A General Approach to Eliminating Downfield Interference in Bioanalysis of Amines by SCX Chromatography - Application to Oxybutynin and NNAL

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OVERVIEW

- Two separate methods were simultaneously being modified:
  - Changing analytical range for desethyloxybutynin in human plasma
  - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine to harmonize tobacco specific nitrosamine analyses
- Highly retained matrix components in isocratic SCX analysis can eventually elute disturbing instrument response
  - Different strategies were utilized in the original methods to eliminate the undesirable effects
  - Pre-column stripping using separate analytical and cleaning mobile phases provided a general approach for both modified methods

INTRODUCTION

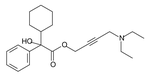
Oxybutynin is a muscarinic antagonist used to treat urinary urgency and incontinence. NNAL is a carcinogenic tobacco specific nitrosamine measured as a marker of exposure to nicotine. The existing SCX methods for these small molecule weak amines exhibited very highly retained matrix components, which were being managed by separate approaches. We describe a generalized method for the rapid elimination of highly retained sample components using strong mobile phase and pre-column stripping, which may be applied to the bioanalysis of polar small molecule weak amines. Method improvements decreased turnaround time, reduced solvent usage, and provided a turn-key solution for analyses that exhibit similar issues. Validated methods for analyses of oxybutynin and desethyloxybutynin in human plasma and NNAL in human urine demonstrate application of the approach.

METHODS

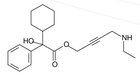
All methods utilized an AB SCIEX API 4000™ mass spectrometer with an ESI source operated in positive MRM mode monitoring the following ions:

Ions monitored (m/z)				Dwell time (msec. Old/New)
Oxybutynin:	358.3	→	142.1	300 / 150
d_{10}-Oxybutynin (IS):	368.3	→	152.2	150 / 75
Desethyloxybutynin:	330.3	→	96.1	300 / 150
d_5-Desethyloxybutynin (IS):	335.3	→	101.1	150 / 75
NNAL:	210.1	→	180.1	400 / 300
Old d_3-NNAL (IS):	213.1	→	183.1	200
New d_4-NNAL (IS):	214.1	→	184.1	150

OXYBUTYNIN AND METABOLITE



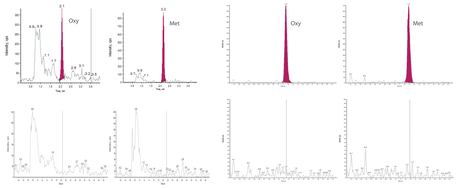
Oxybutynin



Desethyloxybutynin (Met)

The old method of extraction was mixed-mode RP/SCX SPE. The new method utilizes a 96-well neutral liq-liq extraction from 0.2 mL human plasma into MtBE.

	Old LC-MS/MS Method	New LC-MS/MS Method
Mobile Phase 1	80:20 ACN:20 mM CH_3COONH_4, pH 2.5 w/ CH_3COOH	80:20 ACN:5 mM $HCOONH_4$, pH 2.5 w/ HCOOH
Mobile Phase 2	N/AP	70:30 MeOH:25 mM CH_3COONH_4
Pre-column	Phenomenex SecurityGuard™ C_{18}, 4.0 x 3.0 mm	Thermo Scientific, BioBasic SCX, 10 x 3.0 mm, 5 µm, ambient temperature
Analytical Column	Agilent Technologies, Zorbax 300-SCX, 50 x 3 mm, 5 µm	Thermo Scientific, BioBasic SCX, 50 x 3.0 mm, 5 µm, ambient temperature
Event	Flow Stepping	Pre-column Stripping
Runtime	5.5 minutes	2.0 minutes
Solvent	7.7 mL/sample	3.2 mL/sample
K'	~2.4	~3.3



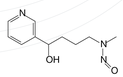
Old Method LLOQ/Blank

New Method LLOQ/Blank

The sample was injected, and after the analytes passed through the pre-column to the analytical column, the pre-column was switched to waste and elution to the mass spectrometer continued from the analytical column.

The wash mobile phase was directed to the pre-column off-line, where the neutral strong buffer in methanol promoted rapid elution of highly retained cationic species to waste.

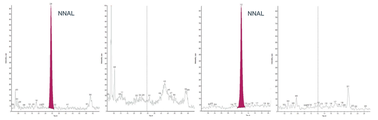
NNAL



4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

Total NNAL was generated from human urine (2.0 mL) with ß-glucuronidase and isolated using a mixed mode RP/SCX SPE in both the old and new methods.

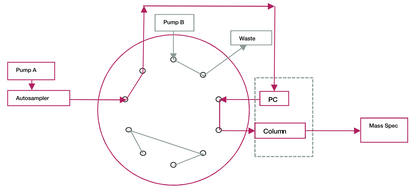
	Old LC-MS/MS Method	New LC-MS/MS Method
Mobile Phase 1	80:20 ACN:5 mM $HCOONH_4$, pH 2.5 w/ HCOOH	80:20 ACN:5 mM $HCOONH_4$, pH 2.5 w/ HCOOH
Mobile Phase 2	70:30 MeOH:25 mM CH_3COONH_4	70:30 MeOH:25 mM CH_3COONH_4
Pre-column	N/AP	Thermo Scientific, BioBasic SCX, 10 x 3.0 mm, 5 µm, 45°C
Analytical Column	Agilent Technologies, Zorbax 300-SCX, 50 x 4.6 mm, 5 µm	Thermo Scientific, BioBasic SCX, 50 x 2.1 mm, 5 µm, 45°C
Event	Strong Mobile Phase Wash	Pre-column Stripping
Runtime	6.5 minutes	2.8 minutes
Solvent	12.5 mL/sample	3.9 mL/sample
K'	~9.8	~6.4



UriSub® LLOQ/Urine Blank

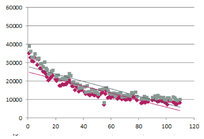
Old Method

New Method

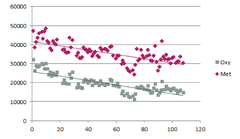


RESULTS

Old Method for Oxybutynin and Metabolite:
(IS Peak Area, with unspiked samples removed)

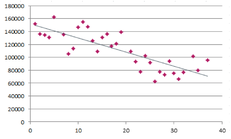


Severe Drift

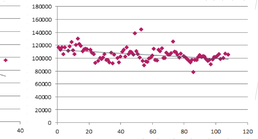


Acceptable Drift

Old Method for NNAL:
(IS Peak Area, with unspiked samples removed)

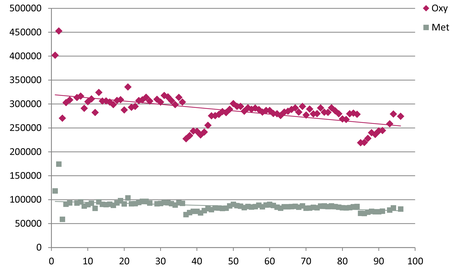


Severe Drift

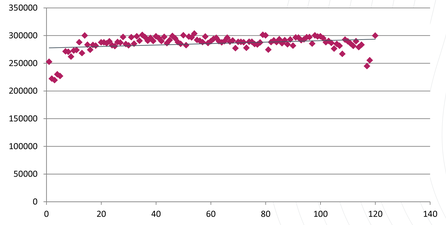


Acceptable Drift

New Method for Oxybutynin and Metabolite:
(IS Peak Area, with unspiked samples removed)



New Method for NNAL:
(IS Peak Area, with unspiked samples removed)



- The acidic weak buffer and high percentage of acetonitrile provided moderate retention and high sensitivity using ESI in positive ion mode.
- The validated analytical range for both oxybutynin and its desethyl-metabolite was 0.050 - 10.0 ng/mL using 0.200 mL of human plasma (EDTA).
- The validated analytical range for NNAL was 5.00 - 1000 pg/mL.
- Both methods were fully validated and conform to applicable international bioanalytical guidelines of the FDA and EMA.
- The oxybutynin method has been applied to over 2000 samples with greater than 99% batch success rate.
- The NNAL method has been applied to over 3600 samples with a 100% batch success rate and more studies are on-going.
- The incurred sample reanalysis met industry wide standards.

CONCLUSIONS

A general approach to eliminating highly retained matrix interference in SCX bioanalysis has been developed and applied successfully. This will become a go-to approach to resolve similar issues in future method development of polar amines exhibiting otherwise undesirable SCX chromatographic properties.

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