Evaluation of Chemical Stability and Bioanalytical Assay Quality Using Adaptive Focused Acoustic Energy Tissue Homogenization and LC-MS/MS Analysis

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Introduction

In the past, a popular technology used for tissue homogenization prior to analysis was to use rotary blade (Polytron) homogenization devices. Polytrons, many are either hand-held single probe or automatic array probes (such as a TOMTEC Autogizer® shown in Fig.1). This technology presents challenges in sample throughput, efficiency and safety, as well as the requirement of thorough cleaning rotary blades between samples; which has potential for analyte cross-contamination in tissue samples. In addition, they have difficulties in processing certain types of tissues (eg. muscle and heart).

Recently, a novel technology has been introduced in this field to overcome the challenges mentioned above. Adaptive Focused Acoustics (AFA) instrument is designed to use megasonic frequency (Fig.2) for tissue homogenization as alternative approach to process and extract pharmaceutical compounds from tissue samples prior to LC/MS/MS analysis. These new devices, E200 and S2 (Covaris, Inc.) provide many improvements in tissue homogenization, including: automated robotic sample handling; increased extraction levels, recovery and reproducibility for pharmaceutical compounds. The most appealing advantage is the "Non-contact" sample processing – therefore NO cleaning (rotary blade) required, and eliminates cross-contamination potential of analytes between samples. Coupled with a Cryo-system, this instrument has more power to efficiently process tissues. Most recent literature have suggested that use of Adaptive Focused Acoustics Energy for tissue homogenization result in greatly enhanced extraction efficiency of drugs from tissue samples, especially more difficult hard tissue types, compared with Polytron technology.

In addition to assay quality, chemical stability of analytes processed by AFA was evaluated. As a result, the new technology by Covaris has been routinely used in authors' laboratory for tissue analysis to support PK/TK studies in preclinical development.

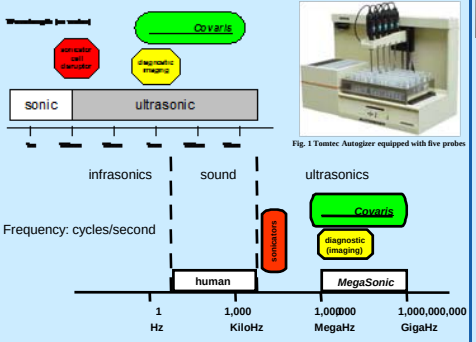

Fig. 2 Scheme of Acoustic Wavelength and Frequency, Covaris Uses Mega-Sonic Frequency Range

Fig. 1 Tomtec Autogizer equipped with five probes

Methods

Instrumentation

- Covaris E200 or S2 Automated AFA System (Covaris, Inc. Woburn, MA), Fig. 3
- Tomtec Quatra 96 Automated Liquid Handler
- HPLC pumps from Cohesive Technology or Perkin-Elmer
- API-4000 Mass Spectrometer (Applied Biosystems, Foster City, CA)

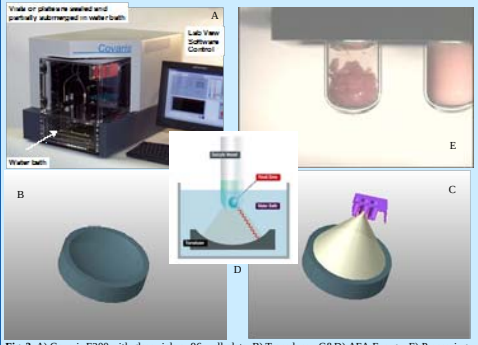

Fig. 3 A) Covaris E200 with glass vials or 96-well plate, B) Transducer, C&D) AFA Energy, E) Processing

Sample Preparation

- Tissue sample was cut and weighed out about 0.5g in a 13x100 glass tube
- Sample was dissolved in 3 volumes (vs. weight) of a solution (10% ACN in buffer)
- Sample tube was capped and placed on the process position in Covaris E200 or S2
- Applied AFAE to break tissue, Covaris processing time took about 2 min/sample
- Tissue homogenate was transferred with a pipette into a 96-Well plate (Costar cluster deep wells, capacity of 1.2 mL)
- Homogenate sample size, 50 µL for standards, QCs and unknown samples
- Added spiking solution (50 µL) and a internal standard (IS) of 25 µL,vortex-mixed
- Extraction from tissue homogenate:
  a) liquid-liquid Ext. (LLE): 0.8 mL of MTBE was used for extraction; a Tomtec liquid handle was used for mixing and transferring
  b) protein precipitation (PP) precipitated with 250 µL 100% acetonitrile
  c) PP followed by high flow column switching online extraction.

LC/MS/MS Analysis

- Options for LC:
  a) Isocratic MP, adjust organic/aqueous ratio is needed within a 2 min window
  b) Gradient MP, sharp gradient of ACN from 10-95 % used
  c) Online Extraction LC added extra cleaning step for sample analysis
- MS/MS Detection: Data acquired were from API3000 and API4000 Mass Spec

Results

Table 1 Summary of Chemical Stability of Five Generic Drugs and Six Discovery New Molecule Entities (NME) in Rat Brain Homogenate

Generic Analyte	Percent Different in Responses of AFA Treated Sample vs. without AFA (N=5)*						Ave. %Diff
	0.1 ug/mL	0.4 ug/mL	1 ug/mL	2 ug/mL	5 ug/mL	10 ug/mL	
Benzoylecgonine	-0.9	1.6	1.5	1.6	0.5	-3.1	0.2
Fenfluramine	-7.0	2.6	-1.7	3.2	5.6	-1.6	0.2
Haloperidol	0.8	-0.1	-0.8	0.7	1.4	1	0.5
Oxazepam	4.2	-1.7	-1.5	1.1	1.6	-2.7	0.2
Puromycin	-5.6	-0.3	-1.6	-2.9	0.1	-0.5	-1.8

NME Analyte	Percent Different in Responses of AFA Treated Sample vs. without AFA (N=5)*						Ave. %Diff
	0.1 ug/mL	0.4 ug/mL	1 ug/mL	2 ug/mL	5 ug/mL	10 ug/mL	
Compound-A	0.5	0.3	-0.3	0.4	-0.1	2.3	0.5
Compound-B	3.9	-0.3	-0.2	-0.4	0.3	2.4	1.0
Compound-C	0.9	0.8	-0.3	1.8	1.1	2	1.0
Compound-D	0.6	1.5	-0.7	-1.3	0.1	1.0	0.2
Compound-E	1.1	1.3	-0.6	-0.2	-0.4	0.1	0.2
Compound-F	-0.1	-0.4	-0.2	-0.2	0.4	0.2	-0.1

* The response difference were calculated using the following formula:
% Rep. Diff. = 100 x (Post AFA Treated Sample – Controls Without AFA) / Controls Without AFA

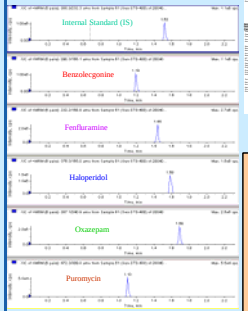

Fig. 4 XIC Chromatogram of FiveGeneric Analytes From a Rat Brain Homogenate (PP+online) During the Chemical Stability Test

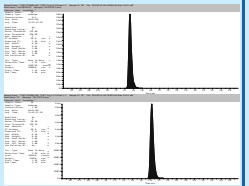

Fig. 6 Chromatogram of Compound B from a Typical Mouse Brain Homogenate (LLE)

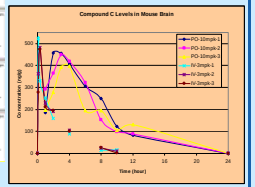

Fig. 7 Brain Conc.-Time Profile of Compound C from a PK study After IV and Oral Doses in Mice

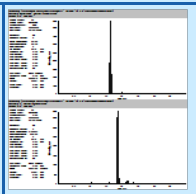

Fig. 5 Chromatogram of Compound A from a Typical Rat Brain Homogenate (PP+online)

Table 2 Summary of Quality Control Concentrations for Compound A from Rat Brain during Sample Analysis

Run Date	Curve Number	Low (2.50 ng/mL)	Mid (50.0 ng/mL)	High (500 ng/mL)
26-May-2005	16	2.40	51.5	424
		NV	70.1[a]	473
		1.69	44.3	531
27-May-2005	18	2.96	48.6	460
		2.89	56.2	455
01-Jun-2005	20	2.23	59.4	441
		2.18	52.7	354[a]
		2.19	53.6	462
06-Jun-2005	22	1.99[a]	50.5	405
		2.24	57.0	425
Mean		2.44	52.6	453
S.D.		0.339	4.61	36.6
%C.V.		13.9	8.8	8.1
%Bias		-2.4	5.2	-9.4
N		7	9	9

Table 3 Summary of Quality Control Concentrations for Compound B in Mouse Brain during Sample Analysis

Run Date	Curve Number	QC-5 10.0 ng/mL	QC-40 400 ng/mL	QC-400 1000 ng/mL
11-Apr-2005	7	9.53	373	967
		8.63	383	964
		9.16	332	755[a]
12-Apr-2005	8	9.23	426	997
		9.16	364	982
Mean		9.14	376	978
S.D.		0.324	34.0	15.2
%C.V.		3.5	9.0	1.6
%Accuracy		91.4	94.0	97.8
%Bias		-8.6	-6.0	-2.2
n		5	5	4

[a] Value exceeds acceptance criteria, %Bias>20%, not included in statistics

Table 4 Summary of Quality Control Concentrations for Compound C in Monkey Brain during Sample Analysis

Analytical Run Date	Curve Number	Theoretical Concentration of Quality Control (ng/mL) QC-2.5 2.50 ng/mL	QC-5 5.00 ng/mL	QC-40 40.0 ng/mL	QC-400 400 ng/mL
13-Apr-2005	11	2.67	4.78	42.7	378
		2.70	5.34	44.7	399
Mean		2.69	5.06	43.7	389
S.D.		0.0212	0.396	1.41	14.8
%C.V.		0.8	7.8	3.2	3.8
%Bias		7.6	1.2	9.3	-2.8
N		2	2	2	2

Conclusions

- The analytes tested are stable after the AFA process; there was no major impact on chemical stability(%Diff<1.8%). Therefore AFA is suitable for tissue analysis
- The new tissue homogenization technique has been successfully applied to support many preclinical studies from different species for several NMEs
- This novel technology using Adaptive Focused Acoustics Energy increases sample throughput by reducing process time; the non-contact closed vessel process eliminates carryover/cross-contamination between samples and provides safety work environment
- Covaris instrument (E200 and S2) can be easily operated; E200 offers complete automation for 12/24 samples/load; standardized AFAE transfer ensures uniform sample process with high recovery and efficiency.