DEVELOPING A HIGHLY SENSITIVE FOR THE ANALYSIS OF A SERIES OF β-BLOCKERS BY UPLC WITH FLUORESCENCE AND PHOTODIODE ARRAY DETECTION

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INTRODUCTION

Chromatographic method development is often a long, tedious process and when coupled with the need for fluorescence detection can be challenging. By employing a systematic chromatographic screening process and simple-to-use detection method development tools, the time needed to develop a robust method which meets the separation and sensitivity criteria can be dramatically reduced. In order to achieve the highest sensitivity for fluorescence detection, the optimal excitation and emission wavelengths for each component must be determined. The scanning capabilities of the ACQUITY UPLC FLR detector simplify the optimization of these parameters. When this systematic approach to fluorescence methods development is applied, the final method can be developed in a significantly shortened time-line. This methodology was applied to the development of an UltraPerformance LC (UPLC) method for a series of β-blockers. The final UPLC method was under four minutes with baseline resolution of all eleven β-blockers. β-Blockers are a class of drugs that are used to deal with different types of heart complications such as the management of cardiac arrhythmias, cardio protection following a heart attack, and hypertension by blocking beta-adrenergic substances like epinephrine. Clinical screening methods for β-Blockers require a method which resolves all drugs of interest and has a detection method which is selective and sensitive such as fluorescence detection.

Figure 1. The ACQUITY UPLC FLR Detector

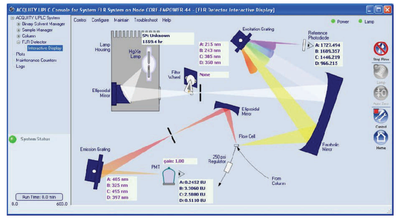

Figure 2. The interactive page for the ACQUITY UPLC FLR in Console gives the user a real time view of the FLR optics

METHODS

Screening Method Conditions

System:	ACQUITY UPLC (BSM, SM, CM, PDA)
Data:	Empower 2 Software
Sample:	11 β-Blockers @ 0.1mg/mL in Water
Column:	ACQUITY UPLC® BEH C_{18}, BEH RP_{18}, BEH Phenyl, HSS T3; 2.1x 50mm
Injection Volume:	2 µL
Temperature:	30°C
Mobile Phase A1:	10mM Ammonium Formate pH 3
Mobile Phase A2:	10mM Ammonium Bicarbonate pH 10
Mobile Phase B1:	Acetonitrile
Mobile Phase B2:	Methanol
Weak Wash:	600µL Water/Methanol (95/5)
Strong Wash:	200µL Water/ACN/IPA (15/70/15)
Flow Rate:	0.500 mL/min
Gradient:	5% to 90% B over 5 minutes
Wavelength:	275 nm
Data Rate:	20 Hz (Normal)

Final Method Conditions

System:	ACQUITY UPLC (BSM, SM, CM, PDA, FLR)
Data:	Empower 2 Software
Column:	ACQUITY UPLC® BEH C18 2.1x 100mm
Injection Volume:	2 µL
Temperature:	40°C
Mobile Phase A:	20mM Ammonium Formate pH3
Mobile Phase B:	Acetonitrile
Weak Wash:	600µL Water/Methanol (95/5)
Strong Wash:	200µL Water/ACN/IPA (15/70/15)
Flow Rate:	0.500 mL/min
Gradient:	11% to 30% B over 2 minutes, hold @ 30% B for 2 minutes
PDA Wavelength:	275 nm
FLR Wavelength:	See Table 1
FLR PMT Gain:	10.0
Data Rate:	20 Hz (Normal)

Time (min)	Excitation Wavelength	Emission Wavelength	Compounds in Window
0.00	229	300	Atenolol Sotalol
1.20	250	320	Nadolol Pindolol
1.70	311	470	Acebutolol
2.03	230	295	Metoprolol
2.40	275	308	Oxprenolol
2.60	305	415	Labetalol
2.90	270	320	Propranolol
3.25	230	300	Betaxolol

Table 1. FLR wavelength settings for final β-Blocker method

METHOD DEVELOPMENT AND OPTIMIZATION

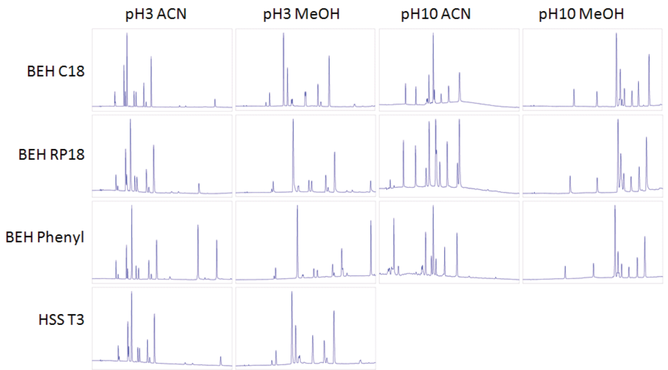

Figure 3. Automated method screening across 4 column chemistries, 2 pHs, and 2 organic modifiers generated a matrix of separations. From these separations the best set of starting conditions was selected for further optimization. The separation on the BEH C18 at pH 3 with ACN was selected as it gave the best combination of resolution and peak shape.

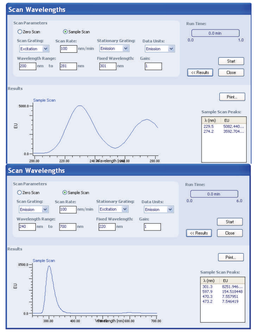

Figure 4. These plots show the excitation and emission spectra for Atenolol. Software features in the Console allow for the scanning of both the excitation and emission spectra. This information can then be used to generate a FLR detection method that yields the highest sensitivity for the compound(s) of interest. Additionally, Empower Software provides the capabilities of collecting 3D spectral scan and λ-λ plots for detection method development.

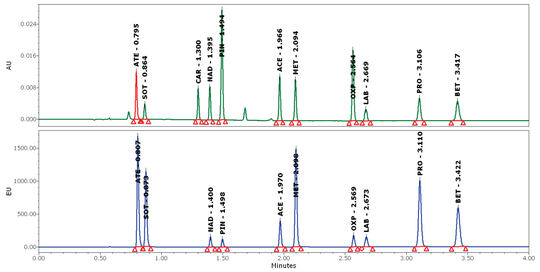

Figure 5. The final optimized separation by UV and FLR detection. Optimization steps included taking the selected result from the automated method screening and importing it into a method development software program. A theoretical optimization was performed where the conditions of the method were altered to achieve maximum resolution and minimized run time. In the final method the temperature was increased from 30°C to 40°C and the gradient composition was changed from 5%-90% B over 5 minutes to 11%-30% B over 2 minutes, hold at 30% for 2 minutes, and the column length was increased from 50mm to 100mm. The mobile phase was changed from a 10mM to a 20mM Ammonium Formate to improve peak shape. The resulting method had baseline resolution (>2.5) of all 11 β-Blockers.

LIMITS OF DETECTION AND QUANTIFICATION

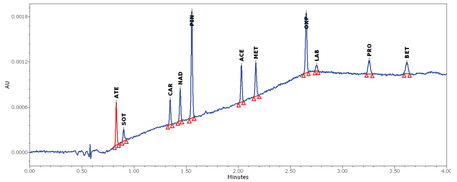

Figure 6. Separation of all 11 β-Blockers with PDA detection near the limits of detection (ATE, ACE, BET, LAB, MET, NAD, OXP, SOT @ 0.4µg/mL; PIN @ 0.2µg/mL; CAR, PRO @ 0.1µg/mL)

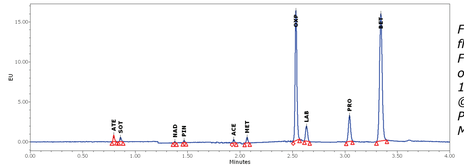

Figure 7. Separation of 10 fluorescing β-Blockers with FLR detection near the limits of detection (OXP @ 1000ng/mL; BET, LAB @200ng/mL; ACE, NAD, PRO, PIN @ 10ng/mL; ATE, MET, SOT @ 4ng/mL)

Compound	UV LOD (µg/mL)	UV LOQ (µg/mL)	FLR LOD (ng/mL)	FLR LOQ (ng/mL)	Increased Sensitivity by FLR
Atenolol	0.059	0.197	0.85	2.83	70x
Sotalol	0.240	0.800	1.30	4.33	185x
Carteolol	0.029	0.097	--	--	--
Nadolol	0.081	0.270	10.6	35.2	7.8x
Pindolol	0.011	0.037	6.47	21.6	1.7x
Acebutolol	0.084	0.280	10.4	34.7	8.1x
Metoprolol	0.081	0.270	1.25	4.17	65x
Oxeprenolol	0.056	0.187	76.7	256	0.7x
Labetalol	0.436	1.453	43.4	145	10x
Propranolol	0.064	0.213	3.33	11.1	19x
Betaxolol	0.279	0.930	52.7	176	5.3x

Table 2. Limits of detection and quantification (LOD and LOQ) for each of the β-Blockers by both UV and fluorescence. All compounds were more sensitive by FLR except carteolol which does not fluoresce and oxprenolol which had excessive noise in its FLR detection window. LOD was defined as 3x s/n and LOQ was defined as 10x s/n.

CONCLUSION

- **A UPLC method with combined PDA and FLR detection was developed for a series of eleven β-Blockers**
- **The final method was 4 minutes with baseline resolution of all eleven β-Blockers (>2.5)**
- **Automated method screening with the ACQUITY UPLC System dramatically reduced the method development time**
- **The scanning capabilities of the ACQUITY UPLC FLR in the Console greatly reduced the time needed to optimize the excitation and emission wavelengths to create a highly sensitive FLR detection method**

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