin	0.251 ± 0.006[h]	100.36 ± 2.43[h]
		DBD	<0.002	<1.0
		Gitoxin	<0.0002	<0.1

[a] Mean ± S.D. ($n = 15$).
[b] Mean ± S.D. ($n = 15$).
[c] % (w/w) of DBD based on digoxin content; value is below quantifiable level ($n = 16$).
[d] % (w/w) of gitoxin based on digoxin content; value is below quantifiable level ($n = 16$).
[e] $n = 6$.
[f] Mean ± S.D. ($n = 6$).
[g] $n = 10$.
[h] $n = 15$.

development but incomplete resolution of bands. If the chamber pre-equilibration time was longer than 6 min, curved bands resulted on plate development. A fluorophore development time of 30 min at 120°C was preferred for the method as longer times showed decreases in fluorescence intensity for the gitoxin band but increases in fluorescence intensity for the DBD band. A 40-μl band loading gave the best detection with minimum diffusion of the analyte bands. The absorbance of the digoxin band at 218 nm was stable for up to 11 h and the fluorescence of the DBD and gitoxin bands was stable for up to 14 h. Standards and samples must be developed and scanned on the same plate for optimum accuracy and precision.

In conclusion, an accurate and precise HPTLC method has been developed for the simultaneous determination of digoxin, DBD and gitoxin in both digoxin drug substance and commercial digoxin tablets. The analytes are separated on a 100% wettable octadecylsilane plate and assayed for digoxin by absorbance densitometry and DBD and gitoxin by fluorescence densitometry.

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