Novel *in vivo* procedure for rapid pharmacokinetic screening of discovery compounds in rats

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In therapeutic areas aimed at developing an orally administered drug, the pharmacokinetic profile of a drug candidate after oral administration *in vivo* is pivotal in evaluating its success. This can be done by monitoring the plasma concentration versus time after dosing and calculating the area under the curve (AUC). The authors describe a novel screening protocol in which an estimated AUC can be determined, allowing the rapid evaluation of large numbers of compounds and providing a rank order of estimated AUC values to prioritize compounds for further investigation.

As automatic synthesis methods such as combinatorial chemistry and parallel synthesis become more routine in the generation of potential drug candidates, there is a need for techniques that can be used to increase sample throughput in the analysis of samples from pharmacokinetic (PK) studies of new compounds. Mass spectrometry (MS) has proved to be a powerful analytical technique in the analysis of samples generated from PK studies. The use of MS for the support of drug metabolism studies for drugs in development is well documented[1–6]. The introduction of HPLC coupled with atmospheric pressure ionization (API) and tandem MS (HPLC–API–MS/MS) has provided new opportunities for this technology to be applied to the participation of drug metabolism in drug discovery[7–11]. Now that the technology has become an established tool for supporting drug discovery efforts, the issue of increasing throughput has arisen. One advantage of HPLC–API–MS/MS is that it can be used for multiple analysis, which can be defined as the determination of more than one compound in one chromatographic assay, and has led to its use in multidrug studies in which several compounds are dosed simultaneously in a laboratory animal[12–17]. These studies are also referred to as combinatorial pharmacokinetic studies, 'cassette dosing' or 'N-in-one' dosing studies[13]. The advantages of a multidrug study are that more compounds can be dosed in a shorter time using fewer animals and that the sample analysis is more efficient. On the other hand, drug–drug interactions could lead to misleading PK results[13]. One way to minimize potential drug–drug interactions is to reduce the dosing level. A recent approach is to dose at $10/n$ mg kg^{-1}, where n is the number of compounds being dosed. Another disadvantage of multidrug dosing studies is that the time for method development and data calculation is increased over the more common single-dosing/single-assay studies. Further, metabolite identification is not practical with multidrug studies. A

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Box 1. Animal dosing and sample collection

Following an overnight fast, male Sprague-Dawley rats (Charles River Company) receive a test compound at a dose of 10 mg kg^{-1} orally in a 5 ml kg^{-1} dose volume. For each compound two rats were dosed. Serial blood samples were collected hourly into heparin-containing tubes from each animal at 1–6 h postdosing and centrifuged to generate plasma. From each time point, 30 μl of plasma were pooled to yield one pooled sample per animal. Approximately 50 μl of the plasma samples collected at the individual time points remained in case further analysis was required. The plasma samples were stored at −20°C until analysis.

Box 2. Sample and standard curve preparation

The pooled samples (n = 2/compound) and the 6-h plasma samples (n = 2/compound) were assayed. A 40 μl aliquot of each plasma sample was precipitated with 100 μl of acetonitrile containing 1 ng μl^{-1} of an internal standard. A structural analog of the compounds tested was used as an internal standard and was the same for all compounds assayed. A mini calibration curve was prepared (two points plus a zero) for each compound assayed. Drug-free rat plasma was measured into 1-ml aliquots and each aliquot was spiked with known concentrations of the compounds to generate standards of the desired concentrations. The concentrations of the standards were chosen to encompass the expected concentration of the pooled samples based on historical data. Standards were set to contain concentrations of 25 and 250 ng ml^{-1} plasma. The plasma standards were precipitated in duplicate along with the samples. The precipitated samples and standards were vortexed and centrifuged. Approximately 50–100 μl of the supernatant were removed and placed in an autosampler glass insert. A volume of 25 μl of the supernatant was used for analysis by HPLC–API–MS/MS. The mini standard curve was run in duplicate, once before the samples and once after the samples. Thus a total of ten samples plus standards were analyzed per compound. Solvent blanks were injected after the highest standard concentration for each compound assayed. Up to eight compounds can be analyzed in one overnight assay using an autosampler that holds 96 samples.

Box 3. HPLC–API–MS/MS equipment

The HPLC–API–MS/MS system consisted of a Waters HPLC pump and controller, and a Finnigan TSQ 7000 mass spectrometer. Chromatographic separation of the plasma samples was carried out on a Waters Symmetry C_{18} column (3.9 mm × 50 mm, 5 μm particle size) using a linear gradient of two solvents. Solvent A consisted of 0.01 M ammonium acetate in 20% methanol in 80% water; solvent B consisted of 0.01 M ammonium acetate in methanol acidified with 600 μl l^{-1} of 10% acetic acid solution. A simple reverse-phase linear gradient was used in the evaluation of all compounds. The total run time was 8 min per sample.

The compounds were analyzed by positive-ion API–MS/MS. Selected reaction monitoring methods were developed for each compound prior to analysis of the plasma samples. A 100 ng μl^{-1} solution of each compound in methanol was infused into the mass spectrometer and a product ion spectrum was taken to determine the proper transition for analysis. A transition from the protonated parent mass (MH^+) to a characteristic fragment ion mass was chosen for each compound analyzed. This provides a selective and sensitive method of analysis that is unique for each compound. A selected reaction monitoring procedure was established for each compound; this measured a specific fragmentation pathway for the compound of interest and the internal standard throughout the chromatographic run.

metabolite might have the same mass as another drug in the assay. For these reasons, the utility of multidrug studies is still unclear.

An alternative to these multidrug studies is the use of PK screening, which is a systematic procedure for rapidly assessing one PK parameter, such as oral area under the curve (AUC) or intravenous clearance, of a series of related compounds. This article describes a PK screening experiment for the determination of oral plasma drug levels (AUC) in rats and provides validation of its utility to screen many compounds rapidly. Rats are dosed with a compound and plasma is collected at equal time intervals over a period of 6 h (Box 1). The plasma samples collected from each of the time points of an individual rat are then pooled and analyzed along with a mini standard curve of concentrations encompassing the concentration of the pooled plasma sample (Box 2). An estimated AUC can be calculated from this pooled plasma concentration. Samples are analyzed by HPLC–API–MS/MS (Box 3). Analysis of a pooled plasma sample and use of an abbreviated standard curve per compound greatly reduces the time required to obtain PK data and a large number of compounds can thus be evaluated fairly quickly. A rank order of estimated AUC values can be constructed from this screening process to prioritize compounds for further investigation.

Determination of AUC and simplification for screening

The area under the plasma concentration–time curve (AUC) is a pharmacokinetically relevant parameter that is based on the concentration of a drug in plasma over a given time course. Ideally, the AUC is determined by measuring plasma concentrations continuously over time and integrating the concentrations over this time course. For practical applications, plasma is typically collected at various times and the concentration of each sample is determined and plotted. The AUC can be calculated using the trapezoidal rule:

$$A_i = \tfrac{1}{2}\Delta t(C_{i-1} + C_i) \quad (1)$$

When n samples of plasma are taken at (n = 1) equal time intervals of Δt, the trapezoidal rule predicts that the area under the plasma concentration time curve for the time 0 to t_{n-1} will be:

$$[\mathrm{AUC}]_0^{t_{n-1}} = \Delta t\left(\tfrac{1}{2}C_0 + C_1 + C_2 \ldots C_{(n-1)} + \tfrac{1}{2}C_n\right) \quad (2)$$

When the concentrations C_0 and C_n are zero or very low, the samples of equal volume (V_i) can be pooled and only one analytical determination is required to obtain the AUC (Ref. 18). However, when C_0 and C_n are not zero, it is possible to take equal concentrations (V_i) of samples 1 to (n – 1) and take one-half of the volume (V_i/2) for C_0 and C_n, the first and last samples, respectively, and pool them to obtain an observed mean plasma concentration (C_{obs}), which can be corrected for the differences in volume obtained for C_0 and C_n. The correction factor to give the actual mean concentration (C_{ave}) is simply (n – 1)/n, thus:

$$C_{ave} = \frac{n \pm 1}{nC_{obs}} \quad (3)$$

and the AUC is:

$$[\mathrm{AUC}]_0^n = \Delta t(nC_{ave}) = \Delta t(n-1)C_{obs} \quad (4)$$

These simple relationships require that the pooled sample volumes (V_i) are the same, except the first and last samples which use half the volume (V_i/2), and that the samples are all equally spaced (Δt). Hop *et al.*[19] have presented procedures using different volumes when unequal time intervals are used. We prefer to use a simpler method, however, to accelerate screening. The method described here has made additional assumptions to provide the simplest approach possible for rapid screening. It is assumed that the concentration at the first time point (t_0) is zero and therefore a sample is not taken. It is also assumed that the concentration of the last sample is sufficiently low that the same sample volume can be used for all time points. While this will slightly overestimate the AUC (<5%), it simplifies the procedure. The volume correction factor is the same as above (Eqn 3) and Eqn 4 also holds. In addition, all the compounds are tested in the same way and the rankings are not significantly affected. However, to implement this procedure for samples resulting from an intravenous dose, it might be necessary to use half the volume of the last concentration to obtain more accurate estimates of AUC.

Analysis of a pooled plasma sample generated by pooling equal volumes of plasma from equally spaced time points gives essentially an average concentration over the entire time course monitored. Multiplication of this average plasma concentration by the total time gives a rectangular area that roughly estimates the actual AUC (Fig. 1). Here, plasma drug concentrations are measured at equally spaced time points from 1 to 6 h and the AUC is calculated. Equal volumes of these plasma samples are then pooled and the concentration of this single sample is measured. The area encompassed by the pooled plasma concentration over the 6-h time course gives a rough estimate of the AUC determined by measuring the individual plasma concentrations. An initial plasma sample (t = 0 h) is not analyzed or used in generating the pooled sample. The concentration at t = 0 h is assumed to be zero and the calculation for the AUC from the individual samples and the pooled samples takes this into consideration. The pooled plasma concentration is

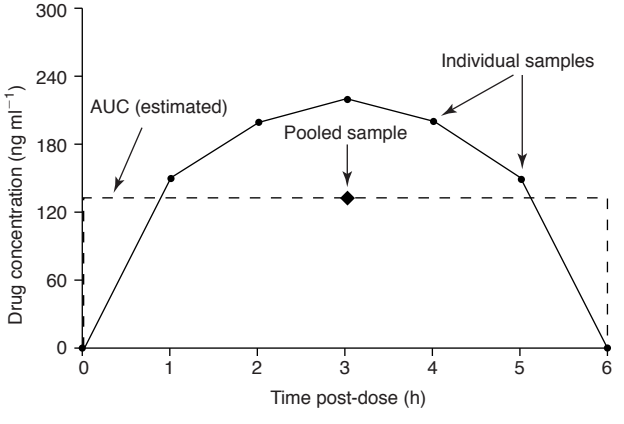

Figure 1. *Illustration of the area under the curve (AUC) determination using a single pooled plasma measurement versus analysis of individual time points.*

Table 1. Determination of estimated AUC

Drug evaluation	Rat A	Rat B	Mean
Pooled plasma: measured (ng ml^{-1})	330	281	306
Pooled plasma: corrected (ng ml^{-1})[a]	283	241	262
Estimated $AUC_{(0-6\ h)}$ (ng.h ml^{-1})[b]	1698	1446	1572

[a]Corrected = measured × 6/7.

[b]Estimated AUC = corrected pooled sample × 6 h.

multiplied by 6/7 to account for the volume difference that results from pooling from six time points and integrating over the time course 0–6 h. This corrects for the missing $t = 0$ sample which would be the seventh time point with a concentration of zero. An example of the calculations used for the pooled plasma samples is shown in Table 1.

Practical implications of an oral AUC PK screen

Analysis of pooled samples provides a rapid procedure for obtaining PK data for many compounds. Determination of relative AUC values after oral dosing is one way of evaluating compounds to screen out those that give poor PK profiles and to concentrate on those that give good profiles. As discussed above, this method does not provide an actual AUC value – rather it gives an estimated value. Large numbers of compounds evaluated in this manner should provide a rank order of estimated AUC values. This rapid PK-profiling method has been used successfully to provide a rank order of over 200 compounds in a specific therapeutic area. A summary of the estimated AUC values obtained for 30 compounds in a specific therapeutic area is provided in Table 2. The compounds that gave estimated AUC values above 900 ng.h ml^{-1} were of interest as potential leads, so the plasma samples collected at the individual time points were analyzed to provide a calculated AUC value over the 6-h time window. The AUC values calculated from the analysis of plasma collected at the individual time points is shown in Table 3 for the first four compounds. There is a fairly good correlation between the estimated AUC values and the calculated AUC values.

Additional information provided by examining the plasma concentration at 6 h

In the example illustrated in Fig. 2, plasma levels are still high at the end of the 6-h time course. As discussed above, this adds only a minimal error to the AUC calculation, and the fact that this drug remains in the plasma for a relatively long time might be important in evaluating the duration of action. If so, then individual plasma samples collected at the end of the time course ($t = 6$ h) can be analyzed along with the pooled sample. This does not add a significant

Table 2. Results of rapid pharmacokinetic screen[a]

Compound	Estimated $AUC_{(0-6\ h)}$ (ng.h ml^{-1})	% CV ($n = 2$)	Concentration at 6 h (ng ml^{-1})
1	5149	56	384
2	2162	17	525
3	1572	11	280
4	908	48	200
5	770	65	72
6	724	22	99
7	704	40	99
8	601	9	72
9	585	54	79
10	562	8	107
11	535	75	22
12	511	11	63
13	490	3	68
14	438	5	54
15	431	42	37
16	381	10	68
17	352	15	95
18	309	9	0
19	301	12	28
20	280	7	37
21	254	58	34
22	247	22	34
23	203	23	29
24	176	42	15
25	136	23	19
26	129	7	21
27	91	24	4
28	62	67	7
29	42	74	2
30	17	14	0

[a]10 mg kg^{-1} oral administration in rats ($n = 2$); estimated AUC calculation from pooled samples.

Table 3. Estimated AUC vs. AUC values calculated from individual plasma concentrations

Compound	Estimated $AUC_{(0-6\ h)}$ (ng.h ml^{-1})	% CV	Measured $AUC_{(0-6\ h)}$ (ng.h ml^{-1})[a]	% CV	Δ%[b]
1	5149	56	4916	47	−5
2	2162	17	2496	19	13
3	1572	11	1920	12	18
4	908	48	855	45	−6

[a]Calculated using plasma concentrations from individual time points (0–6 h) and a full standard curve (0–5000 ng ml^{-1}) in duplicate.

[b]Δ% = % difference = [(measured AUC − estimated AUC) ÷ measured AUC] ×

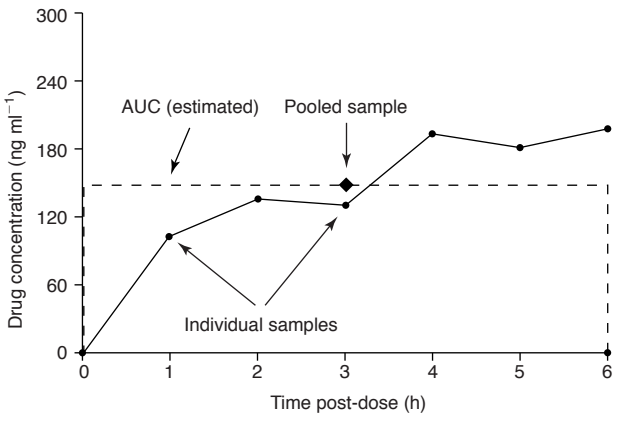

Figure 2. *Comparison of estimated area under the curve (AUC) values calculated from pooled plasma concentrations with AUC values calculated from plasma concentrations measured at individual time points.*

amount of time to the analytical method (n = 2/compound), yet can provide valuable information for compound screening. A low plasma concentration at 6 h indicates that a compound might have a short oral half-life. A 6-h plasma concentration that is roughly equal to the pooled plasma concentration indicates that the compound has probably reached its C_{max} and has a relatively long oral half-life. A 6-h plasma concentration that is greater than the pooled plasma concentration indicates either a long half-life or a slow rate of absorption. The plasma concentrations at 6 h are listed for each of the compounds in Table 2. Compound **1** shows the highest AUC, but its concentration at 6 h is lower than that of compound **2**, indicating that **2** gives lower plasma levels overall but probably has a longer half-life. Both compounds would be considered lead compounds as a result of this rapid PK screen.

One of the biggest advantages of this rapid PK screen is the time saved during the analysis. Typical PK profiling of compounds from an oral dosing study involves the analysis of a 12-point standard curve in duplicate (n = 24 standards/compound) plus samples gathered at numerous time points (n = 24 samples/compound). Utilization of the rapid PK screen allows a reduction in the number of standards to n = 6/compound and the number of samples to n = 4/compound. This results in a dramatic increase in the number of compounds that can be assayed. A throughput comparison (per LC–MS/MS system) of a standard PK study versus the rapid PK screen is provided in Box 4.

Box 4. Comparison of standard PK study and rapid PK screen

Standard rat oral PK analysis

Dosing and sample collection
10 mg kg^{-1} oral administration (n = 3 rats)
Collect blood 0–6 h post dosing

Analysis
Full standard curve (n = 24 standards per compound)
Analyze plasma from individual time points
(n = 24 samples per compound)
Calculate AUC values

Throughput
Four compounds per week (per LC–MS/MS system)

Rapid rat oral PK screen

Dosing and sample collection
10 mg kg^{-1} oral administration (n = 2 rats)
Collect blood hourly from 1 to 6 h post dosing
Pool plasma from individual time points

Analysis
Mini standard curve (n = 6 standards per compound)
Analyse pooled plasma samples (n = 2 per compound)
and 6 h time point (n = 2 per compound)
Calculate estimated AUC values

Throughput
Sixteen compounds per week (per LC–MS/MS system)

Conclusions

At the early stages in drug discovery it is not essential to obtain a full PK profile but rather a rank order of compounds. In a program aimed at developing an orally administered drug, it is important to obtain oral PK information on potential drug candidates as quickly as possible. This type of information can also be useful in screening compounds, because those that exhibit poor oral PK profiles early in the drug discovery phase are not of interest. A screen that eliminates these compounds allows attention to be focused on fewer potential lead compounds for which more detailed PK profiles can be obtained. With this goal in mind, a screening method for evaluating the oral AUC of compounds has been developed. By pooling plasma samples collected at equally spaced time points over a specific period, an average plasma concentration can be determined. Multiplication of this average concentration by the time period gives an estimated AUC, and provides a means of ranking compounds based on early PK information. Clearly, the analysis of more plasma samples throughout the time course will give a

more accurate PK profile of each compound. Analysis of the plasma collected at the last time point can give a more accurate AUC determination and can also give some insight into the oral half-life of each compound. While a more comprehensive PK profile is necessary for the full evaluation of lead compounds, the basic, simple screening procedure described in this report, in which the analysis of only a single sample from each compound is required, allows the evaluation of many compounds in a relatively short time and provides valuable information as to which compounds should be evaluated further and which compounds do not meet the criteria of a potential lead drug candidate.

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REFERENCES

1 Kebarle, P. and Tang. L. (1993) *Anal. Chem.* 22, 972A–986A
2 Gelpi, E. (1995) *J. Chromatogr. A* 703, 59–80
3 Covey, T.R., Lee, E.D. and Henion, J.D. (1986) *Anal. Chem.* 58, 2453–2460
4 Korfmacher, W.A. *et al.* (1995) in *Encyclopedia of Analytical Science*, pp. 3027–3034, Academic Press
5 Korfmacher, W.A. *et al.* (1990) *Biomed. Environ. Mass Spectrom.* 19, 191–201
6 Baillie, T.A. (1992) *Int. J. Mass Spectrom. Ion Processes* 118/119, 289–314
7 Allen, G.D. *et al.* (1996) *LC·GC* 14, 510–514
8 Volmer, D.A. and Vollmer, D.L. (1996) *LC·GC* 14, 236–242
9 Korfmacher, W.A. *et al.* (1997) *Drug Discovery Today* 2, 532–537
10 Bryant, M.S. *et al.* (1997) *J. Chromatogr. A* 777, 61–66
11 Brewer, E. and Henion, J. (1998) *J. Pharm. Sci.* 87, 395–402
12 Halm, K. (1996) *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics*, Portland, OR, USA 12–16 May, p. 1465, ASMS
13 Berman, J. *et al.* (1997) *J. Med. Chem.* 40, 829–831
14 Olah, T.V. *et al.* (1997) *Rapid Commun. Mass Spectrom.* 11, 17–23
15 Allen, M.C., Shah, T.S. and Day, W.W. (1998) *Pharm. Res.* 15, 93–97
16 Adkinson, K.K. *et al.* (1996) *Pharm. Res.* 13, S486
17 Berman, J. *et al.* (1997) *J. Med. Chem.* 40, 827–829
18 Orr, J.S., Shimmins, J. and Spiers, C.F. (1969) *Lancet* 2, 771–773
19 Hop, E.C.A. *et al.* (1998) *J. Pharm. Sci.* 87, 901–903

In short...

Gail Wertz, a professor of microbiology at the University of Alabama (Birmingham, AL, USA), has received a $500,000 award from the Bristol-Myers Squibb Foundation (New York, NY, USA). Wertz is one of only two researchers selected this year by Bristol-Myers Squibb to receive a five-year, unrestricted infectious disease research grant. At present, only nine researchers worldwide hold such grants, which may be used to fund any research into the field of infectious disease. 'Unrestricted grants are especially valuable', Wertz says, 'because they allow you to put resources into studies which the traditional grant system may not be able to support, although they may be of great benefit.'

Wertz plans to use the grant to fund further research into the area of viral replication and gene expression. Wertz pioneered genetic engineering of RNA-based viruses – those that carry their genetic information on strands of RNA, not DNA – such as rabies, mumps and measles. The technique of genetic engineering, used to make changes within the genetic sequence of a virus, is a powerful method used to learn about the virus, how it works and how infections may be prevented or treated.

At present, Wertz's laboratory is working on respiratory syncytail virus, the major cause of childhood bronchiolitis and pneumonia, and vesicular stomatitis virus (VSV), a pathogen of cattle, horses and swine. In 1998, Wertz used the new genetic engineering technology to develop and test a new type of vaccine for VSV. She explains, 'We found that by moving a single viral gene which is required for replication, we could reduce the virus' ability to cause disease in a stepwise manner until it failed to cause any disease at all. But it still stimulated a protective immune response – just what you want in a vaccine.

The infectious disease grants program is one of six biomedical research grants programmes funded by Bristol-Meyers Squibb. Other programmes support research into the areas of cancer, cardiovascular/metabolic diseases, neuroscience, nutrition and orthopaedics.