USE OF DRIED BLOOD SPOT SAMPLING FOR STABILISATION OF A CHOLINESTERASE SENSITIVE ANALYTE RIVASTIGMINE.

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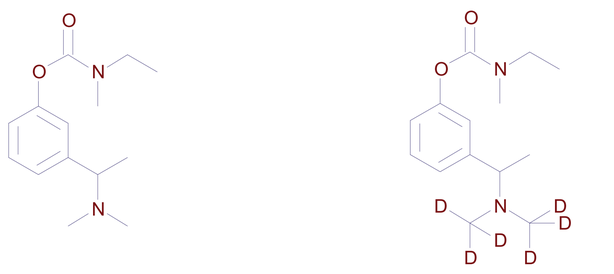
INTRODUCTION

Rivastigmine,[2-[(1S)-1-dimethylaminoethyl]phenyl]-N-ethyl-N-methylcarbamate (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.

Rivastigmine has been previously determined in human plasma in our laboratoryusing a sensitive and robust Turbulent Flow Chromatography (TFC)-MS/MS method. Rivastigmine, however, is rapidly metabolised in the presenceof plasma cholinesterases requiring sample pre-treatment with esterase inhibitor prior to analysis.

This poster explores the potential use of dried blood spots (DBS) as an alternative and simplified method to stabilise the analyte in clinical samples. Comparative short term stability data is presented to illustrate stabilisation of the analyte in dried format and also in whole blood to account for spotting procedure and blood spot drying time. The quantitation in DBS also used a d6-labeled internal standard targeting the same LLOQ of 0.100 ng/mL Rivastigmine as in the in human plasma assay.

Figure 1: Molecular structures of Rivastigmine and Rivastigmine-d6



Rivastigmine
$C_{14}H_{22}N_2O_2$
MW = 250.34 g/mol

Rivastigmine-d6
$C_{14}H_{16}D_6N_2O_2$
MW = 256.37 g/mol

METHODOLOGY FOR DRIED BLOOD SPOTS

Human whole blood (K3-EDTA) containing Rivastigmine was spotted (25 µL) onto DBS cards (IDBS-1004 Bioanalysis card). Cards were dried at ambient temperature for a minimum of two hours and 6.0 mm diameter spot was punched into a 96-well plate using automated punching (figure 2). Spots were then extracted by the addition of 300 µL of Methanol containing d6-labeled internal standard at appropriate concentration. The well plate was sealed and mixed on a horizontal shaker for 10 min at ambient temperature. A 200 µL subaliquot of supernatant was transferred to a separate plate and evaporated under nitrogen prior to reconstitution in 0.1% Formic acid (aq.) and submission for TFC-MS/MS analysis using conditions as noted in table 1.

Figure 2: Automated punching by BSD 600 from IDBS-1004 Bioanalysis card

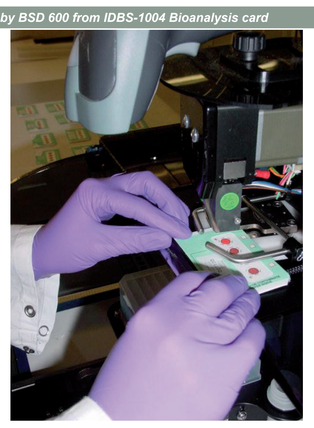
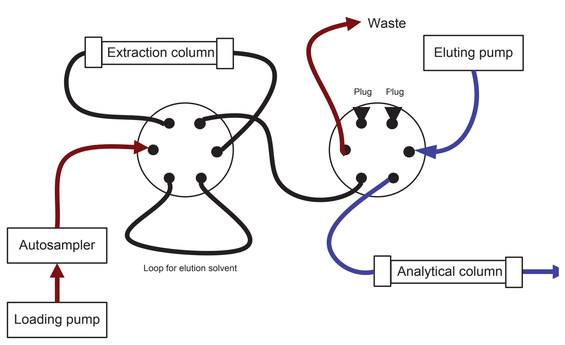


Table 1: TFC-MS/MS conditions

TFC conditions	
Extraction column:	Turboflow® Cyclone, 50 x 0.5 mm (Thermo Scientific)
Loading solution A:	Water / Formic acid, 100:0.1, v/v (pH 3)
Loading solution B:	Methanol / Formic acid, 100:0.1, v/v (pH 3)
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 (pH 3)
Eluting solution B:	Methanol / Formic acid, 100:0.1, v/v (pH 3)
Eluting flow rate:	1.0 mL/min, linear gradient
Injection volume	20 µL
Total cycle time:	5.0 min per sample
MS/MS Conditions	
Instrument:	AB / MDS Sciex API 4000
Source:	ESI positive
Scan mode:	MRM
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

Figure 3: Turboflow® (TFC) configuration for on-line extraction (using dual column focusing mode)



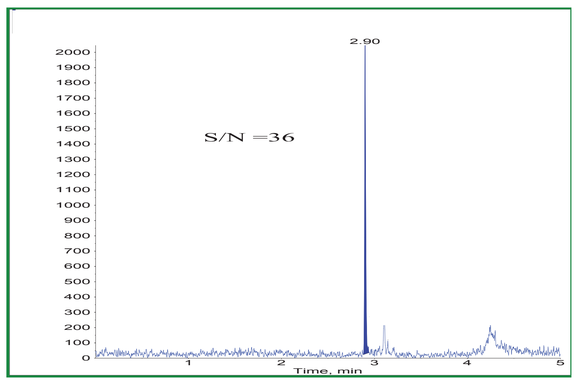
METHOD PERFORMANCE

The method was qualified over a range of 0.100 – 40.0 ng/mL Rivastigmine in Human whole blood. This range was the same as previously applied to Human plasma.
The DBS method showed good precision and accuracy over the applied range using a punch spot size of 6.0 mm (equivalent to 11 µL Human whole blood) see table 2. The recovery from blood spot card was evaluated to be approximately 55%. An example of a typical LLOQ chromatogram is presented in figure 4.

Table 2: Method performance

		Precision (%)	Accuracy (%)
Intra run (n=6)	LLOQ	8.4	101
	Low	3.6	93.7
	Medium	4.0	101
	High	3.0	103
Recovery		Recovery (%)	
	Low	52	
	High	57	
Carry-Over	Response < 20%, relative to mean LLOQ response		
Bench-top Stability	Demonstrated for 24 hours at room temperature		
Batch Size	96 Injections		

Figure 4: Example chromatogram of LLOQ, 0.100 ng/mL Rivastigmine in DBS

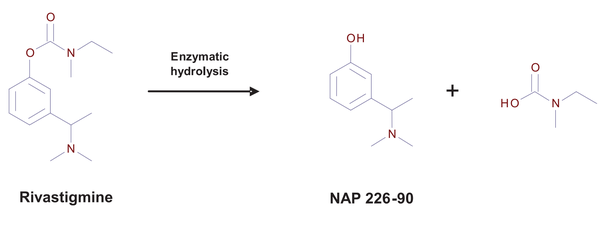


RIVASTIGMINE STABILITY INVESTIGATIONS IN PLASMA SAMPLES

In vivo / in vitro enzymatic cholinesterase activity leads to the hydrolysis of Rivastigmine and the formation of the decarbamylated metabolite NAP 226-90 in human plasma (figure 5). This inherent instability must be addressed during design of the bioanalytical methodology (see reference).

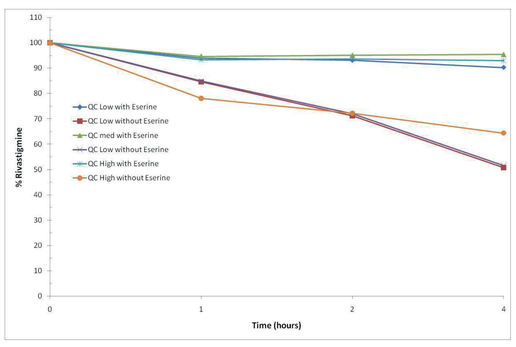
The stability of Rivastigmine was previously evaluated under sample processing and analysis conditions. The effect of pH during sample processing was assessed and it was demonstrated that once plasma samples were maintained at pH 3 (by addition of an acidified solution containing internal standard), ongoing stability could be assumed. A similar procedure was subsequently applied to extracted DBS samples.

Figure 5: Enzymatic hydrosis of Rivastigmine to its metabolite NAP 226-90



In order to investigate Rivastigmine stability in spiked untreated human plasma and plasma treated with an esterase inhibitor (Addition of 1% by volume of a 10 mM Eserine hemisulfate aqueous solution) degradation kinetic experiments were performed at room temperature (RT). These were performed at three concentrations and multiple replicates were analysed using the previously validated plasma method.
Untreated plasma showed approximately 45% analyte degradation within 4 hours (4 hours representing a realistic plasma handling time prior to addition of acid), whereas plasma spiked after addition of enzyme inhibitor was shown to be stable within +/-10% over the same time (figure 6).

Figure 6: Rivastigmine stability kinetics in human plasma untreated and treated with Eserine hemisulfate enzyme inhibitor

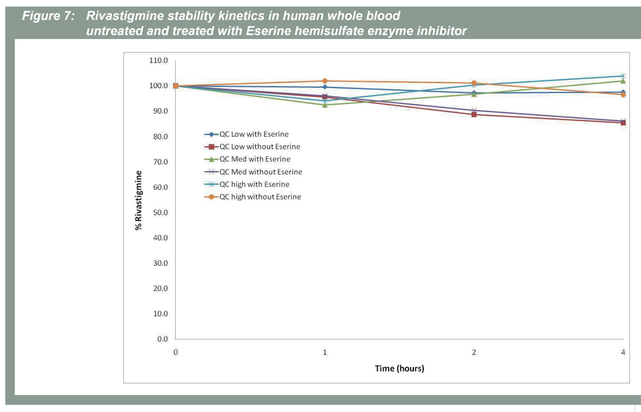


RIVASTIGMINE STABILITY INVESTIGATIONS IN WHOLE BLOOD SAMPLES

The aim of this work was to explore the potential use of dried blood spots as an alternative and simplified method to stabilise the compound without the addition of an enzyme inhibitor in clinical samples.

In order to demonstrate stability in the DBS format, some stability needed to be shown in human whole blood prior to spotting onto untreated DBS cards. This would account for time taken to spot samples onto cards and subsequent drying time. Untreated and treated (1% by volume of a 10 mM Eserine hemisulfate aqueous solution) human whole blood was spiked with Rivastigmine and stored at RT prior to spotting onto cards. Multiple replicates at three concentrations were then extracted and analyse as described in the methodology section.
Untreated whole blood showed less than 10 % analyte degradation after 2 hours which represents a typical combined card spotting and drying time. This was inline with eserine treated blood indicating blood stability prior to dried format without requirement for eserine treatment (figure 7).

Figure 7: Rivastigmine stability kinetics in human whole blood untreated and treated with Eserine hemisulfate enzyme inhibitor

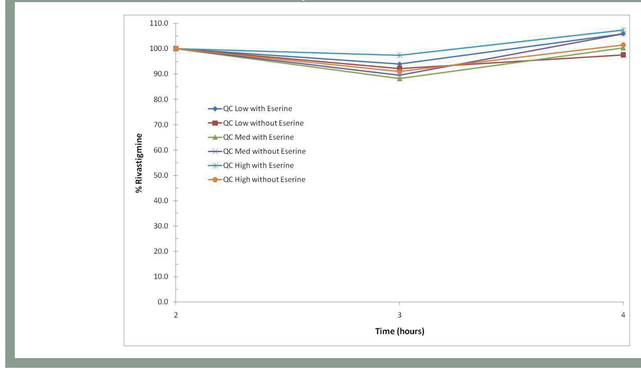


RIVASTIGMINE STABILITY INVESTIGATIONS IN DBS SAMPLES

Once stability during blood sampling and spotting was demonstrated, further stability in dried format as blood spots could be evaluated.

Untreated and treated (1% by volume of a 10 mM Eserine hemisulfate aqueous solution) whole blood was fortified with Rivastigmine and immediately spotted onto DBS cards. Cards were dried at RT and spots extracted after 2, 3 and 4 hours to investigate some short term on card stability.
No difference between inhibitor treated and untreated DBS samples could be observed. This indicates viable short term stability in DBS format in absence of inhibitor (figure 8). Additional QC samples freshly spotted and stored for 24 hours at RT were measured vs. QC samples freshly spotted. Results confirmed stability of the 24 hour DBS samples (results not shown).

Figure 8: Rivastigmine stability kinetics in DBS untreated and treated with Eserine hemislufate enzyme inhibitor



CONCLUSION

A sensitive and robust method was developed and qualified for Rivastigmine in dried blood spots (DBS). This method facilitated the assessment of Rivastigmine stability in a DBS format and whole blood prior to spotting and drying. Comparision to human plasma stability determined using a validated plasma assay could then be performed.
Both short term blood stability and DBS stability were demonstrated without requirement for analyte stabilisation with enzyme inhibitor.
This approach could potentially simplify the blood sampling process at the clinical site and remove the requirement for sample stabilisation with enzyme inhibitor for this analyte. It is assumed the dried format inhibits or significantly slows enzyme activity. No enzyme degradation is assumed as untreated DBS card material was used throughout this evaluation

REFERENCE

A TFC MS/MS method for the determination of the cholinesterase sensitive analyte rivastigmine in human plasma presented at the European Bioanalytical Forum, Barcelona, December 2-4 2009

www.celerion.com

Poster presentation at the 19th International Reid Bioanalytical Forum, Guildford, 4-7 July 2011