Improvement of Sensitivity and Robustness of an LCMSMS Quantitation Method for Digoxin, Controlling the Reactivity of the Deuterated Internal Standard

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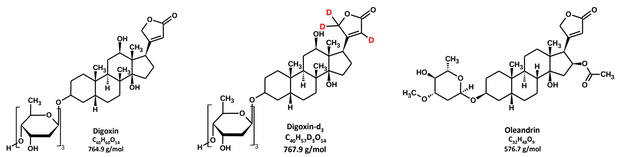
Introduction

The use of deuterated internal standards (IS) is a widespread practice in bioanalytical LC/MS/MS analysis mainly because both the IS and the parent analyte share similar physico-chemical properties and usually behave in the same way under different sample processing conditions. The stability of these molecules during sample processing could limit their efficiency as for Digoxin-d_3 which can react during the sample processing to generate Digoxin.



Method

The former method used for the determination of Digoxin in human plasma used a structural analogue (Oleandrin) as internal standard. The replacement of the IS by Digoxin-d_3 during method improvement lead to interfering zero standard samples (blanks containing only IS) during the sample processing by solid phase extraction (polymeric reverse phase). Following the investigation, the sample processing was modified to a liquid-liquid extraction procedure. After evaporation and reconstitution steps, samples were injected onto an ACE column and the mobile phase was composed of methanol/water, ammonium formate and formic acid. Analysis was performed on an API 5000 LCMSMS equipped with a TurboIonSpray source.

Extraction Procedure

Method Parameters	Old Extraction	New Extraction
Internal Standard	Oleandrin	Digoxin-d_3
Sample Volume	0.200 mL	0.200 mL
Extraction Type	Solid-Phase Extraction	Liquid-Liquid Extraction
Dilution Factor	1	0.75
Analytical Range	50-5000 pg/mL	10-10000 pg/mL

LC-MS/MS Analysis

	Old Extraction	New Extraction
Chromatographic Mode	Reversed Phase	Reversed Phase
Analytical Column	Zorbax SB-CN	ACE 3 C18
Elution Mode	Isocratic	Gradient
Mobile Phase	Water/Methanol/Ammonium Formate/Formic Acid	Water/Methanol/Ammonium Formate/Formic Acid
Flow Rate	1.0 mL/min	0.5 mL/min
Injection Volume	30 µL	30 µL
Retention Time IS	1.68 min	1.34 min
Retention Time Digoxin	2.44 min	1.32 min
Acquisition Time	4.00 min	5.00 min
Detector	AB Sciex API 4000	AB Sciex API 5000
Source	TurboIonSpray	TurboIonSpray
Ion Monitored		
Digoxin	781→651	781→651
Digoxin + NH_4	798→651	798→651
IS	577→433	784→506
IS + NH_4	594→433	801→506

Results

The initial use of Digoxin-d_3 as internal standard led to an important interfering peak in the Digoxin channel at Digoxin retention time. This interference limited the sensitivity of the method (LLOQ = 50 pg/mL).

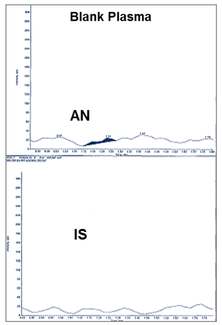


Figure 1. Representative Chromatogram of Blank Human Plasma

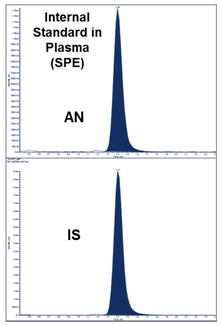


Figure 2. Representative Chromatogram of Blank Human Plasma Containing Digoxin d_3 Extracted by SPE (ZS Sample)

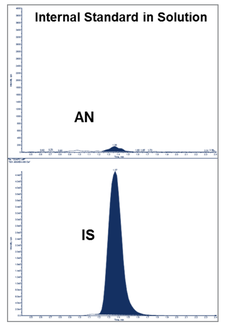
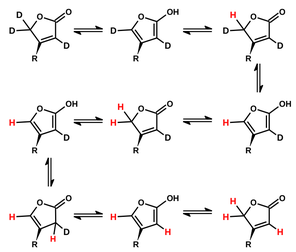


Figure 3. Representative Chromatogram of Digoxin d_3 in Solution (Not Extracted)

An assumption was made that some Digoxin was generated by a deuterium/hydrogen exchange due to a keto-enol tautomerism of Digoxin-d_3 **(Scheme 1)**.



Scheme 1. Hypothetical D/H Exchange Mechanism

The sample extraction procedure was modified and a liquid/liquid extraction was finally selected. To avoid the D/H exchange reaction, the slightly polar, non protic and low boiling point Methyl t-Butyl Ether (MTBE) was used and the temperature was controlled during critical steps. The successful use of the deuterated IS allowed the improvement of the chromatographic conditions.

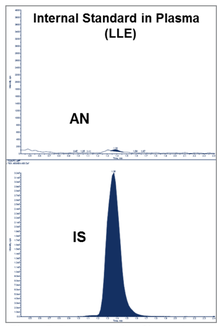


Figure 5. Representative Chromatogram of Blank Human Plasma Containing Digoxin d_3 Extracted by LLE (ZS Sample).

Figure 4. Sample SPE Extraction Process Investigation Workflow

Table 1. Results of Sample Extraction Process Investigation

Step	IS addition	Analyte Peak Response	IS Peak Response	Conversion (% area)
1	IS added to plasma sample	27351	483713	5.35
2	IS added to buffered sample	26512	490139	5.13
3	IS added to SPE extract	21334	564223	3.64
4	IS added to concentrated extract	854	3030086	0.03

The sensitivity of the method was increased and the LOQ could be decreased down to 10 pg/mL with the implantation of the new extraction and chromatographic conditions. Accuracy and precision with the new IS and extraction procedure were demonstrated at low, middle and high concentration levels. Specifically, precision was improved from 14.4% at 50 pg/mL with the SPE method to about 5% at 10 pg/mL with the improved method. The addition of a deuterated internal standard improved the robustness of the methods well as sensitivity and precision at the low end of the calibration curve.

Results

Table 2. Recovery of Digoxin and IS in Human Plasma by Liquid-Liquid Extraction

	From Human Plasma EDTA K_2		
	Digoxin		Internal Standard
Mean Recovery (%) (n=6)	62.63	(Low Level)	71.09
	62.39	(Medium Level)	
	63.97	(High Level)	

Table 3. Evaluation of Lower Limit of Quantification of Digoxin in Human Plasma by Liquid-Liquid Extraction

	Digoxin in Human Plasma EDTA K_2			
	LLOQ			Blank Sample
	Mesured Conc. (pg/mL)	% Bias	Height (cps)	Height (cps)
	11.89	18.90	232.019	12.725
	11.82	18.20	182.235	12.540
	10.91	9.10	226.364	1.000
	12.84	28.40	142.358	7.866
	12.26	22.60	184.239	1.000
	11.89	18.90	187.453	1.000
N	6	6	6	6
Mean	11.935	19.35	192.4447	6.0218
SD(±)	0.6313			
CV(%)	5.29			
Signal/Noise Ratio	32			

Table 4. Accuracy and Precision of Determination of Digoxin in Human Plasma by Liquid-Liquid Extraction

	Digoxin in Human Plasma EDTA K_2			
Calibration range	10 to 10 000 pg/mL			
Correlation coefficient (13 runs)	≥ 0.9932			
Accuracy and Precision	QC	Conc. (pg/mL)	Accuracy Bias (%)	Precision CV (%)
Between-run (n=78)	LLOQ	10	0.86	12.51
	Low	30	6.91	6.34
	Medium	5000	0.68	4.97
	High	7500	4.81	5.68

Conclusion

The systematic investigation of the extraction process and the control of the reactivity of the deuterated internal standard allowed the development of sensitive assay for the analysis of Digoxin in human plasma. The analytical method was validated according to the most current guidelines.

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