A TFC MS/MS Method for the Determination of the Cholinesterase Sensitive Analyte Rivastigmine in Human Plasma

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Introduction

Rivastigmine [3-[(1S)-1-dimethylaminoethyl] phenyl] N-ethyl-N-methyl-carbamate, Figure 1] is a parasympathomimetic agent for the treatment of mild to moderate dementia of the Alzheimer's type. It acts as a reversible cholinesterase inhibitor of the carbamate type that inhibits both butyrylcholinesterase and acetylcholinesterase which would otherwise break down the brain chemical acetylcholine. Therefore, Rivastigmine increases the concentration of acetylcholine which acts to enhance cholinergic function.

Oral doses of Rivastigmine are typically in the 1.5 to 6 mg range, resulting in relatively low plasma concentration levels (in the pg/mL range in the terminal phase). Therefore a 100 pg/mL LLOQ was required to generate pharmacokinetic human plasma concentration data for bioequivalence studies.

Rivastigmine is rapidly metabolised by cholinesterase-mediated hydrolysis, therefore special caution must be paid to the analyte stability during sample processing and measurement.

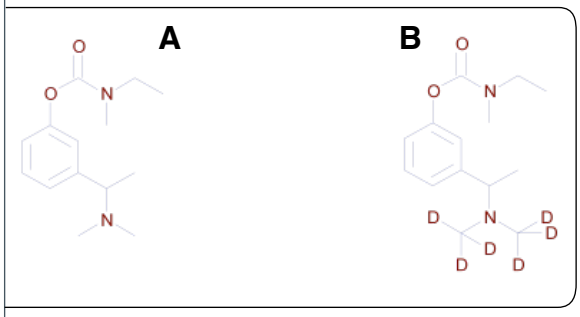

Figure 1. *Molecular structures of Rivastigmine and Rivastigmine-D6*
A: Rivastigmine $C_{14}H_{22}N_2O_2$ MW = 250.34 g/mol
B: Rivastigmine-D6 $C_{14}H_{16}D_6N_2O_2$ MW = 256.37 g/mol

Method Summary

Rivastigmine and its D6-labeled IS (Figure 1) were extracted from diluted human plasma using a Cohesive Turboflow® (TFC) on-line extraction system configured in dual column focusing mode (Figure 2). Extraction conditions were optimized in terms of extraction column selectivity, loading and eluting mobile phase composition and pH. Conditions as described in Table 1 were found to give best overall results for total recovery, peak shape and sensitivity. Further improvements in peak shape could be achieved by addition of an analytical column post extraction system. The dual column configuration allows elution of analyte and IS from the extraction column in a small volume of high organic mobile phase which is subsequently diluted by aqueous mobile phase provided by the eluting pump. This mechanism allows focusing of the compound onto the analytical column, resulting in a very sharp peak shape with positive benefit for MS sensitivity (Figure 3).

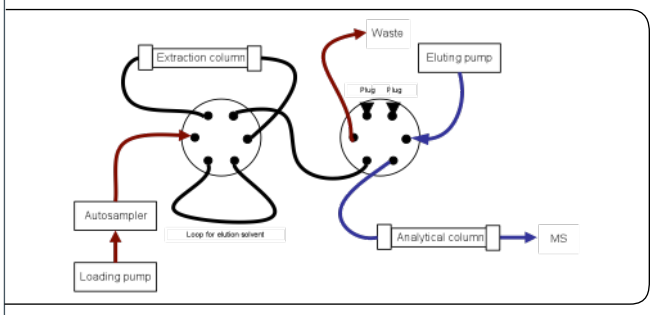

Figure 2. *Turboflow® (TFC) configuration for on-line extraction (using dual column focusing mode)*

Extraction method:	Turbulent flow (TFC) on-line extraction
Sample preparation:	Human plasma (K3 EDTA), diluted 1:4 with 0.1% Formic acid (aq.)
Analytical range:	0.100 – 40.0 ng/mL
Injection volume:	10 µL
TFC conditions	
Extraction column:	Turboflow® Cyclone, 50 x 0.5 mm (Thermo Scientific)
Loading solution A:	Water / Formic acid, 100:0.1, v/v
Loading solution B:	Methanol / Formic acid, 100:0.1, v/v
Loading flow rate:	1.5 mL/min
Analytical column:	XBridge C18, 2.5 µm, 50 x 4.6 mm (Waters)
Eluting solution A:	Water / Formic acid, 100:0.1, v/v
Eluting solution B:	Methanol / Formic acid, 100:0.1, v/v
Eluting flow rate:	1.0 mL/min, linear gradient
Total cycle time:	5.0 min per sample
MS/MS Conditions	
Instrument:	AB / MDS Sciex API 4000
Source:	ESI
Scan mode:	MRM, positive
Resolution:	unit / unit
Dwell time:	75 ms
MS/MS transitions:	251.1/206.1 amu for Rivastigmine 257.2/206.1 amu for Rivastigmine-D6

Table 1. *TFC-MS/MS conditions*

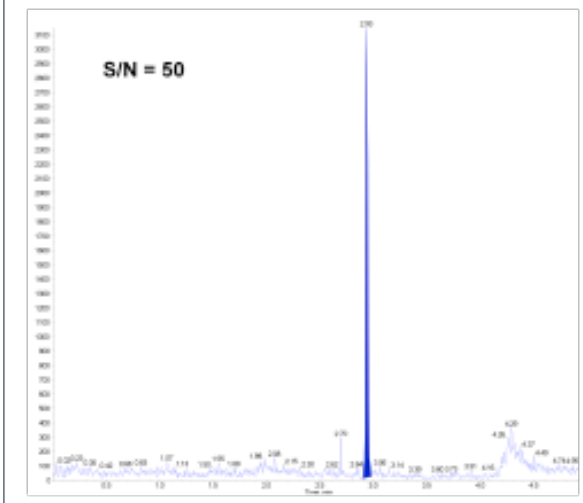

Figure 3. *Example chromatogram*
LLOQ, 0.100 ng/mL Rivastigmine in extracted human plasma (K3 EDTA), peak width of 0.08 min resulting in approximately 30 data points per peak

Rivastigmine Stability Investigations

In-vivo/in-vitro enzymatic cholinesterase activity leads to the hydrolysis of Rivastigmine and the formation of the decarbamylated metabolite NAP 226-90 (Figure 4). This inherent instability must be addressed during design of the bioanalytical methodology.

A series of experiments was conducted to evaluate the stability of Rivastigmine under sample processing and analysis conditions. Initially stability experiments were designed to determine if human plasma samples could be processed under standard laboratory conditions (i.e. room temperature or on ice). Additionally the effect of pH during sample processing was optimised. It was demonstrated that an enzyme inhibitor was required for effective analyte stabilisation.

It was demonstrated in spiked untreated human plasma that the formation of metabolite NAP 226-90 was 16 times lower at pH 3 than at pH 7. This implies at least some degree of enzyme inhibition at low pH and is in line with the optimal pH for on-line extraction recovery. Therefore it was considered that once plasma samples were maintained at pH 3 (by addition of an acidified solution containing internal standard), ongoing stability could be assumed.

In order to investigate Rivastigmine stability prior to acidification kinetics in untreated human plasma were performed at room temperature (RT) and on ice. Spiked plasma maintained at RT presented 22-35% analyte degradation after 3 hours (3 hours representing a realistic sample pre-treatment time prior to addition of acid). On ice stability was proven for 3 hours since degradation was less than 10% (Figure 5).

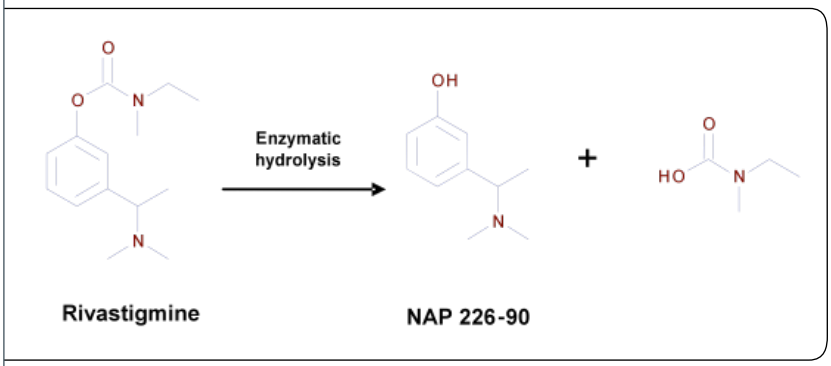

Figure 4. *Enzymatic hydrolysis of Rivastigmine to its metabolite NAP 226-90*

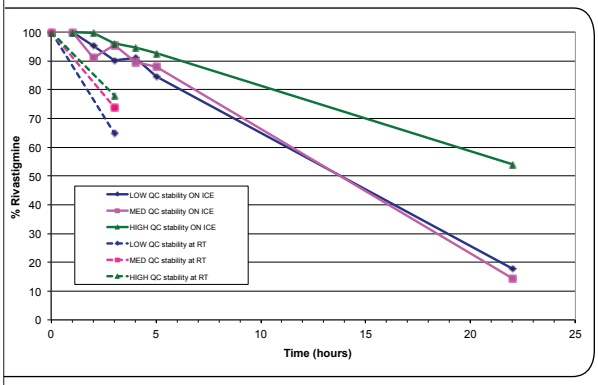

Figure 5. *Rivastigmine stability kinetics in untreated human plasma*

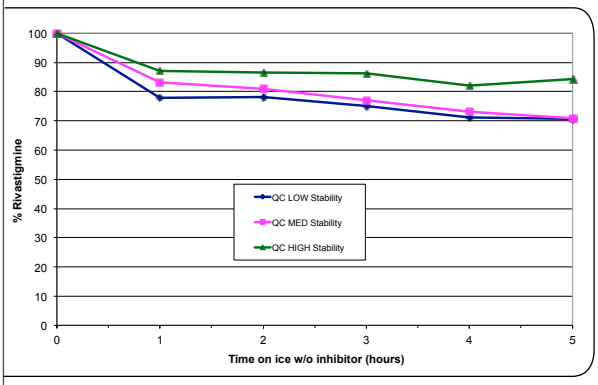

Figure 6. *Rivastigmine stability kinetics prior to inhibitor addition*

However, in order to stabilise Rivastigmine in human plasma beyond 3 hours to allow multiple analysis from the same sample aliquot, the use of an esterase inhibitor was investigated. It was demonstrated that following addition of inhibitor plasma samples were stable for at least 24 hours at RT. As inhibitor could not be added at the clinical site detailed stability kinetics were required to determine how samples should be treated prior to inhibitor addition. For this purpose samples were spiked into untreated human plasma and kept 30 min at RT before flash freezing them and storing them at -20°C for 24 hours (in order to mimic sample collection treatment at clinical site). Thereafter, these samples were thawed and kept on ice before inhibitor addition at increasing time points. Resulting kinetics indicated that the critical steps are the processing steps within the first hour of sample generation. After addition of inhibitor samples were shown to be stable within +/-10% for at least 3 hours (Figure 6).

It was concluded that clinical samples should therefore be flash frozen (within 30 min of collection) and stored at -20°C pending inhibitor addition. In the bioanalytical laboratory, clinical samples and quality control plasma samples were thawed within 10 min and kept on ice during addition of 1% by volume of a 10 mM Eserine hemisulfate aqueous solution within 3 hours. Samples were stored at -20°C pending analysis.

Method Validation Results

The analytical method was successfully validated over a range of 0.100 to 40.0 ng/mL using an assay volume of 50 µL human plasma stabilised with Eserine hemisulfate. Key validation parameters are presented in Table 2.

		Precision (%)	Accuracy (%)
Intra run 1	LLOQ	7.1	95.2
	Low	4.6	102.8
	Medium	4.2	101.6
	High	2.8	97.6
Intra run 2	LLOQ	9.5	95.9
	Low	5.3	103.3
	Medium	3.8	98.5
	High	2.7	96.2
Intra run 3	LLOQ	4.5	96.8
	Low	5.0	103.3
	Medium	1.5	99.9
	High	2.2	100.2
Inter run	LLOQ	7.1	92.8
	Low	4.3	102.0
	Medium	1.4	106.3
	High	0.6	96.3
Selectivity	No Interference, response < 20%, relative to mean LLOQ response in 10 different matrix lots		
Carry-Over	Response < 20%, relative to mean LLOQ response		
Cross-well contamination (96 well format)	Response < 20%, relative to mean LLOQ response in blank samples positioned adjacent to wells containing QC high samples		
Matrix-Effect	Concentration within ± 15% nominal at LLOQ and QC high level in 10 different matrix lots		
Bench-top stability	Demonstrated for 27 hours at room temperature		
Freeze and thaw stability	Demonstrated for 3 freeze (at -20°C) and thaw cycles		
Post-preparative stability	Demonstrated for 41 hours at 5°C		
Dilution integrity	Up to 200 ng/mL		
Processed sample integrity	Demonstrated for 125 hours at 5°C		
Batch size	192 Injections		

Table 2. *Validation parameters*

The validated method was applied to the analysis of clinical study samples collected after oral administration of Rivastigmine. As the method was applied to bioequivalence studies, quantitation of the metabolite NAP 226-90 was not required. Method modification would have been necessary to simultaneously determine Rivastigmine and NAP 226-90 in the same assay. Plasma samples were stabilised with Eserine hemisulfate and were analysed within multiple analytical runs using multiple LC-MS/MS systems. All runs met acceptance. Incurred sample reproducibility was investigated by reanalysis of selected study samples (n = 130). The difference between original and repeated value was < 20% in 96.9% of the reanalysed study samples and was ≤ 24.4% in the remaining 3.1%.

Method Robustness and Performance

Robustness is always a goal for bioanalytical assays, particularly when they are to be applied to clinical studies (perhaps run over a timeframe of years and many thousands of samples). It is therefore important to consider method robustness from an early stage in the method development process.

This method was shown to be extremely robust in our laboratory (100% batch pass rate). It is worth highlighting the factors which lead to this robust method performance. It was an aim to establish a good performing method before any stability experiments were conducted in order to generate reliable stability information. Subsequently effective sample pre-treatment could then be adapted to stabilise the analyte prior to analysis.

Important considerations for the selected methodology included analyte pH stability, robustness benefits from automation (TFC on-line extraction) and a sensitive and stable MS system. TFC allows potential for sample dilution and low injection volumes (absolute plasma volume). Good quality chromatography (peak shape and retentivity from focusing mode) are necessary for integration consistency eliminating potential manual reintegration. Special attention was made to potential matrix effects (from co-eluting or late eluting compounds) – a series of suppression evaluation experiments were conducted (post column infusion and qualitative phospholipids evaluations).

Multiple MS and TFC systems were tested to ensure column reproducibility and maintenance of MS signal. Column lifetime and consistency of chromatographic performance (TFC and analytical column) was demonstrated over several 1000 matrix sample injections.

Conclusion

A method for the determination of the cholinesterase sensitive analyte Rivastigmine in human plasma was developed and validated. The method demonstrated extremely good performance and robustness during its application to clinical studies.