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Whatever happened to cassette-dosing pharmacokinetics?

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Cassette dosing is a procedure that is used for rapidly assessing the pharmacokinetics of a series of discovery drug candidates by dosing a mixture of compounds rather than a single compound. Cassette dosing has advantages and disadvantages associated with its use, which leads to controversy about how and if it should be used. To assess the current practices of the pharmaceutical industry regarding cassette dosing, a survey of several pharmaceutical companies was conducted. Analysis of the survey revealed that opinion on this subject is divided within the pharmaceutical industry. In addition, it was determined that approximately only a half of those companies that perform *in vivo* pharmacokinetic screening use cassette dosing for this purpose.

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▼ Over the past decade, it has been widely recognized that the absorption, distribution, metabolism, excretion (ADME) and pharmacokinetic (PK) properties of drug candidates are as important as pharmacological activity and selectivity in the design of clinically useful therapeutic agents [1,2]. Inappropriate ADME and PK properties are among the major reasons for the failure of a compound to reach the market. This drives pharmaceutical companies to profile ADME and PK properties in the early discovery phase to enable the selection of compounds that have a greater probability of success. With the advance of drug discovery technology, numerous *in vitro* and *in silico* methods have become available to help discovery scientists predict the ADME properties of compounds [3]. However, without *in vivo* animal experiments, our understanding of the integration of biochemical and physiological process at the whole-body level remains incomplete. Animal experiments are generally expensive and labor- and time-intensive. Therefore, to aid selection and add value to drug development candidates, the level of throughput of *in vivo* pharmacokinetic optimization studies must be increased [4].

Cassette dosing (CD, also called 'N-in-one' dosing) was introduced as a solution to the problem of low-throughput screening and exploits the capability of modern mass spectrometers to measure simultaneously the individual concentrations of chemical compounds in a plasma sample that contains multiple drugs [5]. A mixture of test compounds, which typically comprises five to ten compounds, is dosed to a group of animals and the pharmacokinetics of all the compounds are determined in parallel, which substantially reduces the time required for the overall procedure compared with conventional serial determinations [6] (Figure 1).

The potential of CD to yield false PK information as the result of drug–drug interactions was recognized immediately after its introduction [5,7]. To minimize the errors observed, safeguards were introduced, including administering only low doses of compounds and eliminating known inhibitors of drug-metabolizing enzymes. Furthermore, most scientists believed that only false positives could occur, which means that drug–drug interactions, if present, would result in deceptively enhanced PK properties for a compound, thus 'poor' compounds would appear to be better candidates than they actually are. In this case, the error would be detected at the next stage when the compound is administered as a single dose. However, we performed a theoretical PK analysis and evaluation of CD that demonstrated that the commonly used safeguards were not necessarily effective and that the probability of the occurrence of false negatives was equivalent to that of false positives [8]. For example, if the compounds in a cassette compete for binding to plasma protein, then clearance of one or more of the drugs could be higher when that compound is dosed in combination with other drugs, thus some drugs

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would appear to be eliminated more rapidly than they would if dosed singly. This analysis also resulted in the development of several recommendations, including limiting cassette size and using low doses of compounds. Subsequent to the publication of our analysis [8], we wondered if drug discovery companies would discontinue the use of CD or, at least, alter the way that it is used. Accordingly, we reviewed the current state of CD in the pharmaceutical industry.

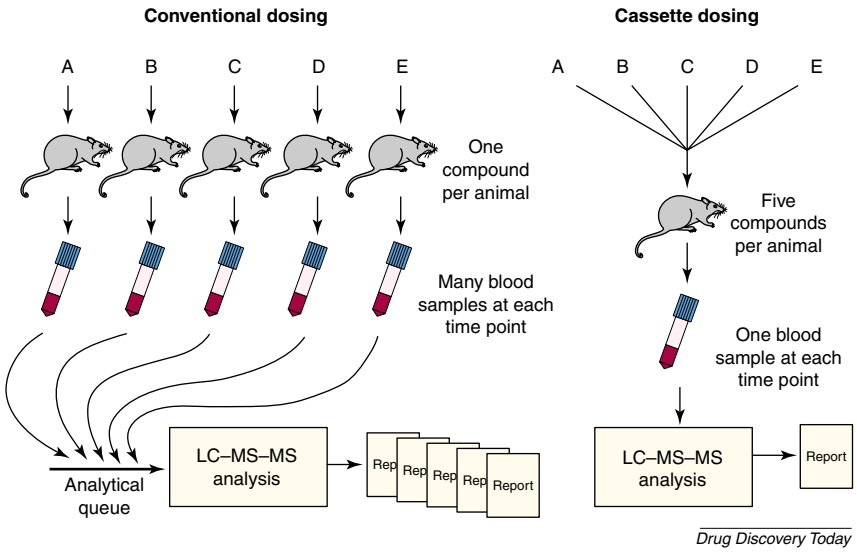

Figure 1. Conventional dosing versus cassette dosing in the pharmacokinetic screening of five compounds. In conventional (or discrete) dosing, five formulations containing one compound each (A, B, C, D and E) are separately dosed to five animals. At selected time points, blood samples are drawn from each animal and assayed by LC–MS–MS for the particular compound that the animal received. The analysis of one compound per sample permits the use of a simple assay method. The data are analyzed and a report of the calculated PK parameters is issued for each compound. As a consequence of there being only one compound present in a particular animal, these parameters are the true values for that animal. However, five animals have to be dosed and five sets of samples have to be analyzed. In cassette dosing, the five compounds are mixed to produce a single formulation for dosing (usually a solid-liquid suspension or solution), which is then dosed to a single animal. At selected time points, blood samples are drawn from the animal and assayed by LC–MS–MS for all five compounds. The assay of five compounds simultaneously typically requires a complex assay method. The data are analyzed and a single report containing the calculated PK parameters for all five compounds is issued. The simultaneous presence of five compounds in the animal could potentially result in the distortion of the parameters by PK interactions among the compounds. However, this approach requires the use of only one animal and the analysis of only one set of samples, which lowers the total number of animals required and potentially leads to a reduction in costs. In addition, cassette dosing is generally significantly faster than conventional dosing. Abbreviations: LC–MS–MS, liquid chromatography–mass spectrometry–mass spectrometry; PK, pharmacokinetic.

Current cassette dosing industrial practices

To address the current status of the cassette-dosing pharmacokinetic technique, we decided to poll industry-based colleagues directly. Thirty-one international pharmaceutical companies (ranging from small to large companies) were queried about their past experience of and present use of CD. Twenty-five completed and returned the questionnaire, giving an overall response rate of 81%. Therefore, the data should present an accurate representation of the pharmaceutical industry. Twenty-two (88%) of the companies surveyed have tried CD, but only 12 of these continue to use CD (48%). Although these findings indicate that CD remains a viable approach, the discontinued use of CD by ten (40%) companies suggests that there has been a substantial decline in the popularity of this technique (Figure 2).

The frequency of the implementation of CD ranged from 'rarely' to 'six times per week', with each cassette comprising between two and ten compounds (average number of compounds per cassette was five). Hence, despite some early publications demonstrating the analytical feasibility of much larger cassettes, most companies prefer to use modest-sized cassettes, even though these cassettes would appear to limit the productivity gain. The majority of companies recognize the importance of keeping dose size small and the dose size that was most frequently chosen was ~1 mg of compound per kg of body weight for each compound (mg/kg/compound). However, as a result of assay-sensitivity limitations with smaller doses, two companies still use dose sizes of 10 mg/kg/compound. Companies that routinely employ cassette dosing appear satisfied with the results and report an average reliability of 80% for derived PK parameters (range 50–100%). Although the exact meaning of 'reliability' was generally not well-defined by our respondents, it could be taken qualitatively to mean that the results obtained using CD were comparable to the results that would have been generated ~80% of the time with a discrete dosing approach.

It is interesting to consider the responses of the 13 (52%) companies that do not use CD today. The majority of these companies (ten, 40%) have tried CD and abandoned it. These organizations reported opposite experiences with CD to those that continue to use this method. The primary reason cited for abandoning CD (80%) was that the results generated had proven to be unreliable; other reasons cited

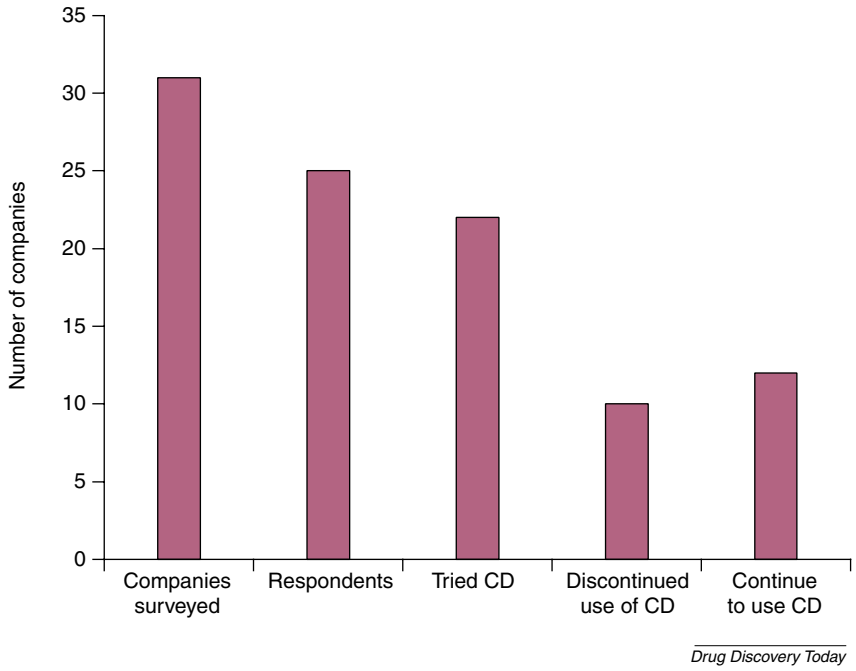

Figure 2. Results of industry survey on the use of cassette dosing. Thirty-one pharmaceutical companies were polled about their experience of using CD. Of the 25 respondents, 22 companies reported that they had tried CD, with 12 companies continuing to use CD and 10 completely abandoning the use of CD. Thus, approximately only a half of respondents use CD for rapid *in vivo* pharmacokinetic screening. Abbreviation: CD, cassette dosing.

included that the analytical requirements were too demanding of analyst time and that CD took too long compared with other available rapid PK screening techniques (Figure 3). The remaining three companies (12%) indicated that they had never tried CD, with one stating that they had never trusted the technique. In addition, two representatives of companies that are actively implementing CD implied that this technique is only used because the director of the department has mandated it. In these two cases, the bench scientists themselves preferred to use CD as little as possible because of the unreliability of the data produced. These views indicate that the industry is surprisingly polarized on the use of CD, with researchers either strongly advocating or opposing the employment of this approach in the drug discovery process.

What is the problem with using cassette dosing?

The diversity in opinion on the use of CD can be understood by examining the advantages and disadvantages of this technique. Features that make the CD approach attractive to a drug discovery team include:

- Rapid analysis time resulting from a reduced analytical sample load. Several respondents reported impressive numbers of candidates being screened each week.
- Reduction in interanimal variability in comparisons of compounds. The testing of several compounds simultaneously in a single animal results in the reduction or elimination of individual differences among animals; interanimal variability could lead to ambiguous results with conventional dosing.
- Consolidated report output. A single experiment performed on many compounds leads to the generation of a single report. By contrast, conventional dosing could potentially result in the production of numerous reports over several weeks.
- Reduction in animal usage. In addition to saving on the animal purchase and husbandry budget, CD can be an important indication of good-faith efforts to an institutional animal care and use committee.

However, some serious practical difficulties with using CD were also reported by researchers. The three most important problems encountered that influenced the decision of PK scientists not to perform investigations using CD or to abandon the use of CD completely were:

- Drug–drug interactions. Data acquired from CD can suggest PK properties for compounds that are different from the PK properties indicated by the discrete dosing of individual compounds. Several scientists told that PK values generated from a CD experiment had later been identified as erroneous and indicated that the inaccuracy had been caused by the occurrence of a significant kinetic interaction.
- Excessive time required by the liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) analyst for the setting up of assay conditions for ten compounds. Often a cassette of five compounds (four unknowns and one standard) is the compromise approach. However, in this scenario, the potential productivity gain of CD is never fully achieved.
- Difficulties in preparing a usable dosing formulation from a mixture. The poor water solubility of many contemporary discovery candidates requires their formulation as suspensions in a vehicle such as methylcellulose or polyethylene glycol (PEG), which could make it difficult to obtain uniformity of the component compounds. For *intra venous* dosing, which requires a solution formulation,

the dissolution of a cassette of compounds might require an organic solvent, even if doses are low.

What is the solution?

It must be remembered that CD was invented to provide rapid *in vivo* PK data to keep up with the fast pace of candidate production and evaluation in drug discovery [9]. That need has not changed. Therefore, there is a continued active publication of CD-based screening programs, but now investigators are more aware of the dangers and design their studies more carefully to incorporate the precautions outlined [8]. For example, Hasegawa *et al.* [10] reported good results in evaluating the PK of antifungals in rats using CD. To avoid major problems with drug–drug interactions, Hasegawa and colleagues used only four unknown compounds, plus a reference compound, per cassette. In addition, they limited the dose to 2 mg/kg/compound and used organic solvents to dissolve the compounds. However, the approach of dissolving compounds in organic solvents could produce misleading results for programs seeking an orally bioavailable drug. Similarly, Mallis *et al.* [11] applied CD to the study of phytoestrogens in rats at low doses and used a cassette of only five compounds. Unfortunately, they reported several specific PK parameters, including maximal concentration in plasma (C_{max}), half-life, area under curve (AUC) and bioavailability, as the definitive values, a step that most PK scientists are reluctant to carry out with the results of CD because of the possibility that the parameters contain substantial errors.

The CD method has been extended to measure brain uptake of a compound, as well as plasma PK [12]. Rats were orally dosed (1 mg/kg/compound) with a cassette comprising four drug candidates and a reference compound. Groups of three rats were sacrificed at selected time points and plasma and brain samples were collected for LC–MS–MS analysis of drug levels. The results obtained from CD were in good agreement with those obtained from single-compound administration. Zhang *et al.* [13] recently reported a similar brain penetration study in which the contemporary standard practice of low dose and small cassette size was adopted. It can be noted that the CD approach is particularly useful for brain penetration studies because the time expended on the labor-intensive procedures of brain removal, homogenization and extraction is minimized. However, the brain uptake component introduces an additional level of drug–drug interaction, specifically, the competitive inhibition of central nervous system-efflux transporters. For example, inhibition of P-glycoprotein (PGP) has been demonstrated to have a significant affect on net brain uptake with some compounds that act as PGP substrates [14]. Thus, the use of a 'biological internal standard' to demonstrate the fidelity of brain uptake results is only valid when the standard is known to be a PGP substrate.

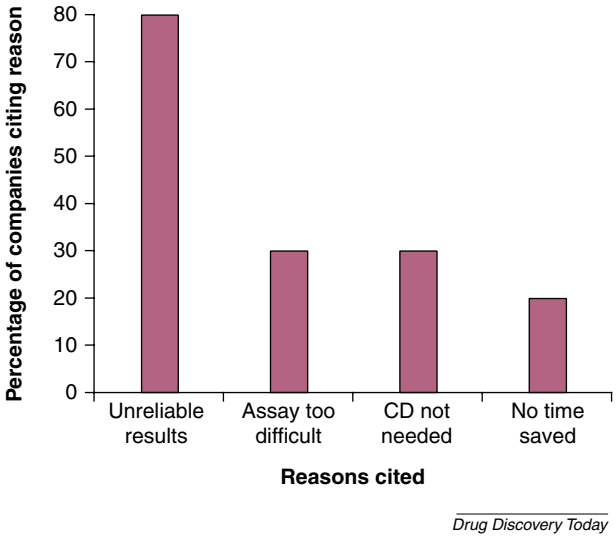

Figure 3. Reason for discontinuing cassette dosing. Among those companies that have tried CD and discontinued its use, 80% indicated that the primary reason for abandoning CD was that the results generated were too unreliable. The next most common reasons cited for discontinuing CD were that the complex LC–MS–MS assays required were too difficult to set up regularly and that CD was not needed because the company had solved its rapid *in vivo* pharmacokinetic requirement with a different approach, such as 'rapid rat'. Two companies (20%) merely stated that CD did not save them time compared to other methods. Most companies indicated more than one reason for discontinuing the use of CD and therefore the response distribution does not total 100%. Abbreviations: CD, cassette dosing; LC–MS–MS, liquid chromatography–mass spectrometry–mass spectrometry.

CD continues to be used in large animals, where animal usage is more of an issue than with rodents. Macdonald *et al.* [15] dosed dogs with an eight-compound cassette at a low dose of 0.2 mg/kg/compound, which again illustrates the need to deal with the practical difficulties of CD. Other researchers used CD technology to screen antivirals and achieved generally good results [16]. However, results produced from the subsequent examination of six of the compounds using discrete dosing indicated a 4.5-fold difference in the half-life of one of the compounds tested [16]. In studies that comprise the dosing of only small total numbers of compounds, the issue of the sustainability of weekly LC–MS–MS assay development is irrelevant. However, Ward *et al.* [17] reported a large study involving CD in monkeys that comprised 218 compounds dosed at approximately 2 mg/kg/compound. Although errors were observed (e.g. >6-fold variation in the bioavailability for one compound in CD versus discrete dosing), compounds were typically classified into the correct bioavailability quintile.

Interestingly, cassette dosing is also being used in humans; however, the purpose of CD in humans is not drug discovery and it is called 'cocktail' dosing. For example, cocktail dosing was used to investigate the influence of hepatic extraction ratio on the magnitude of drug–drug interactions [18]. More recently, several enzyme-specific probe compounds (as many as six) were dosed to human subjects concomitantly with an investigational drug to determine if the new compound inhibited specific drug metabolism pathways [19]. These two examples illustrate the contrast between drug discovery CD and clinical cocktail dosing. Although these two techniques provide rapid answers and direct comparisons within a single subject, in drug discovery CD it is hoped that drug–drug interactions are avoided, which is in contrast to cocktail dosing where the purpose is to identify drug–drug interactions.

There is a continued exploration of the methodology of CD. At least one study avoided using expensive LC–MS–MS equipment by employing an alternative LC-fluorescence method [20]. However, the limitation of this approach was that only three compounds could be assayed simultaneously and, even without this drawback, it is doubtful that this method could be generally applied to other investigations encompassing CD. Keseru and Molnar [21] recently reported the creation of a computational method (METAPRINT) for the design of cassettes that considers chemical diversity and the possible production of metabolites that could potentially interfere with the compound of interest [21]. Although no examples of the implementation of METAPRINT have as yet been presented, noncybernetic consideration of possible interfering metabolites has been reported [13]. Finally, a new column-switching method that enables chromatographic method development and helps to minimize mass spectrometric interference by PEG, which is used in as a suspension agent in some CD formulations, has been reported [22]. Ohkawa *et al*. [22] stated that this approach facilitated result analysis, a claim that was subsequently proved by the application of the new method to 50 cassettes and a total of 200 compounds.

'Right box' analysis

The term 'right box' analysis was introduced in 2001 to denote the placement of a drug candidate into the correct broad category (i.e. the right box) [8]. The suitability of a drug candidate can be assessed in terms of PK parameters, such as clearance, half-life and oral bioavailability, which have clearly defined ranges of acceptable values. For instance, oral bioavailability could be divided into four categories – poor (0–10%), moderate (10–50%), good (50–80%) and excellent (80–100%). If the right box screening technique successfully placed a candidate into the correct category of bioavailability, it is not important for the numerical value to be exact – it only matters that the candidate is correctly identified as having 'poor' bioavailability to enable the deprioritization of the compound for further screening. For example, if the correct bioavailability of the candidate was ~3% but it appeared to have 9% bioavailability in CD (because of a drug–drug interaction), then CD correctly placed the candidate into the right box even though the observed value includes a 200% error. Accordingly, following our 2001 publication [8], it has become increasingly common for researchers to use the right box analysis to interpret CD results [16,17].

Alternatives to cassette dosing

The 50% of scientists that choose not to use CD have had to explore other options for the acquisition of rapid, *in vivo* PK information, and several innovations have been reported. The two general approaches described are: (i) assay enhancement, or the use of various technological tricks such as rapid sample preparation and parallel HPLC, to accelerate the analytical step; and (ii) sample reduction, or minimizing the number of samples that must be assayed by such means as pooling plasma samples or truncating the period of PK sample collection [23]. The pooling of plasma samples from individually dosed animals to achieve reduced analysis times while avoiding drug–drug interactions has proved particularly popular (also called cassette analysis [24]). The group that instigated sample pooling for plasma PK measurements also showed the utility of sample pooling in expediting the determination of brain penetration while avoiding the complications associated with CD [25]. Another variation on rapid *in vivo* PK determination through sample pooling is the use of ion trap mass spectrometry (rather than triple-quadrupole mass spectrometry), which enables the concurrent assay of the parent drug and the detection and identification of metabolites [26]. Hsieh *et al*. [27] have taken sample pooling a step further with the use of direct plasma injection technology to eliminate the sample preparation step and, thus, achieve shorter total analysis times.

The 'in-life' phase of rapid *in vivo* PK determination has also been accelerated. The head of a prominent contract research organization offering ADME services related that, until a few years ago, the company received many requests for CD, but such enquiries have now become rare. Instead, demand has switched to rapid, discrete dosing and this organization now offers PK screening with a one-week turnaround time. A comprehensive approach that provides higher-throughput screening with individually dosed animals was introduced a few years ago [28]. This approach, which is called cassette-accelerated rapid rat screen (CARRS) or

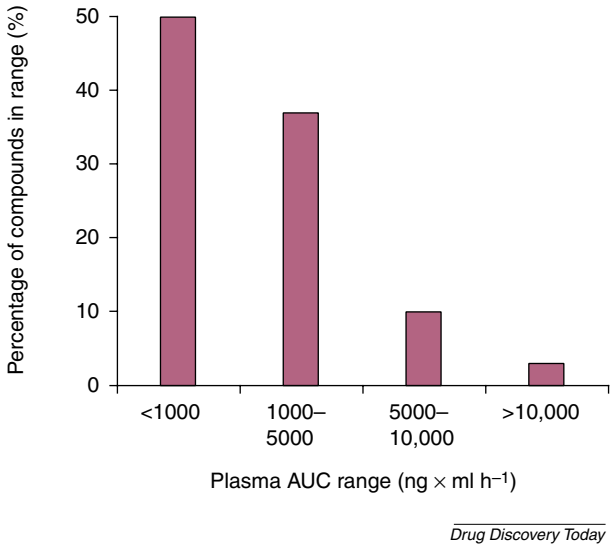

Figure 4. Distribution of plasma area under curve-values among drug discovery compounds. Discovery compounds will typically display a distribution in which most of the compounds have poor PK properties [17]. In this example, plasma AUC-values that were generated from the dosing of rats at 10 mg/kg are illustrated. Although exact cut-offs are somewhat arbitrary, it is clear that the 50% of compounds having AUC-values of <1000 ng × ml h^{-1} are unlikely to be considered as candidates for further development compared with compounds having AUC-values of >5000 ng × ml h^{-1}. To ensure that resources are conserved for drug development candidates, compounds that show no promise must be quickly identified and eliminated from consideration. The cut-off AUC for progressing compounds to the next stage will vary from program to program and will depend on many factors. In the representative example shown, the 'rapid rat' screen facilitated the testing of 600 compounds from a single discovery program over a period of months. Although approximately only 13% of the compounds had AUC-values >5000 ng × ml h^{-1}, the large number of candidates tested led to the identification of 75 compounds with excellent PK parameters for subsequent screening in pharmacological models. Abbreviations: AUC, area under curve; PK, pharmacokinetic.

just 'rapid rat', involves complete regimentation of the entire process of *in vivo* PK screening, including weekly designation of candidate compounds, regularly scheduled animal procurement and dosing, coordinated LC–MS–MS plasma sample analysis and automated result calculation, display and report generation. Rapid rat is similar to CD in that all aspects of the experiment are performed with cassettes of compounds, with the exception of the in-life phase.

In the Schering-Plough (http://www.sch-plough.com) implementation of rapid rat, medicinal chemists select 48 compounds for PK screening in rats each week and the candidates are organized into groups of six compounds from the same drug discovery program (cassettes). Therefore, eight cassettes are designated on a weekly basis, which potentially affords several combinations of cassettes and programs, for example, eight programs with one cassette per program or one program with eight cassettes. Following assembly and delivery of the cassettes by the medicinal chemistry department, each of the 48 compounds to be tested is dosed to two rats (dosing occurs over three consecutive days) and blood is sampled from each rat at six specific times over a 6 h time period after administration. The blood samples from each time point are pooled, plasma is prepared and the samples are transferred to the bioanalytical group. Thus, the samples from each cassette, which has six time points per compound, standard and blank, exactly fill a 96-well plate, which significantly simplifies sample handling, analysis and data work-up. Dedicated mass spectrometers analyze the eight plates in automated overnight runs and the report is issued within two weeks from the original cassette designation. The coordination of the procedure ensures that while the dosing group is performing the in-life phase, the bioanalytical group is assaying samples of the dosings from the previous week. Hence, eight reports on the eight cassettes (48 compounds) are issued each week. The data output for each compound is a graphical representation of the 6 h time-concentration profile, an estimate of C_{max} and a 6 h AUC. These parameters are virtually always sufficient to differentiate those compounds exhibiting poor bioavailability in a series of discovery candidates (Figure 4).

The rapid rat system has proven to be robust and indefinitely sustainable. Indeed, Walter Korfmacher, the director of the exploratory bioanalytical group at Schering-Plough, reports that the weekly running of the 'rapid-rat train' has enabled the screening of over 7000 compounds in the four years since its inception. Chemists are enthusiastic because they can rely on acquiring results from large numbers of compounds on a fast and predictable schedule, whereas PK scientists are satisfied because they can be certain that the results are not distorted by the occurrence of drug–drug interactions.

Conclusion

Based on a survey of the pharmaceutical industry, we identified a decline in the frequency of use of CD in drug discovery, a finding that is supported by the recent literature. Only a half of the companies surveyed are still actively and extensively using CD. More importantly, following the publication of our analysis of PK theory and consequent recommendations [8], the application of CD has been modified. Specifically, we discovered that the majority of CD users now limit cassettes to five or fewer compounds, use doses in the 1 mg/kg/compound range or lower and use CD-derived PK parameters only semi-quantitatively, for example, for the ranking of compounds or for 'right box'

Box 1. Recommendations to follow when applying cassette dosing to drug discovery

- Limit the total number of compounds in a cassette to five or less.
- Use the lowest detectable dose size for individual compounds.
- Include a benchmark compound as an internal standard to verify the results obtained.
- Never accept the absolute values of pharmacokinetic parameters from cassette dosing as definitive.
- Use 'right box' analysis whenever possible.

analysis. Therefore, we recommend that those who choose to use CD adopt the 'best practices' outlined in Box 1.

Approximately 50% of the industry use alternatives to CD, such as variations on the plasma-sample-pooling technique following the discrete dosing of individual compounds. With all the current innovations in place, including a cassette approach that excludes the dosing step, these methods exhibit a sustained throughput that exceeds the level of throughput of CD but has none of the associated problems. However, rapid discrete dosing comes with its own set of disadvantages, and the procedure that a research group elects will depend on their particular tolerance for ambiguity, access to LC–MS–MS resources and capacity for animal dosing.

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