Advances in Sample Preparation for Metabolite Profiling

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Introduction:

Nerviano Medical Sciences (NMS) is a new company created following the spin-off by Pfizer of their R&D centre in Nerviano (Milan, Italy). Through its Preclinical Development (PCD) unit, NMS specialises in toxicology, drug metabolism and pharmacokinetics services to pharmaceutical and biotechnology companies. These PCD capabilities are used to support the selection of candidate drugs, predict clinical outcomes from preclinical data (*in silico*, *in vitro*, *in vivo*) and then perform preclinical studies to reduce the attrition rate in the clinic and accelerate the whole drug development process

One of the key stages of the journey of a new chemical entity from initial screening hit, through optimisation, into clinical trials, and ultimately to launch as a new block buster drug, is assessment of the activation pathway and toxicity. These stages are coming under increasing focus as regulatory authorities and patients groups alike are demanding more information about toxic side effects, hence the barrier to launch is raised yet higher. Within every stage of the assessment of the possible new drug, stringent controls are in place to prevent false positive results, or worse still, false negatives. The focus of such controls has traditionally been on the analytical technique and the calibration of systems used. A recent study carried out at Nerviano Medical Sciences has shown that the sample preparation steps employed can be just as important as the analytical steps in generating errors in the process, and require just as much attention when setting up the process. Critical to the process being established within Nerviano's laboratory is the evaporation methodology used when preparing the samples.

Project Outline:

The aim of the project was to set up a procedure, based on a semi-preparative LC-MS-MS system to allow the determination of the biological activity and the definitive structure of metabolites taken from in *vitro* assays or in *vivo* studies. Purified metabolites could then be tested in several ways, including:

- Tested on target enzymes for activity
- Tested on non-target enzymes for activity
- Definitively identified with NMR
- Entered into toxicological studies
- Assessed for possible drug-drug interaction
- Tested for reactivity
- Used as standards for pharmacokinetic determinations

There is a significant advantage in being able to extract the sample from the original assay, purify and identify it so that it can then be used for further study. Typically, metabolites that require further study are synthesised following determination, which extends the time taken for metabolite evaluation considerably. To enable further studies to take place, the goal of the project was to establish a system to provide 50 to 100ul of a 1mM solution of purified identified metabolite in DMSO solution. This solution can then be screened for activity against a number of different targets. The procedure was established using a series of Tyrosine Kinases targets and a compound with well-established metabolism leading to two main metabolites, one of which is active, and another which is inactive against specific targets.

Process:

Samples were taken from the assay and an aliquot presented to the LC-MS-MS system, first running an analytical column to determine the optimal conditions for preparative separation. Next, the bulk of the sample is separated using the preparative column, and the fractions collected. LC solvents were water and methanol, containing 0.1% formic acid as a modifier. After separation the samples were dried and then diluted to known concentration and reanalysed by LC-MS-MS and NMR.

It is at the evaporation stage of the process that problems were initially encountered.

First, one of the drying methods trialled blew nitrogen onto the samples to hasten evaporation, however this resulted in much of the sample drying and sticking to the sides of the tube which made dissolution in minimal (50 to 100ul) DMSO very difficult. This lead to use of a centrifugal concentration system which evaporates the dried sample into a small area at the base of the tube, which is far better for redissolving in minimal solvent.

The second drying issue that was encountered was in screening samples post drying. At first, blank samples (containing no compound) were run through the whole process and via both drying methods showed up false positives in the screening trials, this was attributed to residual modifier from the LC solvents.

Thirdly, with both of the methods tried, some compound degradation was observed, attributable to poor temperature control of the samples in the evaporator. Comparisons between standards and pilot samples showed lower activity of the samples that has been dried. Clearly further method development needed to be done as sample degradation is unacceptable due to the sensitive and exacting nature of the toxicologists work.

These issues led Nerviano to search for a more efficient evaporation system, one that concentrated the sample to the bottom of the tube leaving little or none on the tube walls, removed all the modifier from the LC solvents thereby eliminating a cause of false positives in the screens, and did not degrade the compounds with excessive heat. Trials with the Genevac EZ-2 for the evaporation of the purified parent and metabolite fractions proved very successful with the activity results very close to those obtained with the respective analytical standards. False positives were eliminated and a low extent of compound degradation was observed. Minor variations seen were acceptable considering the intrinsic variability of the activity as determined by high throughput screening. Above and beyond the greatly improved screening results, an additional benefit was that the evaporation time was sensibly lower compared with the other centrifugal system tried, or the blow down method.

Conclusions:

The novel semi-preparative auto-purification system including on-line LC-MS-MS analysis was successfully installed and setup. The new system allows the isolation and identification of pharmacologically active compounds and their metabolites. The Genevac evaporator provided the optimal balance between speed and minimal compound degradation during the evaporation step, and eliminated interferences with mobile phase buffers from the LC solvents. The validity of this procedure was confirmed by subsequent biological activity tests and by proton NMR. Validation of sample preparation techniques is as important as analytical methods to because they either may be the source of erroneous results.