Development and optimization of a liquid chromatography- mass spectrometry assay for the quantification of fludarabine in human CSF

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INTRODUCTION

- Fludarabine is a chemotherapeutic often utilised as a lymphodepletion agent in paediatric patients prior to CAR T-cell therapy and haemopoietic stem cell transplantation (HSCT).
- Fludarabine exposure has been linked to clinical outcomes for patients, lower exposure to Fludarabine is linked to disease relapse and treatment failure and higher exposure is associated with drug toxicity and increased non-relapse mortality.[1,2]
- CAR-T Cell therapy has been increasingly utilised in solid brain tumours, with fludarabine utilised as a lymphodepleting agent.
- Little is known about Cerebral Spinal Fluid (CSF) penetration of Fludarabine and if this is adequate to lymphodeplete the brain.
- Previous attempts to develop a method for the quantification of Fludarabine in CSF were made difficult due to the limited availability of blank human CSF and the high salt and low protein composition of artificial CSF making it unsuitable as a surrogate matrix.
- The aim of this study was to identify an appropriate substitute matrix and optimise the current method to be suitable for Fludarabine quantification in human CSF.

METHODS

- An LC-MS/MS assay with a range of 5-1000ng/mL was developed.
- For extraction, 25µL of each plasma sample was mixed with 37.5 µL of internal standard ([13C,15N3]-Fludarabine in water) and 37.5 µL 20% trichloroacetic acid.
- Samples were spun at 20,814 g for 3 minutes. Resulting supernatant was diluted 1:2 in water.
- 10 µL of each sample was injected onto an Agilent 1260 Infinity HPLC chromatography instrument with an Agilent 6460 triple quadrupole mass spectrometer.
- Mobile phase A was 1mM ammonium hydroxide (NH4OH) and mobile phase B was 100% acetonitrile.
- Human plasma, human plasma diluted 1:200 in water, artificial CSF, water, human CSF diluted in water, human CSF spiked 1:4 into 1:200 plasma and CSF spiked into extracted 1:200 plasma were tested for suitability as surrogate matrices.
- Suitability was further confirmed by measuring the accuracy of QCs prepared in CSF.
- Further method validation was conducted according to ICH M10 guidelines. Parameters validated include lower limit of quantification, selectivity, linearity, accuracy and precision, matrix effect, recovery and short-term stability.

RESULTS

- 1:200 plasma and human CSF spiked 1:4 into 1:200 plasma were identified as the closest match to human CSF with percentage similarity in fludarabine response of 93.9% and 98.6% respectively.
- QCs prepared by spiking CSF into blank 1:200 plasma match those prepared in human CSF (Table 1).

Spiked CSF Diluted 1:4 in 1:200 Plasma			
Name	**Expected Conc (ng/mL)**	**Mean Conc (ng/mL)**	**% Accuracy**
QCL	45	43.86	97.46
QCM	400	397.99	99.50
QCH	800	732.76	91.60
CSF			
Name	**Expected Conc (ng/mL)**	**Mean Conc (ng/mL)**	**% Accuracy**
QCL	45	43.16	95.91
QCM	400	352.59	88.15
QCH	800	758.21	94.78

Table 1: QCs prepared in human CSF and human CSF spiked 1:4 in 1:200 plasma quantified against a curve made in 1:200 plasma were within acceptable accuracy with a range of mean accuracy 88.2-99.5%. As a result of this it was determined that 1:200 plasma could be utilised to prepare standard and QC samples, while patient CSF samples could be spiked 1:4 in 1:200 plasma for quantitation.]

- The method was linear with a range of 5-1000ng/mL across 5 separate runs, with all R^2 >0.99. (Figure 1).

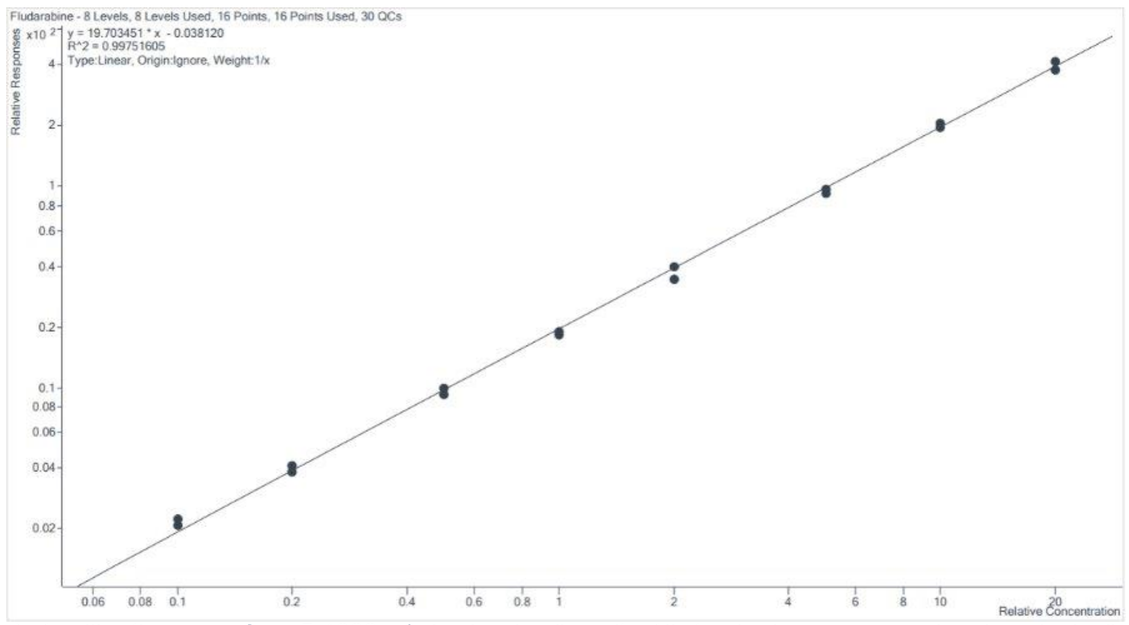

Figure 1- A calibration curve from 5-1000ng/mL, showing linearity. All standard were prepared and run in duplicate using 1:200 plasma. All data in acceptable range (85-115%) and R^2 < 0.9

- The LOD of the assay according to the concentration that resulted in a signal to noise ratio of 1:10 was <0.5 ng/mL. The LOQ of the assay was determined at 5 ng/mL during selectivity validation.
- The method was shown to be selective for fludarabine. Mean Fludarabine response in 6 individual batches of blank plasma was 18.9% of the response at the LOQ.
- The method was accurate across 3 separate runs (Table 2).

LLOQ (5.0 ng/mL)				
Run	**Mean (ng/mL)**	**SD**	**CV(%)**	**Accuracy(%)**
1	5.44	0.18	3.29	108.88
2	5.39	0.27	4.96	107.72
3	5.57	0.30	5.30	111.43
Mean	5.47	0.25	4.57	109.34
QCL (15.0 ng/mL)				
Run	**Mean (ng/mL)**	**SD**	**CV(%)**	**Accuracy(%)**
1	14.34	0.37	2.60	95.61
2	15.38	0.61	3.97	102.52
3	14.07	1.60	11.36	93.81
Mean	14.57	1.05	7.19	97.16
QCM (400 ng/mL)				
Run	**Mean(ng/mL)**	**SD**	**CV(%)**	**Accuracy(%)**
1	402.26	11.40	2.83	100.57
2	412.96	22.24	5.38	103.24
3	370.24	10.97	2.96	92.56
Mean	395.15	23.83	6.03	98.79
QCH (800 ng/mL)				
Run	**Mean(ng/mL)**	**SD**	**CV(%)**	**Accuracy(%)**
1	794.69	42.52	5.35	99.34
2	769.37	38.97	5.07	96.17
3	815.06	33.06	4.06	101.88
Mean	793.04	40.87	5.15	99.13
ULOQ (1000 ng/mL)				
Run	**Mean(ng/mL)**	**SD**	**CV(%)**	**Accuracy(%)**
1	960.92	31.64	3.29	96.09
2	918.30	34.58	3.77	91.83
3	934.85	44.15	4.72	93.49
Mean	938.03	39.31	4.19	93.80

Table 2- Intra-assay accuracy was tested by running six replicates of QC samples, the accuracy range was 96.1 - 109.0 % (%CV 2.6-5.4%). Inter-assay precision and accuracy was determined by running 6 sets of QCs across 3 separate analytical runs. The mean accuracy for each level ranged from 91.8- 109.0%.

- Matrix effect was tested for both 1:200 plasma and human CSF spiked 1:4 into 1:200 plasma. Mean IS normalised matrix factor ranged from 0.23-0.57 in 1:200 plasma with %CV <13.5%. For CSF spiked 1:4 in 1:200, mean IS normalised matrix factor ranged from 0.99-1.03, with %CV <14.1%.
- Recovery from 1:200 plasma was tested at 3 QC levels, with recovery ranging from 100.2- 208.4% respectively, with %CV <12.8.
- Fludarabine in 1:200 plasma was stable for 24 hours at 4°C (%difference from T_0 <4.3%) and 24 hours at room temperature (%difference from T_0 <15.5%).

CONCLUSIONS

An accurate and sensitive LC-MS/MS method has been developed and partially validated for the quantification of fludarabine in human CSF. Further validation tests such as dilution integrity, carryover and long-term stability, recovery from CSF spiked in 1:200 plasma, and short-term stability tests for CSF spiked 1:4 in 1:200 plasma should be conducted to fully validate this method for clinical use.

REFERENCES

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