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# Effective strategies for the development of specific, sensitive and rapid multiple-component assays for cassette dosing pharmacokinetic screening

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**Abstract**

Enabled by liquid chromatography/tandem mass spectrometry (LC/MS/MS) based multiple-component assays, cassette dosing is an effective tool to significantly improve the throughput for pharmacokinetic (PK) screening. However, this higher throughput approach also carries its limitations. In addition to potential drug–drug interactions, the multiple-component assays used to analyze cassette-dosing samples are also subject to potentially serious errors. In this work, a systematic approach has been taken to investigate each critical step in the development of a specific, accurate, and rapid multiple-component assay. These steps include pool selection, sample preparation, chromatographic separation and assay qualification. Based on the results, effective strategies for the development of multiple-component assays are proposed which involve proactive pool assignment, on-line extraction, monolithic-column separation and cassette/discrete response comparison. The effectiveness of these strategies was evaluated by comparing the pharmacokinetic results between discrete and cassette studies for over 20 compounds. Statistically good correlation between discrete and cassette data was established.
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*Keywords:* Cassette dosing; Multiple-component assays; LC/MS/MS; Bioanalysis

## 1. Introduction

In an effort to reduce the ever-increasing cost associated with the high attrition rate in drug development, the evaluation and optimization of absorption, distribution, metabolism, and elimination (ADME) properties of new chemical entities has been shifted upstream into the early discovery stage in the pharmaceutical industry [1,2]. This shift has significantly increased the workload on ADME screening. Particularly, with the implementation of combinatorial chemistry and parallel assays for in vitro potency screening, the number of new chemical entities that are put into ADME screening has increased dramatically. While numerous high-throughput in vitro ADME studies have been developed and successfully implemented, the prediction of in vivo ADME properties based on in vitro methods has not always been successful [3,4]. Therefore, pharmacokinetic (PK) screening in vivo remains to be the primary screening method in many drug discovery programs. To improve the throughput of the animal studies and the subsequent bioanalysis, a new PK screening approach involving a parallel concept, namely cassette dosing was first reported in 1995 [5]. In this approach, multiple compounds are dosed to the same animal followed by bioanalysis using liquid chromatography/tandem mass spectrometry (LC/MS/MS) based multiple-component assays. By going through a similar work cycle of a conventional discrete PK study, the PK properties for multiple compounds can be obtained simultaneously, resulting in considerably enhanced throughput. Since its introduction, the use of the cassette dosing approach has been reported by several groups [6–12].

Like any other high-throughput strategies, cassette dosing carries its limitations and risks. The PK properties ob-

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tained from cassette dosing could potentially deviate significantly from those in discrete studies if certain biological concerns are not addressed properly [13,14]. Among these concerns, drug–drug interactions due to the inhibition of P450 enzymes or drug transporters are potentially a major source of error. In addition to potential errors from these biological concerns, serious error can also occur if the specificity, accuracy, and sensitivity of the multiple-component assays are not ensured during analytical method development. First, compounds in the cassette may interfere directly with each other, if they overlap in mass and chromatographic retention time. This kind of interference is relatively easy to detect. Also, interference can result from mass and retention time overlap with a metabolite of another compound in the cassette. It is sometimes difficult to predict this kind of interference since the metabolic profile and the retention time of metabolites are usually unknown at this stage. In addition, analytical results may be biased due to the differentiation of ion suppression effects with and without the other compounds in the cassette. These kinds of errors are sometimes overlooked but can become significant in some cases. Finally, another potential limitation is related to the assay sensitivity. Late time point concentration information may be lost since dose levels are often considerably reduced in cassette dosing.

In this work, we studied each critical step in the development of a multiple-component LC/MS/MS method and proposed strategies to develop specific, accurate and rapid multiple-component assays in support of cassette dosing. We also report our evaluation of the results of cassette dosing performed using these strategies.

## 2. Experimental

### 2.1. LC/MS/MS system

The LC/MS/MS system consisted of a binary HPLC pump (10 AD, Shimadzu Corporation, Columbia, MD, USA), an autosampler (HTS PAL, Leap Technologies, Carrboro, NC, USA) and a triple-quadrupole mass spectrometer (API-4000, Applied Biosystems Inc., Foster City, CA, USA). All these components were controlled through Analyst software (version 1.2, Applied Biosystems Inc.) on a Pentium IV computer. Unless otherwise noted in the text, formic acid (0.1%) in water and acetonitrile were used as mobile phases A and B, respectively. For separations that use conventional particulate columns (YMC AQ column, 2 mm × 50 mm, Waters Corporation, unless otherwise noted in the text), gradient elution mode was used with a total flow rate of 400 μL/min. For separations that use monolithic columns (Chromolith SpeedROD, RP-18e, 4.6 mm × 50 mm, Merck KgaA, Darmstadt, Germany), gradient elution with a total flow rate of 4 mL/min was used for separation and the flow was split down to 0.4 mL/min via a Tee before introducing into the mass spectrometer. For this work, the mass spectrometer was operated in multiple reaction monitoring (MRM) mode using positive ion electrospray ionization.

### 2.2. On-line extraction system

A home-built on-line extraction system similar to the one previously reported was set up by making some minor modification of the LC/MS/MS system described above [10]. Briefly, an additional binary HPLC pump (1100, Agilent Technologies, Palo Alto, CA, USA) was employed to deliver solvent for on-line extraction. An extraction column (Cyclone, 0.5 mm × 50 mm, Cohesive Technologies, Franklin, MA, USA) with smaller column i.d. than previously reported was interfaced to the LC/MS/MS system via an electrically actuated six-port valve preinstalled in the Agilent pump column oven. Samples in their biological matrices were injected directly onto the system after mixing with an equal volume of internal standard in high aqueous solution. The injection volume was 50 μL. The sample was loaded and washed on the extraction column with water at 1 mL/min for 1 min. The retained analyte was then back-flushed and eluted onto the separation column via column switching using a gradient at a flow rate of 400 μL/min. The extraction column was recycled with methanol and water at 1 mL/min for 1 min, respectively.

### 2.3. Sample preparation

For the protein precipitation method, an aliquot of 50 μL of the plasma sample was transferred to a 96-deep-well plate. The sample was then mixed with 150 μL of acetonitrile containing the internal standard at 100 nM. After vortexing, the samples were centrifuged at 3000 × $g$ for 10 min. The supernatant was dried down and reconstituted in 150 μL of 5% acetonitrile with 0.1% formic acid. An injection volume of 50 μL was used.

For the on-line extraction method, an aliquot of 50 μL of the plasma sample was mixed with 50 μL of an aqueous internal standard solution (200 nM) in a 96-deep-well plate. The samples were ready for injection after vortexing.

### 2.4. Chemicals and materials

All commercially available chemicals were purchased from Sigma (St. Louis, MO, USA) and they were used without further purification. All proprietary compounds used in this work were synthesized at Millennium Pharmaceuticals Inc. (Cambridge, MA, USA).

### 2.5. Animal study

Three male Sprague–Dawley rats were used in each cassette dosing study. Each of these rats was dosed with a mixture of compounds at 0.5 mg/kg each in 50% HP cyclodextrin as an IV bolus. Plasma samples were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 8 and 24 h, respectively.

## 3. Results and discussion

As an approach to perform PK screening in a parallel mode, cassette dosing can significantly improve throughput while saving both animal and bioanalytical resources. Even a relatively small cassette size, such as a five-in-one cassette, allow PK screening at more than twice the speed as with discrete studies. This includes confirming the winner from each cassette in a subsequent discrete study. However, cassette dosing is a highly specialized technique and should never be implemented without a set of optimized strategies in place. As part of the effort to develop strategies to minimize risks associated with cassette dosing, we studied the steps involved in the development of a multiple-component LC/MS/MS method for cassette dosing.

The first step in the method development for multiple-component LC/MS/MS method is to choose which compounds can be dosed in one cassette. This is often referred to as pool selection. A good pool selection strategy can significantly reduce the method development time in the subsequent steps and increase the success rate of a multiple-component method. The major challenge in this step is to set the right selection criteria that maintain a balance between quality and throughput. For example, compounds that have potential for interference may be omitted from the cassette to ensure quality. Also, as many compounds as possible should remain in the pool to maintain the throughput. In this work, a proactive pool assignment strategy was established to address this dilemma starting with a pool of compounds in sufficient quantity to dose in multiple cassettes. Compounds with any slight potential for interferences were proactively assigned to different pools rather than being dropped. The pool selection process usually involves both calculation and experimental testing. Table 1 list 12 test compounds and a PK reference compound that were submitted for pool selection for three five-in-one cassettes. The compounds were sorted according to the molecular weight of the parent compound. During the calculation step, the molecular weight for some potential metabolites were calculated and listed. Since the metabolic profile for the series of compounds were unknown, only mono- and di-hydroxylation metabolites were listed. Phase II metabolites such as glucuronides were not listed in Table 1 because the range of the molecular weight for these compounds is relatively narrow and not likely to have mass overlap between parent compounds and any conjugates. Compounds were assigned to different pools based on the criterion that the mass difference among any parent compounds and potential metabolites in the same cassette should be 3 Da or larger (for compounds containing chlorine or bromine, it should be 5 Da or larger). In this case, a few compounds did not meet this criterion, and thus, they were subject to experimental testing. The PK reference compound and compounds D, I, F, and H have the same or similar molecular weight. These compounds were infused into the mass spectrometer and it was found that the product ions of the PK reference compound are largely different from those from compounds D, I, F, and H. Therefore, the PK reference compound was still used. Compounds I and H were also tested since they were assigned in the same pool and only differ by 2 Da. The final pool assignment was listed in the last column of Table 1.

An important step in multiple-component LC/MS/MS analysis is sample preparation. Sample preparation for cassette dosing has different requirements compared with single-component assays. Because of the potentially diversified chemical properties of the compounds in the cassette, it is important that generic and relatively non-selective sample preparation methods are used. Among the commonly used sample preparation methods, protein precipitation method generally offers good recovery on a wide range of compounds, and therefore, is suitable for multiple-component assays. An alternative method for sample preparation is the on-line high-flow extraction method. In this method, samples in their biological matrices are injected directly onto an extraction column operated under a high linear flow rate. The analytes are eluted onto a second column via column switching for separation. This method is relatively non-selective, offers great simplicity as samples in their original biological matrices can be injected directly onto the system. Fig. 1 shows the MRM chromatograms for a 250 nM five-in-one standard analyzed on the on-line high-flow column switching system.

Table 1
Pool assignment for 12 compounds

| Compound | MW (parent, free base) | MW (mono-hydroxylation, parent + 16) | MW (di-hydroxylation, parent + 32) | Pool assignment |
|---|---|---|---|---|
| C | 366 | 382 | 398 | #1 |
| D | 379 | 395 | 411 | #1 |
| I | 380 | 396 | 412 | #3 |
| PK reference | 380 | 396 | 412 | #1, #2, and #3 |
| F | 382 | 398 | 414 | #2 |
| H | 382 | 398 | 414 | #3 |
| G | 390 | 406 | 422 | #3 |
| J | 393 | 409 | 425 | #2 |
| A | 407 | 423 | 439 | #1 |
| B | 407 | 423 | 439 | #2 |
| L | 472 | 488 | 504 | #2 |
| K | 474 | 490 | 506 | #3 |
| E | 505 | 521 | 537 | #1 |

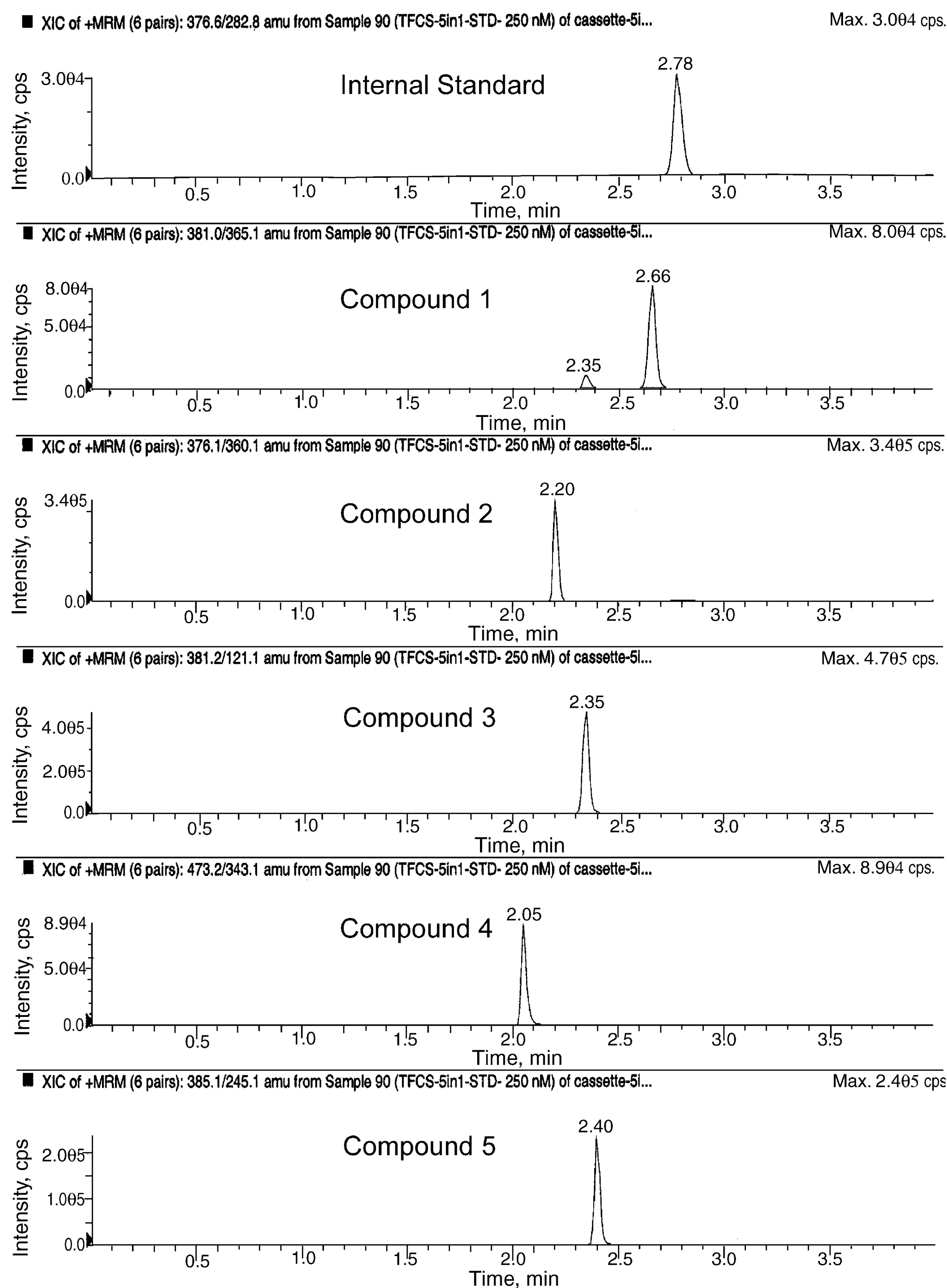


Fig. 1. MRM chromatograms for a 250 nM five-in-one rat plasma sample injected directly onto the high-flow on-line extraction with column switching system.

All five components were resolved with a total runtime of four minutes including extraction. It should be noted that compound 3 contained a MRM transition that was identical to the MRM transition used for monitoring compound 1. The high separation efficiency provided by this column switching method was able to fully resolve the interfering peak.

To minimize the potential risks for interference and ion suppression in multiple-component assays, it is important to obtain sufficient separation of the analytes from endogenous components and other analytes and their metabolites in the pool. Theoretically, separation can be achieved through either extraction or chromatography. Since relatively nonselective extraction methods such as protein precipitation and on-line extraction are used, good chromatographic separation becomes crucial to achieve this goal. As it is difficult to predict the presence and the retention time of all potential metabolites, an effective approach used to minimize potential interference is to increase the number of theoretical plates of the separation. However, an increase in theoretical plates is usually achieved through employing longer columns and

longer runtime, resulting in reduced throughput. Recently, the use of monolithic columns for bioanalysis has been reported [15–17]. The major advantages of the monolithic column include a lower column backpressure and a flatter van Deemter curve [18]. Therefore, a considerably higher flow rate can be employed without generating a high backpressure or compromising separation efficiency, resulting in higher speed and higher-quality separation. This unique feature makes monolithic column a suitable tool for multiple-component assays. Fig. 2 shows the total ion MRM chromatograms of a five-in-one mixture using a monolithic column and a conventional particulate column. Both columns offered a runtime

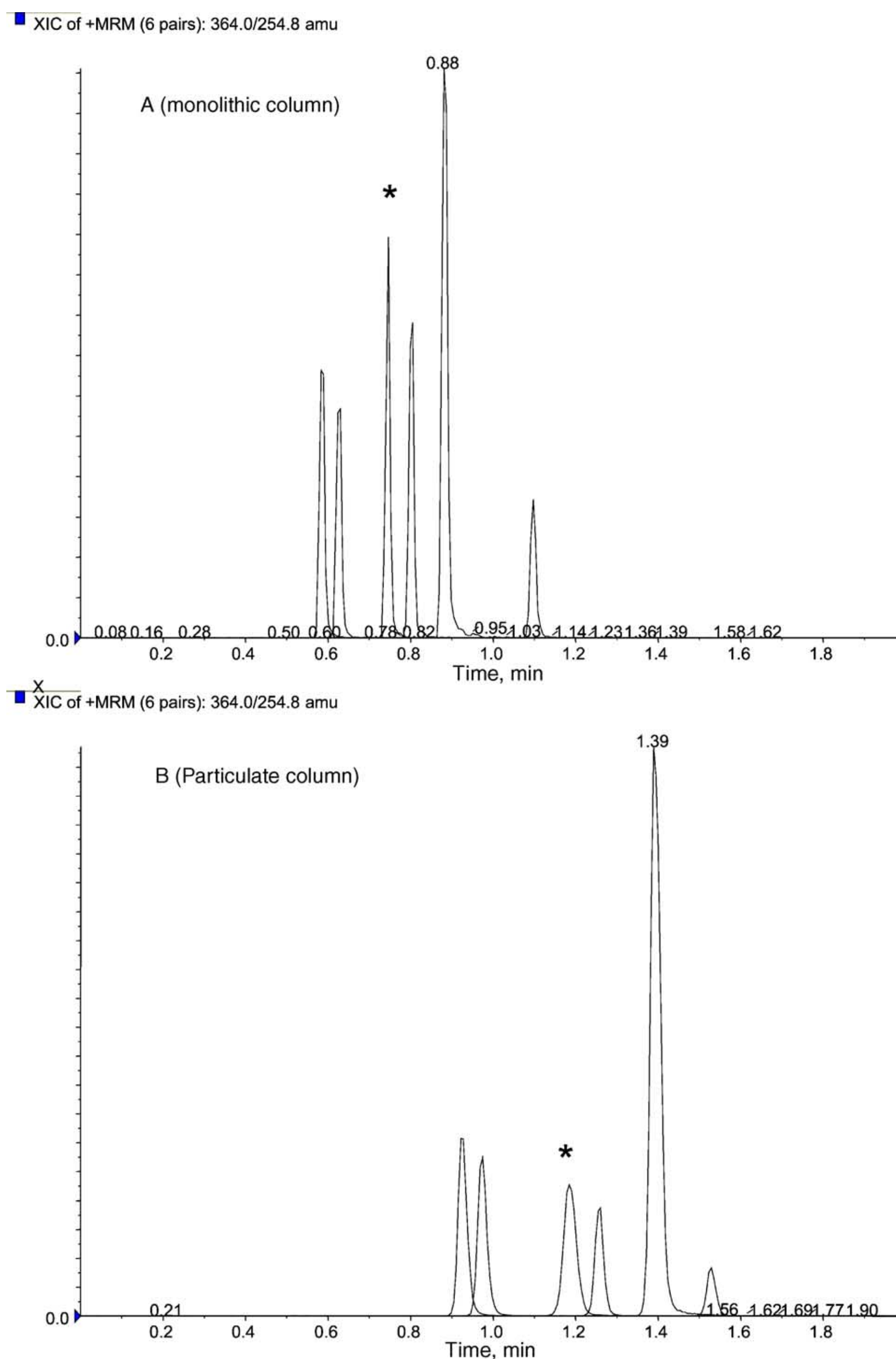


Fig. 2. Comparison of the chromatographic resolving power within the same runtime between a monolithic column (A) and a conventional particulate column (B) for a five-in-one cassette dosing study. The monolithic column was a 4.6 mm × 50 mm Chromolith SpeedROD C18 column (Merck KgaA, Darmstadt, Germany). The particulate column was a 2.1 mm × 20 mm DASH-18 column (Thermo Electron Corporation, Waltham, MA, USA).

Table 2
Comparison of ULOQ standard (2500 nM) responses as a single component and as a part of a mixture with other compounds in the cassette

| Sample name | Area ratio (analyte/IS) before optimization | | | Area ratio (analyte/IS) after optimization | | |
|---|---|---|---|---|---|---|
| | Single component | Mixture | Difference (%) | Single component | Mixture | Difference (%) |
| Compound 1 | 2.14 | 1.84 | −14.0 | 1.61 | 1.58 | −1.9 |
| Compound 2 | 2.37 | 1.98 | −16.5 | 2.09 | 1.83 | −12.4 |
| Compound 3 | 1.72 | 1.03 | −40.1 | 0.98 | 0.8 | −18.4 |
| Compound 4 | 1.67 | 1.00 | −40.1 | 1.04 | 1.00 | −3.8 |

of two min. However, using the peak marked by an asterisk as an example, the particulate column only offered a total of 4821 theoretical plates while the monolithic column offered a total of 25,754 theoretical plates. Given the same runtime, the monolithic column offered five-fold more resolving power and therefore, reduced the risk of potential interferences.

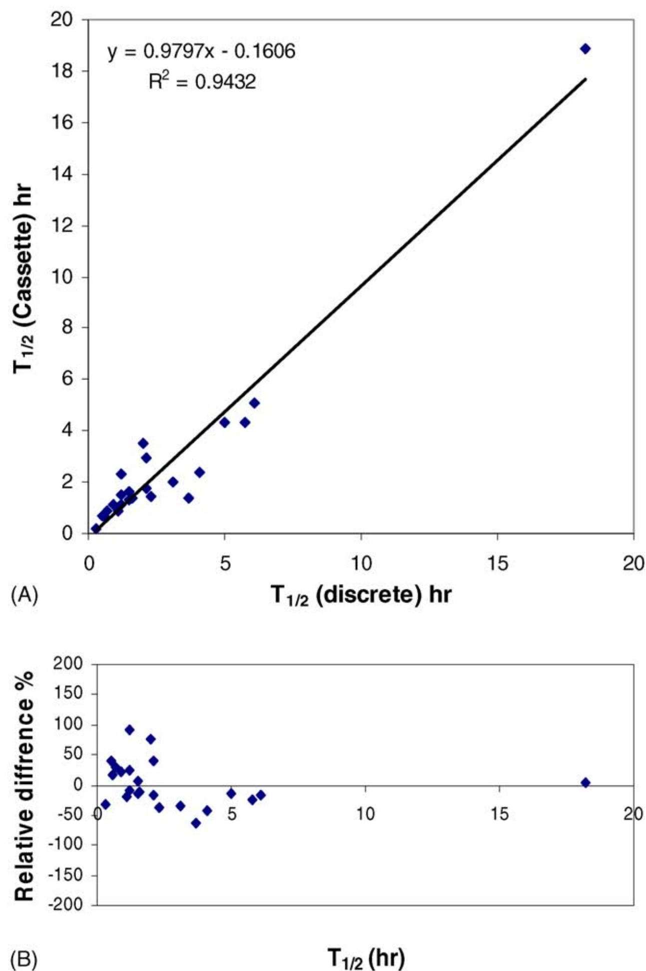


Fig. 3. Comparison of half-life values determined by discrete and cassette dosing studies. (A) The correlation of half-life values between discrete and cassette dosing studies. (B) The relative percentage difference of half-life values between discrete and cassette dosing studies. The relative difference % is calculated as (cassette half-life − discrete half-life)/discrete half-life × 100%.

Another potential source of error for multiple-component LC/MS/MS assays are the differences in the matrix between the standards and the samples. In the standards, the concentrations of all analytes are at the same concentration. In the samples, however, the concentrations of different analytes are usually significantly different. Sometimes one or all of the other analytes may not be present at all. If the analyte response is dependent upon the concentration of other analytes due to ion suppression, then errors could occur as the ion suppression effect is different between samples and standards. To evaluate and minimize the risks for this type of error, a test was added to the standard method development procedures for multiple-component assays. In this test, two standards at the upper limit of quantitation (ULOQ) were prepared in pre-extracted blank matrix. One of the standards was prepared with all the other analytes present and at the ULOQ and the other standard was prepared without any other analytes. The responses of these two standards were compared. Any significant discrepancies in response (>30% of the discrete response) between the analyte alone and the analyte in mixture indicate the potential of ion suppression. One of these examples is shown in Table 2, where the response comparisons for four compounds in a cassette dosing study were listed. With the original multiple-component LC/MS/MS method, there was a response discrepancy for compounds 3 and 4. An inspection of the chromatograms indicated this might result from ion suppression as compounds 3 and 4 co-elute (chromatograms not shown). An effort was made to chromatographically resolve compounds 3 and 4 but was not successful. Since all of the compounds showed reasonably good sensitivity, the injection volume was thus reduced from 50 to 10 μL and the response comparison was re-conducted. The results for the optimized method are also shown in Table 2. With the reduced injection volume, the response discrepancies for all compounds in this cassette were less than 30%. Therefore, the final sample assay was performed using the reduced injection volume.

The strategies described above have been used to develop multiple-component LC/MS/MS methods to analyze plasma samples collected from cassette dosing studies. Other strategies have also been developed to improve the in-life portion of cassette dosing studies. These strategies include dose reduction, cassette size control, and P-450 inhibition screening. A PK reference compound is always included and the dose is limited to i.v. route only. Both the assay related and in-life strategies form a systematic approach for conducting

cassette-dosing PK screening at the authors' laboratories. These strategies are continuously evaluated and improved on an on-going basis. Fig. 3A lists the half-life values between discrete and cassette dosing studies for a total of 23 compounds across different therapeutic targets. A correlation factor of 0.9432 was observed between discrete and cassette data. These data were further investigated in more detail by plotting the cassette-dosing half-life values as relative percentage difference from discrete-dosing half-life values (Fig. 3B). As shown in Fig. 3B, the test compounds distributed evenly on both sides of the baseline (discrete values), indicating there was no systematic bias in this cassette dosing approach. It is also worth noting that the magnitude of the relative differences was slightly different across the baseline. There appeared to be more false positives (cassette half-life values are 50% or more higher than discrete ones) than false negatives (cassette half-life values are 50% or more less than discrete ones). More data points need to be collected to prove the statistical significance of this observation.

## 4. Conclusions

Four critical steps have been investigated for the method development of multiple-component assays for cassette dosing. These steps include pool selection, sample preparation, chromatographic separation, and method qualification. Based on the results of this investigation, it is proposed that pool selection be performed with a sufficiently large number of compounds. Protein precipitation or on-line high-flow extraction is the method of choice for sample preparation. Monolithic columns provide good separation without compromising throughput. A comparison of the responses of a ULOQ standard in the presence and absence of other analytes in the sample is an important step to qualify a multiple-component assay. A comparison of PK parameters between discrete and cassette studies for over 20 compounds indicate that these strategies are effective.

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