Cardiac Glycosides from Food Products

1 Introduction

The cardiac glycosides digoxin, digitoxin and oleandrin are naturally occurring chemicals that are found in various plants. Digitoxin and digoxin are found in *Digitalis lanata* and *Digitalis purpurea* (commonly known as foxglove); they are also used in the treatment of congestive heart failure and atrial fibrillation. Oleandrin is found in *Nerium oleander* (bay laurel). Toxic effects of cardiac glycosides include nausea, vomiting, visual disturbances and cardiac arrhythmias.

2 Scope

This procedure is designed to detect digoxin, digitoxin and/or oleandrin in food products, beverages and plant materials.

3 Principle

Samples are alkalinized with a buffer and extracted into a mixture of chloroform and isopropanol. Resulting extracts are taken to dryness, reconstituted, and analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) in the Fourier Transform (FT) mode.

4 Specimens

This procedure has been validated for a variety of food products, beverages and plant materials. It can easily be validated for new matrices by determining the limit of detection of the analyte(s) of interest in a specific matrix. Typically, 0.5 mL of a 1:1 aqueous homogenate will be extracted and analyzed.

5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

a. Screw-top test tubes (16 x 100 mm) with caps and Teflon inserts

b. Disposable glass pipettes with rubber bulbs

c. Volumetric flasks

d. Pipetters with disposable tips

e. pH paper

f. Vortex mixer

g. Centrifuge

h. Evaporator with nitrogen

i. Blender

j. Chloroform (HPLC grade)

k. Isopropanol (HPLC grade)

l. Cardiac Glycoside Extraction Solvent (95:5 chloroform:isopropanol):
Combine 5 mL isopropanol and 95 mL chloroform and mix well. Store at room temperature in brown glass. Stable 1 month.

m. Deionized water

n. Ammonium chloride (Reagent grade)

o. Ammonium hydroxide (ACS grade)

p. Ammonium Chloride Buffer (1.2 M, pH 9.5):
Add 6.25 g ammonium chloride to a 100-mL volumetric flask. Add approximately 50 mL deionized water and mix. Add 6.0 mL of ammonium hydroxide. Bring to the mark with deionized water and mix well. Verify that pH is between 9 and 10. Store in plastic at room temperature. Stable for six months.

q. Methanol (Optima grade)

r. Acetonitrile (Optima grade)

s. Ammonium formate (99%+)

t. Formic acid (99.8%+)

u. Water (Optima grade)

v. Ammonium Formate (2 mM, pH 3):
Add 0.126 g ammonium formate to a 1-L graduated cylinder, bring to the 1-L mark with Optima grade water, and mix well. Add 0.2 mL formic acid. Verify pH is between 2.5 and 3.5. Store in glass at room temperature. Stable for one month.

w. Liquid chromatograph/mass spectrometer capable of high mass resolution equipped with a 15 cm x 2.1 mm x 5 µm Altima-C18 (or equivalent) column.

6 Standards and Controls

a. Digoxin: Purchased as a 1.0 mg/mL solution from Cerilliant Corporation in Round Rock, Texas. Storage and stability determined by manufacturer.

b. Digitoxin: Purchased from ChromaDex in Irvine, California. Storage and stability determined by manufacturer.

c. Oleandrin: Purchased from ChromaDex in Irvine, California. Storage and stability determined by manufacturer.

d. Digoxin Working Solution (5 PPM):
Add 0.050 mL of the digoxin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.

e. Digitoxin Stock Solution (1 mg/mL):
Weigh 10 mg digitoxin. Dilute to 10 mL with methanol in a 10 mL volumetric flask. Store refrigerated in glass or plastic. Stable at least one year.

f. Oleandrin Stock Solution (1 mg/mL):
Weigh 10 mg oleandrin. Dilute to 10 mL with methanol in a 10 mL volumetric flask. Store refrigerated in glass or plastic. Stable at least one year.

g. Digitoxin Working Solution (5 PPM):
Add 0.050 mL of the digitoxin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.

h. Oleandrin Working Solution (5 PPM):
Add 0.050 mL of the oleandrin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.

i. Negative Control:
Typically, deionized water will be used as a negative control. A 1:1 deionized water homogenate of a blank matrix similar to the questioned item may also be used as a negative control. A negative control will be extracted and analyzed with every assay.

j. Positive Control:
When sample size permits, a portion of the questioned sample will be spiked with the analyte of interest to prepare the positive control. The concentration of the positive control will be based on limit of detection experiments in that matrix, but 1.0 PPM is a good starting point for most matrices. A positive control will be extracted and analyzed with every assay.

k. Cardiac Glycoside Column Check Mix (1 PPM each component):
Combine 0.1 mL of each appropriate Working Solution above. (Only analytes of interest for the day's batch must be in the column check mix.) Bring to 0.5 mL with methanol. Prepare fresh.

7 Calibration

If quantitative results are required, consult the *Guidelines for Toxicological Quantitations* procedure (Tox 101) for guidance.

8 Sampling

Not applicable.

9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

a. For food and plant material specimens, homogenize the sample 1:1 with deionized water. Beverages may be analyzed directly.

b. Into properly-labeled test tubes (16 x 100 mm), add 0.5 mL homogenate, or 0.25 mL undiluted liquid sample. For confirmatory, targeted analyses, a cardiac glycoside not present in the sample may be added as an internal standard at a concentration of 1 PPM by adding 50 µL of the Working Solution of the compound. For a screen, the unknown may be analyzed in duplicate; one sample will be run without an internal standard and one will be spiked with one of the three analytes.

c. Add 1.5 mL deionized water, 0.5 mL ammonium chloride buffer, and vortex.

d. Add 5 mL Cardiac Glycoside Extraction Solvent and mix via gentle inversion for 30 minutes.

e. Centrifuge at approximately 3000 rpm for 5 minutes.

f. Remove organic layer (bottom) to a clean test tube (12 x 75 mm) and evaporate to dryness under nitrogen at approximately 45 °C.

g. Reconstitute extracts in 0.12 mL methanol and analyze by LC/MS/MS using the conditions in Section 10.

10 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

10.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
B: Acetonitrile	flow rate	0.3 mL/min		type	C-18
	time (min)	%B	%C	length	15 cm
C: Ammonium Formate	0	20	80	internal diameter	2.1 mm
	5	38	62	particle size	5 µm
	6	65	35	temperature	30 °C
	9	70	30		
	12	70	30	autosampler T	15 °C
	13	20	80		
	25	20	80		

10.2 Mass Spectral Parameters

Ionization mode	electrospray (+)
Scan Events	2
Scan Event #1	FTMS; res=30000; 450-900 amu
Scan Event #2	ms/ms of the most intense of the following: 594.3637 (oleandrin), 798.4634 (digoxin) and 782.4685 (digitoxin) amu; res = 7500; CID CE = 60%; isolation width = 8.0

11 Decision Criteria

11.1 Column Check Mix Suitability

Proper calibration and sensitivity of the LC/MS/MS are demonstrated each day samples are analyzed. The Cardiac Glycoside Column Check Mix effectively evaluates system suitability. By analyzing this standard mix the analytes can be evaluated for proper mass assignments, elution times and signal to noise responses. Table 1 shows the parameters evaluated by use of this Mix.

Table 1: Parameters used in evaluating system suitability

Analyte	Retention Time	Full Scan Ammoniated Adduct Ion	Product Ions
Digoxin	7.64 min	798.4634	651.374, 391.248, 521.311
Digitoxin	9.11 min	782.4685	375.253, 505.316, 635.379
●leandrin	9.26 min	594.3637	433.258, 373.238, 517.316

Retention times may shift as an analytical column ages or with minor differences in mobile phase composition. From run to run, retention times should vary less than 0.5 minute.

High resolution mass spectrometric data is used in this procedure. Therefore, the full scan ammoniated adduct ion for each analyte in full scan mode should be within 5 mmu of the expected value. Additionally, product ions should be within 5 mmu of the expected value.

11.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the below should be met in order to identify a cardiac glycoside within a biological specimen:

11.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

11.2.1.1 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control of the analyte of interest.

11.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank sample injected just prior to that sample. Note: Visual evaluation of S/N is acceptable as reconstructed ion chromatograms in FTMS may have mathematically nonsensical S/N values.

11.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of the appropriate reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See Table 1 for expected ions. Full scan ammoniated adduct ions and product ions should agree with the expected values within 5 mmu.

12 Calculations

Not applicable.

13 Uncertainty of Measurement

Not applicable.

14 Limitations

a. Limits of Detection: Limits of detection for the cardiac glycosides will vary according to matrix, and will be determined with each new matrix analyzed. The following detection limits have been determined in the following matrices:

Oleandrin	- 1 PPM in coffee
	- 2 PPM in pancakes and salad
Digoxin	- 0.2 PPM in pancakes
	- 0.25 PPM in salad
Digitoxin	- 0.25 PPM in salad
	- 25 PPM in dried leaf material (based on 5 mg sample size)

b. Interferences: None known.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. See the *FBI Laboratory Safety Manual* for further guidance.

16 References

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Guidelines for Toxicological Quantitations (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Preparation of Chemical Reagents (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Safety Manual.

Rev. #	Issue Date	History
0	05/19/10	New document.
1	08/23/12	Updated chromatography decision criteria in Section 11.2.1.

Approval

Redacted - Signatures on File

Appendix 1: Abbreviated version of the Cardiac Glycoside Procedure for bench use.

Redacted - Form on File