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LTQ FT MS Accurate Mass and SRM Data Dependent Exclusion MS\textsuperscript{o} Measurements for Structure Determination of FK228 and Its Metabolites

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MS\textsuperscript{2} fragments are produced by cleavage of all protonated molecules at the lone electron pairs of heteroatoms or the π electrons of double bonds of FK228. Usually, data dependent MS\textsuperscript{2} measurement cleaves only MS\textsuperscript{2} fragment of highest intensity that normally does not contain important metabolic sites. New SRM (selected reaction monitoring) data dependent exclusion MS\textsuperscript{o} measurement makes it possible to obtain MS\textsuperscript{2} fragmentation data for all MS\textsuperscript{2} fragments, useful for structural determination of FK228 and its metabolites using ESI ion trap. The two different fragmentation pathways of FK228, of which fragment ions had the different elemental composition, were estimated. The accurate masses of FK228 fragment ions Fa1 (or Fb1) and Fa2 (or Fb2) were measured by LTQ FT MS to compare with those calculated exact masses. The fragmentation pathway of former was correct as the result. LTQ FT MS accurate mass and SRM data dependent exclusion MS\textsuperscript{o} measurements are required for the structure determination of FK228 and its metabolites.

1. Introduction

The strategy for structural determination of drug metabolites is based on theory associated with protonated molecules and MS\textsuperscript{o} fragmentation of drug metabolites in ESI Ion Trap MS\textsuperscript{1} and reactivity of unchanged drug with reactive oxygen diradicals involved in cytochrome P-450 cycles.

The strategy for structural determination of drug metabolites derived from protonated molecules and MS\textsuperscript{o} fragmentation is as follows:

1) Drugs with many lone electron pairs in heteroatoms (e.g., N, O, S, and X) and the π electrons of double and triple bonds, benzene rings, and hetero-rings characterize functional groups because of interactive pharmaceutical efficacy with biological receptors or junctions of these functional groups.

Protonation of molecules by ESI MS occurs at either the lone electron pairs of heteroatoms in Fig. 1 or the π electrons of double and triple bonds, benzene rings, and hetero-rings in Fig. 2.

Fragmentation of protonated molecules with isolated lone pair electrons and π electrons occur at many protonation sites. In contrast, protonated molecules with conjugated lone pair electrons and π electrons cleave at only a few protonation sites, at weak bonds and/or produce a few cations and neutral molecules with more stable energy levels (fragmentation pathways are shown in Figs. 1 and 2). The protonated molecular cation dissociates to produce a cation and a neutral species. Protonated positions are closely related to the positions of fragmentation. The protonated ion of a drug having heteroatoms with lone pair electrons (N, O, and S) or isolated functional groups (OH, SH, NH\textsubscript{2}, ROR, RSR, RCONH\textsubscript{2}, RCOOR, RCOR) cleaves to form cations and their complementary neutral molecules during ESI MS/MS (MS\textsuperscript{3}). In addition, allylic cleavage, benzylic cleavage,\textsuperscript{3} acetylene cleavage\textsuperscript{6} and propargyl cleavage\textsuperscript{6} are observed as fragmentation pathways of the protonated molecules with π electrons and hetero-rings during ESI MS/MS (MS\textsuperscript{3}) (Figs. 1 and 2), analogous to fragmentation of radical cations.

We measured MS\textsuperscript{o} of many drugs and their metabolites using ion trap MS and found that the mechanism of ionization and fragmentation of drug metabolites varied according to the ionization method, e.g., chemical ionization (CI), fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and atmospheric pressure ionization (API) (especially electrospray ionization (ESI)) in combination with a mass analyzer, such as Magnetic sector, TSQ, Ion Trap, TOF (time-of-flight) and FT-ICR (Fourier transform ion cyclotron resonance)\textsuperscript{5}. Fragmentation of drugs is different by ionization and analyzer of MS. Ionization of EI, FAB, and MALDI is high energy ionization and accompanies with collision. In generally, soft ionization of CI, APCI, and ESI observe only molecular related ion. In the case of using much amount of sample, in-source fragmentation is observed. Magnetic sector-type mass spectrometers and TOF mass spectrometers observe fragmentation of unstable ions during flight (meta-stable ions). These fragmentation are different from CID.

Few studies on chemical structures of protonated molecules and their fragmentation in ESI MS have
been reported. Some theories about protonated molecules and fragmentation of simple molecules have been proposed, such as amines, ketones and ethers, mono substituted cyclohexane derivatives, X-Cn-Y (X, Y: functional groups, Cn: Carbon skeleton) and xylene derivatives with methane CI MS, although they were studied between 1970–1985, before the appearance of ESI TSQ MS and ESI ion trap MS. CI MS produces [M+H]+ via the reaction (CH5++M) in gas phase. ESI MS produces the protonated molecules by 3–5 keV ionization on the way of vaporization from the solution. So pH of the solution affects the stereo conformation of the protonated molecules. Amines are basic compounds and protonation of amines depend on pH of the solution. The cleavage of protonated amines is more difficult than that of protonated alcohol. The easiness of protonation is different from the easiness of cleavage of the protonated molecules. Conjugation or localization of heteroatoms, double and triple bonds, benzene rings, and hetero-rings affect cleavage of the protonated molecules. In addition to in-source protonation, ESI ion trap MS observes the internal protonation of cleaved ions in an ion trap under acidic condition of LC mobile phase containing 0.1% formic acid.

ESI ion trap MS is possible to measure MS^n fragmentation. MS^3 is not observed by TSQ MS although MS^2 corresponds to MS/MS by TSQ MS. The protonated ion of a drug having heteroatoms with lone pair ele-

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Fig. 1. Fragmentation of protonated molecules adducting proton at lone pair electrons of heteroatoms of various functional groups of drugs.

Fig. 2. Fragmentation of protonated molecules adducting proton at π electrons of benzene rings, double and triple bonds.
trons (N, O, and S) or isolated functional groups (OH, SH, NH₂, ROR, RSR, RCONHR, RCOOR, RCOR) cleaves to form carbo-cations and their complementary neutral molecules during ESI MS/MS (MS²). MS³ is carbo-cation cleavage to produce additional carbo-cation (Fig. 3). Drugs have many heteroatoms that sometimes contribute to carbo-cation cleavage as neighboring groups to produce the onium cation. MS⁴ of the onium cation is onium cleavage and McLafferty cleavage. It is very interesting that MSⁿ (n ≥ 3) fragmentation of ESI ion trap MS looks like EI fragmentation.

Fig. 3. MSⁿ fragmentation of protonated molecules by ESI ion trap MS: MS² fragmentation is carbo-cation cleavage, MS³ fragmentations are carbo-cation cleavage, onium cleavage, McLafferty cleavage and so on as same as EI fragmentation.

Fig. 4. Reactivity of drugs with reactive diradicals of oxygen produced during a CYP reaction cycle.
bo-cation cleavage and McLafferty cleavage, though EI ionization produces radical cation (odd electron), on the contrary, protonated molecules (even electron) by ESI.

(2) On the basis of reactivity of a drug with reactive diradicals of oxygen produced during a CYP reaction cycle, the structure of metabolites can be determined, as in Fig. 4.

(3) Using the following procedure incorporating protonated molecules and MS methodology, the sub-structural connectivity of the drug and its metabolites can be estimated by ESI ion trap MS:

a. Measure the mass spectra of the unchanged drug and its metabolites.

b. Prepare a table comparing MS fragment ions of a drug with those of its metabolites.

c. Compare the spectral patterns of the drug before and after metabolism.

d. Assign the characteristic peaks of each metabolite in the mass spectrum.

e. Elucidate the structure of the metabolites.

This strategy is useful for rapid structural determination of in vitro metabolites of drugs in the early stages of testing using human and animal liver microsomes in high throughput screening.

FK228 (Fig. 5), (E)-(1S,4S,10S,21R)-7-[(Z)-ethylidene]4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraaza-bicyclo[8,7,6]tricos-16-ene-3,6,9,19,22-pentanone, was isolated as a fermentation product of Chromobacterium violaceum. FK228 is a potent histone deacetylase inhibitor under development as an anticancer agent. Recently, we described a strategy for effectively measuring drugs and small amounts of their metabolites using ESI Ion Trap MS, plus propose new methods for SRM data dependent MS measurement and SRM data dependent exclusion MS measurement. We measured the mass spectra of FK228, its in vitro metabolites, and in vivo metabolites according to SRM data dependent exclusion MS measurement and analyzed their fragmentation to determine their chemical structures. FK228 was estimated the two different fragmentation pathways of which fragment ions had the different composition of atoms. Recently, LTQ FT MS (Fig. 6) has developed and is possible to measure accurate mass spectrometry of both molecular ion and fragment ions. In this paper, we describe LTQ FT MS accurate mass measurement and SRM data dependent exclusion MS measurement for effectively measuring FK228 using ESI Ion Trap MS. As FK228 is on the way of Phase II study and its metabolites are estimated active metabolites, we can not describe their chemical structures now.

2. Materials and Methods

2.1 Chemicals

$^{14}$C-FK228 (Fig. 1) was synthesized at Amersham Biosciences UK Limited. Specific activities of $^{14}$C-FK228 of Code CFQ13065 and Code CFQ13380 were 3.53 and 1.39 MBq/mg, respectively. Radiochemical purities of both labeled compounds checked by TLC and HPLC were greater than 98% when used. FK228 (Lot No. 100118G; chemical purity, 99.6%) was synthesized at Fujisawa Pharmaceutical Co., Ltd. $^{14}$C-FR 901228 was dissolved in a mixture of methanol-acetonitrile (1:1, v/v) and stored at −80°C. Acetonitrile for HPLC was purchased from Wako Pure Chemical Industries, Ltd., and methanol for HPLC was purchased from...
Merck (Germany). β-Nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate (G-6-P), and glucose 6-phosphate dehydrogenase (G-6-P DH) were obtained from Sigma Chemical Co. (U.S.A.).

An HPLC analytical column TSKgel ODS-80T (5 μm, 4.6 mm i.d. x 150 mm), (5 μm, 2.1 mm i.d. x 150 mm), and a TSKguardgel ODS-80T cartridge guard column (3.2 mm i.d. x 15 mm) were purchased from Tosoh Corp. (Japan). Inertsil ODS-3 columns (5 μm, 4.6 mm i.d. x 150 mm and 5 μm, 10 mm i.d. x 250 mm) and a YMC ODS-AM AM-302 column (5 μm, 4.6 mm x 150 mm) were obtained from GL Sciences Inc. (Japan) and YMC Co., Ltd. (Japan), respectively.

2.2 In vitro metabolism

Seven-week-old male Sprague-Dawley strain SPF [Crj:CD(SD)IGS] rats (n = 5) were obtained from Charles River Japan Inc., and used after quarantine and acclimatization for 1 week. Body weights of animals were 270–300 g when used. The livers from 5 rats were pooled and all subsequent steps were carried out at 4°C.

The livers were homogenized with 3 volumes of 1.15 KCl using a Teflon-glass homogenizer (0.25 g tissue eq./mL). The homogenate was centrifuged at 9,000 x g for 20 min. The supernatant was centrifuged at 105,000 x g for 60 min. The resulting pellet was suspended in 1.15% KCl using a Teflon-glass homogenizer (0.25 g tissue eq./mL). The supernatant was centrifuged at 105,000 x g for 60 min. Then the resulting pellet was re-suspended in 1.15% KCl, to a concentration of 1 g tissue eq./mL, and used as microsomes.

Pooled human liver microsomes prepared from 46 different individuals were obtained from XenoTech (USA, Lot No. 0210171, 20 mg protein/mL). All liver microsomal preparations were stored at -80°C until use. An NADPH generating system (10 components) was freshly prepared with 8 mg NADP, 17 μg glucose-6-phosphate, 296 mL water, 500 mL of 0.2 M potassium phosphate buffer (pH 7.4), 200 μL of 125 mM MgCl2, and 3.4 mL (1 unit/mL) glucose-6-phosphate dehydrogenase. Drug (1–5 mg) was dissolved in methanol–acetonitrile (1 : 1) solution to produce 10 mM solution. A 5 μL aliquot was added to 45 mL of 50% acetonitrile to produce 1 mM solution.

To each of 10 mL round-bottom glass tube (total 6 tubes), 400 μL of 500 mmol/L potassium phosphate buffer (pH 7.4), 20 μL of 6 mmol/L 14C-FK228, 200 μL of human liver microsomes (20 mg protein/mL), 200 μL of the NADPH-generating system, and 1,180 μL of water were added to make a total volume of 2 mL. After incubation, 2 mL of a mixture of methanol–acetonitrile (1 : 1, v/v) was added to each reaction tube and mixed well. The sample was centrifuged at 12,000 rpm for 2 min. The supernatant was collected and evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 50% acetonitrile. The solution was injected onto HPLC. The column eluate was collected using a fraction collector (Retriever IV, Isco, USA). A portion of each fraction was collected to a pony vial (Perkin...
Elmer Life Sciences) and mixed with 4 mL of Hionic-Fluor (Perkin Elmer Life Sciences) to measure the radioactivity. Each fraction was stored at −80°C.

At least 10 kinds of metabolites were formed by liver microsomes of various animal species, including humans. A recent study demonstrated that reduction of an intramolecular disulfide bond of FK228 greatly enhanced its inhibitory activity and that the disulfide bond was rapidly reduced in cells by glutathione (GSH). Moreover, FK228 has been found to undergo conjugation with GSH by incubation with rat or human plasma in the presence of GSH. These results suggest that GSH play an important role in the metabolism and antitumor activity of FK228. Chemical structures of FK228 metabolites formed by in vitro systems were investigated using rat and human liver subcellular preparations. Additionally, structural elucidation of radioactive metabolites in the bile from rats dosed intravenously with 14C-FK228 was also conducted. Because isolation of the large amounts of metabolites seemed to be difficult, especially in vivo, LC/electrospray ionization multi-stage mass spectrometry (LC/MSn) was utilized to the analytical technique. We measured the mass spectra of in vitro 14 metabolites of FK228 according to SRM data dependent exclusion MSn measurement and analyzed their fragmentation to determine their chemical structures.

2.3 In vivo metabolism

After intravenous administration of 14C-FK228 to bile-duct cannulated male rats at a dose of 0.3 mg/kg, the excretion of radioactivity in the bile was 66% of the dose, up to 48 hr after dosing. Biliary excretion of unchanged FK228 accounted for 3% of the administered radioactivity and more than 30 kinds of metabolites were detected in the bile, indicating that FK228 is extensively metabolized in vivo. We measured the mass spectra of in vivo 8 metabolites according to SRM data dependent exclusion MSn measurement and analyzed their fragmentation to determine their chemical structures.

2.4 Measurement of LC MS

Electrospray ionization (ESI) MSn study was performed on a Thermo-Finnigan LCQ Deca ion trap mass spectrometer fitted with an ESI source (San Jose, CA). The electrospray voltage and heated capillary temperature were set at 4.5 kV and 250°C (unstable metabolites at 200°C), respectively. The sheath gas (N2) pressure was 70 psi with an auxiliary gas (N2) flow of 0–7 units, a collision gas (He) pressure of 2 mTorr and a Fig. 8. Mass chromatogram of FK228 at m/z 541 (upper row), mass spectra of MS² fragment ions at m/z 541 (second row), and MS³ fragment ions (third row) by SRM (m/z 541) data dependent exclusion MSn measurement using infusion ESI ion trap MS.
suitable collision offset voltage for each metabolite, ranging from 25 to 40%. The positive ion mode was used. FK228 and its metabolites were measured using a Magic 2002 HPLC system (MRS Japan, Tokyo, Japan) equipped with LC Column (TSKgel ODS-80TM (5 μm, 2.1 mm I.D. × 150 mm)) (flow rate: 200 μL/min, gradient system (Mobile phase A: 20 mmol/L acetic acid–ammonium acetate buffer (pH 4.0)) and B: acetonitrile, % of
mobile phase B; 2% for 0–1 min, 2–45% for 1–35 min, 45–80% for 35–40 min, 80% for 40–45 min, and 80–2% for 45–45.1 min) and connected with an electrospray ionization (ESI) interface. Samples were injected by Microsyringe injector.

**Accurate mass determination** was performed using a Thermo electron LTQ-FT MS (Thermo electron, Bremen) equipped with LC/ESI interface (The electrospray voltage and heated capillary temperature were set at 4.5 kV and 320°C, respectively). The sheath gas (N₂) pressure was 40 psi with an auxiliary gas (N₂) flow of 6 units, a collision gas (He) pressure of 2 mTorr and a suitable collision offset voltage 35%). The positive ion mode was used. Before accurate mass measurement, LTQ FT MS was tuned automatically using Ultrimark as internal standard. Accurate mass was measured using outside standards.

**The data dependent MS* measurement** was programmed using 5 times data dependent CID (collision induced dissociation) measurement to cleave automatically the highest intensity peak at each scan. The higher peaks of biological matrix inhibit usually the data dependent MS* measurement of metabolites and an unchanged drug. In this case, we can find metabolites by scanning the mass chromatogram.

**The SRM data dependent MS* measurement** was programmed using the first scan at the selected m/z ions of the protonated molecules, the second CID scan at the selected m/z ions of the protonated molecules, and then 3 times data dependent CID measurement to cleave automatically only one fragment of the protonated molecule with the highest intensity.

**The SRM data dependent exclusion MS* measurement** was programmed using the first scan at the selected m/z ions, the second MS/MS scan at the selected m/z ions of the protonated molecules, and then 3 times data dependent exclusion measurement to cleave automatically the all fragment of the protonated molecules. The SRM data dependent MS* measurement and the SRM data dependent exclusion MS* measurement were established for the structure determination of metabolites and an unchanged drug using MS* fragmentation in an ESI Ion Trap.19, 25)

### 3. Results and Discussion

#### 3.1 New SRM data dependent exclusion measurement of FK228 and its metabolites for structural determination using LC/ESI/Ion Trap MS

We established the new SRM data dependent exclusion MS* measurement for structure determination of FK228 and its metabolites according to the following steps:

1. At first, we measured usual data dependent MS* measurement of the in vitro metabolites.
2. We searched the estimated m/z of metabolites by mass chromatography.

![Fig. 10. Two possible fragmentation pathways of FK228.](image-url)
3) We programmed the automatic SRM estimated m/z of metabolites and then data dependent exclusion (Repeat Count 1, Repeat Duration 0.1 minutes, Exclusion Duration 0.3 minutes, Exclusion Mass Width 3.0) MS^n measurement to obtain MS^3 fragmentation data of all MS^2 fragments of FK228.

4) We measured new SRM data dependent exclusion MS^n measurement.

5) We compared variously data obtained by both measurements to determine the structure of metabolites.

In the case of infusion MS measurement, we can manually set each m/z of MS^2 fragments and measure MS^3 many times, something that is impossible for LC/MS measurement. Automatic measurement of all MS^2 and MS^3 fragments would be most useful for elucidating the many components of the chemical structure of metabolites. In general, the exclusion measurement is used to avoid m/z of the interfere peaks and the data dependent MS/MS exclusion measurement is used for only MS^n measurement of the mixture having the same retention time of LC/MS. We attempted to adopt the data dependent MS/MS exclusion scan to measure MS 3 fragmentation of all MS^2 fragments. SRM data dependent exclusion MS^n measurement using the SRM MS^2 scan program and then data dependent exclusion MS^n scan enable automatic analysis of all MS^2 and MS^3 fragments using LC/ESI/Ion Trap MS.

1) Data dependent MS^n measurement

As a first, usual basic measurement, data dependent MS^n measurement is used for analysis of FK228 and its metabolites in the supernatant after centrifugation of in vitro metabolism reaction mixture, using LC/ESI/Ion Trap MS. Although the total ion chromatogram (TIC) (regular mode) shows no peak when using 1 mM substrate, many peaks from metabolites and the biological matrix were observed in the base peak chromatogram. Fragmentation of unchanged drug had previously been characterized before beginning the metabolism study. We checked each peak against characteristic fragmentation of the parent drug to characterize metabolites. Most large peaks showed no characteristic fragmentation because of interference by the biological matrix. Metabolite structure can be determined using m/z of the metabolites. However, in the case of metabolites with lower intensity than the biological matrix at the same retention time in LC, the next step of SRM data dependent MS^n measurement is necessary for structural characterization of FK228 and its metabolites.

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Fig. 11. LTQ FT MS accurate mass spectrum of FK228 fragment ion Fa1.

Fig. 12. LTQ FT MS accurate mass spectrum of FK228 fragment ion Fa2 using different external standards.
2) SRM data dependent MS\textsuperscript{n} measurement

Data dependent MS\textsuperscript{n} measurement generally measures MS/MS at m/z of the highest intensity ion peak (base ion peak). Sometimes metabolites do not have the highest intensity ion peak at their retention time, when using LC/ESI/MS and do not produce MS\textsuperscript{n} fragmentation data, although the mass chromatogram shows the possibility of presence of metabolites. SRM data dependent MS\textsuperscript{n} measurement using the first SRM scan program and then data dependent MS\textsuperscript{n} scanning allows analysis of the in vitro metabolism sample, producing MS\textsuperscript{n} fragmentation of metabolites for structural determination with LC/ESI/Ion Trap MS.

3) SRM data dependent exclusion MS\textsuperscript{n} measurement

SRM data dependent exclusion MS\textsuperscript{n} measurement produces MS\textsuperscript{n} fragmentation data for all MS\textsuperscript{m} fragments that is useful for structural determination of drug metabolites using ESI ion trap, as MS\textsuperscript{n} fragments are produced by cleavage of all protonated molecules at the lone electron pairs of heteroatoms or π electrons of double and triple bonds, benzene rings and hetero-rings of drugs. Usually, data dependent MS\textsuperscript{n} measurement cleaves only one MS\textsuperscript{n} fragment of the highest intensity that does not contain metabolic sites. Comparing the MS\textsuperscript{n} fragments between metabolites and parent drug, the different MS\textsuperscript{n} fragments are related to metabolism and their MS\textsuperscript{n} data are meaningful for characterizing metabolic sites. Examining both MS\textsuperscript{n} and MS\textsuperscript{n} fragmentation data of drug metabolites enables structural determination.

3.2 FK228

The base ion and m/z 541 mass chromatogram of FK228 are observed in the supernatant after centrifugation of in vitro metabolism reaction mixture by usual data dependent MS\textsuperscript{n} measurement using LC/ESI/Ion Trap MS, but the characteristic fragment ions of FK228 were not detected. SRM data dependent MS\textsuperscript{n} measurement of observed the protonated molecule of FK228 at m/z 541 ion by ESI ion trap mass spectrometer (Fig. 7). Collision-induced dissociation (CID) of FK228 cleaved between the ester bond at \(\text{C}^2\)-\(\text{C}^3\) and \(\text{C}^3\)-\(\text{C}^4\), \(\text{N}^5\)-\(\text{C}^6\) (main) and \(\text{N}^6\)-\(\text{C}^7\) to produce m/z 523, 495, 442, 424, and 341 ions accompanying dehydration almost. The numbering and notation system of FK228 is indicated in Fig. 5.\textsuperscript{4}

MS\textsuperscript{n} fragmentation of FK228 was studied using data dependent MS\textsuperscript{n} measurement, SRM data dependent MS\textsuperscript{n} measurement (Fig. 7) and SRM data dependent exclusion MS\textsuperscript{n} measurement (Fig. 8). When subjected to SRM data dependent MS\textsuperscript{n} measurement (Fig. 7), MS 2 scan at m/z 541 ion (protonated molecules of FK228) detected 3 ions at m/z 523 (5\%), m/z 424 (100\%), and m/z 341 (40\%). MS\textsuperscript{3} scan at m/z 424 detected 2 ions at m/z 272 (100\%) and m/z 244 (60\%). MS\textsuperscript{4} scan at m/z 272 detected only one ion at m/z 244 (100\%). MS\textsuperscript{5} scan at m/z 244 detected 2 ions at m/z 180 (100\%), and m/z 173 (75\%).

When subjected to SRM data dependent exclusion MS\textsuperscript{n} measurement (Fig. 8), the expanded mass chromatogram of FK228 at the upper row show the retention time for 0.6~0.8 minutes period and the decrease of ion intensity by SRM at m/z 541 and data dependent exclusion MS\textsuperscript{n} measurement (the retention time of the first column at 4.80 min, the second column at 4.87 min, and the third column at 4.95 min). The each column of the second row shows the same mass spectra of MS\textsuperscript{n} fragment ions at m/z 541. The each column of the third row shows the different MS\textsuperscript{n} fragment ions at the different MS\textsuperscript{n} fragment ions (the first column at m/z 424, the second column at m/z 341 and the third column at m/z 302 by SRM (m/z 541)) data dependent exclusion MS\textsuperscript{n} measurement using Infusion ESI ion trap MS.

SRM data dependent MS\textsuperscript{n} measurement of the supernatant after in vitro metabolism reaction gave same data as that of data dependent MS\textsuperscript{n} measurement of FK228. SRM data dependent exclusion MS\textsuperscript{n} measurement (black arrow) gave more data than those of SRM data dependent MS\textsuperscript{n} measurement (white arrow) described in Fig. 9. Manual MS\textsuperscript{n} measurement by infusion MS gave same data as those of SRM data dependent exclusion MS\textsuperscript{n} measurement, but Manual MS\textsuperscript{n} measurement by LC/MS is impossible to use. SRM data dependent exclusion MS\textsuperscript{n} measurement is possible to combine with infusion MS and LC/MS to determine the structure of FK228 and its metabolites. MS\textsuperscript{n} fragmentation of FK228 is base to compare with that of FK228 metabolites.

We measured the mass spectra of FK228, its in vitro 14 metabolites, and in vivo 8 metabolites according to SRM data dependent exclusion MS\textsuperscript{n} measurement and analyzed their fragmentation to determine their chemical structures.\textsuperscript{25} As FK228 is on the way of Phase II study and its metabolites are estimated active metabolites, we can not describe their chemical structures now.

3.3 LTQ FT MS accurate mass measurement of FK228 fragment ions for structure determination

The two different fragmentation pathways of FK228 (Fa1~5 (or Fb1~5) in Fig. 10), of which fragment ions had the different elemental composition (Fig. 10 and Table 1), were estimated. Recently, LTQ FT MS (Fig. 6) has developed and is possible to measure accurate mass spectrometry of both molecular ion and fragment ions. The exact masses of FK228 fragment ions Fa1 (or Fb1) and Fa2 (or Fb2) in Fig. 10 were measured by LTQ FT MS.

### Table 1. The Calculated Exact Mass of FK228 Fragment Ions Fa1, Fb1, Fa2, and Fb2

<table>
<thead>
<tr>
<th></th>
<th>C 12</th>
<th>H 1.0078</th>
<th>N 14.0031</th>
<th>O 15.9949</th>
<th>S 31.9721</th>
<th>Total</th>
<th>Difference</th>
<th>PPM</th>
</tr>
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<tr>
<td>Fa1</td>
<td>C\textsubscript{9}H\textsubscript{10}N\textsubscript{2}O\textsubscript{3}</td>
<td>144</td>
<td>18.1404</td>
<td>14.0031</td>
<td>31.9898</td>
<td>63.9442</td>
<td>272.0775</td>
<td>0.0415</td>
</tr>
<tr>
<td>Fb1</td>
<td>C\textsubscript{9}H\textsubscript{10}N\textsubscript{2}O\textsubscript{3}S</td>
<td>144</td>
<td>20.1560</td>
<td>28.0062</td>
<td>47.9847</td>
<td>31.9721</td>
<td>272.1190</td>
<td>0.0415</td>
</tr>
<tr>
<td>Fa2</td>
<td>C\textsubscript{9}H\textsubscript{10}N\textsubscript{2}O\textsubscript{3}S</td>
<td>132</td>
<td>18.1404</td>
<td>14.0031</td>
<td>31.9898</td>
<td>63.9442</td>
<td>244.0826</td>
<td>0.0415</td>
</tr>
<tr>
<td>Fb2</td>
<td>C\textsubscript{9}H\textsubscript{10}N\textsubscript{2}O\textsubscript{3}S</td>
<td>132</td>
<td>20.1560</td>
<td>28.0062</td>
<td>31.9898</td>
<td>31.9721</td>
<td>244.1241</td>
<td>0.0415</td>
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FT MS to compare with those calculated exact masses (Table 1).

The calculated exact masses of Fa1 and Fb1 are 272.0775 and 272.1190, respectively. The measured accurate mass of the fragment ion was 272.0775 as shown in Fig. 11 and coincided with the calculated exact masses of Fa1 completely. The calculated exact masses of Fa2 and Fb2 are 244.0826 and 244.1241, respectively. The measured accurate mass of the fragment ion was 244.0821 or 244.0824 using different external standards in Fig.12 and coincided with the calculated exact masses of Fa1. The fragmentation pathway of formers of Fig. 10 was correct as the result.

LTQ FT MS accurate mass was required to determine for structure of FK228 and its metabolites.

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References

Keywords: Protonated molecules, MS* Fragmentation, Data dependent measurement, ESI MS, Drug metabolism, Structural determination