AUTOMATED LC/MS/MS HIGH THROUGHPUT MULTI-MODE IONIZATION QUANTIFICATION PROTOCOL APPLIED FOR MICROSOME STABILITY TEST IN DRUG DISCOVERY AND DEVELOPMENT

Waters

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INTRODUCTION

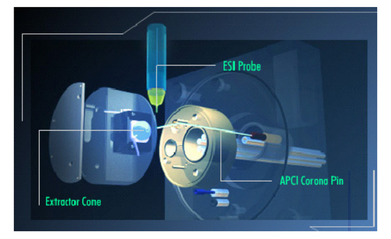
Highlights

- Multi-mode ionization: ESCi®
  - No need to physically change ionization sources for ESI/ APCI switching.
- Automated LC/MS/MS analytical protocol (from method development to report generation)
  - Automated MS and MSMS scans for optimization
  - Automated MRM MS acquisition method generation
  - Automated data acquisition
  - Automated quantification and report generation
- Application example presented for microsome-stability test in drug discovery

Background

- LC/MS analysis typically utilizes atmospheric pressure ionization (API) as the ionization source with two options:
  - Electrospray (ESI): favors polar compounds
  - Atmospheric pressure chemical ionization (APCI): favors non-polar compounds and relative small molecules.
- The best ionization mode for any compound is determined by optimizing MS conditions in both ESI and APCI.
  - Generally, about 80% compounds can be analyzed by ESI[1].
  - APCI is the common alternative option for non=ESI compounds.
- Traditionally, to change between ESI and APCI
  - Analysts need to physically change the ionization source
  - All ion source parameters need to be re-optimized
  - This results in lost analysis time and reduces analytical throughput significantly[1].

ESCi Multi-mode Ionization



- "ESCi multi-mode ionization source" refers to a combination ESI & APCI capability available on Waters mass spectrometers.
- The enabling of the ESCi source is driven by specifically designed electronics capitalizing on unique attributes of the source geometry.
- In ESCi, both ESI and the APCI modes can function by alternating at high MS switching speeds in both positive & negative polarities[4].

Challenges for ESCi Applied to Drug Discovery

- The characteristics for analyses carried out in drug discovery are:
  - Many new compounds to be analyzed for screening purpose
  - Typically, each sample solution will contain a single compound for the analysis
  - Many injections will be required. Each injection will contain a single target analyte.
- The bottle necks are:
  - MS optimization including tuning each analyte for MRM transition, cone voltage (CV), and collision energy (CE)
  - Method creation:
    MRM MS acquisition method: each analyte requires a separate MS acquisition method
    MS quantification method for post run quantification
- The instrument feasibility as well as the advantages of ESCi Multi-mode ionization have been previously demonstrated[5]. In reality, to apply ESCi in a drug discovery lab, an automated protocol is crucial to minimize the instrument time required for analysts.

Project Goal

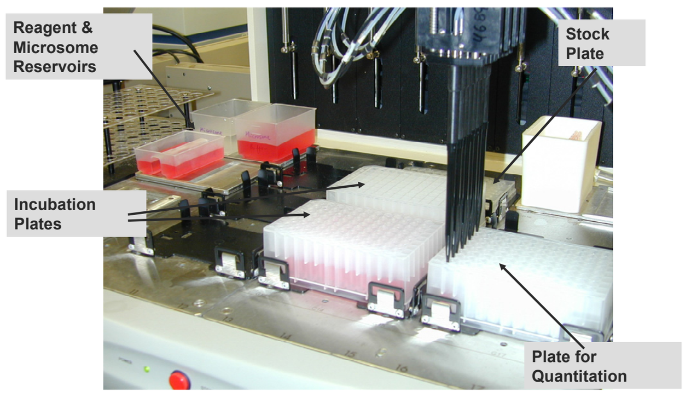
To develop an automated ESCi-LC/MS/MS analytical protocol for the *in vitro* metabolic stability test using the 96 well plate to demonstrate the advantages of ESCi multimode ionization and the Waters ACQUITY UPLC™ for higher throughput and broader compound coverage in drug discovery.

EXPERIMENTAL CONDITIONS

In-Vitro Metabolic Stability Screening

- The test method is a routine method in the Wyeth Research Lead Optimization Lab
  - Rat liver microsomes
  - Single time point incubation: 15 min
  - Physiological level: 1 μM
- Samples ready for LC/MS/MS analysis after the microsome incubation
  - Samples are in 96 well plate format
- 48 different compounds per plate, different RT and MS conditions for each compound
  - One compound per well
  - Each compound appeared in two wells for duplicated injections
  - Typically, 45 new analytes and 3 reference standards

Microsomal Incubation: Plate Layout



Di, J Biomolec Screening (2003) 8, 453
Di, J Pharm Sci (2004) 93, 1537

UPLC Conditions

- Waters® ACQUITY UPLC™ Binary Solvent Manager
- Mobile Phase:
  - A: 10 mM NH_4OAc in ACN/H_2O 10/90
  - B: 10 mM NH_4OAc in ACN/MeOH 80/20
- Column: ACQUITY UPLC™ C_{18} BEH Column, 1.7 μm, 2.1 x 50 mm, 40°C
- Sample Temperature: 5°C
- Flow Rate: 0.6 ml/min.
- Optimization: 90%B Isocratic elution
- Quantification Gradient:

Time (min.)	%A	Curve
0.0	95%	6
0.8	5%	6
1.0	5%	1
3.0	95%	1

MS Conditions

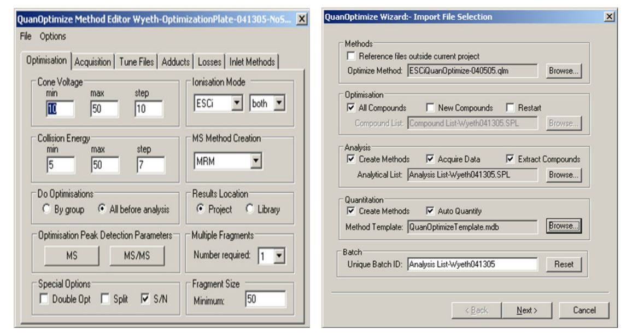
- Waters Micromass® Quattro Premier™ Triple Quadrupole MS
- Tune Page Parameters:
  - ESCi Multi-Mode Ionization enabled
  - ESI capillary voltage (kV): 3.00
  - APCI corona current (μA): 4.0
  - Source Temperature (°C): 130
  - Desolvation Temperature (°C): 420
  - Desolvation Flow (L/Hr): 980
  - Cone Gas Flow (L/Hr): 50
- Optimization and Quantification performed by QuanOptimize
  - MS and MSMS scans for optimization
  - MRM for quantification

MS OPTIMIZATION

QuanOptimize in MassLynx™

- QuanOptimize is a MassLynx optional application package. It enables a complete automatic MS quantification process.
- Once the LC conditions for the target analytes were determined, only one analyte needs to be infused into the mass spectrometer (T with the LC mobile phase at the operating LC flow rate) to optimize the tune page parameters (everything except the cone voltages for parent ions and collision energies for daughter ions).
- QuanOptimize can be set up with all necessary methods and sample lists specified, the LC/MS/MS run can be initiated. QuanOptimize will then perform the following tasks:
  - Perform MS scan and Daughter scan to obtain MS data acquisition conditions for each analyte by injecting individual standard solutions.
  - Create MS acquisition method based on the optimization injections

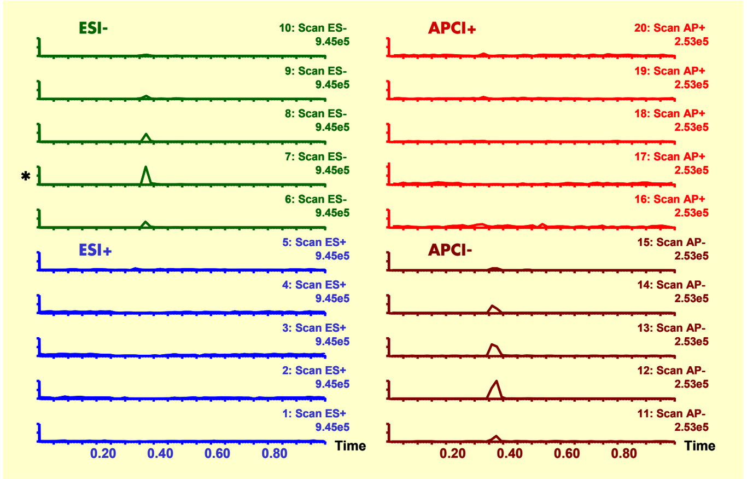
QuanOptimize Method Set Up



- Acquire quantification data for sample analysis
- Create MS quantification method for post run processing
- Complete post run quantification and generate report in QuanLynx browser

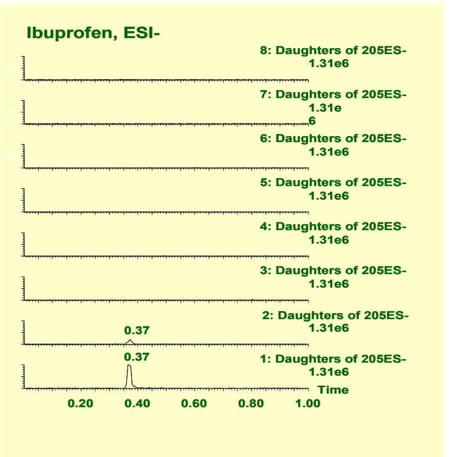
MS Scan in 4 Ionization Modes in a Single Injection

Ibuprofen, Extracted MS Chromatograms at m/z 205



ESI-
APCI+
ESI+
APCI-
10: Scan ES- 9.45e5
9: Scan ES- 9.45e5
8: Scan ES- 9.45e5
7: Scan ES- 9.45e5
6: Scan ES- 9.45e5
5: Scan ES+ 9.45e5
4: Scan ES+ 9.45e5
3: Scan ES+ 9.45e5
2: Scan ES+ 9.45e5
1: Scan ES+ 9.45e5
20: Scan AP+ 2.53e5
19: Scan AP+ 2.53e5
18: Scan AP+ 2.53e5
17: Scan AP+ 2.53e5
16: Scan AP+ 2.53e5
15: Scan AP- 2.53e5
14: Scan AP- 2.53e5
13: Scan AP- 2.53e5
12: Scan AP- 2.53e5
11: Scan AP- 2.53e5
0.20 0.40 0.60 0.80
Time

Daughter Scan with the Optimized Ionization Mode



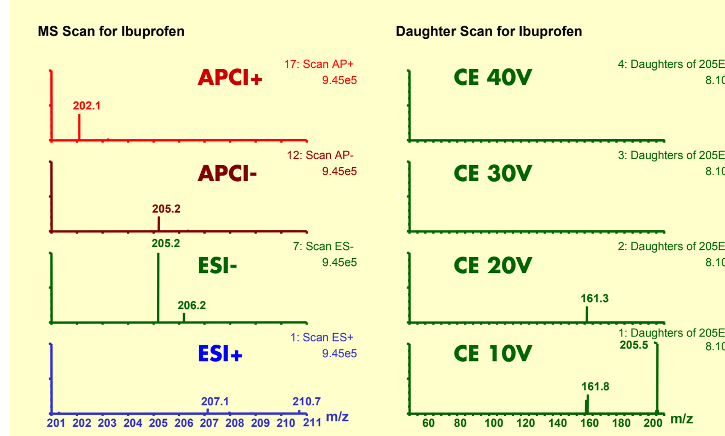
Ibuprofen, ESI-
8: Daughters of 205ES- 1.31e6
7: Daughters of 205ES- 1.31e6
6: Daughters of 205ES- 1.31e6
5: Daughters of 205ES- 1.31e6
4: Daughters of 205ES- 1.31e6
3: Daughters of 205ES- 1.31e6
2: Daughters of 205ES- 1.31e6
1: Daughters of 205ES- 1.31e6
0.37
0.37
0.20 0.40 0.60 0.80 1.00
Time

During a QuanOptimize run, two sets of samples are required per analyte: the first set is the pure standard solution at high concentration, which is to be used for the MS the scan optimizations. The second set is the calibration solutions of the analyte, they can be a mixture if the project requires the analysis of a mixture with certain concentration series. They are to be injected for the calibration.
The number of injections required per compound during the optimization stage will depend on the QuanOptimize method. For non-split optimization, there will be two injections total: The first injection is for MS scan of all four mode (ESI+/ESI-/APCI+/APCI-), and the second injection will be the daughter ion scan at the optimum ionization mode. For split optimization, there will be three injections total: The first injection is for MS scan in positive mode (ESI+/APCI+), the second injection is for MS scan in negative mode (ESI-/APCI-), and the third injection is for daughter ion scan at the optimum ionization mode.

MS Optimization Results

No	Name	MW	Mode	MRM	CV (V)	CE (V)
1	Alprazolam	308	ESP+	309.27 > 205.37	50	40
2	Amoxicillin	365	APcl+	366.38 > 126.84	20	26
3	Astemizole	458	ESP+	459.46 > 135.28	50	40
4	Atenolol	266	ESP+	267.33 > 145.40	30	26
5	Bifonazole	310	ESP+	311.33 > 243.59	10	12
6	Bupivacaine	288	ESP+	289.43 > 139.90	30	26
7	Cephalexin	347	ESP+	348.26 > 158.34	30	12
8	Chlorampehnicol	323	APcl-	322.22 > 121.30	40	40
9	Chlorpromazine	318	ESP+	319.27 > 85.79	30	19
10	Chlorzoxazone	169	ESP-	167.92 > 132.25	40	19
11	Cimetidine	252	ESP+	253.26 > 95.24	30	26
12	Clozapine	326	ESP+	327.27 > 270.57	40	19
13	Debrisoquine Sulfate	175	ESP+	176.01 > 98.45	20	12
14	Desipramine	266	ESP+	267.33 > 72.18	30	12
15	Dexamethasone	392	ESP-	390.53 > 293.62	10	40
16	Diclofenac Sodium	318	ESP+	319.18 > 163.36	10	19
17	Diltiazem	414	ESP+	415.33 > 178.12	40	26
18	Diphenhydramine	255	APcl+	256.28 > 167.49	20	12
19	Ibuprofen	206	ESP-	205.18 > 161.83	20	5
20	Imipramine	280	ESP+	281.36 > 85.76	30	19
21	Isoxicam	335	ESP-	334.25 > 270.57	30	12
22	Ketamine	237	ESP+	238.20 > 124.75	30	33
23	Labetalol	328	ESP-	327.40 > 309.78	50	19
24	Lorcainide	370	ESP+	371.33 > 125.85	40	26
25	Miconazole	416	ESP+	417.14 > 159.19	50	26
26	Midazolam	325	ESP+	326.25 > 291.58	40	26
27	Norfloxacin	319	ESP+	320.27 > 302.62	50	19
28	Nortriptyline	263	ESP+	264.33 > 90.99	30	26
29	Ofloxacin	361	ESP+	362.27 > 318.70	40	19
30	Piroxicam	331	ESP+	332.20 > 95.18	30	19
31	Prednisone	358	ESP+	359.36>147.28	30	12
32	Probenecid	285	ESP-	284.36 > 240.66	30	19
33	Promethazine	284	APcl+	285.32 > 85.83	30	19
34	Propranolol	259	ESP+	260.31 > 115.76	40	19
35	Quinidine	324	ESP+	325.33 > 81.43	50	33
36	Sufamethoxazole	253	ESP+	254.15 > 92.17	40	33
37	Tenidap	320	APCI–	319.15>276.58	20	19
38	tenoxicam	337	ESP-	336.22 > 272.54	20	12
39	Terbutaline	225	ESP+	226.20 > 152.03	30	19
40	Terfenadine	471	ESP+	472.51 > 436.92	30	26
41	Thiamphenicol	356	ESP-	355.18 > 194.70	20	33
42	Thymidine	242	ESP-	241.24 > 174.09	40	33
43	Tolbutamide	270	ESP+	271.26 > 91.25	30	33
44	Triflupromazine	352	ESP+	353.29 > 86.33	40	19
45	Wafarin	308	ESP-	307.37 > 161.18	40	19
46	Zolpidem	306	ESP+	307.92>235.16	30	36

MS and Daughter Scan Spectra



MS Scan for Ibuprofen
Daughter Scan for Ibuprofen
APCI+
17: Scan AP+ 9.45e5
202.1
APCI-
12: Scan AP- 9.45e5
205.2
ESI-
7: Scan ES- 9.45e5
205.2
206.2
ESI+
1: Scan ES+ 9.45e5
207.1
210.7
201 202 203 204 205 206 207 208 209 210 211 m/z
CE 40V
4: Daughters of 205ES- 8.10e5
CE 30V
3: Daughters of 205ES- 8.10e5
CE 20V
2: Daughters of 205ES- 8.10e5
161.3
CE 10V
1: Daughters of 205ES- 8.10e5
205.5
161.8
60 80 100 120 140 160 180 200 m/z

Example MS spectra for Ibuprofen . One in each of the 4 ionization mode.

Daughter ion scan spectra in the optimized ionization mode (ESI–) at different collision energies.

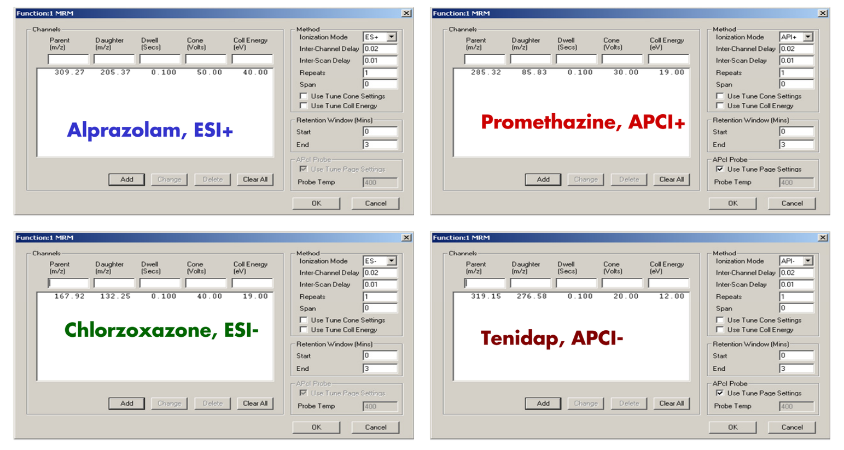
- There are a few different ways that optimization to be carried on by QuanOptimize.
- In this project, the optimization was performed this way:
  - A single injection for MS scan in all four ionization mode (ESI+/ESI-/APCI+/APCI-).
  - The optimum ionization mode was decided based on the integration of the peak area. The ionization mode and cone voltage that resulted in the highest peak area was chosen to be the optimum MS condition. In the example demonstrated, the ESI- at function 7 (CV 20) was chosen to be the optimum MS condition.
  - A second injection was made for daughter ion scan at ESI– with the MS cone voltage set at 20v.
  - The best fragment ion and collision energy was again chosen based on the integration of peak area. In the example demonstrated, collision energy of 5v and daughter ion 161 was chosen as the optimum condition.

RESULTS AND DISCUSSION

QuanOptimize ESCi Application for *in-vitro* Microsome Stability Test

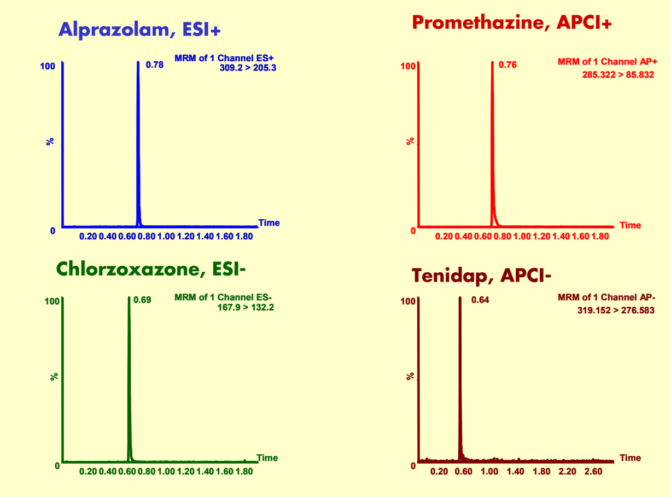
- The application of ESCi places significant demands on data acquisition of the mass spectrometer. This is specifically true for MS and MSMS scan experiments during the optimization stage.
- However, the actual requirements for any experiment are dependent on the fitness of purpose. "All factors bearing on any analysis must be consistent with the overall purpose of the task"[6].
- For ESCi MS and MSMS scan experiments, the purpose was to choose the optimum ionization mode as well as the optimum MRM parameters. This type of experiment is typically qualitative. Even though it is always better to obtain more data point across the peak, for qualitative work, a few data points across the peak should be sufficient for the purpose. As shown in the sample MS and daughter ion scan spectra, we were able to obtain spectra with sufficient quality to allow optimization to be completed automatically.
- For ESCi MRM experiments, the number of data points obtained varied depending on the number of channels to be monitored in a single injection. Less data points were obtained when more MRM channels were to be monitored, especially when ionization mode switching is required during a single injection.
- Mixture analysis can be useful for cassette dosing experiments. In drug discovery, the analytical requirement may be semi-quantitative estimation of the ADME properties. This is less demanding in figure of merit allowing more compounds to be analyzed simultaneously. Even with mixture and 4 mode simultaneous switching, Quattro Premier still has the capability for mixture analysis[5].
- For the microsomal stability test, there is no need for multi-analyte analysis. A typical experiment is carried out for a single unknown compound per well. Therefore, the need is simply to analyze a single compound per injection. With the ESCi capability, compounds in a 96 well plate can be analyzed in a single analytical batch with both ESI and APCI.

Example MRM MS Acquisition Methods



These MRM methods were generated automatically by QuanOptimize after the MS optimization was completed. The MRM ionization mode was chosen based on the integration of peak area. The scan conditions that resulted in the highest peak area were chosen to be the MRM conditions.

Analyte MRM Chromatograms



Microsome Stability Test Results

No.	Name	MW	Mode	%Remaining	Metabolic Half-life (min.)
1	Alprazolam	308	ESP+	37.06	10
2	Amoxicillin	365	APcl+	90.32	>30
3	Astemizole	458	ESP+	8.24	4
4	Atenolol	266	ESP+	91.95	>30
5	Bifonazole	310	ESP+	1.83	3
6	Bupivacaine	288	ESP+	0.03	1
7	Cephalexin	347	ESP+	66.93	26
8	Chlorampehnicol	323	APcl-	53.02	16
9	Chlorpromazine	318	ESP+	0.52	2
10	Chlorzoxazone	169	ESP-	110.42	<1
11	Cimetidine	252	ESP+	83.66	>30
12	Clozapine	326	ESP+	0.53	2
13	Debrisoquine Sulfate	175	ESP+	118.58	<1
14	Desipramine	266	ESP+	16.08	6
15	Dexamethasone	392	ESP-	29.85	9
16	Diclofenac Sodium	318	ESP+	14.09	5
17	Diltiazem	414	ESP+	3.10	3
18	Diphenhydramine	255	APcl+	9.01	4
19	Ibuprofen	206	ESP-	86.89	>30
20	Imipramine	280	ESP+	0.22	2
21	Isoxicam	335	ESP-	68.39	27
22	Ketamine	237	ESP+	0.39	2
23	Labetalol	328	ESP-	0.57	2
24	Lorcainide	370	ESP+	0.06	1
25	Miconazole	416	ESP+	9.74	4
26	Midazolam	325	ESP+	0.53	2
27	Norfloxacin	319	ESP+	105.41	<1
28	Nortriptyline	263	ESP+	2.60	3
29	Ofloxacin	361	ESP+	96.81	>30
30	Piroxicam	331	ESP+	72.96	>30
31	Prednisone	358	ESP+	76.23	2
32	Probenecid	285	ESP-	53.39	17
33	Promethazine	284	APcl+	0.13	2
34	Propranolol	259	ESP+	0.79	2
35	Quinidine	324	ESP+	2.26	3
36	Sufamethoxazole	253	ESP+	87.67	>30
37	Tenidap	320	APCI-	46.52	14
38	tenoxicam	337	ESP-	74.44	>30
39	Terbutaline	225	ESP+	98.45	>30
40	Terfenadine	471	ESP+	8.73	4
41	Thiamphenicol	356	ESP-	65.95	25
42	Thymidine	242	ESP-	66.98	26
43	Tolbutamide	270	ESP+	97.26	>30
44	Triflupromazine	352	ESP+	2.01	3
45	Wafarin	308	ESP-	63.86	23
46	Zolpidem	306	ESP+	46.52	14

- $\%Remaining = (Peak\ Area\ T_{20}/Peak\ Area\ T_0) \times 100$
- *In-Vitro* Metabolite Half-life = (ln2 x Incubation Time)/ln(%Remaining/100)

CONCLUSIONS

- We have developed an automated HT ESCi-LC/MS/MS analytical protocol for the *in vitro* metabolic stability test:
  - HT separation enabled by the Waters ACQUITY UPLC™ Technology.
  - High speed capability of the Waters Quattro Premier allowing simultaneous four mode ESCi switching during a single injection
- This method can be applied in screening a large number of compounds with high speed and covering diverse structures for drug discovery and development.
- The protocol will allow the analytical test to be run completely automated so that
  - The instrument time required for analyst is minimized
  - Some bottle necks are eliminated
  - Method transfer is made easy
  - The protocol is easily adapted into the current lab routine

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