Chapter

Bioactive Indanes: Development and Validation of a Bioanalytical Method of LC-MS/MS for the Determination of PH46A, a New Potential Anti-Inflammatory Agent, in Human Plasma, Urine and Faeces

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Abstract

PH46A, a new chemical entity developed by our group, has shown potent anti-inflammatory activities through various pre-preclinical studies. The aim of this work was to develop and validate a sensitive and robust LC-MS/MS analytical method to determine the levels of PH46 in human plasma, urine and faeces. The linearity (0.5–500 ng/mL for plasma/urine, and 10–2000 ng/g for human faeces), accuracy (within 100 ± 15% for plasma/urine or 100 ± 20% for faeces), precision (≤ 15% CV for plasma/urine or ≤ 20% CV for faeces) and the method's specificity were demonstrated to be acceptable. No significant matrix effects or carry-over was observed for PH46 and IStd, and the recovery was consistent. About 10- and 100-fold dilutions in control matrix were found not to affect the assays' performance. PH46 was proven to be stable: at room temperature for >24 hrs in plasma through 3 freeze-thaw cycles, at –20°C for 83 days in plasma/32 days in urine/33 days in faeces, and at –80°C for 154 days in plasma/33 days in faeces. The re-injection reproducibility of PH46 in matrix extracts was at least 239 hrs at 4°C in plasma/25 days in urine/6.5 days in faeces. This method was successfully applied to the pharmacokinetic evaluation of the Phase I clinical studies.

Keywords: bioanalytical, development, validation, LC-MS/MS, PH46A, human plasma, urine, faeces

1. Introduction

The therapeutic effect of indane derived molecules has been clinically evident for treatment of many disease conditions, ranging from inflammation [1], cancer [2],

neurological conditions [3] to HIV [4]. Several classes of indane dimers have been developed, characterised and investigated by our research group [5–7] for various biological activities, including smooth muscle relaxation, mediator release inhibition and inflammatory conditions [8–14]. In particular, A lead, first-in-class molecule, [6-(Methylamino)hexane-1,2,3,4,5-pentanol 4-(((1S,2S)-1-hydroxy-2,3-dihydro-1H,1′H-[2,2-biinden]-2-yl)methyl)benzoate (PH46A) (**Figure 1**) with S, S configuration [15] has been considered as a potential new treatment for inflammatory bowel disease (IBD) based on the observation of its biological effect in two different well-established preclinical models of murine colitis: the acute dextran sodium sulphate model and the chronic and spontaneous Interleukin-10 (IL-$10^{-/-}$) knock-out mouse model. During the course of our work, PH46A was subject to a range of preclinical studies [16–19] prior to entering a Phase I clinical trial which has recently been completed [20].

This manuscript describes the development and validation of a sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalytical method for determining PH46 (the free acid form of PH46A salt) (**Figure 1**) in human plasma, urine and faeces samples according to the US Food and Drug Administration (FDA) Guidance Document on Bioanalytical Method Validation [21] and the European Medicines Agency (EMA) Guidelines on Bioanalytical Method validation [22]. The method was subsequently used to analyse the clinical samples from healthy volunteers in the Phase 1 trial [20]. The lower limit of quantification (LLOQ) in plasma and urine was 0.5 ng/mL and the LLOQ in faeces was 10 ng/g faecal equivalent.

2. Results and discussion

2.1 Stability of PH46 and IStd in stored stock solutions

The mean accuracy of the stored solutions compared to freshly prepared solutions met the acceptance criteria. Both PH46 and Compound 1 (Internal Standard, IStd) (**Figure 1**) stock solutions were found to be stable at 4°C for at least 200 and 250 days, respectively. The stability of IStd working solutions for at least 192 days at 4°C, and PH46 stock solutions for at least 24 h at room temperature (RT) was demonstrated.

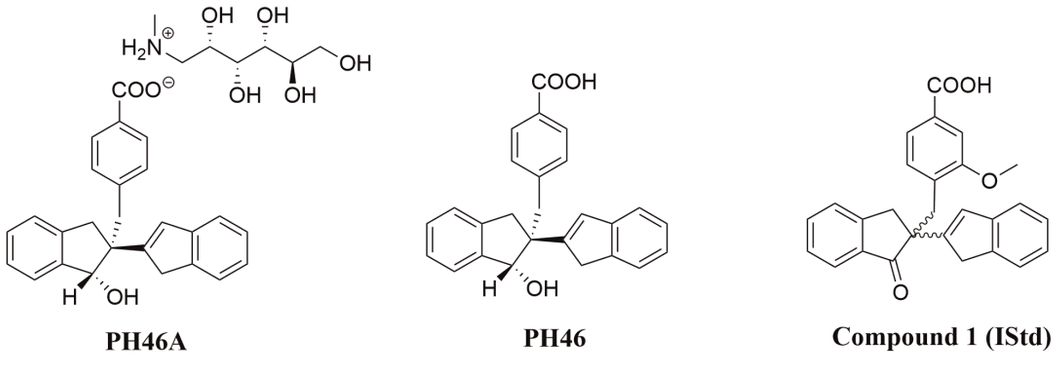

Figure 1.
Chemical structures of PH46A, PH46 and compound 1 (internal standard, IStd).

2.2 Linearity and specificity

The calibration data from reported human plasma (12 batches), urine (3 batches) and faeces (3 batches) standards over a range of 0.5–500 ng/mL (plasma or urine) or 10–2000 ng/g (faecal equivalent), duplicate run at each concertation, were analysed. The data are presented in **Table 1** for human plasma and **Table 2** for human urine and faeces batches.

In all cases, all concentrations determined for these standards met the acceptance criteria being at least 75% of standards [including at least one replicate at the LLOQ and the upper limit of quantification (ULOQ) levels] used to construct each calibration line met the acceptance criterion of the determined concentrations being within 100 ± 15% (plasma and urine batches) or 100 ± 20% (faeces batches) of the nominal concentrations (100 ± 20% for plasma and urine batches or 100 ± 25% for faeces batches of the nominal concentration at the LLOQ).

Regression analysis of the peak area ratios of PH46:IStd against the concentration showed good linearity for human plasma, urine and faeces over the range of concentration tested (0.5–500 ng/mL for plasma/urine samples and 10–2000 ng/g faecal equivalent for faeces samples) using a linear regression with a weighting factor of $1/x^2$ (**Tables 3** and **4**). All calculated concentrations for these standards met the acceptance criteria.

The quality control (QC) sample data for the supporting QC samples are presented in **Table 5**, which met the acceptance criteria. The selectivity of the assay for PH46 and IStd was determined by extraction and analysis of the following samples: one double blank (DB) sample (control matrix only without PH46 or IStd) from six independent sources of control matrix; three single blank (SB) samples (control matrix with IStd only) in a single source of control matrix; three ULOQ samples (no IStd) in the same source of control matrix as for the SB samples. The assay specificity demonstrated there were no significant interfering substances (IStd, PH46 or any impurity) at the retention times of PH46 and IStd, respectively. Samples containing either PH46 or IStd showed no significant interfering substances at the retention time of the other compound. Therefore, the assays were deemed to be specific to PH46. Representative chromatograms of human plasma extracts containing PH46 and IStd (LLOQ and ULOQ) are shown in **Figure 2**.

2.3 Assay recovery and matrix effects

For PH46 and IStd in plasma, the recovery was consistent. When the concentration of PH46 at 1.25 ng/mL, the IStd-normalised Matric Factors (MFs) (six sources) were calculated: 1.40, 1.35, 1.46, 1.51, 1.57, 1.25, 1.37 and the associated precision was determined to be 7.8%. When the PH46 concentration at 400 ng/mL, the IStd-normalised MFs were 1.35, 1.26, 1.45, 1.38, 1.44, 1.21, 1.25 and the corresponding precision was found to be 7.2%. These results confirmed that the matrix effects met the acceptance criteria, and no significant matrix effect was present for PH46 and IStd in human plasma by the current method.

2.4 Assay carry-over and matrix dilution

For human plasma, urine and faeces samples, no significant carry-over was observed in the matrix blank and solvent samples injected after a ULOQ sample for PH46 or IStd using needle-wash solution: [H_2O:acetonitrile (ACN):trifluoroacetic acid

Batch	Nominal concentration of PH46 (ng/mL)								Calibration curve parameters		
	0.500	**1.00**	**3.25**	**12.5**	**50.0**	**150**	**450**	**500**	**Slope**	**Intercept**	**R^2**
1	0.524	1.08	2.99	13.5	51.8	158	444	469	0.05576	0.002248	0.9951
	0.458	1.01	3.34	11.3	50.1	157	414	513			
2	0.489	0.894	2.99	15.6*	55.0	185*	400	471	0.07431	0.005518	0.9908
	0.534	1.03	3.31	14.7*	52.2	171	441	NR			
4	0.493	0.929	3.28	13.3	53.7	159	445	468	0.09615	0.002045	0.9958
	0.510	1.03	3.41	12.8	52.0	145	404	458			
5	0.454	0.897	3.63	12.2	48.5	157	444	466	0.08515	0.007061	0.9948
	0.548	1.04	3.46	12.2	49.6	156	442	484			
6	0.563	1.07	3.28	13.7	51.6	166	420	445	0.1433	0.001421	0.9909
	0.427	0.964	3.22	12.7	46.7	147	372*	397*			
7	0.521	1.08	3.48	14.6*	53.3	163	427	457	0.1846	0.006788	0.9919
	0.471	0.931	3.15	13.9	48.3	152	386	401*			
8	0.425	0.935	3.18	12.3	50.4	145	408	460	0.1267	0.002473	0.9909
	0.577	1.03	3.57	13.6	53.7	163	443	465			
9	0.500	1.01	3.22	12.7	52.0	150	459	491	0.06133	0.001111	0.9981
	0.490	1.02	3.29	13.3	50.1	153	425	449			
10	0.495	0.986	3.28	12.5	50.7	148	426	468	0.05657	0.002131	0.9987
	0.496	1.03	3.42	12.6	52.4	153	446	492			
11	0.465	0.959	3.52	12.6	49.1	147	406	486	0.04653	0.003935	0.9952
	0.540	0.964	3.56	12.7	54.4	145	465	471			
12	0.505	1.03	3.48	13.3	51.3	155	419	478	0.07467	0.005944	0.9971
	0.485	0.982	3.17	13.4	49.9	148	416	486			
Mean	0.499	0.996	3.33	12.9	51.2	154	428	472	0.09137	0.003698	0.9945
Precision (%)	8.0	5.6	5.3	5.0	4.1	4.8	4.8	3.5	–	—	—
Accuracy (%)	99.8	99.6	102.5	103.2	102.4	102.7	95.1	94.4	—	—	—

–: not applicable.

**Outside acceptance criteria (100 ± 15%), value not used in statistical calculations or regression analysis. NR: no result as no internal standard peak detected in sample, value not used in statistical calculations.*

Table 1.
Calibration curve data and parameters for PH46 in human plasma.

(TFA) (25:75:0.1, *v/v/v*); H_2O:ACN:formic acid (FA):TFA (90:10:0.1:0.005, *v/v/v/v*)]. The accuracy and precision for plasma and urine samples prepared at 4000 ng/mL or faeces samples at 16000 ng/g eq., and diluted at 10- and 100-fold, also met the acceptance criteria (**Table 6**). The results showed that dilutions of samples in control plasma, urine and homogenised faeces had no effect on the accuracy and precision of the method.

2.5 Intra- and inter-batch assay accuracy and precision

The intra-batch accuracy and precision of the assay for PH46 in plasma, urine and faeces met the acceptance criteria at each of the four concentrations assessed. The inter-batch accuracy and precision for PH46 in plasma also meet the acceptance criteria on each occasion (**Table 7**).

Urine	Batch	Nominal concentration of PH46 (ng/mL)								Calibration curve parameters		
		0.500	**1.00**	**3.25**	**12.5**	**50.0**	**150**	**450**	**500**	**Slope**	**Intercept**	**R^2**
	1	0.525	1.02	2.60*	10.7	49.7	153	471	538	0.03028	−0.002197	0.9910
		0.502	0.923	2.90	11.0	48.6	153	513	554			
	4	0.503	1.07	2.80	10.8	48.2	152	482	536	0.03211	−0.001705	0.9920
		0.313*	0.983	2.69*	11.1	50.4	156	477	556			
	5	0.471	0.948	2.61*	14.0	50.7	161	423	435	0.03061	0.0004537	0.9909
		0.557	0.898	3.58	12.8	53.5	156	417	460			
	Mean	0.512	0.974	3.09	11.7	50.2	155	464	513	0.03100	−0.001149	0.9913
	Precision (%)	6.2	6.6	13.7	11.5	3.8	2.1	8.0	10.2	—	—	—
	Accuracy (%)	102.4	97.4	95.1	93.6	100.4	103.3	103.1	102.6	—	—	—
Faeces	**Batch**	**Nominal concentration of PH46 (ng/g eq.)**								**Calibration curve parameters**		
		10.0	**20.0**	**48.0**	**120**	**280**	**720**	**1800**	**2000**	**Slope**	**Intercept**	**R^2**
	2	9.54	18.5	43.9	115	254	708	1930	1890	0.01759	0.01151	0.9922
		10.4	21.9	49.1	139	267	793	1810	1930			
	3	10.2	19.1	45.0	112	251	693	2090	1900	0.01539	0.01445	0.9908
		9.15	23.8	49.1	124	287	696	1840	2000			
	6	10.1	20.6	42.9	74.4*	276	693	1750	1850	0.01116	−0.002342	0.9939
		9.73	10.2*	51.3	127	315	441*	1920	1860			
	Mean	9.85	20.8	46.9	123	275	717	1890	1910	0.01471	0.007873	0.9923
	Precision (%)	4.7	10.3	7.2	8.7	8.7	6.0	6.3	2.9	—	—	—
	Accuracy (%)	98.5	104.1	97.7	102.5	98.2	99.6	105.1	95.5	—	—	—

–: not applicable.

**Outside acceptance criteria 100 ± 15% (urine) or 100 ± 20% (faeces) [100 ± 20% (urine) or 100 ± 25% (faeces) at the LLOQ], value not used in regression analysis. LLOQ: at the lower limit of quantification.*

Table 2.
Calibration curve data and parameters for PH46 in human urine and faeces.

Nominal conc. of PH46 (ng/mL)	Occasion 1			Occasion 2			Occasion 3		
	Response	Calculated conc. (ng/mL)	Bias (%)	Response	Calculated conc. (ng/mL)	Bias (%)	Response	Calculated conc. (ng/mL)	Bias (%)
0.500	0.031488	0.524	4.8	0.041840	0.489	−2.2	0.045759	0.454	−9.2
	0.027788	0.458	−8.4	0.045210	0.534	6.8	0.053705	0.548	9.6
1.00	0.062580	1.08	8.0	0.071948	0.894	−10.6	0.083474	0.897	−10.3
	0.058307	1.01	1.0	0.082009	1.03	3.0	0.095815	1.04	4.0
3.25	0.168746	2.99	−8.0	0.227887	2.99	−8.0	0.316055	3.63	11.7
	0.188249	3.34	2.8	0.251369	3.31	1.8	0.301471	3.46	6.5
12.5	0.756801	13.5	8.0	1.166293	15.6*	24.8*	1.049172	12.2	−2.4
	0.633890	11.3	−9.6	1.101343	14.7*	17.6*	1.043108	12.2	−2.4
50.0	2.888743	51.8	3.6	4.093857	55.0	10.0	4.138322	48.5	−3.0
	2.798080	50.1	0.2	3.883057	52.2	4.4	4.230480	49.6	−0.8
150	8.833344	158	5.3	13.718101	185*	23.3*	13.394936	157	4.7
	8.769691	157	4.7	12.682625	171	14.0	13.315633	156	4.0
450	24.759200	444	−1.3	29.696339	400	−11.1	37.845875	444	−1.3
	23.116439	414	−8.0	32.792890	441	−2.0	37.649477	442	−1.8
500	26.174887	469	−6.2	34.993741	471	−5.8	39.725235	466	−6.8
	28.612686	513	2.6	—	—	—	41.206685	484	−3.2
Slope	0.05576			0.07431			0.08515		
Intercept	0.002248			0.005518			0.007061		
R^2	0.9951			0.9908			0.9948		

–: no result, no internal standard detected in sample. R^2: coefficient of determination. Bias: difference between determined concentration and nominal concentration. Conc.: concentration.
**Value outside acceptance criteria (100 ± 15%) and not used in regression analysis.*

Table 3.
Assay linearity for PH46 in human plasma.

Nominal conc. (ng/mL)	PH46 in human urine				PH46 in human faeces		
	Response	Calculated conc. (ng/mL)	Bias (%)	Nominal conc. (ng/g eq.)	Response	Calculated conc. (ng/g eq.)	Bias (%)
0.500	0.013704	0.525	5.0	10.0	0.179251	9.54	−4.6
	0.012990	0.502	0.4		0.195100	10.4	4.0
1.00	0.028823	1.02	2.0	20.0	0.336200	18.5	−7.5
	0.025742	0.923	−7.7		0.397292	21.9	9.5
3.25	0.076650	2.60*	−20.0*	48.0	0.784065	43.9	−8.5
	0.085737	2.90	−10.8		0.874742	49.1	2.3
12.5	0.320860	10.7	−14.4	120	2.033762	115	−4.2
	0.329679	11.0	−12.0		2.459854	139	15.8
50.0	0.502113	49.7	−0.6	280	4.485738	254	−9.3
	1.470794	48.6	−2.8		4.704624	267	−4.6
150	4.622468	153	2.0	720	12.464152	708	−1.7
	4.632629	153	2.0		13.952398	793	10.1
450	14.258525	471	4.7	1800	33.899934	1930	7.2
	15.531203	513	14.0		31.771859	1810	0.6
500	16.297231	538	7.6	2000	33.258315	1890	−5.5
	16.758430	554	10.8		33.933302	1930	−3.5
Slope	0.03028				0.01759		
Intercept	−0.002197				0.01151		
R^2	0.9910				0.9922		

Bias: difference between determined concentration and nominal concentration. Conc.: concentration.
**Value outside acceptance criteria 100 ± 15% (urine) and not used in regression analysis.*

Table 4.
Assay linearity for PH46 in human urine and faeces.

2.6 Stability experiments of PH46 in matrix

The results of different stability experiments are summarised in **Table 8** with the detailed data presented in **Tables 9–11**.

The mean stability results of PH46 in human plasma, at low, high and diluted concentrations, after at least one-month (33 days) storage at −20°C did not meet the acceptance criteria. This experiment was repeated after 35 days storage and similar results were obtained (**Table 11**). The data were unexpected as no stability issues had been observed in the previous studies of PH46 in dog and rat plasma [16]. Stability studies of PH46 in human plasma at −80°C, was subsequently assessed after at least one month (30 days) storage alongside the assessment of the −20°C stability after at least two months (83 days) storage (**Table 11**). The mean stability results at all concentration levels met the acceptance criteria for both storage temperatures and durations. The data generated for the two-month stability timepoint at −20°C contradict that of the one-month timepoint, but provide confidence that there should not be stability issue at −20°C. At one-month timepoint at −20°C, fresh calibration

Nominal conc. (ng/mL)		**1.25**	**15**	**400**
PH46 in plasma	Batch 6	1.22	15.3	354
		1.20	14.9	368
	Batch 7	1.20	15.4	341
		1.17	14.6	347
	Batch 8	1.14	14.7	387
		1.35	14.5	382
	Batch 9	1.16	15.6	392
		1.28	15.6	378
	Batch 10	1.28	15.0	400
		1.22	15.5	388
	Batch 11	1.33	16.1	362
		1.18	15.0	410
	Batch 12	1.24	14.8	373
		1.30	15.3	374
	Mean	1.23	15.2	375
	Precision (%)	5.3	3.0	5.3
	Accuracy (%)	98.4	101.3	93.8
Nominal conc. (ng/mL)		**1.25**	**15**	**400**
PH46 in urine	Batch 5	1.24	15.1	412
		1.28	9.23*	388
	Mean	1.26	12.2	400
	Precision (%)	—	—	—
	Accuracy (%)	100.8	81.3	100.0
Nominal conc. (ng/g eq.)		**25.0**	**100**	**1600**
PH46 in faeces	Batch 6	21.2	69.4*	1180*
		25.2	91.1	1780
	Mean	23.2	80.3	1480
	Precision (%)	—	—	—
	Accuracy (%)	92.8	80.3	92.5

-: no applicable. Conc.: concentration.
**Outside acceptance criteria value included in statistical analysis.*

Table 5.
Quality control data relating to PH46 in human plasma, urine and faeces.

standard (CS) and QC stock solutions were prepared and tested to ensure the stocks were suitable for use. The stock solutions were then used to prepare fresh bulk CSs and QC samples in control matrix., and the stability samples were extracted alongside these fresh CS and QC samples. The acceptance criteria for both CSs and supporting QC samples were met for each of the stability batches, therefore giving no reason to suspect the failing stability data after one-month storage at −20°C.

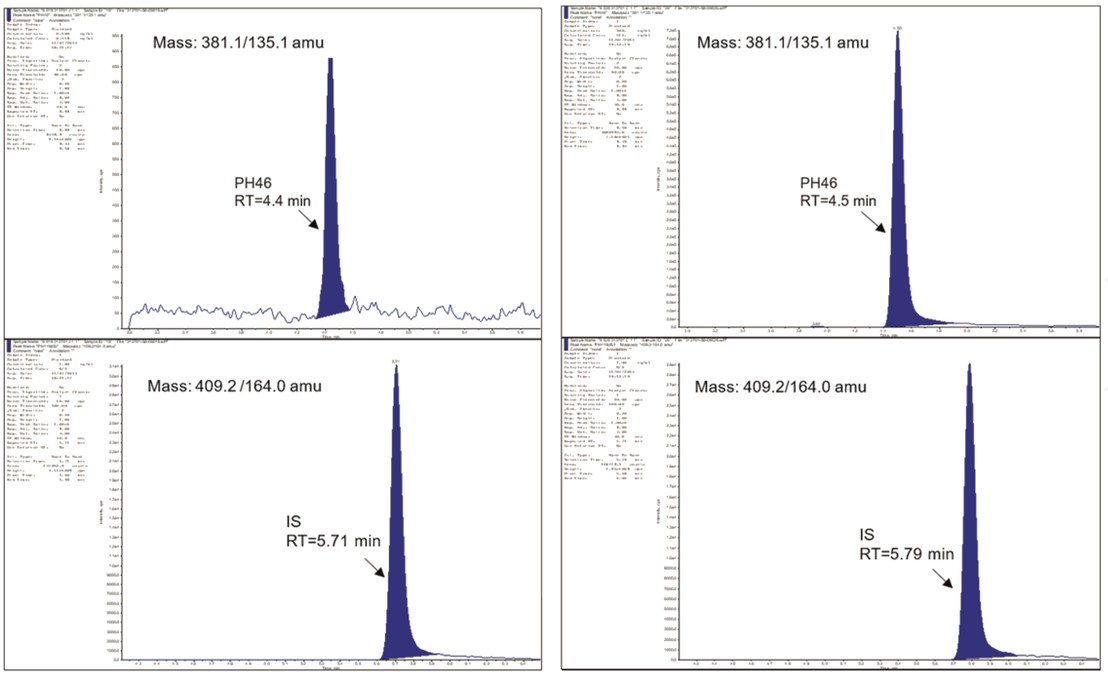

Figure 2.
Chromatograms of a human plasma extract (left) containing PH46 (LLOQ, 0.5 ng/mL) & internal standard and a human plasma extract (right) containing PH46 (ULOQ, 500 ng/mL) & internal standard.

Replicate	Plasma		Urine		Faeces	
	DF		DF		DF	
	10	100	10	100	10	100
1	3550	4260	4520	3940	16,800	15,600
2	3950	3660	4530	3910	16,500	15,600
3	3160	3830	4480	4100	17,800	19,400
4	3450	5000	4430	4050	18,700	16,200
5	4210	3320	4360	4000	17,100	19,400
6	3890	4310	4540	3820	18,100	14,600
Mean	3700	4060	4480	3970	17,500	16,800
Precision (%)	10.4	14.6	1.6	2.5	4.8	12.4
Accuracy (%)	92.5	101.5	112.0	99.3	109.4	105.0

Initial concentration of PH46: 4000 ng/mL (plasma and urine) or 16,000 ng/g eq. (faeces). DF: dilution factor.

Table 6.
Effects of dilution of PH46 with human plasma, urine and faeces homogenate.

At the one-month timepoint for −80°C and two-month timepoint for −20°C, a further fresh set of CS and QC stock solutions were made and used to prepare the fresh bulk CS and QC samples extracted alongside the stability samples. The acceptance criteria for these CSs and supporting QC samples were met. As the stability samples stored at −20°C and −80°C extracted in this batch also met the acceptance criteria, this demonstrates that both sets of samples prepared at different times were appropriate for use. Although there is still no explanation for the anomalous results at

	Nominal conc. (ng/mL) Occasion	0.5			1.25			15			400		
		1	2	3	1	2	3	1	2	3	1	2	3
A	Intra-assay Mean (n = 6)	0.443	0.487	0.476	1.21	1.30	1.22	15.7	14.0	14.8	404	354	371
	Intra-assay precision (%)	10.0	4.6	14.5	6.8	6.8	4.5	6.8	5.2	4.4	5.5	3.8	5.1
	Intra-assay accuracy (%)	88.6	97.4	95.2	96.8	104.0	97.6	104.7	93.3	98.7	101.0	88.5	92.8
	Inter-assay Mean		0.469			1.24			14.8			376	
	Inter-assay precision (%)		10.6			6.7			7.2			7.4	
	Inter-assay accuracy (%)		93.8			99.2			98.7			94.0	
B	**Nominal conc. (ng/mL)**		**0.5**			**1.25**			**15**			**400**	
	Intra-assay mean (n = 6)		0.566			1.38			16.7			429	
	Intra-assay precision (%)		6.4			4.1			1.9			1.5	
	Intra-assay accuracy (%)		113.2			110.4			111.3			107.3	
C	**Nominal conc. (ng/g eq.)**		**10**			**25**			**100**			**1600**	
	Intra-assay mean (n = 6)		9.56			26.5			111			1500	
	Intra-assay precision (%)		7.3			8.5			11.0			5.7	
	Intra-assay accuracy (%)		95.6			106.0			111.0			93.8	

A: human plasma assay. B: human urine assay. C: human faeces assay. Conc.: concentration.

Table 7.
Intra- and inter-assay accuracy and precision for PH46 in human plasma, urine, and faeces.

one-month timepoint for −20°C storage, it does provide confidence that there is not a stability issue for PH46 in human plasma at −20°C for the timeframe tested (**Table 11**). After at least four months (153 days) storage at −20°C, the mean stability results met the acceptance criteria at high and diluted concentrations but did not meet at the low concentration. The low concentration samples were repeated and the failure to meet the acceptance criteria was confirmed. The mean stability results, after storing PH46 in human plasma −80°C for at least three months (100 days) and five months (154 days) met the acceptance criteria at all levels of low, high and diluted concentration (**Table 11**).

Following the successful method establishment, this validated bioanalytical method was applied to determine PH46 in human samples obtained from the healthy volunteering during the phase I clinical study (ISRCTN90725219) [20].

Stability study	Results
Short-term frozen storage stability and freeze/thaw stability of PH46 in matrix	At low, high and diluted concentrations, the mean stability results (after 3 freeze/thaw cycles in plasma, **Table 9A**, one month storage at −20°C and −80°C in urine and faeces, **Table 10A** and **B**) met the acceptance criteria.
Ambient room temperature stability of PH46	The results of the 24-h ambient room temperature stability of PH46 in human plasma at low, high and dilution concentrations also met the acceptance criteria (**Table 9B**).
Stability of PH46 in human plasma, urine and faeces extracts (re-injection reproducibility)	The mean reinjection reproducibility of PH46 extracts of plasma for 239 h, urine for 25 days and faeces for 6.5 days, stored in an autosampler at 4°C before being re-injected, also met the acceptance criteria (**Tables 9C** and **10C, D**).
Extended frozen storage stability	PH46 is stable in human plasma after two months (83 days) at −20 to 80°C and at least 5 months (154 days) at −80°C (**Table 11**).

Table 8.
Summary of stability results of PH46 in human plasma, urine and faeces.

	0.5*		1.25*		400*		4000*, #	
A.	B.L.	3 Cycles	B.L.	3 Cycles	B.L.	3 Cycles	B.L.	3 Cycles
Mean (n = 6)	–	–	1.30	1.22	354	365	3750	3510
Precision (%)	–	–	6.8	5.8	3.8	7.2	2.5	6.4
Accuracy (%)	–	–	97.6		91.3		87.8	
B.	**B.L.**	**24 h**	**B.L.**	**24 h**	**B.L.**	**24 h**	**B.L.**	**24 h**
Mean (n = 6)	–	–	1.30	1.21	354	369	3750	3610
Precision (%)	–	–	6.8	7.0	3.8	2.7	2.5	4.1
Accuracy (%)	–	–	96.8		92.3		90.3	
C.	**0.5***		**1.25***		**15.0***		**400***	
Mean (n = 6)	0.512		1.24		15.2		378	
Precision (%)	7.2		6.9		2.3		4.6	
Accuracy (%)	102.4		99.2		101.3		94.5	

A. Freeze/thaw stability; B. Ambient temperature stability; C. Autosampler stability (plasmas extract stored at 4°C, reinjection reproducibility). B.L. Baseline.
**Nominal concentration of PH46. #Samples diluted 10-fold prior to extraction and analysis.*

Table 9.
Stability tests (freeze/thaw, ambient temperature & reinjection reproducibility) of PH46 in human plasma.

3. Materials and methods

The research work reported in this article was carried out following the Principles of the Organisation for Economic Co-operation and Development (OECD) of Good Laboratory Practice (GLP) as accepted by international regulatory authorities, including EMA (EU), FDA & EPA (USA) and MHLW, MAFF & METI (Japan).

	−20°C			−80°C		
A.	1.25*	400*	4000*,#	1.25*	400*	4000*,#
Mean (n = 6)	1.23	402	3990	1.18	405	4100
Precision (%)	3.8	4.3	2.5	2.9	1.6	13.9
Accuracy (%)	98.4	100.5	99.8	94.4	101.3	102.5
	−20°C			**−80°C**		
B.	25.0*	1600*	16,000*,#	25.0*	1600*	16,000*,#
Mean (n = 6)	23.1	1400	15,200	23.8	1480	13,800
Precision (%)	10.3	15.0	6.2	12.0	8.2	9.1
Accuracy (%)	92.4	87.5	95.0	95.2	92.5	86.3
C.	**0.5***	**1.25***		**15***	**400**	
Mean (n = 6)	0.544	1.36		16.4	424	
Precision (%)	6.4	2.6		2.5	1.2	
Accuracy (%)	108.8	108.8		109.3	106.0	
D.	**10***	**25***		**100***	**1600**	
Mean (n = 6)	8.76	24.4		103	1460	
Precision (%)	7.5	10.4		10.1	5.3	
Accuracy (%)	87.6	97.6		103.0	91.3	

**Nominal concentration of PH46 (ng/ml for urine and ng/g eq. for faeces). #samples diluted 10-fold prior to extraction and analysis. A. freezing stability (urine); B. freezing stability (faeces); C. reinjection reproducibility (urine); D. reinjection reproducibility (faeces).*

Table 10.
Stability tests (one month storage and reinjection reproducibility) of PH46 in human urine and faeces.

Ethical approval was obtained through Trinity College Dublin which complies with the Council for International Organisations of Medical Sciences' (CIOMS), International Guiding Principles for Biomedical Research.

3.1 Chemical and reagents

PH46A [purity 97.1% with moisture and solvent free; correction factor (purity and salt content): 0.6429] and Compound 1 as IStd (**Figure 1**) were obtained from Trino Therapeutics Ltd. (Ireland). Control human plasma (using lithium heparin as anticoagulant), urine and faeces were obtained from Charles River Laboratories (UK) and stored at −20°C when not in use. All control plasma was mixed and centrifuged prior to use. Control human faeces was homogenised prior to use with addition of deionised H_2O (1:3, faeces:H_2O, *w/v*). HPLC grade solvents and additives, including methanol (MeOH), ACN, H_2O, ammonia solution (28.0–30.0%), acetic acid (AA), acetone, dimethylsulphoxide (DMSO), chloroform ($CHCl_3$), TFA and FA, were purchased commercially between VWR (Ireland), Fisher Scientific (Ireland) and Sigma-Aldrich (Ireland) and were of analytical/HPLC grade or equivalent.

	Baseline	1 M	1 M^{+}	2 M	3 M	4 M	4 M^{+}	5 M
A.								
1.25*								
Mean (n = 6)	1.30	1.06	0.918	1.22	–	1.03	1.01	–
Precision (%)	6.8	5.9	5.8	6.8		5.6	2.9	
Accuracy (%)	n/a	84.8^	73.4^	97.6		82.4^	80.8^	
400*								
Mean (n = 6)	354	321	268	389	–	355	–	–
Precision (%)	3.8	2.9	2.4	2.2		1.2		
Accuracy (%)	n/a	80.3^	67.0^	97.3		88.8		
4000$^{*,\#}$								
Mean (n = 6)	3750	3310	3140	3940	–	3810	–	–
Precision (%)	2.5	2.7	3.5	4.8		1.4		
Accuracy (%)	n/a	82.8^	78.5^	98.5		95.3		
B.								
1.25*								
Mean (n = 6)	1.22	1.19	–	–	1.15	–	–	1.21
Precision (%)	9.2	2.9			3.3			5.7
Accuracy (%)	n/a	95.2			92.0			96.8
400*								
Mean (n = 6)	371	363	–	–	389	–	–	377
Precision (%)	2.1	3.7			1.5			2.8
Accuracy (%)	n/a	90.8			97.3			94.3
4000$^{*,\#}$								
Mean (n = 6)	3820	3640	–	–	3740	–	–	3720
Precision (%)	1.8	1.6			0.7			2.2
Accuracy (%)	n/a	91.0			93.5			93.0

M: month. – : not applicable. A. stability test at −20°C; B. stability test at −80°C.
*Nominal concentration of PH46. $^{+}$Repeat timepoint experiment. $^{\#}$Samples diluted 10-fold prior to extraction and analysis.^Does not meet acceptance criteria (100 ± 15%).

Table 11.
Storage stability tests of PH46 in human plasma at −20°C and −80°C.

3.2 Instrumentation and operation conditions

AB Sciex API3000 LC/MS/MS spectrometer system: HPLC pump, vacuum degasser, column oven (Series 200, Perkin Elmer), autosampler (HTS Pal, CTC Analytics) along with data handling system (Analyst Version 1.4.2, AB Sciex) and Laboratory Information Management System (Watson 7.0, Thermo Fisher Scientific) for operations and data analysis. Halo C18 LC column (75 × 2.1 mm, 2.7 μm, HiChrom) and PreFrit Filter guard column (0.5 μm, 6.35 × 1.57 mm, Anachem) were used. Column and autosampler temperatures were 60°C and 4°C, respectively. Mobile

phase A: MeOH:AA (100:0.2, *v/v*) and mobile phase B: H_2O:AA (100:0.2, *v/v*). The gradient was 60% A (0 min), 80% A (5.0 min), 100% A (6.0 min), 60% A (6.5 min). Flow rate was 0.3 mL/min with injection volume of 10 μL and run time of 6.5 min. The solvent mixtures as needle wash were H_2O:ACN/TFA (25:75:0.1, *v/v/v*) and H_2O:ACN:FA:TFA (90:10:0.1:0.005, *v/v/v/v*). Sample diluent was MeOH:H_2O:AA (50:50:0.2, *v/v/v*). TurboIonSpray negative ionisation mode was applied to carry out the detection of mass spectrometry. Nitrogen (API3000) was used for nebulizing and drying, with 700°C of ion spray temperature and −4500 V of ion spray voltage. PH46 and IStd ions monitored were 381.10–135.10 (±0.5) and 409.20–164.00 (±0.5) dwell time 200 ms.

3.3 Preparation of CSs and QC samples

The PH46 and IStd primary stock solutions were prepared at 1 mg/mL in ACN:DMSO (50:50, *v/v*). The plasma/urine CSs at concentrations of 0.5, 1, 3.25, 12.5, 50, 150, 450 and 500 ng/mL) were made by serial dilutions of the primary stock in control matrix. For each batch of plasma/urine samples, 200 μL aliquots of the bulk CSs were extract (duplicate) resulting in different concentrations of PH6 within the linear concentration range of the assay. 15 ng/mL of IStd solution was also made from the primary IStd stock solution using a mixture of MeOH:$CHCl_3$ (50:50, *v/v*). The bulk faeces CSs were prepared by serially diluting the stock solution in homogenised control faeces to give 10, 20, 48, 120, 280, 720, 1800 and 2000 ng/g faecal equivalent. For each batch of faeces samples, aliquots (1 mL, equivalent to 250 mg of faecal material) of the bulk CSs were extracted in duplicate to give a range of concentrations of PH46 within the linear range of the assay and a fixed concentration of IStd (60 ng/g faecal equivalent).

Plasma/urine QC samples were prepared in control matrix from the primary PH46 stock solution in similar manner as CSs, to give QC samples at 0.5 (LLOQ), 1.25 (low), 15 (medium) and 400 (high) ng/mL plasma/urine concentrations and aliquots (200 μL) of the bulk QC samples were extracted for analysis. For faeces QC samples, concentrations were made at 10 (LLOQ), 25 (low), 100 (medium) and 1600 (high) ng/mL faecal equivalent, and aliquots (1 mL, equivalent to 250 ng of faecal material) of the bulk QC samples were extracted for analysis. All sample solutions were stored in a freezer set to maintain a temperature of −20°C in dark when not use and brought to RT before analysis. To all weighing, a correction for batch specific purity and a correction for salt content (free acid (382.5)/salt (577.7)) were applied.

3.4 Preparation of control matrix and sample extraction

Control plasma (Lithium Heparin anticoagulant), urine and homogenised faeces (1:3 faecal/H_2O slurry, *w/v* by blending until a smooth consistency was obtained), stored at −20°C when not in use, were removed from the freezer and allowed to thaw at RT. Control matrix (pooled) was vortex mixed and centrifuged (3500 rpm, 4°C & 5 min) prior to use. 200 μL (1 mL in the case of homogenised faeces, equivalent to 250 mg faeces) was added to a clean, dry screw cap glass tube (12 × 75 mm) for DB (control matrix only without PH46/IStd) and SB (control matrix with IStd only) samples. CSs, QC and test samples were removed from the freezer and allowed to thaw at RT and vortex mixed prior to aliquoting. 200 μL of aliquots (1 mL in the case of faeces) of each sample solution were added to clean glass tubes (12 × 75 mm). 50 μL (10 μL in the case of in the case of faeces) of IStd in MeOH:$CHCl_3$ (50:50, *v/v*) at a working concentration of 0.06 μg/mL (1.5 μg/mL) was added to all samples except for

the DB samples, where 50 μL (10 μL instead in the case of faeces) of MeOH:$CHCl_3$ (50:50, *v/v*) was added. A further 2 mL of MeOH:$CHCl_3$ mixture (50:50, *v/v*) (4 mL of $CHCl_3$ in the case of faeces) was added to all samples and the tubes were sealed and vortex mixed thoroughly prior to centrifugation (3500 rpm, 5 min, 4°C). The supernatant from all samples (the lower $CHCl_3$ layer in the case of faeces) was transferred to clean tubes, dried under nitrogen at 60°C and then reconstituted in 100 μL of MeOH:H_2O:AA (50:50:0.2, *v/v/v*). The extracts were then vortex-mixed and transferred into plastic matrix tubes before being centrifuged for 5 min at 3500 rpm at 4°C.

3.5 Validation procedures

When the reference standard PH46A was weighed, a correction factor was made for the salt content. Therefore, all peak area measurement and determined concentrations reported in the study were for PH46 (the free acid form). Each batch of samples had matrix DB, matrix SB, CSs, QC and test sample extracts. Duplicate DB and SB samples and CSs at each level were extracted. The CSs which met the acceptance criteria were used to construct the calibration curve. Two replicates were injected: one at the start and one at the end of the run. Concentration order was used for CS injections in each section of the run. QC samples were made in control matrix at three concentrations: low, medium and high ($n \geq 2$ at each level). Where $n = 2$ at each level were prepared, the samples were injected as follows: one low-QC and one medium-QC level samples were injected at the start of the run following the first set of CSs, one Low-QC and one High-QC level samples were injected at the end of the run prior to the second set, and the remaining Medium-QC and High-QC level samples were injected midway through the run. In the case of $n > 2$ at each level were prepared, extra QC samples were appropriately distributed through the run. In order to avoid potential assay carry-over, the injections of solvent or extracted matrix DB samples were made after the high concentration extracts before the injection of low concentration extract.

The acceptance criteria were: (i) at least 75% of the CSs must back-calculate to within 100 ± 15% (plasma and urine assays) or 100 ± 20% (faeces assay) of the nominal concentrations (100 ± 20% at the LLOQ for plasma and urine assays or 100 ± 25% at the LLOQ for faeces assay); (ii) at least 67% of the total number of QC samples in each batch had to be within 100 ± 15% (plasma and urine) or 100 ± 20% (faeces) of the nominal concentrations for the determined concentrations, including at least one QC sample at each concentration had to meet this criterion. Precision was calculated as CV of mean. Accuracy was calculated as mean determined concentration/nominal concentration.

3.5.1 Determination of stability of stored spiking solutions

The stability of the PH46 storing stock solutions in ACN:DMSO (50:50, *v/v*) in a refrigerator set to maintain 4°C was examined by comparing the peak area ratios of the fresh stock solutions with the stock solutions on 200 days storage. The preparation of all stock solutions were performed in the same way. The ULOQ solutions were prepared by diluting the stock solutions using an appropriate diluent. The stability of the IStd storing solutions in ACN:DMSO, (50:50, *v/v*) for the stock solution and MeOH:$CHCl_3$ (50:50, *v/v*) for the working solution, in a refrigerator set to maintain 4° C was also studied by comparing the peak area ratios of the freshly prepared and diluted stock and working solutions with the previous stocks on 250 days storage and

previous working solutions on 192 days storage, respectively. PH46 and IStd were considered to be stable if the mean responses (MRs) for the stored solutions were within 100 ± 10% of the MRs of the fresh solutions and ≤ 10% for the precision.

The stability of the PH46 storing stocks at ambient RT was also investigated by comparing the peak area ratios of an aliquot of the stock solution (4°C) against an aliquot of the same stock solution on storage at ambient RT for 24 h. Dilutions were made to both solutions to the ULOQ level with an appropriate diluent. PH46 was considered to be stable at RT when the MRs of the solutions stored at RT were within 100 ± 10% of the MRs from the solutions at 4°C and ≤ 10% for the precision.

3.5.2 Linearity and specificity

For each batch, the calibration curve over 0.5–500 ng/mL (plasma and urine assays) or 10–2000 ng/g (faeces assay) was constructed from PH46:IStd area response ratios plotted against the nominal matrix concentrations of PH46 to determine the optimum regression parameters, using linear regression with $1/x^2$ weighting factor. The matrix concentration of PH46 from each CSs was calculated from the corresponding curve. DB and SB samples were also extracted and analysed; but not included in the regression analysis. For the batch to be acceptable the determined matrix concentration of PH46 for each sample used to construct the calibration curve, had to be 100 ± 15% (plasma and urine assays) or 100 ± 20% (faeces assay) of the nominal matrix concentration (100 ± 20% (plasma and urine assays) or 100 ± 25% (faeces assay) of the nominal matrix concentration at LLOQ). At least 75% (including at least one LLOQ and one ULOQ samples) of the CSs had to meet the above criteria.

The specificity of the assay for PH46 and IStd was determined by extraction and analysis of one DB sample from six independent sources of control matrix, three SB samples in a single source of control matrix and three ULOQ samples (without IStd) in the same source of control matrix as for SBs. If interfering peaks at the retention time of PH46 and/or IStd were noted, these were deemed to be insignificant if the response at the retention time of PH46 in the DB and SB samples was ≤20% of the average analyte response in LLOQ CSs and if the response at the retention time of IStd in DB and ULOQ samples was ≤5% of the IStd response accepted in the calibration curve, including SBs.

3.5.3 Assay recovery and matrix effect

The matrix effects on the plasma assay were determined by extracting replicate (n = 6) samples of control matrix, from six individual sources with one of these sources also presented as haemolysed plasma and spiking appropriate volumes of PH46 and IStd solutions (prepared in MeOH:H_2O:AA (50:50:0.2, *v/v/v*) following extraction. Three replicate matrix effect samples at each of the matrix equivalent concentrations (1.25 and 400 ng/mL) and IStd concentration (15 ng/mL) were generated. Spiking appropriate volumes (as above) of the PH46 and IStd solutions in the same ratio as the extract samples resulted in replicate (n = 3) non-extracted QC samples. The ratio of the MR of replicate samples spiked into matrix to the MR of the non-extracted samples was calculated to determine the MF for PH46 and IStd in each source of matrix. The value of (PH46-MF)/(IStd-MF) from the same source was calculated to determine the IStd-normalised MF. There was deemed to be no matrix effect in the samples when the precision of the IStd-normalised MF (calculated from the six sources) was ≤15% at each level.

The recovery of the plasma assay was investigated following the preparation, extraction and analysis of replicate (n = 3) matrix-effect samples, at low, medium and high levels, from a single matrix source. Replicate (n = 3) extracted samples were prepared following the details in Section 3.5.4 at the same concentration levels (low, medium and high) as the matrix-effect samples. The recoveries of PH46 and IStd were calculated as: MRs in the extracted samples/MRs in the matrix-effect samples.

3.5.4 Intra-batch assay accuracy and precision

The accuracy and precision of intra-batch assay were assessed using replicate (n = 6) QC samples prepared in control matrix at 0.5 (LLOQ), 1.25 (low), 15 (medium) and 400 (high) ng/mL for the plasma and urine assays, and 10 (LLOQ), 25 (low), 100 (medium) and 1600 (high) ng/g faecal equivalent for the faeces assay. The intra-batch accuracy expressed as the mean percentage determined concentration/nominal concentration. For the plasma and urine assays, the acceptance criteria were expected to be within 100 ± 15% at each concentration level (100 ± 20% at LLOQ level) for all three occasions. For the faeces assay, the criteria for acceptance at each level were within 100 ± 20% (100 ± 25% at the LLOQ level). The intra-batch precision was determined by the CV of the mean determined concentration. The criteria for acceptance should be ≤15% at each level (≤20% at LLOQ level) for all three occasions for the plasma and urine assays or ≤ 20% (≤25% at the LLOQ level) for the faeces assay. The inter-batch assay accuracy and precision were determined on three occasions (on different days). The acceptance criteria at each level were that assay accuracy for all three occasions was within 100 ± 15% (100 ± 20% at the LLOQ level), and that the assay precision for all three occasions was ≤15% (≤20% at the LLOQ level).

3.5.5 Assay carry-over and matrix dilution

Carry-over was assessed by the preparation, extraction and analysis of replicate (n = 2) samples prepared in control matrix at concentrations of 0.5 ng/mL (assay LLOQ) and 500 ng/mL (assay ULOQ). Additional replicate (n = 2) DB samples were also prepared, extracted and analysed. The samples were analysed in the following sequence: ULOQ (x1), DB (x2), LLOQ (x1), ULOQ (x1), solvent samples (MeOH: H_2O:AA, 50:50:0.2, *v/v/v*) (x2) and LLOQ (x1). The assay was deemed to have no carry-over for PH46 when the responses for PH46 in DB and solvent samples were ≤ 20% of the detector responses for PH46 in the next LLOQ samples. The assay was deemed to have no carry-over for IStd if the responses for IStd in DB and solvent samples were ≤ 5% of the detector responses for the IStd in the previous ULOQ samples. A bulk stock (4 mg/mL of PH46 in control plasma/urine or 16 mg/g faecal equivalents in control homogenised faeces) was prepared in control matrix. Replicate (n = 6) QC samples from this stock were diluted 10- and 100-fold with control matrix. Aliquots (200 μL for plasma/urine assays or 1 mL equivalent to 250 mg faecal material for faecal assay) (n = 6 of each dilution factor) were then extracted (nominal concentrations of 400 and 40 ng/mL respectively for plasma/urine assays or 1.6 mg/g and 160 ng/g faecal equivalent respectively for the faecal assay). The determined concentrations were corrected for the dilution factors. Matrix dilution was considered to be acceptable if the assay accuracy was within 100 ± 15% for the plasma/urine assays or 100 ± 20% for the faeces assay, and the assay precision was ≤15% for the urine assay and ≤ 20% for the faeces assay.

3.5.6 Stability experiments

For the following stability experiments, bulk QC samples were prepared at the appropriate low, high and dilution levels for each assay. A 10-fold dilution in control matrix was carried out on the dilution level QC before extraction and analysis.

3.5.6.1 Determination of short-term frozen storage stability and freeze/thaw stability of PH46 in matrix

Six aliquots (200 μL) at each concentration were extracted and analysed immediately to confirm the suitability of the stability samples. The remainder of each bulk was aliquoted into tubes (n = 18 for plasma, n = 48 for urine and n = 46 for faeces) containing a greater volume than required for analysis. Half of the replicates in each matrix (urine and faeces) were stored at −20°C and the other half of the replicates in each matrix were stored at −80°C. The effects of the corresponding stability samples were investigated after at least 1 month storage. The freeze/thaw stability of PH46 in plasma matrix assay was performed by using QCs samples (n = 6) at each concentration subjecting to freeze/thaw cycles (×3) in a freezer set to maintain −20°C (initial freeze cycle 24 h as minimum, freeze cycles 2 and 3 at least 12 h and thaw cycle for one h ± 6 min at RT unprotected from light). At the time point, replicate samples (n = 6) at each concentration and each condition were then extracted and analysed with fresh CSs and QCs. The samples were deemed to be stable if the accuracy was within 100 ± 15% (urine and plasma) or 100 ± 20% (faeces) of the nominal concentration and the CV was ≤15% (urine and plasma) or ≤ 20% (faeces) at each concentration level.

3.5.6.2 Determination of re-injection reproducibility

The accuracy and precision batch for each assay was re-injected, having stored the extracts in an autosampler at 4°C for at least 239 h for plasma extracts, at least 25 days for urine extracts and at least 6.5 days for faeces extracts.

3.5.6.3 Determination of ambient RT stability of PH46 in matrix

Ambient RT stability of PH46 in plasma was assessed on further QCs (n = 6) at each level after being left at ambient temperature (unprotected from light) for 24 h ± 30 min before extraction with fresh CSs and QCs.

3.5.6.4 Determination of extended frozen storage stability of PH46 in matrix

Six aliquots (200 μL) at each concentration were extracted and analysed immediately to show that the stability samples had been suitably prepared. The remainder of each bulk was aliquoted into glass tubes (n ≥ 48) containing a greater volume than required for analysis. The extended frozen storage stability of PH46 in human plasma was investigated after at least one, two and four months at −20°C and the effects of storing samples at −80°C were also assessed after at least one, three and five months. At each time point, replicates (n = 6) at each concentration were extracted and analysed with fresh CSs and QC samples. The samples were deemed to be stable if the

accuracy was within 100 ± 15% of the nominal concentration and the CV was ≤15% at each concentration level.

3.6 Data handling, processing and calculations

Analyst® 1.4.2 software was used for data collection during the study. Thermo Fisher Scientific Watson™ 7.0 software was employed to determine the calibration parameters/values, including slope, intercept and coefficient of determination, the mean, standard deviation and accuracy data. Three significant digits were presented for the nominal matrix concentration (CS and QC samples) and the determined concentration results. The calculations of the accuracy and precision data were made from the rounded mean values and from the unrounded determined concentration data and peak area data, respectively, and were displayed to one decimal place. Four significant digits were reported for slopes and intercepts, four decimal places were for coefficients of determination, and six decimal places for instrument responses.

4. Conclusions

In summary, we developed and validated a sensitive and specific bioanalytical method for the determination of PH46, a potential anti-inflammatory bowel disease agent, in human plasma, urine and faeces. A range of testing, including solution stability, specificity, assay linearity, accuracy, precision, recovery, matrix effect, carry-over, dilution effect and stabilities, were established for the matrices. The method meets EMA validation criteria. This method was successfully applied to the clinical pharmacokinetic study of PH46A in human.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualisation, N.F. and H.S.; investigation, H.S.; funding acquisition, N.F. and H.S.; data curation, G.A.S. and T.Z.; data analysis, T.Z. and G.A.S.; methodology, G.A.S. and T.Z.; data supervision, G.A.S.; writing—original draft, T.Z.; writing—review and editing: T.Z., G.A.S., N.F., H.S. All authors have read and agreed to the published version of the manuscript.

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