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Investigation of low-abundant *in vitro* metabolites of stable isotope-labelled BAL4815 by accurate mass capillary-LC-ESI-qTof-MS and MS/MS

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The metabolic profile of BAL4815, an antifungal azole drug, was determined using *in vitro* rat hepatocyte incubations and subsequent analysis by capillary LC-qTof-MS and MS/MS including accurate mass determination. For the detection of the metabolites, a mixture of the drug and its deuterium-labelled analogue was used for incubations. Metabolic stability of BAL4815 was high in cultured rat hepatocytes. However, several low-abundant metabolites were detected by the use of capillary LC-qTof-MS and manual investigation of the data. The peak intensity of the most abundant metabolite was close to the limit of detection. Except for an apparent oxidation product, the masses of the other detected metabolites could not be assigned to a single and frequently occurring biotransformation. Accurate mass determination and possible elemental compositions suggested that metabolism occurred through a combination of glutathionylation and defluorination. This was verified using accurate mass MS/MS. The use of accurate mass measurements and the derived suggestions for the elemental compositions were essential to elucidate this atypical metabolic pathway. A mass accuracy better than 8 ppm could be achieved for most assigned MS and MS/MS signals with intensities less than 6 cps in the spectra. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: glutathione; accurate mass; metabolism; mass spectrometry; electrospray; azole

INTRODUCTION

Metabolic profiling provides insight into functional biology and is therefore used as a powerful tool in early drug development.[1] The antifungal azole BAL4815 is mainly eliminated in bile. Therefore, to investigate the metabolic profile of BAL4815 *in vitro* assays were performed in which various drug concentrations were incubated in the presence of rat hepatocytes. These assays represent a widely used relevant model of liver metabolism.[2]

Following incubation, metabolite assay normally entails two steps: (1) to identify the tentative metabolites in complex incubation mixtures or biofluids ('metabolite spotting') and (2) to elucidate their structure.

These biological fluids of *in vivo* samples or *in vitro* incubations are highly complex and also change their pattern over the incubation time. In particular for non-abundant metabolites, it is mostly a challenge to distinguish the significant signals of the metabolites from the matrix background. A specific indicator is required to help spot the significant drug related metabolites in this complex dataset.

Various strategies and possibilities have been successfully used to spot abundant metabolites. However, methodologies for the characterization of low-abundant metabolites derived from highly stable drugs are often limited. The determination and identification of low-abundant metabolites is important, in particular, for low intrinsic clearance drugs with formation of minor metabolites. However, despite the low abundance of the metabolites, it is essential to demonstrate that the metabolites formed in humans were also investigated in preclinical toxicological studies, e.g. in the toxicological species.

Use of radioactively labelled drugs is often the most sensitive approach for metabolite spotting, provided that radioactive drug analogue is available and depending on the isotope that is chosen.[3] On-line and off-line radioactivity detection coupled to LC is independent of possible ionizability by MS and delivers a direct quantitative result. However, this technique does not reveal the metabolite structure and a further analytical technique, usually MS detection, is required for this purpose.[3–5]

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Mass spectrometry, in particular, selective scan-modes coupled to HPLC for different Phase II conjugates,[6–8] or a directed search for pre-calculated, tentatively expected metabolites, can be used to find metabolites.[9] For this purpose, the comparison to suitable blank incubations is essential. Generally, however, these techniques are useful only in identifying the metabolic products of expected biotransformations.

Stable isotope-labelled drug offers an elegant alternative to earlier techniques.[6] This approach entails the investigation of MS spectra to elucidate an expected and unique isotopic pattern, and is particularly helpful if biotransformations are complex, e.g. single and evident biotransformations are combined to effect a complex structural change.

Deuterated drug analogues are mostly available quite early during drug development. Therefore, the use of deuterium-labelled drug analogues is highly interesting and wildly applicable for the elucidation of the metabolic pattern in hepatocyte and microsome incubations.

Various software packages are available for spotting compounds having a characteristic isotopic pattern.

A final structural elucidation normally requires the use of NMR, but may be impractical if drug stability is high and sample quantities are limited. In these cases, high-resolution MS and MS/MS techniques in combination with capillary LC can determine the metabolite structure. Combination of capillary LC and qTof mass spectrometry is well suited to this approach,[10–12] in particular, if from the extracted accurate mass, suggestions for the elemental compositions of atypical or complex biotransformations are accessible.[13,14]

This report describes the use of these techniques in the investigation of the metabolic profile of BAL4815, a new broad-spectrum antifungal azole drug, after *in vitro* metabolization degradation in rat hepatocytes.

EXPERIMENTAL

Rat hepatocytes incubation

Hepatocytes were freshly isolated from the liver of adult male Wistar rats via different cannulation and perfusion steps. Cells were seeded in collagen coated 24 well plates (Biocoat, Becton & Dickinson) containing 500 µl Medium I, which consisted of 500 ml William's E (Sigma), 10 ml fetal calf serum (Gibco), 0.2 ml insulin (10 mg/ml, Sigma), 2.5 ml streptomycin/penicillin (Gibco) and 1 ml glutamine (200 mM; Gibco).

Approximately 2×10^5 hepatocytes were transferred into each well and allowed to attach over a 2 h period. After that Medium I was discarded and 200 µl Medium II consisting of 500 ml William's E, 0.2 ml insulin, 2.5 ml streptomycin/penicillin, 1 ml 200 mM glutamine, 100 µl hydrocortisone (0.24 mg/ml) and 50 ml fetal calf serum containing 1 mM, 10 mM and 100 mM BAL4815 was added to the respective wells. Diclofenac (10 mM, Sigma) and midazolam (10 mM, B. Braun) were used as controls. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Hepatocytes were exposed to the test compounds up to 60 min. After collecting the supernatant by aspiration, 200 µl MeOH/water (1 : 1) was added to each well, the cell monolayer was scraped off, combined with the supernatant and transferred into a 96 well plate. To assure the chemical stability of the test compounds, spiked Medium II was incubated without any liver cells over 60 min under the same conditions as mentioned above.

Sample preparation

For the incubations, BAL4815 was used as a mixture of the standard (H_4) and fourfold-deuterated (D_4) form in the ratio 1 : 1. Concentrations in this study relate the sum of both forms. Stopped incubations were centrifuged at 14 000 rps at 4 °C for 10 min. The supernatant was dried under a nitrogen flow at 40 °C and resolved in 40% acetonitrile. An incubation volume of about 100 µl was resolved in 25 µl. Thus, a concentration of factor four could be achieved. This solution was again centrifuged for 20 min at 14 000 rps at 4 °C and analyzed by the described LC-MS method without further sample-treatment.

Capillary LC

Samples were analyzed by capillary LC-MS using the CapLC (Waters AG). Mobile phase A consists of 0.1% formic acid in 3% acetonitrile and mobile phase B consists of 0.1% formic acid in 98% acetonitrile. Separation was achieved on a X-terra column, 15 cm, 300 µm ID, 3 µm (Waters AG). One microlitre was injected using the micro pickup-injection mode. A linear gradient starting at 10% B to 95% B over 30 min was applied.

Mass spectrometry

Mass spectrometric detection was carried out on a qTof Ultima system equipped with a nano-Lock spray interface (Waters AG). Erythromycin was used as m/z reference compound in the reference channel. All data shown in this study were obtained in the positive ESI mode. A capillary voltage of +1500 V and a source block temperature of 80 °C were applied. The spray tips were produced in-house by pulling fused silica capillaries.

RESULTS AND DISCUSSION

Strategy

Analysis of tentative metabolites of BAL4815 was done using a non-radioactive, stable isotope-labelled drug. BAL4815 was accessible as analogue carrying four deuterium atoms (D_4). For the experiments, a mixture (1 : 1) of the H_4 and the D_4 from of BAL4815 was used. This mixture results in a highly characteristic isotopic pattern. Structure of BAL4815 and the +ESI-MS spectrum of the used isotope-mixture are given in Fig. 1.

This isotopic pattern was also expected to be found for the metabolites, at least for those, which contain the isotope-labelled aromatic part of the molecule. Therefore, the strategy was to spot the appearance of compounds in the incubations, which possessed this expected isotopic pattern. The MS/MS spectrum of the H_4 BAL4815 is also given in Fig. 1. The ions, which are marked by *, were shifted by +4 Da when selecting the D_4 form for MS/MS. With the used precursor window, it was not possible to fragment H_4 and D_4 simultaneously.

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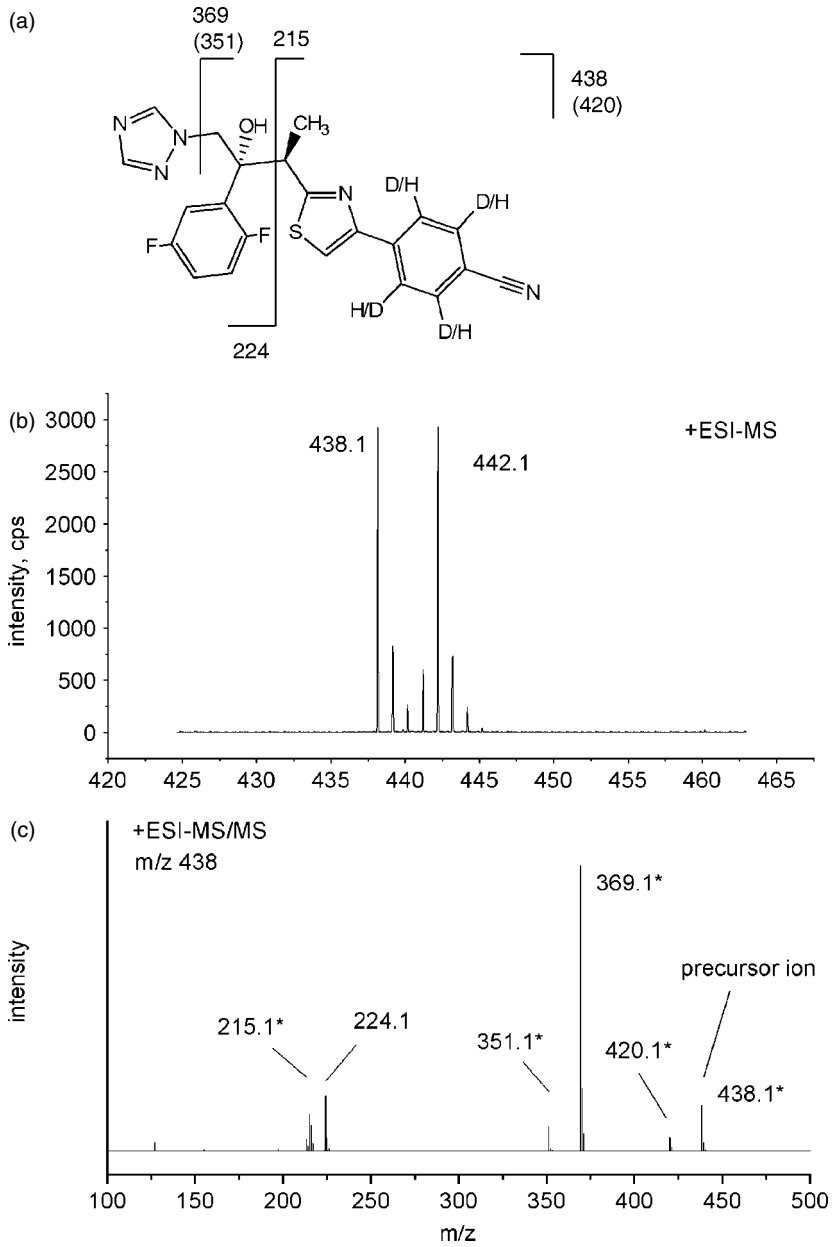

Figure 1. BAL4815 was accessible as hydrogen (H_4) and deuterium (D_4) analogue (a). For the incubations, a mixture of the two forms was used in the ratio 1 : 1, resulting in the characteristic and unique isotopic pattern measured using +ESI-MS (b); corresponding MS/MS spectrum of the H_4 form (c). Fragmentation scheme of BAL4815, m/z values are annotated for the H_4 variant, water loss −18 Da in brackets (a). Peaks that are also shifted by +4 Da when selecting the D_4 analogue are marked by *.

Quantitative estimations

Samples were prepared with drug concentrations of 1, 10 and 100 μM and incubation times from 0 min (blank) up to 24 hours. All samples were analyzed by the above-mentioned LC-MS method. For the analysis of the 1 μM solutions, the sensitivity of the LC-assay was not sufficient even with the described pre-concentration steps. At concentrations of 100 μM no metabolites were found, since for this high substrate concentrations, inhibition of the enzymes can be expected. Therefore, all data shown here are generated from the 10 μM incubations. With respect to the maximum number of metabolites occurring, samples incubated for 24 hours were chosen for further analysis.

All spotted metabolites were found in such low intensities that the processing of the raw-data had to be done manually even though software tools are commercially available for this purpose. The peak top intensity for the most abundant metabolite measured (at m/z 741) in this study was about 5 counts for a 1.5-s spectrum. The particular spectrum is given in Fig. 2(a).

For this single 1.5-s spectrum, the characteristic isotopic pattern is difficult to recognize. After combination of all peak-generating spectra and smoothing, the expected isotopic pattern is more pronounced (Fig. 2(b)). This particular spectrum demonstrates that the use of the highly sensitive capillary LC is needed for the elucidation of those metabolites. For equivalent measurements using a large bore HPLC coupled to MS, no significant signals could be expected.

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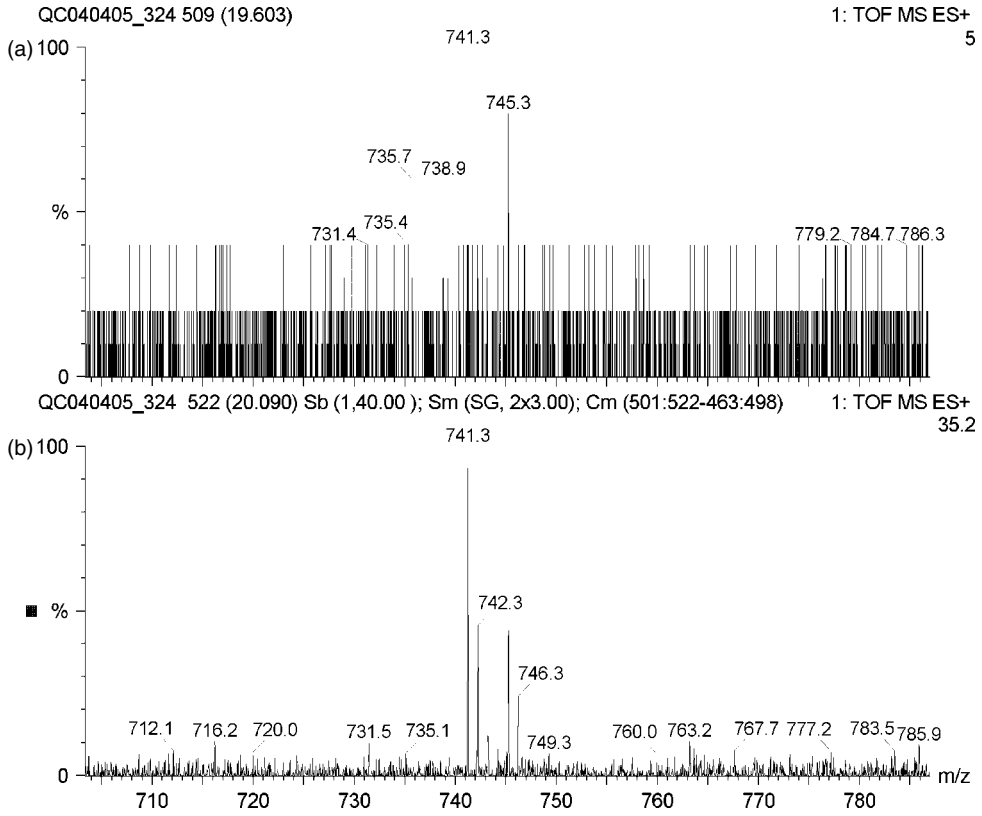

Figure 2. +ESI-MS spectrum of the most abundant metabolite (glutathione conjugate) at the highest intensity, 5 counts/1.5 s (a). Related combined and smoothed spectrum of the compound (b).

LC-MS with accurate *m/z* determination

Incubations were analyzed using the abovementioned procedure. For all spotted compounds showing the expected isotopic pattern, the reconstructed ion traces are displayed in Fig. 3.

Used m/z range for the trace reconstruction was chosen as ±50 mDa. For a wider window, no LC peaks can be found from the background noise. All putative metabolites elute earlier, at more hydrophilic retention times. The peak top intensities of the metabolites ranged between 1.5 and 4 counts/1.5 s. Extracted spectra (combined over the LC peak, background subtracted and smoothed) are given on the left-hand side of the respective ion trace in Fig. 3. After data processing, MS peak intensities between 10 and 30 counts could be found. The metabolite at m/z 454 seems to correlate to oxidation. This trace shows a second peak at about 14 min. The particular component does not show the expected isotopic pattern in the extracted MS spectrum. Except for this evident biotransformation, no other occurring peaks of metabolites could be assigned to an evident and frequently occurring biotransformation.

Therefore, it was chosen to extract the accurate masses from the MS data for the suggestion of elemental composition for these metabolites. The data are summarized in Table 1.

Starting from an oxidation (metabolite at m/z 454), BAL4815 is principally conjugated to glutathione (SG). This SG-adduct is degraded classically into cysteine (SCys) and acetylcysteine (SCysAc) adducts. But based on the suggested elemental compositions, in these conjugated metabolites one fluorine atom is replaced by an −OH group. This shift results in a unique mass difference of −1.9957 Da (+OH: 17.0027 Da/−F: 18.9984 Da). In this case the accurate mass difference is essential, since an oxidation with subsequent

Table 1. Summary of the spotted MS signals that show the expected isotopic pattern. An interpretation is given based on the measured accurate masses. For several of the metabolites, a loss of fluoride in the elemental composition and an additional hydroxy group compared to the standard glutathione pathway was proposed. For compound assignment, see Fig. 3

Compound (RT, min)	Sugg. elem. composition	Mass cal. Da	Mass found Dev. Da	ppm	Assignment
27.5 min	$C_{22}H_{17}F_2N_5OS$	437.1122	437.1142	4	Drug
24.4 min	$C_{22}H_{17}F_2N_5O_2S$	453.1071	453.1121	11	Oxidation
19.7 min	$C_{32}H_{33}FN_8O_8S_2$	740.1847	740.1895	6	F/OH, glutathione
22.8 min	$C_{27}H_{25}FN_6O_5S_2$	596.1312	596.1340	5	F/OH, acetylcysteine
19.6 min	$C_{25}H_{23}FN_6O_4S_2$	554.1206	554.1190	3	F/OH, cysteine

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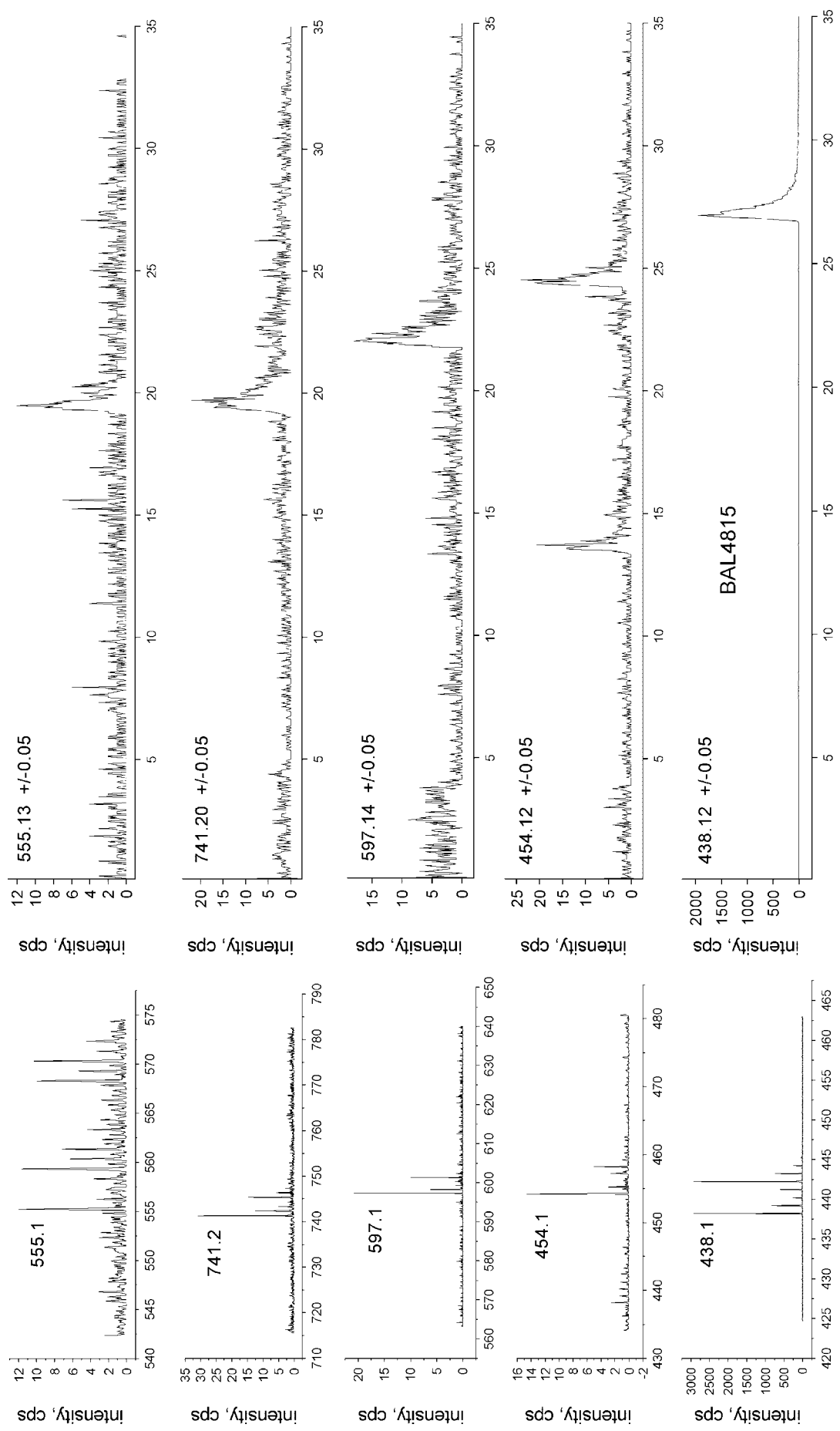

Figure 3. Right: Reconstructed ion traces (+ESI-MS) of all eluting compounds that showed the expected characteristic isotopic pattern. On the left corresponding to each compound: the extracted (combined and smoothed) +ESI-MS spectrum of the compounds.

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water elimination might result in a difference of −2.0157 Da (+O: 15.9949 Da/−H_2O: 18.0106 Da).

All spectra on the left-hand side are extracted from the same experiment. The intense signal of BAL4815 clearly shows the 1:1 ratio of the H_4 and D_4 form. This ratio is changed for the MS spectra of the metabolites. The H_4 form for all the observed metabolites is more pronounced. A preference during biological transformation cannot be excluded, but seems to be less probable. It can be assumed that this ratio is mostly due to instrumental limitations. At this low-intensity level, it is hardly possible to extract accurate intensity ratios. Regarding Fig. 2(a), our intensity is at the limit of detection system. Additionally, the D_4 form of BAL4815 elutes in a broader LC peak compared to the H_4 one. Nevertheless, an unequivocal explanation of this effect cannot be derived from our data.

To verify the SG conjugation, further MS/MS experiments were performed.

LC-MS/MS with accurate *m/z* determination

Occurring metabolites were analyzed by LC-MS/MS. For this purpose only, the H_4 – isotope was selected as the precursor ion. The suggested SG pathway could be confirmed. Figure 4 shows the results for the most abundant metabolite at *m/z* 741.

The expected fragmentation pattern could be assigned to the suggested structure. Above all assigned MS/MS peaks, the *m/z* deviation from the calculated value is given.

The pronounced fragmentation sites for BAL4815 (Fig. 1) were also found at its SG conjugate. But the main fragmentation takes place in the SG part. A significant loss of 129 Da (neutral loss of the glutamic acid part), resulting at *m/z* 612 could be found (as annotated in Fig. 4). This neutral loss could also be found for the fragment ions at *m/z* 527 and *m/z* 672. The ion for *m/z* 672 is hardly found in the MS/MS spectrum and therefore marked (*) but the resulting −129 ion at *m/z* 543 clearly indicates the conjugation site at the fluorine-carrying

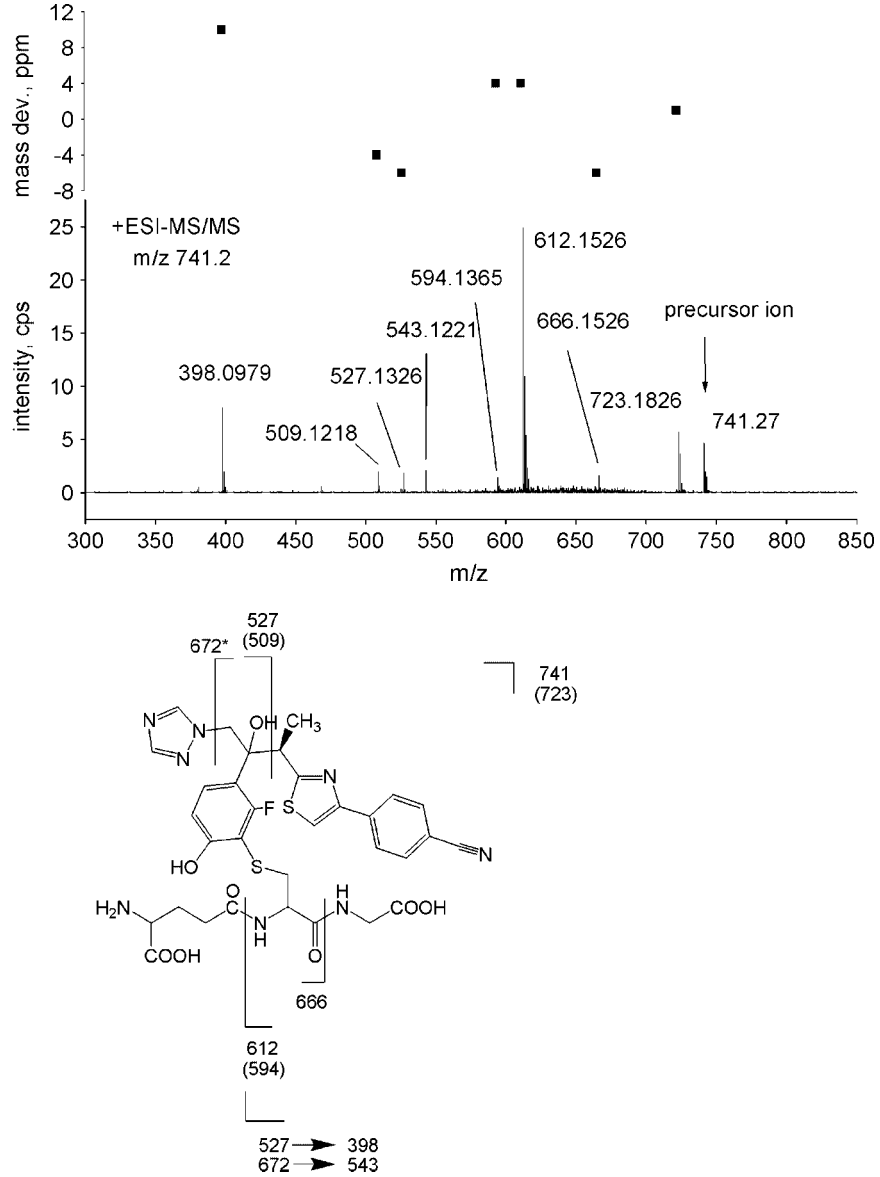

Figure 4. +ESI-MS/MS spectrum of the SG conjugate and its fragmentation scheme. Mass deviation is given at the top of each MS/MS signal. The substitution pattern at the aromatic system is not accessible by MS/MS. One pattern was arbitrarily chosen. In the fragmentation scheme, water loss (−18 Da) is assigned in brackets.

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aromatic system. The MS/MS spectrum was measured in an equivalent LC run using the same amount of injected sample as for the experiments given in Fig. 2. The capability to measure high quality MS/MS spectra from a very low intense signal in the single stage MS is an unequivocal advantage of qTof instruments. The same procedure was also followed for all other metabolites. Those MS/MS spectra were in good agreement with the expected structures of the metabolites. Discussion about the structures of the metabolites is summarized in the following paragraphs.

Mass accuracy

In routine analysis, it is possible to achieve a mass accuracy of about ±4 ppm in positive mode from peak intensities between 10 and 140 cps and ranging from m/z 300 to about m/z 1800.[15] As shown in Fig. 2 and 4, for these low-intensity signals in this study, the accuracy is in the range of 8 ppm between m/z 500 and 1200, and better than 12 ppm below.

Metabolic degradation

Figure 5 summarizes the metabolic pathway for BAL4815 found in this study.

After a typical Phase I oxidation to the epoxide, a SG conjugate is formed including the re-aromatization by the loss of fluoride.[16] After this, Phase II conjugation a classical SG degradation to the SCys as well as SCysAc adduct can be found. Since this study was focused on the elucidation of the

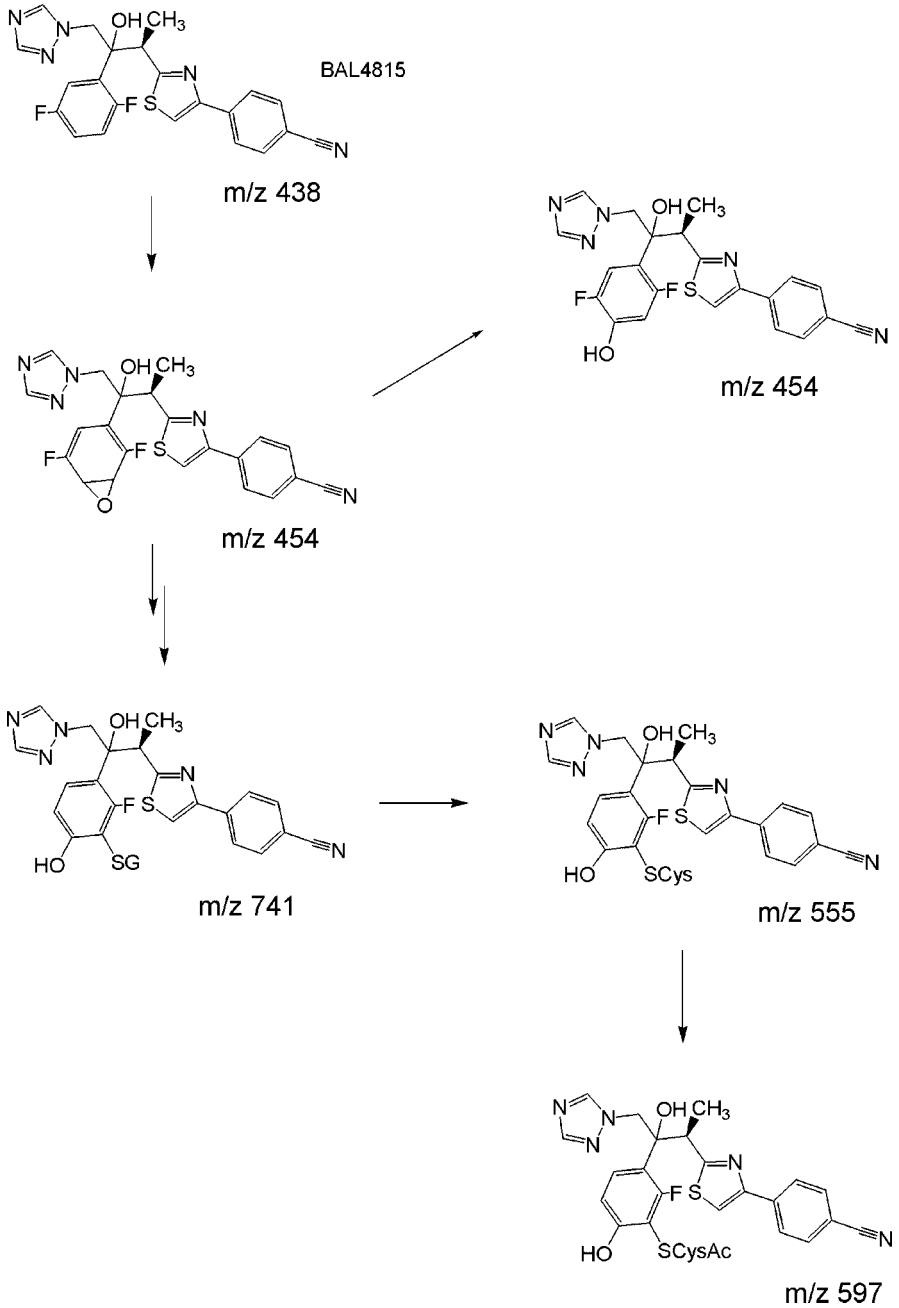

Figure 5. Proposed metabolic pathway in hepatocyte incubations. After the glutathione (SG) addition, the expected degradation to the cysteine (SCys) and acetylcysteine (SCysAc) can be found. The substitution pattern at the aromatic system is not accessible by MS/MS. One substitution pattern was arbitrarily chosen.

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metabolic cascade, the incubation with the highest number of metabolites was chosen. A quantitative conclusion between the ratios of the metabolites cannot be derived. Elucidation of the substitution pattern at the fluorine-carrying aromatic system is evidently not accessible by MS/MS. Because of the low amount of metabolites present in the incubations, other methods for the determination of their structure will fail.

CONCLUSIONS

Combination of capillary LC-qTof-MS and MS/MS with the use of stable isotope-labelled drugs is highly suited for the elucidation of the metabolic pattern. A great advantage of this approach is the accessibility of unexpected biotransformations. Accurate mass determination of these previously unknown compounds turned out to be very helpful for the elucidation of low-abundant metabolites. This approach could help solve several issues of previously unknown and atypical metabolic pathways.

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