OVERVIEW

- Reactive Metabolism plays a very important role in the safety profile of pharmaceuticals.
- Early prediction of reactive metabolites is of great importance for the reduction of attrition rate of a drug candidate at a later stage in drug development.
- An assay has been developed to detect the formation of reactive metabolites by using LC/MS/MS with exact mass measurement to search for metabolites conjugated with alutathione.
- Glutathione conjugations when fragmented by the mass spectrometer give a common loss corresponding to the pyroglutamic acid moiety.
- Until recently, this work has been carried out with triple quadrupole or linear ion trap technology using nominal mass
- The advantage of the hybrid quadrupole time of flight mass spectrometer is the selectivity and sensitivity that can be achieved. Moreover, full scan MS information is retained with this approach.
- Exact mass measurement provided unequivocal results confirming all reactive metabolites

INTRODUCTION

The early prediction of the metabolic fate and interactions of candidate drug molecules is of great importance for the success of the drug discovery process. Factors such as metabolic stability, toxic metabolite production, p450 inhibition and induction are all routinely monitored to prevent compounds with 'poor' pharmacokinetic properties progressing forward onto clinical trials.

Over recent years, a number of pharmaceutical compounds have been withdrawn due to adverse drug reactions. There is no direct link between toxicity and formation of reactive metabolites, but there is plenty of evidence indicating that idiosyncratic drug reactions are due to reactive metabolites. It is rather challenging and difficult to predict which reactive metabolites will be toxic. A major strategy to this problem is to detect these reactive intermediates by using trapping experiments and then follow this by detection and identification by LC/MS. Then, this is followed by the appropriate structural modification during the lead optimisation stage. Research over the past three decades in the field of biological reactive intermediates has yielded a wealth of information on the functional groups that may be converted by either Phase I or Phase II enzymes to electrophilic metabolites. Despite the rather extensive literature on the mechanisms by which drugs and other foreign compounds undergo metabolic activation, it is likely that numerous functional groups which

have not been recognised as precursors to reactive intermediates also can undergo bioactivation. Therefore, the detection and identification of biologically reactive metabolites has become an essential requirement in the discovery and development of drug candidates within the pharmaceutical and biotechnology industry.

In the majority of cases, reactive metabolites are electrophilic species and, as such, are susceptible to covalent binding with nucleophilic portions of proteins, DNA, and cellular glutathione (GSH). The generation of GSH conjugates is a clearance pathway for the body to remove reactive metabolites. Such reactions in vivo may lead to enzyme deactivation, genotoxicity, hapten formation triggering immune responses, compromised cellular function due to GSH depletion and increased oxidative stress, apoptosis, cumulative damage to long-lived proteins and ultimately cause cell death . There are different analytical strategies available to detect reactive metabolite formation. Most prevalently used are GSH depletion assays, time dependent P450 inhibition assays, covalent binding of radiolabeled material, and GSH trapping of reactive species with LC/MS/MS analysis.

Glutathione (GSH) is a tripeptide present in mammalian systems. It plays an important role in the detoxification of electrophilic foreign compounds and chemically reactive intermediates, which may arise during the biotransformation of xenobiotics. There are several methods to identify reactive metabolites by the use of chemical trapping agents, such as reduced GSH or cyanide trapping, to form stable adducts that are amenable to characterization by liquid chromatography-tandem mass spectrometry. The identification and quantitative analysis of GSH conjugates has advanced dramatically as new analytical techniques become available. The extra selectivity and sensitivity made LC/MS the instrument of choice for both quantitative and qualitative analysis. GSH conjugates when fragmented under collision-induced dissociation (CID) give a characteristic loss corresponding to the pyroglutamic acid (129 Da) moiety, which can be monitored (Figure 1). Constant neutral loss scanning for 129 mass units has become a screening tool for GSH conjugates. Exact mass neutral loss detection in the QTof Premier is achieved via sequential low and high energy MS acquisitions (Figure 2). Then, MS/MS is carried out on the parent mass of interest to confirm the common neutral loss. In vitro samples from a microsomal incubation (rat) with Clozapine, was analysed using the ACQUITY UPLC[™]/Q-Tof Premier[™]. Exact mass measurement provided unequivocal results confirming all reactive metabolites with errors less than 3 ppm RMS.



Figure 1. Shows the loss of the pyroglutamic acid moiety which may be monitored in exact mass neutral loss mode.



Figure 2. Shows the high and low collision energy sequence on the Q-Tof Premier™ for exact mass neutral loss acauisitions

METHODS

Gradient:

Mass spectrometer:	Micromass [®] Q-Tof Premier [™]
lonisation mode:	Electrospray positive ion mode
Cone voltage:	40 V
Capillary voltage:	2.7 kV
Source temperature:	120 °C
Desolvation temperature:	450 °C
Lock mass:	Leucine enkephalin m/z 556.2771, concentration 0.2 ng/uL
Solvent delivery system:	ACQUITY UPLC™
Column:	ACQUITY UPLC™ BEH C ₁₈ column, 100 x 2.1 mm id, 1.7 µm particle size
Mobile phase A:	Ammonium formate pH 9
Mobile phase B:	Acetonitrile
Gradient:	0 min 98% A, 7 min 2% A, 8 min 2% A, 8.1 min 98% A
Flow rate:	600 µL/min
Injection volume:	10 µL

Sample details: Incubations containing rat liver microsomes (1 mg/mL protein) were conducted, in 96-deepwell refill tubes, in 50 mM potassium phosphate buffer (pH 7.4, 500 µL total volume), at 37 °C in a water bath shaking at 45 rpm for 90 minutes. The test compound Clozapine, were added as a solution of dimethyl sulfoxide (DMSO) in buffer and glutathione (GSH) was added in buffer to achieve a final concentration of 100 µM of test compounds and 10 mM GSH. The incubation mixture contained a final concentration of 0.5% DMSO. The reaction was initiated by an NADPH-generating system consisting of 0.44 mM NADP+, 5.53 mM glucose-6-phosphate and 1.2 units/mL glucose-6-phosphate dehydrogenase together with 3 mM MgCl₂. The incubation was terminated at 90 minutes by the addition of 100 µL of acetonitrile containing 6% of acetic acid. After guenching the reaction, the samples were placed on ice for approximately 10 minutes. The guenched samples were centrifuged at 2,250 g for 20 minutes. The supernatants of the samples were directly injected into LC/MS system.

RESULTS

- mass neutral loss with the Q-Tof Premier™ (Figure 3).
- The use of ACQUITY UPLC[™] provided very fast run times and vital separation of metabolites (Figure 3).
- This mode of operation provides an information rich approach (Figure 4) whereby all full scan MS, pseudo MS/MS and MS/MS is retained.
- metabolites of interest (Figure 5). Here it can be observed that one of the GSH adducts have been identified in the high energy scan together with the loss of the pyroglutamic acid.



loss screening with the ACQUITY UPLC[™]/Q-Tof Premier[™]. This examples show the different acquisition modes all happening simultaneously from a single injection.

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- All Clozapine GSH adducts were detected by the use of exact
- The use of exact mass is of extreme importance to confirm

- All other phase I metabolites are also detected (Figure 6) in the low energy acquisition mode.
- Moreover, the great advantage of obtaining this information in the low energy mode (Figures 7a, b, c) is that the data may be interrogated at a later stage if the need arises with software packages such as MetaboLynx[™]. These data maybe used in conjunction with exact mass in order to identify putative phase I metabolites.



Figure 4. Shows the individual spectra for each one of the acquisition modes in low energy, high energy full scan mode and MS/MS to confirm the loss of the pyroglutamic acid moiety.



Figure 5. Shows the exact mass obtained for one of the GSH conjugates and the loss of the pyroglutamic acid moiety. As can be observed the measurements are below 3 ppm.



Figure 6. Shows all major metabolites and parent compound from the low energy acquisition mode with exact mass extracted ion chromatogram windows.



Figure 7a. Spectra for parent compound and exact mass from the low energy acquisition mode.



Figure 7b. Spectra for N-dealkylated metabolite with exact mass from the low energy acquisition



Figure 7c. Spectra for hydroxylated metabolite with exact mass from the low energy acquisition

CONCLUSION

- Exact mass neutral loss approach provided excellent selectivity and generated rich structural information from full spectrum MS data (Phase I and other Phase II metabolites) and targeted MS/MS for glutathione.
- Less false positives will be generated in exact mass mode and data maybe searched for other conjugations.
- The use of ACQUITY UPLC[™] enabled GSH screening to be carried out in a very fast and efficient manner. The peak capacity was greatly improved and also it enable us to obtain better chromatographic separation with little system optimisation. The method was directly transferred from the HPLC methodology previously used.
- The turnaround for these experiments were 9 minutes instead of 20 minutes previously used before with an HPLC approach.

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