Approaches to higher-throughput pharmacokinetics (HTPK) in drug discovery

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With pressure on pharmaceutical companies to reduce time-to-market and improve the success rate of new drug candidates, higher-throughput pharmacokinetic (HTPK) support has become an integral part of many drug discovery programmes. This report details the amalgamation of robotics, new sample preparation technologies and highly sensitive and selective mass spectrometric detection systems to deliver the promise of HTPK. A historical perspective on automated bioanalysis with the current approaches and future prospects for the discipline are described.

Discovery pharmacokinetics (PK) has traditionally been regarded as a low-throughput activity, a finding that is directly opposed to the need to address the high attrition rate of compounds in drug development caused by poor PK profiles[1]. Historically, the drug discovery cascade has been established as a sequential process with many drug candidates, which originate from a variety of sources, being initially screened for potency against a biological target. At best, limited PK and metabolism studies would be conducted prior to acceptance of a compound for safety evaluation with, as it transpires, the said high attrition rate.

A new paradigm in drug discovery has emerged in which the entire sample collection is rapidly screened using robotized high-throughput assays at the outset of a programme[2,3], often together with some selectivity testing against other targets. This frequently provides higher quality leads for optimization by the medicinal chemistry department, often employing new combinatorial synthetic technologies[4,5]. Indeed, drug metabolism groups can now offer input even at this early stage[6–8] based on cheminformatic knowledge-based systems that have been developed to provide a portfolio of techniques to highlight those chemical series that might have unwanted properties. For example, structural motifs that are compatible with polymorphic metabolism, cytochrome P450 (CYP) induction, CYP inhibition or metabolic activation to yield a toxic intermediate would be avoided. A new knowledge-based, issue-driven drug design process, incorporating a more parallel rather than sequential approach is increasingly being adopted. Proposed selection criteria for the further progression of drug candidates are presented in Fig. 1.

Higher-throughput PK (HTPK) is being achieved through the introduction of new techniques, including automation for sample preparation and new experimental approaches for the evaluation of many substances in parallel. In addition, the widespread use of liquid chromatography (LC) interfaced to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) allows for the sensitive, selective and rapid quantitative analysis of drugs in biological samples. Application of these technologies can now support multiple projects at a pace appropriate to the output of a synthetic chemistry team, thus providing timely and relevant information that will impact directly on drug design. In this review, the PK process, the historical context of newer developments, current HTPK approaches (including upstream and downstream activities), and future prospects for further improvements are discussed.

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 1359-6446/99/$ – see front matter PII: S1359-6446(99)01434-8

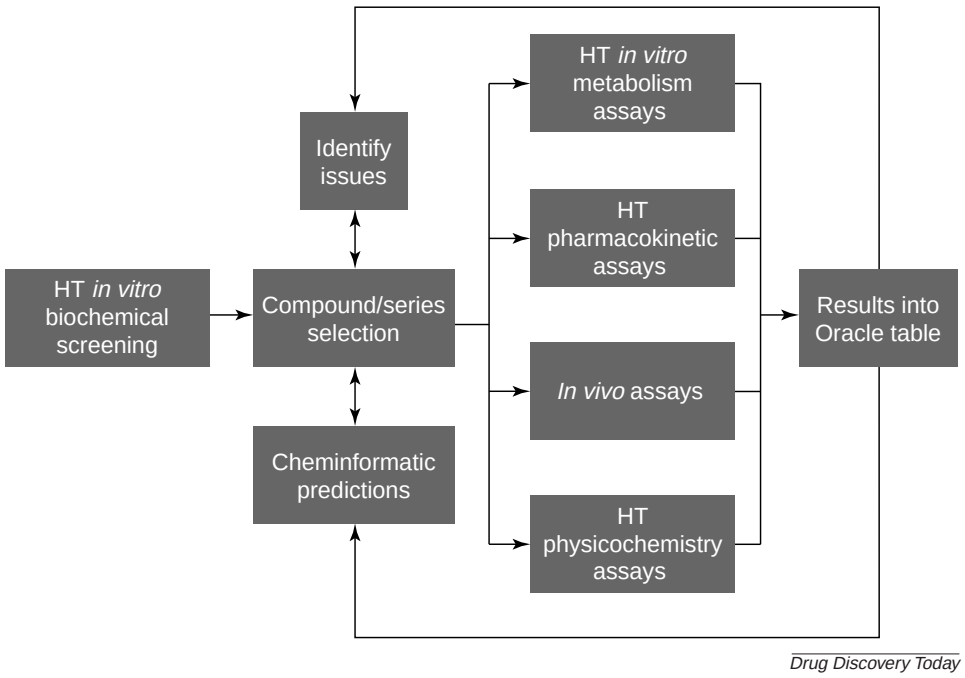

Figure 1. *A new paradigm for drug discovery in which issues can be flagged early through reference to data on previous compounds and/or cheminformatic predictions. To resolve these issues in a timely manner, a higher throughput (HT), highly parallel testing cascade is put in place to address the* in vivo *properties of molecules through suitable* in vitro *and* in vivo *assays.*

Pharmacokinetics in practice

The PK profile of a drug, in which the absorption, distribution, metabolism and excretion processes following *in vivo* administration are mathematically described, is derived from the plot of the systemic drug concentration versus time for that compound. Following either parenteral or oral administration, serial blood samples are collected as a function of time and then analyzed for drug content. The complete process is summarized in Fig. 2 and can be divided into five main areas:

- The *in vivo* work, including the initial surgery required to prepare animals for dosing, actual dosing and collection of blood samples
- The calibration of an analytical method
- The preparation of samples prior to analysis
- The actual quantitative analysis
- Data reduction and reporting.

Consideration of the *in vivo* aspects yields little opportunity for throughput improvements for single-compound studies as, by definition, the experiment must be conducted in real time. However, as will be seen, alternate experimental designs such as multicomponent analysis might provide new options. Clearly, the calibration, sample preparation and analysis steps have represented the major bottlenecks and it is these that have appropriated the most attention for the development of higher-throughput approaches.

Calibration

To ensure that measured concentrations truly reflect the actual sample concentrations, analytical methods are calibrated by adding varying known concentrations of drug into control plasma. These are then prepared and analyzed following a protocol identical to that used for the actual samples. Preparation of appropriate dilutions is time-consuming and laborious, but must be performed to a high degree of accuracy as the integrity of the assay results depends on this. Similarly, quality controls are included to assess the performance of the assay over time as these are interspersed into the analytical run. These values are then compared with those obtained from the calibration standards.

Sample preparation methodologies

The aim of the sample preparation process is to provide a suitable sample, usually for chromatographic analysis, which will not contaminate the instrumentation and where the concentration in the prepared sample is reflective of that found in the original. The method of sample preparation selected is generally dictated by the analytical techniques available and the physical characteristics of the analytes under investigation. The two main sample preparation methods are matrix cleanup or direct injection (Fig. 2). In a matrix cleanup procedure, the aim is to remove as much endogenous material as possible from the drug sample, either by liquid- or solid-phase extraction (SPE) or by precipitation of the plasma proteins. In direct injection, which is usually only amenable to relatively lipophilic compounds, the compound of interest interacts

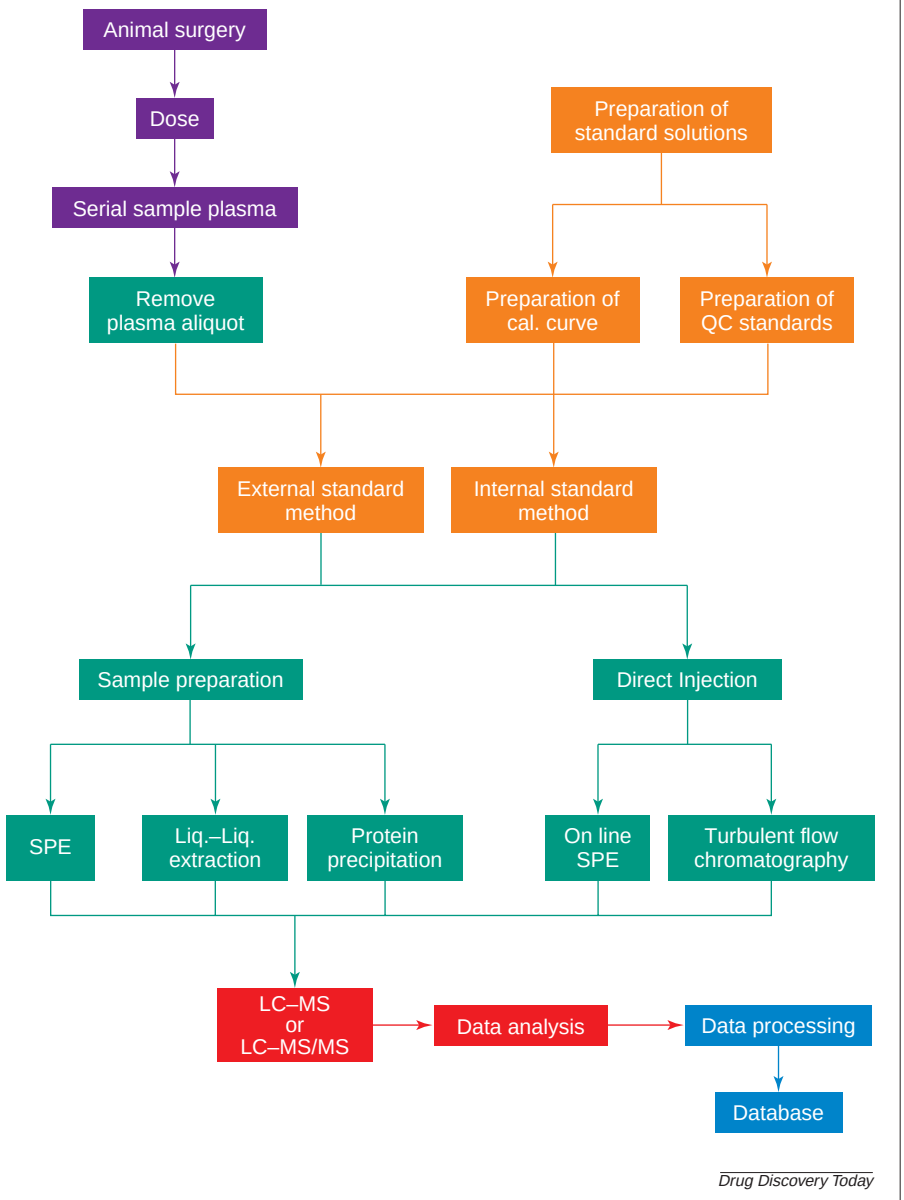

Figure 2. *Flow-diagram representing the processes required to implement a typical pre-clinical pharmacokinetic study. The various components required are colour coded as follows:* in vivo *work (purple), calibration (orange), sample preparation (green), sample analysis (red) and data analysis (blue). Abbreviations: cal. curve, calibration curve; liq.–liq. extraction, liquid–liquid extraction; LC–MS, liquid chromatography coupled to mass spectrometry; LC–MS/MS, liquid chromatography coupled to tandom mass spectrometry; QC, quality control; SPE, solid-phase extraction.*

with a stationary phase, which is then eluted and analyzed, leaving behind the endogenous material.

Although it is imperative that all samples, standards and quality controls are treated identically, processing each sample sequentially is time-consuming and prone to error, even in the hands of a well-skilled analyst, as the operations are highly repetitive. This suggests that the introduction of new technologies and/or automation at the sample preparation stage should help improve throughput and reduce errors.

Analysis

In the early 1990s, there was a vast increase in the analysis of xenobiotics in biofluids with the introduction and widespread adoption of atmospheric pressure ionization (API)-MS and, in particular, MS/MS coupled to HPLC (Refs 9–11). With non-selective analyses such as HPLC–UV, which was the previous mainstay of bioanalysis, all sample components possessing a chromophore could be detected resulting in complex chromatograms in which the peak of interest was often difficult to distinguish from endogenous components. With API-LC–MS, however, detection is performed on the basis of the presence of protonated molecular ion of the parent compound such that only components with that mass (± 0.5 Da) are detected. There is a further gain in specificity through using LC–MS/MS operating in a specific-reaction monitoring (SRM) or multi-reaction monitoring (MRM) mode. Here, the transition from the mass-to-charge ratio of the precursor ion to a specific product ion produced within a collision cell in the mass spectrometer is determined by collisionally induced dissociation (CID). This essentially removes background interference, as it is likely that only the 'parent' ion will generate these precursor and product ions. This is, of course, an oversimplification of the system when dealing with sample matrices and is discussed in more detail later.

Improvements in signal-to-noise ratio result in higher simplified chromatograms with greater sensitivity and selectivity for the compound of interest. Additionally, the ability to rapidly scan different transitions enables the

simultaneous analysis of several components without the requirement for chromatographic resolution, thereby reducing run times. Furthermore, this enables rapid generic gradients to be applied to all samples, therefore eliminating chromatographic method development time.

Automation perspective

From the inception of modern chromatographic science, automation technologies have continuously developed, beginning with the introduction of autosamplers and microprocessor-controlled instrumentation in the late 1970s to early 1980s. This enabled the sequencing of samples, often requiring different analytical instrument characteristics, and this increased the number of samples that could be processed in a batch by enabling unattended, overnight operation. In the mid-1980s, early adopters of robotic technologies were using the newly introduced Zymark Zymate II (Zymark Ltd, Runcorn, Cheshire, UK) to try to further improve throughput by programming a robot to reproduce manual operations from the sample preparation steps[12–14]. Whilst some gains in productivity using this approach were evident, there were several problems with such systems:

- The use of robotics to purely reproduce a manual method is usually not the most efficient or cost-effective way of proceeding.
- Many methods suffered from poor reliability and required frequent maintenance or recalibration.
- At best, most methods were semi-automated, often requiring substantial manual input, and therefore negating the advantages of employing the system.
- The timing of implementation, which was prior to the adoption of LC–MS/MS, was wrong as sample analysis was still the rate-determining step. Hence, automation of sample preparation did not bring substantial gains in productivity.

In fact, the initial slow adoption of systems such as the linear track robots in the late 1980s demonstrates the lack of commitment to automation at the time, as other areas of the infrastructure were not in place to allow this to be fully exploited nor justified from a cost perspective.

With the introduction of the microtitre plate format as the standard platform on which to perform a multitude of assays, it was inevitable that the time gains seen in areas such as biochemical screens through parallel sample processing would be adopted for bioanalytical assays. Furthermore, the shift in formats from vials and tubes to microtitre plates has made it much more viable to apply robotic technologies, as many liquid handling systems, such as the Packard Multiprobe (Canberra Packard Ltd, Pangbourne, Berks, UK) or the Beckman Biomek [Beckman Coulter (UK) Ltd, High Wycombe, Buckinghamshire, UK], are designed to specifically handle these formats.

Current technologies

The current options available to increase the throughput of a PK assay can be divided into the two main areas of sample preparation and experimental design, although consideration should also be given to data handling and utilization, which will also be discussed.

Sample preparation

Henion recently reviewed the options available to the analyst for preparing biological samples for LC/MS analysis, including the use of 96-well plate formats for SPE (Ref. 15). Simply grouping together the cartridges on one analysis plate increases throughput, as it is easier to manually treat several samples simultaneously[16]. However, many groups are using this as the basis for more fully automated analysis[17–19], with typical sample preparation times of under two hours for 96 samples. A further extension of this approach is the use of SPE disk technologies[20,21]. Here, the thickness of the adsorbent bed is dramatically reduced, enabling elution in volumes approximating those used for chromatographic analysis. Hence, the eluent from the SPE disk can be analyzed directly, therefore eliminating steps such as evaporation and reconstitution.

Further improvements in throughput can be made by the removal of a sample preparation step and the direct introduction of plasma into a chromatographic system. One such system, the Prospekt (Spark Holland Instrumenten, Emmen, The Netherlands), consists of small cartridges (10 × 2.0 mm internal diameter) packed with 15–25 μm polymeric or 40 μm silica particles. Plasma samples can be directly injected into the system and the endogenous material removed by elution before switching the flow to an analytical HPLC column prior to detection[22–25]. Some of the newer solid-phase materials, such as the Waters OASIS HLB (Waters Ltd, Watford, Hertfordshire, UK), have additionally been supplied in a column rather than a cartridge format, enabling online analysis of plasma samples without the prerequisite for sample clean-up, and this is known as turbulent-flow chromatography[26]. Here, the use of high flow-rate HPLC coupled with large particle-size columns has demonstrated good chromatographic performance because of the non-laminar flow of the mobile phase. Fast gradients are performed where endogenous material is essentially unretained and total

sample analysis times of approximately 1.2 minutes including re-equilibration are achievable[27,28]. Liquid–liquid extraction can also reliably work in a 96-well plate format[29]. Other techniques, such as trace enrichment[30] or on-line column switching[31], can improve throughput but are usually application specific.

Experimental design

An alternative approach to increasing PK throughput is to reduce sample numbers for analysis. Two common approaches are used. Firstly, samples from identical study designs but that contain different analytes can be pooled to produce a single set of samples. This results in a dramatic decrease in sample preparation and analysis time proportional to the number of studies that are pooled. Secondly, for a single test compound, serial plasma samples can be pooled to produce a single composite sample for analysis. The concentration of this sample is representative of the exposure to the drug over the entire sampling period[32,33], and as the sampling regime is defined, an estimate of the area under the concentration-time curve (AUC) can be determined that is in good agreement with AUC values determined by measuring the concentration in individual samples. This clearly reduces the number of samples requiring analysis and could be used, for example, in bioequivalence studies, where only total exposure levels or a yes/no answer on drug levels following oral administration are required. However, this approach will probably be of limited application, as PK analyses rely on the wealth of information that can be mathematically described by full concentration–time data profiles.

Although the discussion so far has considered a single analyte dosed to several test subjects, the power of MS to detect multiple analytes, in effect simultaneously, highlights additional possibilities for experimental design[34]. Cassette dosing is a technique in which a number of analytes are administered to a single animal and then plasma samples analyzed for each individual analyte[35–38]. This allows construction of PK profiles for several analytes from a single experiment, dramatically improving throughput and reducing animal numbers. The experiment, however, is not without its critics who highlight the possibilities for *in vivo* drug–drug interactions. The role of cassette dosing could be as a compound selection tool, through allowing 'poor' compounds to be eliminated at an early stage, rather than by producing definitive PK data. This is because most experimental errors are caused by false positive rather than false negative results, as drug–drug interactions are more likely to enhance oral bioavailability or reduce clearance (through CYP inhibition) than *vice versa*.

Automated reporting

Although in discussing HTPK, the focus has been primarily on sample preparation and analysis, handling the data and delivering them for rapid assimilation into a research programme are equally essential parts of the operation. As both the number of samples increases and analysis timeframes are condensed, the time spent handling data will be crucial. Laboratory information management systems (LIMS) that facilitate the trafficking of data to Oracle databases[39,40] are an integral part of HTPK. Oracle tables can already be regarded as essential for the utilization of computational data-mining and prediction tools, so as to improve our understanding of the factors influencing PK behaviour and therefore improve the design of future drug candidates.

Limitations of HTPK

Whilst LC–MS/MS has undoubtedly brought great benefits to the analysis of biofluids, it is not without its disadvantages and is far from being a ubiquitous technique for all analytes. In particular, when dealing with the ionization of compounds in the presence of potentially interfering matrix components, ion suppression is sometimes observed, as matrix components can preferentially ionize or ion-pair with the analyte[41,42]. Additionally, many components might be late eluting from the chromatographic system and so could appear almost randomly throughout a sequence, leading to irreproducibility in quantification. Furthermore, the mode of ionization employed [usually electrospray (ESI) or atmospheric pressure chemical ionization (APcI)] is important, as these unwanted effects are more pronounced with ESI than with APcI (Ref. 43). Evidence suggests that the magnitude of the matrix effect depends both on the sample preparation method employed (with protein precipitation the poorest, and liquid–liquid extraction the best) and on the nature of the analyte (with more polar analytes compromised more than lipophilic ones)[44]. This makes generalizations concerning the problem difficult, as each assay has to be considered case-by-case. Furthermore, even common mobile-phase additives such as trifluoroacetic acid (TFA) are known to suppress ionization and so compromise detection limits by strong ion-pairing between the TFA anion and protonated analyte cation[45].

Another possible source of interference is from in-source CID producing fragmentation of the analytes. If metabolic species are not chromatographically resolved[46,47] and fragmentation can lead to regeneration of the parent compound, misleading results could be obtained. For example, an acyl glucuronide formed from a carboxylic

acid could fragment to release the parent acid that, without chromatographic resolution, would be seen as the parent compound using MS. A further example would be a prodrug that is designed to release its parent compound, usually enzymatically. Again, if this is not resolved from the parent compound, it is difficult to ensure that all the parent compound observed has been formed metabolically and not as a consequence of CID.

For validated quantitative work, MS assays should be conducted with an internal standard. One favoured approach involves co-eluting perdeuterated standards with sufficient incorporation to avoid ^{13}C-isotope interference from the parent compound. However, operating in the MRM mode will mean switching between the precursor ion for the parent compound and the internal standard, and the product ions for both species might be the same because of their structural similarities. If the ions are not removed from the collision cell prior to switching to the next precursor ion, crosstalk will occur in which a response will be detected for precursor 2, when the product ion was actually formed from precursor 1. More recently, this has been overcome through software alterations and hardware redesign of collision cells, but on older mass spectrometers (pre-1996), this might still be an issue.

Finally, the benefits from full automation assume system reliability giving reproducible high-quality data all the time. It is recognized that at the threshold of a new era in bioanalysis, system integration, in which all the assay components are required to take samples through a complete cycle, might not yet work optimally. However, with appropriate quality standards, it should be possible to identify the issues and work in an environment of continuous improvement to fulfil the promise of HTPK.

Conclusions and future perspectives

Advances in MS currently offer many intriguing possibilities for the further development of HTPK. Triple quadrupole MS systems continue to improve in sensitivity with the development of enhanced vacuum pumping systems and ion optics. The requirement for method development is negated by improvements in selectivity and detection limits such that instrument optimization for ultimate performance is no longer required. In addition, such instrumentation allows PK investigation at more pharmacologically relevant doses.

Electrospray ionization coupled with time-of-flight (TOF) mass analyzers, accurate to within 5 ppm, could offer the possibility of analyzing crude samples by enabling construction of mass chromatograms with windows of 20 mDa to produce specificity approaching that of triple quadrupole MS. For quantitative work, the linear range of TOF might not be as great as that for triple quadrupole MS (approximately three orders of magnitude)[48], although this range might still be suitable for a large range of applications. Furthermore, multiple inlet systems are being developed for both TOF and triple quadrupole instrumentation, enabling the simultaneous analysis of four or eight separate chromatographic systems, each writing to separate data files[49]. With MS optimization in mind, manufacturers are introducing new software tools to either automatically obtain optimized MS/MS characteristics from an infused sample or simply from a loop injection that, when operated in batch mode for multiple-analyte screening, will dramatically reduce analytical method development time.

There could also be a drug discovery use for the hybrid quadrupole–TOF technology, currently expensive and with a niche role in proteomic analysis. However, it is likely to enter the mainstream in the same manner as MS/MS has done over the past few years. This combines the power of the TOF with the ability to obtain accurate mass MS/MS data on samples of interest by using a quadrupole MS before the TOF analyzer. This might enable simultaneous parent and metabolite information to be easily gleaned from a single chromatographic run, thereby facilitating more data to be obtained from a single biological sample.

A key differentiator in the further development of automation technologies is likely to be the software. The majority of hardware platforms, particularly for the manipulation of microtitre plates, are already highly competent. However, fully unattended operation will only become a reality when intelligent, decision-making software becomes available, as many operations (particularly in the manipulation of plasma samples) require the analyst to make decisions as part of the process. For example, samples might not necessarily be taken for every time point, resulting in empty wells. Ideally therefore, the equipment should not abort in error, but simply log this and then move on to the next sample. This information should then form part of the final report. Such developments are likely to follow when the focus of instrument manufacturers shifts from purely supporting the HTS-end of the operation to encompassing the wider drug discovery arena.

Whilst it is clear that significant advances have been made particularly in the fields of sample preparation and analysis, further improvements are still necessary in the automation and integration of the whole PK process. Many recent technologies, such as the microtitre plate, robotic instrumentation and MS, have all advanced

sufficiently to provide a platform on which to build a more fully automated approach to the analysis of plasma samples, thereby promoting HTPK. However, as yet, the promise is not fully realized, as no solution works 'out-of-the-box', and efforts to fully validate such methodology continue in an attempt to increase throughput whilst retaining the quality of the analysis. This has essentially redefined the skill-base of the modern-day bioanalyst to encompass a high degree of familiarity with robotic instrumentation and software programming.

The drug discovery process is heading towards systems involving a fully robotized process from sample collection through to data reporting, linking together all the necessary processes such as sample preparation and MS. This will ultimately result in a report file, containing concentration data along with all the appropriately logged experimental details, which will feed straight into database fields and create a truly 'turn-key' approach to HTPK.

Acknowledgements

We would like to thank our colleagues from the drug metabolism groups within Merck for many fruitful and positive discussions.

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