Quantitative Determination of Phenoxybenzamine in Human Plasma by LC-MS-MS

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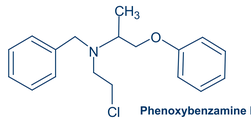
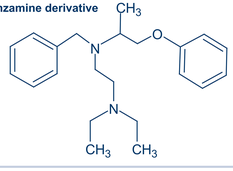
Introduction:

Phenoxybenzamine is an antihypertensive agent which is quite unstable at high pH due to its N-2-chloroethyl substituent. Consequently, the objective was to develop a sensitive method while taking into account the instability of the molecule in the plasma matrix, during the extraction procedure and in the reconstituted extract. This was accomplished by a combined extraction/derivatization procedure outlined below.



Phenoxybenzamine MW = 303.8

Phenoxybenzamine derivative
MW = 340.3



Experimental:

Reference Compounds and Chemicals

Phenoxybenzamine hydrochloride was purchased from Sigma-Aldrich, Inc. The internal standard, Phenoxybenzamine-D_7 HCl was from Alsa-Chim. All the chemicals used were AR grade and all solvents were HPLC grade or better.

Standard Solutions

All solutions of Phenoxybenzamine and Phenoxybenzamine-D_7 were prepared in 0.1M HCl. A series of spiking standard solutions was prepared from dilutions of the stock to cover the entire quantitation range from 0.025 to 5.00 ng/mL.

Quality control (QC) samples were prepared in human plasma at three different concentrations (0.0750, 1.00, and 4.00 ng/mL). These low, medium and high QC samples were stored as 0.500 mL aliquots containing 20% 1M HCl at -70°C and were prepared from different stock solutions than the calibration standard samples.

Extraction

To all samples (0.200 mL plasma, 0.0500 mL 1M HCl) except blank-blank samples, 20 µL of internal standard solution (Phenoxybenzamine-D_7) is added followed by 0.7 mL of 0.1 M HCl.

Extraction Graphic

0.250 mL Sample and Spiked as Above
↓
Applied on OASIS MCX LP cartridges, conditioned with 1mL of methanol followed by 1 mL of water
↓
Washed with 1 mL of 0.1M HCl followed by 1 mL of methanol
↓
Eluted with 1 mL of 5% diethylamine in methanol
↓
Keep at 50ºC for approximately 1h to complete the derivatization
↓
Evaporate and reconstitute

Sample Analysis by LC-MS-MS

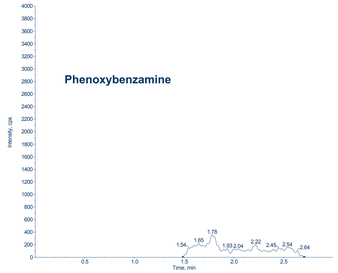
The LC-MS-MS system consisted of Perkin Elmer autosamper, Shimadzu pumps and SCIEX API 4000 mass spectrometer with an ionspray interface. The ion transitions monitored were m/z 341.3→100.1 for derivatized Phenoxybenzamine and m/z 348.3→100.1 for derivatized Phenoxybenzamine-D_7 (internal standard). The samples were injected onto the LC system with a flow rate of 0.700 mL/min. The analytes were separated on an XBridge Phenyl: 3.5 µm, 4.6 × 50 mm column (Waters) using methanol/water/formic acid/ammonium hydroxide as mobile phase in a gradient mode as presented below. Derivatized Phenoxybenzamine and its internal standard were retained at approximately 2 minutes.

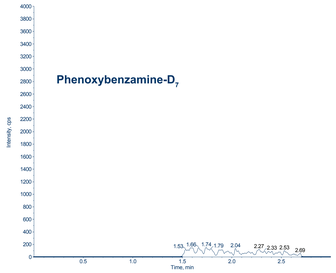
Time (min)	Flow Rate (mL/min)	Func.	Value
Initial	0.700	%B	70
1.0	0.700	%B	70
1.5	0.700	%B	90
2.3	0.700	%B	90
2.5	0.700	%B	70

Mobile Phase A (water/formic acid/ammonium hydroxide; 1000:1.00:0.250)
Mobile Phase B (methanol/formic acid/ammonium hydroxide, 1000:1.00:0.250)

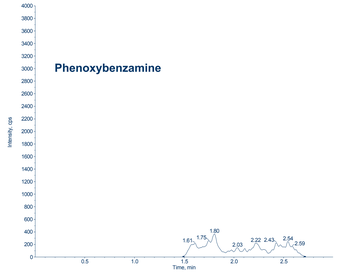
Representative Chromatograms:

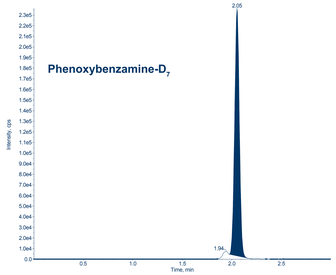
Extracted Blank Plasma Sample



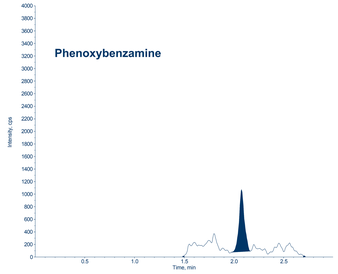


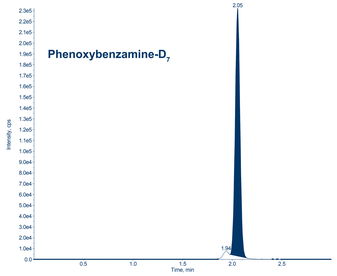
Blank Plasma and Internal Standard





Std A (0.0250 ng/mL) in Plasma





Results:

Standard Precision and Accuracy

Mean of Three Curves Over Three Days of Validation (Table 1)

Amount Added (ng/mL)	0.0250	0.0500	0.100	0.250	0.500	2.0	4.50	5.0
Mean (ng/mL)	0.258	0.0472	0.0956	0.253	0.507	2.01	4.40	5.24
CV (%)	3.9	6.7	*	*	5.3	3.3	7.2	3.6
Bias (%)	3.2	-5.6	-4.4	1.2	1.4	0.5	-2.2	4.8

* >15& bias in one curve. Point dropped

LLOQ and QC

Mean Over Three Days of Validation (Table 2)

		Concentration (ng/mL)			
		0.0250	0.075	1.00	4.00
Intraday	Mean (ng/mL)	0.269	0.0772	1.02	3.99
	CV (%)	8.6	3.8	3.5	2.6
	Bias (%)	7.7	2.9	1.1	-0.2
	n	6	6	6	6
Interday	Mean (ng/mL)	0.269	0.0772	1.01	3.99
	CV (%)	9.1	4.8	6.0	3.9
	Bias (%)	7.6	2.9	1.0	-0.3
	n	17	18	18	18

Stability

(Table 3)

QC Pool Concentration (ng/mL)	0.075	4.0
	Mean % Change from QC Pool Concentration (n=3)	
Benchtop (24 hr)	-0.5	-3.0
Freeze/Thaw (5 cycles)	-10.0	-3.4
Extract Stability (113 hr)	9.4	7.5

LLOQ, QC and ULOQ

Data for Extended Curve (Table 4)

	Concentration (ng/mL)				
	0.0250	0.0750	3.00	12.0	15.0
Mean	0.0223	0.0731	2.79	10.7	14.6
CV (%)	2.2	2.8	2.1	2.0	2.3
Bias (%)	-10.8	-2.5	-7.0	-10.8	-2.7
n	6	6	6	6	6

Stability

Extended Range (Table 5)

QC Pool Concentration (ng/mL)	0.075	12.0
	Mean % Change from QC Pool Concentration (n=3)	
Benchtop (24 hr)	5.1	-3.1
Freeze/Thaw (5 cycles)	5.0	-1.7
Extract Stability (166 hr)	0.3	-8.0
Long-term Stability at -20°C (47 Days)	-	6.4
Long-term Stability at -70°C (51 Days)	-	6.4

The assay was initially validated over a range of 0.025 to 5.00 ng/mL using a plasma volume of 0.200 mL. Three validation runs were performed each on separate days. For the curve CV was between 3.3 and 7.2%. Accuracy (% bias) was between -5.6 and 4.8% across all levels of the calibration range (Table 1). For the LLOQ samples and QC's CV was 2.6 to 8.6% intraday and 3.9 to 9.1% interday and accuracy (% bias) was -0.2 to 7.7 intraday and -0.3 to 7.6% interday (Table 2). No chromatographic interferences were observed in six different lots of plasma were observed indicating the specificity of the method. As for matrix effects, nine out of ten plasma lots tested were within acceptance criteria. Based on these results, analytical recovery was not significantly affected by variations in matrix. Stability of Phenoxybenzamine in acidified plasma was established for 24 hours at room temperature, 5 cycles of freezing and thawing, and over 113 hours in the final extract (Table 3).

As several preliminary samples were found to be above the curve range a one run partial validation was carried out, extending the range to 15.0 ng/mL. New QC pools at 0.075, 3.00 and 12.0 ng/mL were included. Bias of the curve standards were -5.8 to 15.0%. R^2 was 0.9953 using a weighting of $1/x^2$. LLOQ, QC and ULOQ data are in Table 4. New benchtop, freeze-thaw and extract stabilities were established for the new range as well as long-term stability at -20ºC and -70ºC. The results are in Table 5.

Conclusion:

A sensitive and accurate assay was developed. Stability of the drug was maintained by keeping the pH low until the elution step of the extraction. The directly following derivatization step using the elution solution converted Phenoxybenzamine to a stable derivative. This assay has been used to successfully analyze more than 4000 samples.