The method for rapid detection and quantitation of amiodarone in the blood sample by LC/MS/MS was examined. Amiodarone hydrochloride is an effective anti-arrhythmia treatment medicine classified in the Vaughan Williams class III. However, as amiodarone is a lipophilic compound, the disposition patterns of amiodarone in human bodies show the great differences between individuals and TDM (therapeutic drug monitoring) is applied. In this study, following topics for the evaluation of LC/MS/MS method detecting of amiodarone or amiodarone metabolite are examined;  
I) Creating usual LC/MS/MS (MRM) detection method for amiodarone or desethylamiodarone 
II) Usage of d4-amiodarone and d4-desethylamiodarone as the internal standards 
III) Sample preparation by Dried Blood Spot (DBS) as the sample collection device 
IV) Investigation of LC separation and condition suitable for rapid routine work cycles

Creating LC/MS/MS (MRM) detection method for amiodarone or desethylamiodarone

Using the standard reagent of Amiodarone Hydrochloride, LC/MS/MS conditions are examined.
Calibration curve was created by the diluted standard solution.

[ Instruments ]
Mass Spectrometer: Agilent 6460 Triple Quad LC/MS with Agilent Jet Stream Technology
HPLC System: Agilent 1200 SL Binary Pump, Column Oven, Well Plate Autosampler
Ion Source parameters: Agilent Jet Stream Technology ESI positive mode

Optimization of instrument parameters for MRM mode acquisition was done by Agilent MassHunter Optimizer software.
In positive ESI ionization mode, m/z 646.1 were observed as [ M+H ]+ precursor ion.
Precursor ion (m/z) 646.1 Product ion (m/z) 58.1 Fragmentor voltage (V) 160 Collision energy (eV) 45

[ HPLC ]
Column: ZORBAX Eclipse plus C18 (2.1 x 50 mm, 1.8 µm) Column temp. : 50 °C
Mobile Phase; A: 10 molar ammonium formate with 0.02% formic acid B: Acetonitrile Flow rate ; 1 mL/min
Gradient ; Time (min) %B 0min (10%) – 20min (30%) – 30min (60%) – 40min (90%) – 45min(90%)
Injection Volume ; 1 µL

Standard solution of amiodarone was diluted by mobile phase A to create the calibration sample with 0.1pg/mL to 100pg/mL concentration. There is fine linearity through the range 0.1pg/mL to 100pg/mL.

Five replicated injection of 0.5pg/mL sample solution : RSD (%) = 1.05

Reference : For the sample obtained from a patient who was administrated amiodarone, above LC/MS/MS method could detect amiodarone at pg/mL order as a concentration of final sample solution. Also, LC/MS/MS (product ion scan) for desethylamiodarone could be performed to check the fragment ions. Then, desethylamiodarone could be detected with same pg/mL order by LC/MS/MS method. (data not shown.)
**Usage of d4-amiodarone and d4-desethylamiodarone as the internal standards**

Application of deuterium isotopic standards of amiodarone and desethylamiodarone are examined.

< Reagents > Amiodarone Hydrochloride, Amiodarone-d4 Hydrochloride, Desethyl Amiodarone Hydrochloride, Desethyl Amiodarone-d4 Hydrochloride were purchased from Toronto Research Chemicals Inc.

< Optimization of MS/MS conditions > Optimization of MS/MS instrument parameters for MRM mode acquisition was done by Agilent MassHunter Optimizer software.

### Sample preparation by Dried Blood Spot (DBS) as the sample collection device

Rat blood was provided of by the kindly courtesy of Department of Biophysical Chemistry, Kyoto Pharmaceutical Univ. FTA Elute MicroCards (FTA-cards) were provided by the kindly courtesy of GE Healthcare Japan Corporation.

**< Dried Blood Spot (DBS) extraction >**

Standard solutions with each concentration shown below were prepared by dilution with HPLC mobile phase A. 2 μL of internal standard solution (10 μg/mL of d4-amiodarone and d4-desethylamiodarone, respectively) and standard solution (12 concentration steps of amiodarone and desethylamiodarone) are spiked in 96 μL of rat blood. 15 μL of spiked blood was spotted on FTA-cards. FTA-cards were dried at room temperature for about 5 hours.

Optimization of MS/MS instrument parameters for MRM mode acquisition was done by Agilent MassHunter Optimizer software.

### Investigation of LC separation suitable for rapid cycles

HPLC conditions for the separation of amiodarone and desethylamiodarone are examined.

Column : ZORBAX Eclipse plus C18 (2.1 x 30 mm, 1.8 μm)
Column temp. : 50° C
Mobile Phase : A: 2 mM ammonium formate formate with 0.1% formic acid
B: 2 mM ammonium formate with 0.1% formic acid in MeOH
Flow rate : 0.8 ml/min
Injection Volume : 5 μL

Gradient profile is investigated under considerations with the separation and the peak intensities.

One example of gradient profile shown in the left chromatogram:

Time (min) 0 0.2 1.0 2.2 2.21 2.71 2.72 3.0
%B 37.5 37.5 73.5 73.5 97.5 97.5 37.5 37.5

Suppression influence is considered with the gradient profiles; differences of intensity between standard solution and matrix-added sample was observed (next page).
Suppression effect by blood extracted matrix supposed to be changed according to the gradient profile. Peak area differences between standard solution and the standard which is spiked in blank matrix to be the same concentration is compared.

Considering both intensity and suppression ratio results, gradient profile 2 Seemed to be most suitable For further quantitation work.

Suppression ratio is calculated based On the average peak area of three Replicate injections for each sample.

Investigation of LC separation suitable for rapid Cycles (cont.)

Calibration with standard solution or DBS sample

With the standard solution made up with the mobile phase A solution, there was fine linearity through Level 10 (15pg/mL) to Level 1 (300ng/mL) for both amiodarone and desethylamiodarone.

For DBS extracted standard solution there was fine linearity through Level 8 (150pg/mL=1ng/mL blood) to Level 1 (300ng/mL=2μg/mL blood) for both amiodarone and desethylamiodarone.

S/N ratio calicration is based to RMSx5.
Quantitative LC/MS/MS detection for amiodarone and desethylamiodarone was examined. With Agilent 6460 Triplequad LC/MS system, the detection limit of standard amiodarone solution was 0.1pg/mL, and the limit of quantitation was 0.5pg/mL at final concentration. Reproductivity at the quantitation limit was as good as Area%RSD=1.05 (n=5).

Usage of isotopic labeled reagents as the internal standards combined with DBS extraction could be adapted for quantitation analysis.

With the blood-spiked standard, extracted through DBS method, calibration curve showed fine linearity between 1ng/mL blood to 2ug/mL.

Further studies for precision, reproducity, will be expected.

Reference