Electronic Supplementary Material (ESI) for RSC Advances.

Pre-Clinical Pharmacokinetic Pharmacodynamic Modelling study of 4-Hydroxyisoleucine using Validated Ultra Performance Liquid Chromatography-Tandem Mass Spectroscopy

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Supplementary data

Table S1.: Optimization of protein precipitation extraction method for sample preparation

Extracting solvent	Sample volume (µL)	Volume of extraction solvent added (mL)	Vortex time (min)	Centrifugation [speed (g), time (min)]	% Recovery	Remarks
Methanol	300	3	2	15000, 10	28-31	Inconsistent and poor recovery
Acetonitrile	300	3	2	15000, 10	40-42	Consistent recovery was noticed

Table S2.: Optimization parameters of liquid chromatography and mass spectroscopy

Sr.No.	Chromatographic condition	Parameters	Remarks
1.	Mode	ES-	Low intensity of peak
		ES+	High Intensity of peak
Based on the results, ESI+ mode was selected and used further for optimizing the chromatographic conditions			
2.	Mobile Phase (50: 50)	ACN: Water	No proper ionization of analytes which results in decreased peak intensity
		ACN: 0.1% glacial acetic acid	Splitting in the peaks was observed
		ACN: 0.1% formic acid	Symmetrical peak with high peak intensity
From these results, it was conformed that the ACN: 0.1% formic acid was used as mobile phase and further optimized			
3.	Mobile phase composition (ACN: 0.1% formic acid)	40:60	Retention time was more than 2 min
		30: 70	Peak splitting was noticed
		20:80	Symmetric peaks of analyte and IS was observed with proper resolution
Based on the results, it was concluded that ACN:0.1% formic acid with 20:80 was used as the mobile phase for further method development and validation of parameters			

Table S3.: Intellistart MRM Optimization of analytes

Compound Name	Formula	Parent m/z	Cone voltage	Daughters	Collision energy	Ion mode
4-Hydroxy isoleucine	$C_6H_{13}NO_3$	148.19	24	74.02	12	ES+
			24	102.13	10	ES+
			24	84.16	18	ES+
			24	95.88	14	ES+
L-isoleucine	$C_6H_{13}NO_2$	132.17	24	86.19	12	ES+
			24	44.08	18	ES+
			24	69.04	20	ES+
			24	41.08	24	ES+

Table S4.: Regression parameters of the calibration curve generated for each weighting factor.

Wi	b	a	r^2
unweighted	9.65e-06	6.82E-04	0.9993
1/var	1.02e-05	6.86E-04	0.9990
$1/x^2$	1.02e-05	6.49E-04	0.9991
1/x	1.02e-05	6.17E-04	0.9988
$1/y^2$	1.05e-05	6.25E-04	0.9966
1/y	1.03e-05	6.16E-04	0.9291

Wi, weighing factor; b, slope; a, constant; r^2, regression co-efficient

Based on the above results, $1/x^2$ was selected for further analysis based on the regression co-efficient value.[1]

Reference

1 H. Gu, G. Liu, J. Wang, A.-F. Aubry and M. E. Arnold, *Anal. Chem.*, 2014, **86**, 8959–8966.

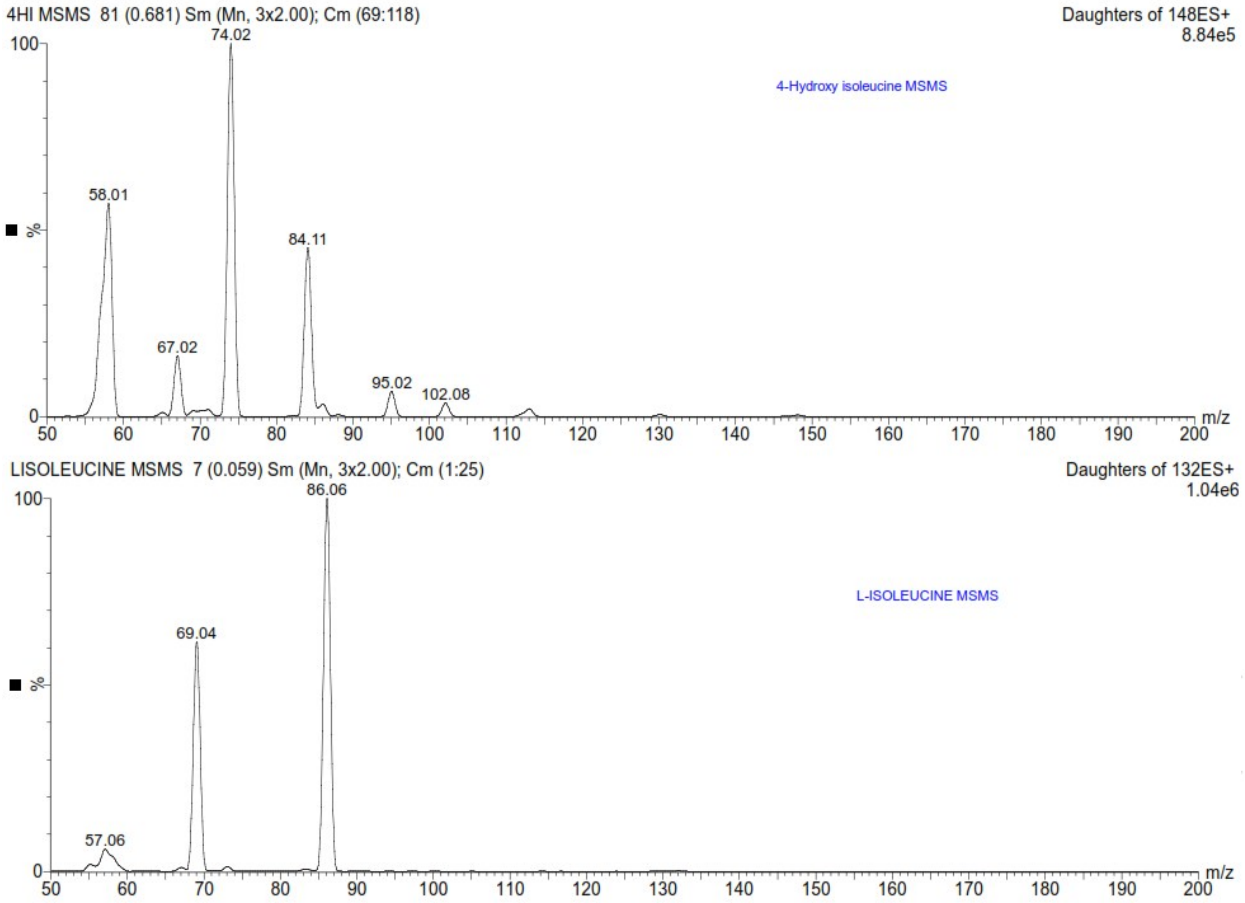

Fig S1.: Splitting pattern for 4-hydroy isoleucine and l-isoleucine

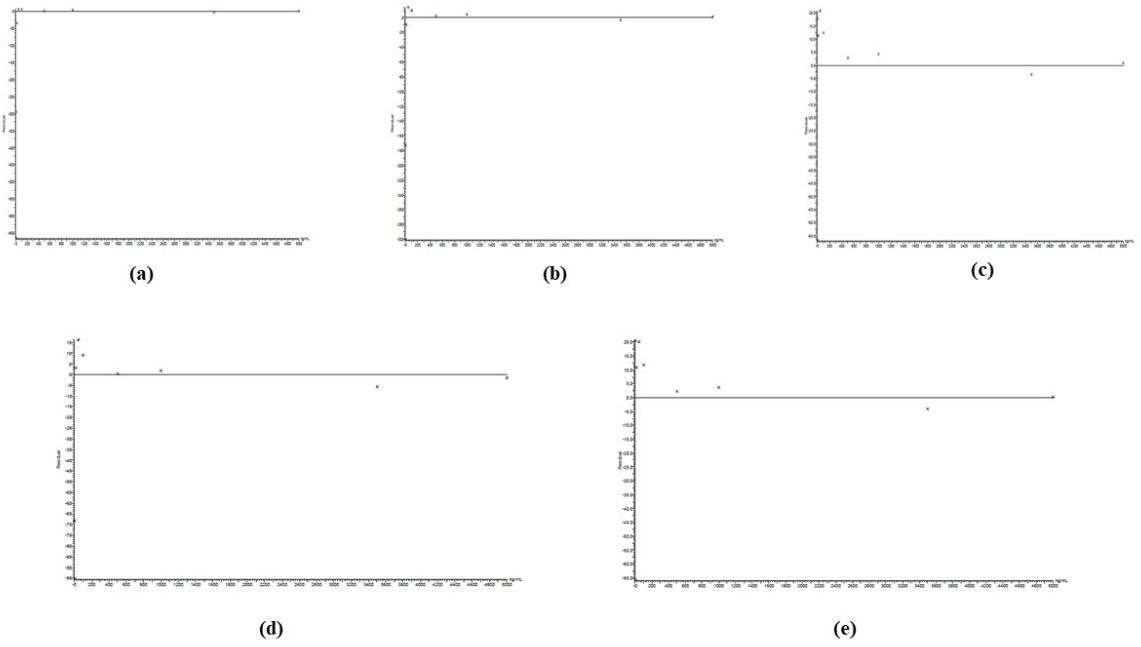

Fig S2.: Statistical residuals plotted against concentrations (ng/mL) (a) 1/variance; (b) $1/x^2$; (c) 1/x; (d) $1/y^2$; (e) 1/y