Panexcell Clinical Lab Private Limited develops a Bio-analytical method for a sensitive molecule – Formoterol and Glycopyrrolate

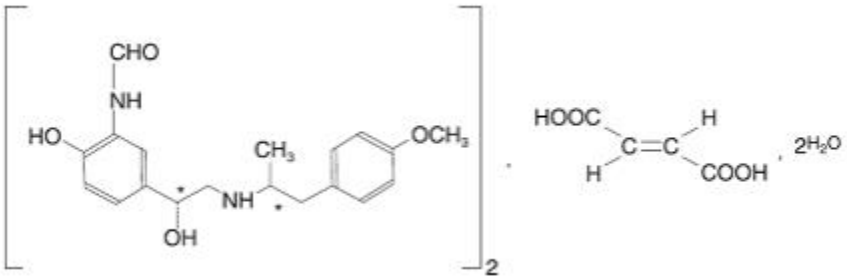
Introduction to the molecule: - Formoterol and Glycopyrrolate combination is used as long-term maintenance treatment of chronic obstructive pulmonary disease (COPD), including chronic bronchitis and emphysema. COPD is a long-term lung disease that causes bronchospasm (wheezing or difficulty with breathing).

Glycopyrrolate and formoterol are long-acting bronchodilators. Bronchodilators are medicines that are breathed in through the mouth to open up the bronchial tubes (air passages) in the lungs. They relieve cough, wheezing, shortness of breath, and troubled breathing by increasing the flow of air through the bronchial tubes.

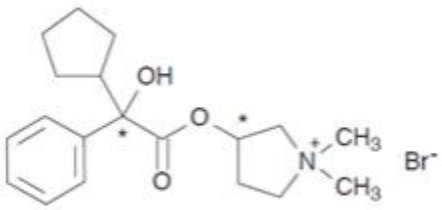
Objective: - To develop an ultra-sensitive, robust, precise, accurate & reproducible method for the quantification of Formoterol & Glycopyrrolate in human plasma using LC-MS/MS.

Chemical Structures of the molecule: -

(A) Formoterol



(B) Glycopyrrolate



Methodology: -

Instrument : AB Sciex API5500 Triple Quad and Shimadzu HPLC

Experimentation: -

Analytes	Formoterol & Glycopyrrolate
Internal standard	Formoterol-D_6 & Glycopyrrolate-D_3
Column	Zorbax SB-CN, (4.6 X 250mm) 5µm
Flow rate	1.000 mL/min
Injection volume	40 µL
Sample preparation	Solid phase extraction
Run time	13.0 mins
Mass polarity	Positive
Mode	Multiple reactions monitoring (MRM)
Transition	345.2 > 149.1 for Formoterol 351.2> 155.1 for Glycopyrrolate 345.2 > 149.1 for Formoterol-D6(ISTD) 351.2> 155.1 for Glycopyrrolate-D3(ISTD)
Calibration range	0.404 to 50.054 pg/mL for Formoterol 0.408 to 50.521 pg/mL for Glycopyrrolate
Matrix	Human plasma having K_3EDTA, 3.2% Sodium Citrate as anticoagulant
Mobile Phase	Buffer (5 mM Ammonium Trifluoroacetate in water), 0.01%Formic Acid in Acetonitrile (70:30) V/V
Extraction Method	Solid Phase Extraction (SPE) using Strata X

Table 1: Curve Parameter Summary

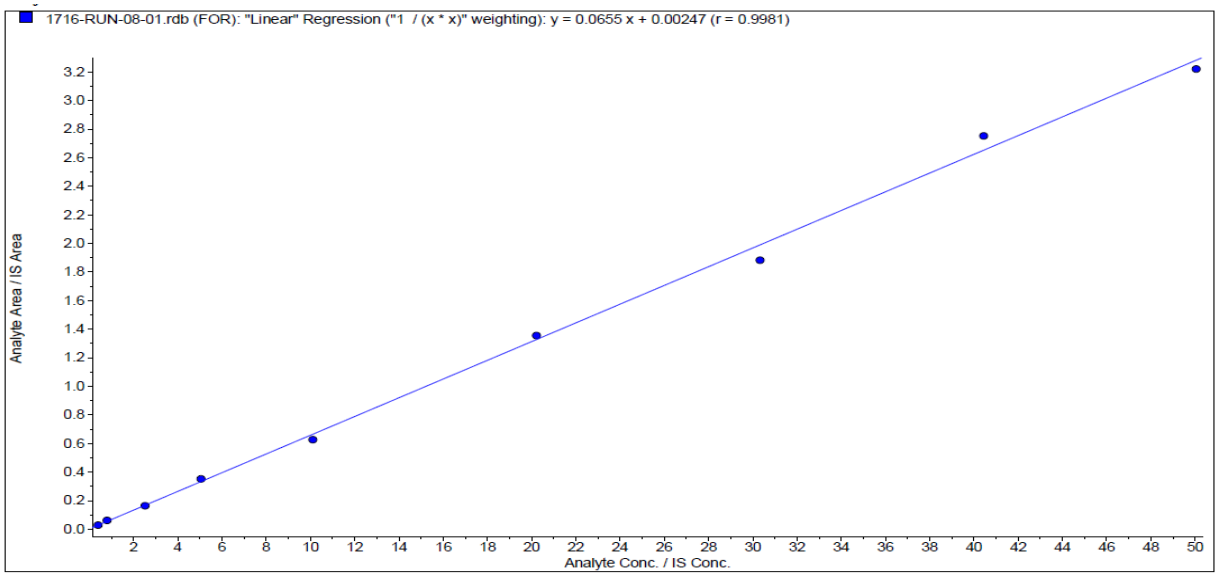
Curve parameter summary and back-calculated calibration curve concentrations for Formoterol in human plasma during method validation.

Nominal concentration (pg/mL)	0.404	0.809	2.528	5.056	10.112	20.224	30.336	40.448	50.054
Mean	0.4000	0.8270	2.5512	4.9588	9.5284	21.1436	31.1512	40.9024	48.6454
SD	0.00886	0.03752	0.05664	0.24009	0.21080	0.64677	1.61216	0.70028	0.85256
% CV	2.22	4.54	2.22	4.84	2.21	3.06	5.18	1.71	1.75
% Nominal	99.01	102.22	100.92	98.08	94.23	104.55	102.69	101.12	97.19
n	03	03	03	03	03	03	03	03	03

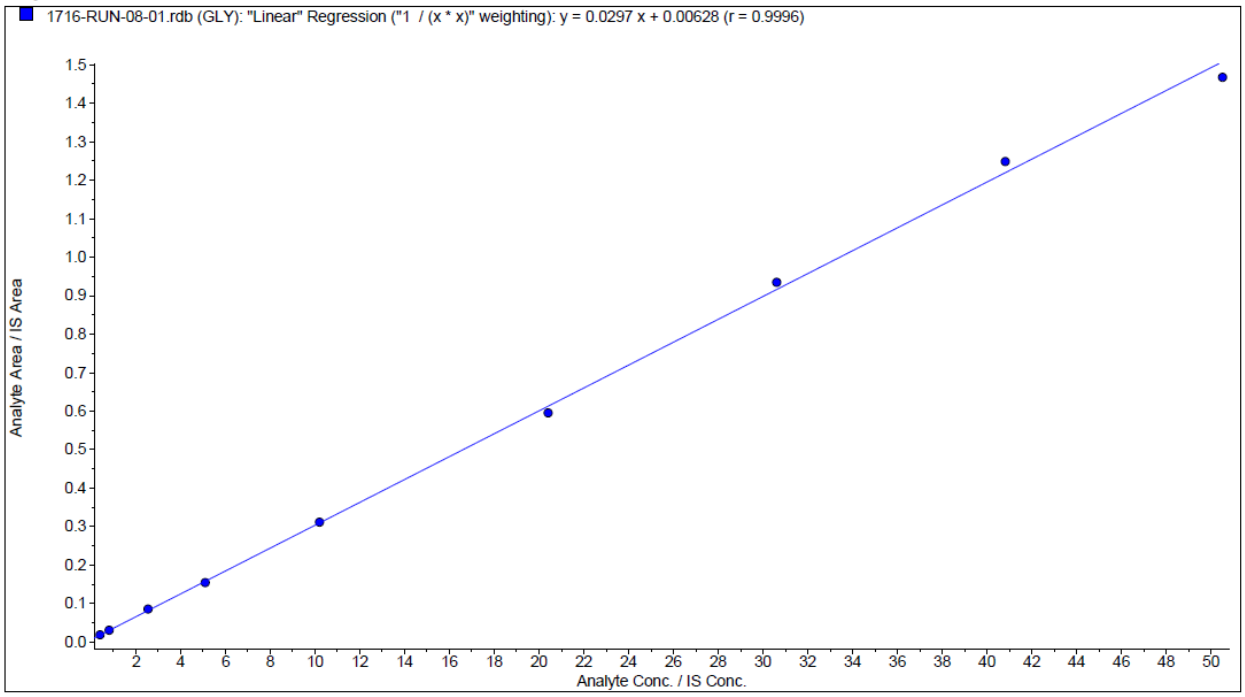
Curve parameter summary and back-calculated calibration curve concentrations for Glycopyrrolate in human plasma during method validation

Nominal concentration (pg/mL)	0.408	0.817	2.552	5.103	10.206	20.413	30.619	40.825	50.521
Mean	0.4068	0.8190	2.6324	4.9670	9.7084	20.9802	32.0282	41.3306	48.4256
SD	0.00217	0.00987	0.03646	0.15882	0.39409	0.98902	0.85450	0.64582	1.21199
% CV	0.53	1.21	1.39	3.20	4.06	4.71	2.67	1.56	2.50
% Nominal	99.71	100.24	103.15	97.33	95.12	102.78	104.60	101.24	95.85
n	03	03	03	03	03	03	03	03	03

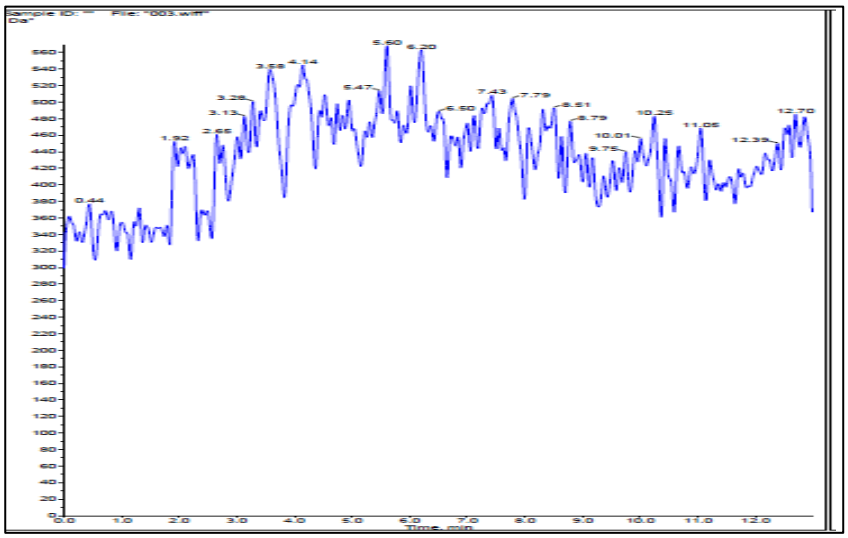
A representative regression analysis of a calibration curve for Formoterol



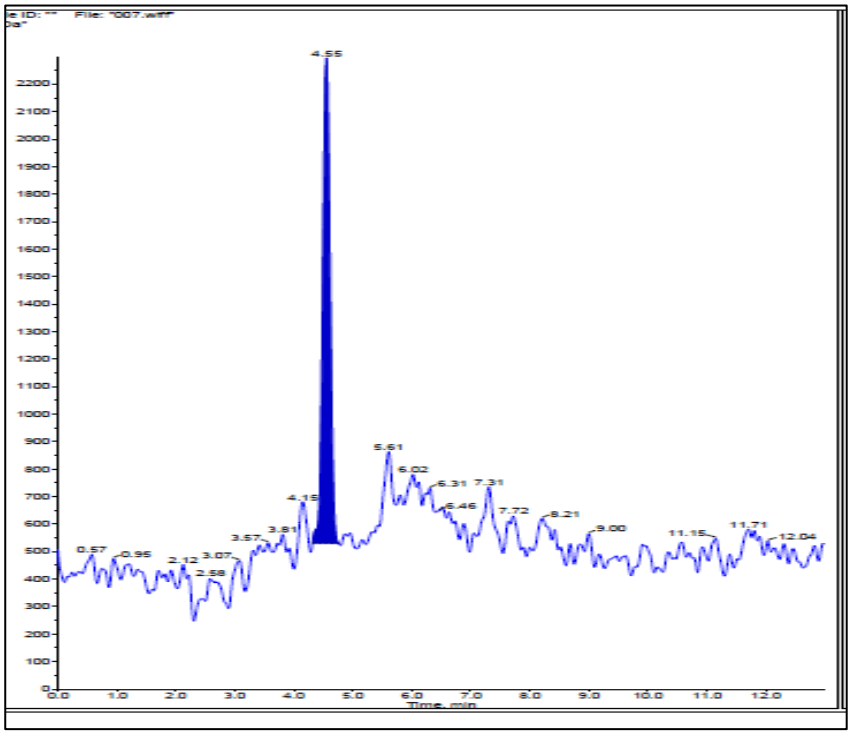
A representative regression analysis of a calibration curve for Glycopyrrolate



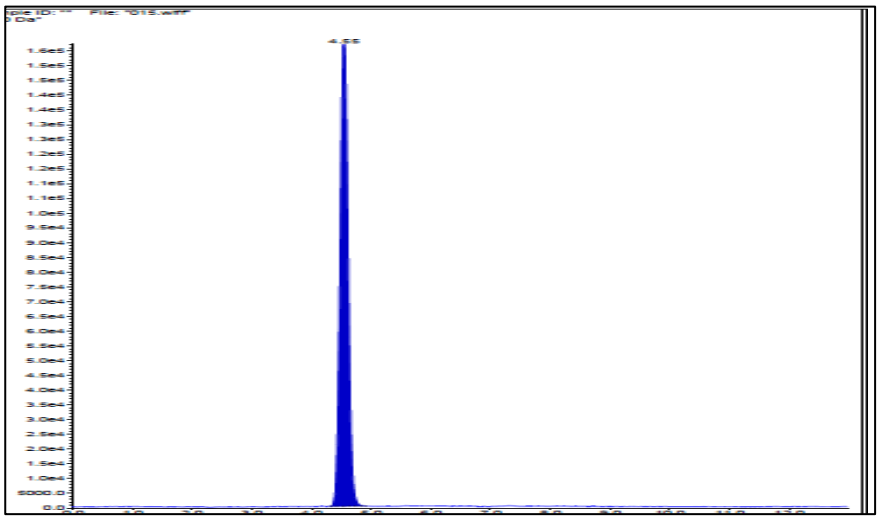
Representative Chromatogram Plasma Blank- Formoterol



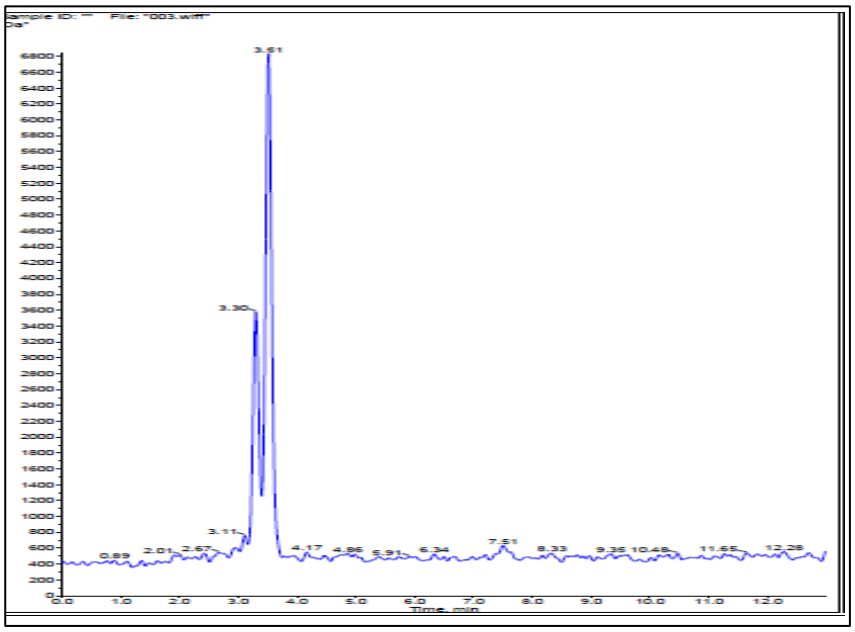
Representative Chromatogram Plasma LLOQ- Formoterol



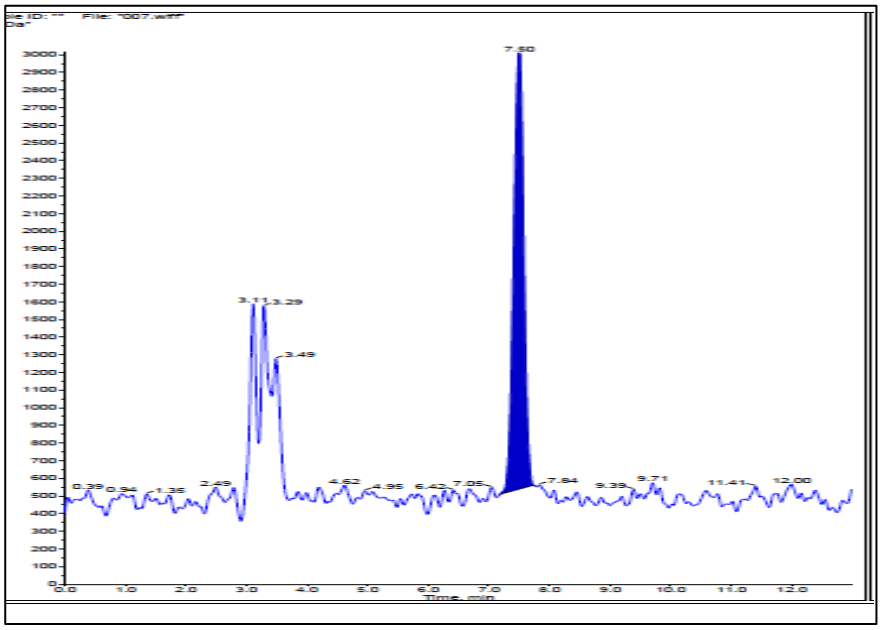
Representative Chromatogram Plasma ULOQ- Formoterol



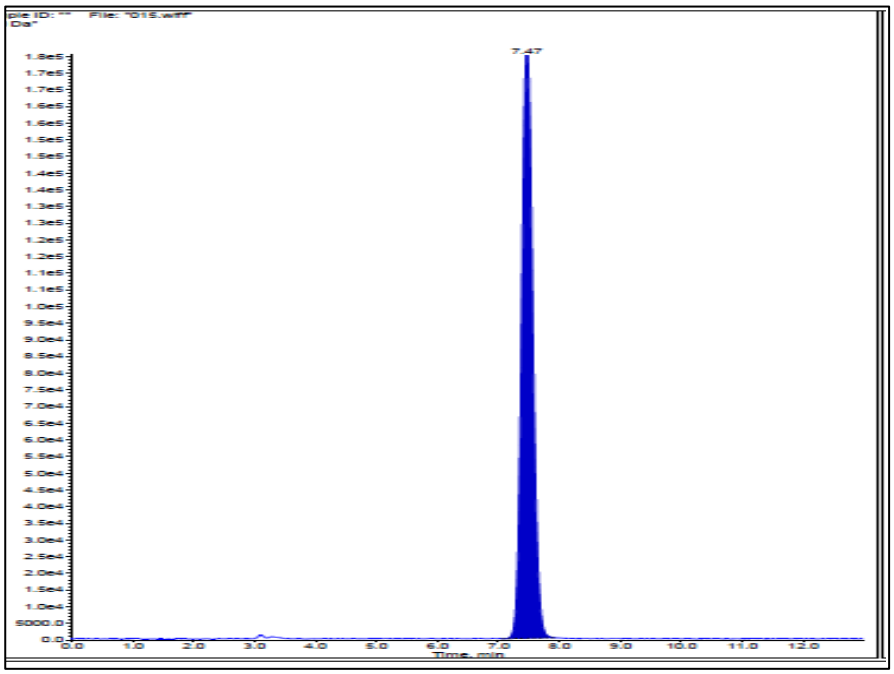
Representative Chromatogram Plasma Blank- Glycopyrrolate



Representative Chromatogram Plasma LLOQ- Glycopyrrolate



Representative Chromatogram Plasma ULOQ- Glycopyrrolate



Specificity / Selectivity: - Eight batches of human blank plasma (six normal, one hemolyzed and one lipemic) containing human plasma having K_3EDTA, 3.2% Sodium Citrate as anticoagulant were chromatographically screened for interfering substances and they did not show significant interference at the retention time of Formoterol & Glycopyrrolate and Formoterol-D_6 & Glycopyrrolate-D_3 (ISTD) respectively.

Sensitivity: -

Lower Limit of quantification (LLOQ) for Formoterol was **0.404 pg/mL**
Lower Limit of Quantification (LLOQ) for Glycopyrrolate was **0.408 pg/mL**

Recovery: -

The mean recovery for the low, medium and high-quality control samples was 89.49 %, 84.98 % and 73.99 % for Formoterol and 93.78 %, 88.35% and 87.83% for Glycopyrrolate respectively.

The precision for mean of % recovery obtained at each QCs level were within the acceptance criteria of ≤ 15 %.

Formoterol –D_6 and Glycopyrrolate –D_3(ISTD):

The recovery for was 88.42, 89.99 and 77.79 % for Formoterol –D_6(ISTD) and 95.02, 93.69 and 92.24 % for Glycopyrrolate-D_3 respectively.

The precision of % recovery was within the acceptance criteria of ≤ 15 % considering the recovery obtained for all samples.

Matrix Effect: -

Matrix effect was evaluated through matrix factor, which is calculated by comparing area response in presence of matrix ions with mean area response in absence of matrix ions.

Matrix (plasma)	IS Normalized Matrix Factor for Formoterol		IS Normalized Matrix Factor for Glycopyrrolate	
	LQC	HQC	LQC	HQC
Normal	0.990	0.992	0.802	1.024
Normal	0.985	1.029	0.796	1.035
Normal	0.987	0.992	0.766	1.022
Normal	0.992	1.012	0.834	1.034
Normal	0.962	1.030	0.829	1.035
Normal	0.974	1.030	0.581	1.003
Haemolysed	1.063	1.001	0.784	1.009
Lipemic	0.991	0.968	0.822	1.021
Mean	0.9930	1.0068	0.7768	1.0229
SD	0.03009	0.02259	0.08242	0.01202
% CV	3.03	2.24	10.61	1.18

The CV (%) for IS normalized matrix factor in eight lots was within the acceptance criteria of ≤ 15 % at each level.

Based on the obtained results it can be concluded that, no significant ion suppression or enhancement was observed during ionization in mass spectrometric detector.

Result table of stability data obtained during validation

Parameters	%Degradation for Formoterol			%Degradation for Glycopyrrolate	
	n	Drug	ISTD	Drug	ISTD
Short term stock solution (after 22.0 hours at room temperature)	6	0.99% and 1.00%	0.10% and 0.10 %	0.99% and 0.99%	0.99% and 0.99%
Long term stock solution (within 2 to 8°C) (After 18 days)	6	1.00% and 1.00%	0.99% and 0.98 %	1.00% and 1.01%	1.03% and 1.02%
Autosampler [L & H] (after 203 hours within 6°C)	6	1.07% and 1.03%	NA	0.99% and 1.00%	NA
Wet Extract [L & H] (after 190 hours within 2-8°C)	6	1.07% and 1.01%	NA	1.02% and 1.02%	NA
Freeze thaw [L & H] (5 Cycles)	6	0.98% and 0.95%	NA	0.99% and 0.98%	NA
Bench top [L & H] (after 5.0 hours at ice water bath)	6	0.97% and 0.95%	NA	0.98% and 0.98%	NA

Application: -

The applicability of this to pharmacokinetic studies has been established after successful application for about 36 subjects in one bioavailability studies. The following assessments were made for Formoterol and Glycopyrrolate in human plasma during study.

Method Ruggedness: -

- Maximum number of injections per subject: 116
- Analytical column lot numbers tested: 2
- Number of LC-MS/MS systems validated: 3
- Number of analysts validated: 4

Conclusion: -

The LC-MS/MS method is ultra- sensitive with LLOQ of 0.404 pg/mL and 0.408 pg/mL for Formoterol & Glycopyrrolate respectively. The robustness of this method has been tested with multiple lots of plasma, multiple analysts and different lots of analytical column. This method has been used for bioequivalence studies of over 36 subjects and was found to be selective, sensitive and robust.