Rapid Determination of Oral Pharmacokinetics and Plasma Free Fraction Using Cocktail Approaches: Methods and Application

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Purpose. To apply cocktail approaches for protein binding (PB) and pharmacokinetics (PK) within a discovery program as a means of providing timely systemic exposure (AUC and C_{max}) data.

Methods. For PB data, a procedure of cocktail ultrafiltration, mixed matrix sample preparation and single quadrupole atmospheric pressure ionization LC/MS analysis was used. *In vivo* PK studies consisted of 4 experimental compounds and a control compound dosed orally at 1 mg/kg (5 mg/kg total dose), with plasma samples obtained at 0.5, 1, 2, 4 and 8 h post dose. For PB and *in vivo* PK analysis, a control compound was tested within each cocktail to ensure consistent reproducibility.

Results. Approximately 2 weeks were spent comparing single and cocktail approaches to determine the feasibility of this method for this project. Comparisons of cocktail data with single compound data revealed no significant differences between the approaches. The oral AUC values ranged from 0.01 to 9.28 μg·hr/ml and the C_{max} values ranged from 0.04 to 2.17 μg/ml. Free fractions of the 44 compounds studied ranged from 0.006 to 0.271. Using the free fraction values to correct for free AUC and C_{max} results in ranges of 0.001 to 0.473 μg·hr/ml, and 0.001 to 0.119 μg/ml, respectively.

Conclusions. All 44 compounds tested had similar potencies *in vivo*. Thus, these results suggest that a respective 400 and 100-fold range in AUC and C_{max} corrected for free fraction exist in the presence of comparable *in vivo* activity. The ability to generate this type of data in a timely manner allowed the selection of a candidate with low peripheral exposure relative to the effective dose. The free fraction and PK data on the 44 compounds described was collected within three work days by 2 lab scientists.

KEY WORDS: cocktail dosing; pharmacokinetics; plasma free fraction; ultrafiltration; HPLC/APCI/MS.

INTRODUCTION

The primary goal for a discovery program was recognized to be reduction of systemic drug exposure (AUC) without loss of *in vivo* activity (i.e. maintain the same ED_{50} in the pharmacology model). The *in vivo* activity of forty-four compounds in a drug discovery program was similar (ED_{50}s ≤ 2 mg/kg for 70% of the compounds tested). No relationship between ED_{50}, IC_{50} and low systemic exposure existed. Differentiation of these compounds for candidate selection based on pharmacokinetic profile was desirable to identify a compound with low peripheral exposure relative to the effective dose.

For this program, the target organ was liver; therefore it was assumed if oral pharmacokinetics were determined for all compounds in parallel with *in vivo* pharmacology (ED_{50}) screening, a short list of compounds with reduced peripheral exposure could be identified without sacrificing efficacy. Inskeep and Day outlined various cocktail strategies which were utilized in the current study (1).

With the need to investigate the pharmacokinetic parameters AUC and C_{max} (PK) of discovery compounds more rapidly comes the need to rapidly measure the free drug fraction (FF) in the *in vivo* models used to generate the PK data. HPLC/atmospheric pressure chemical ionization mass spectrometry (HPLC/APCI/MS) has been shown to be applicable for quantitative determination of biologic samples (2) and allows the simultaneous determination of multiple compounds in a single injection. Cocktail dosing has been shown to effectively reduce the number of animals and samples needed to assess a compound's PK profile (3,4). To increase the throughput of PK and FF samples, cocktail dosing of study animals and cocktail ultrafiltration procedures were developed, validated and applied to the above discovery project area. The cocktail methods and analysis were validated for this discovery project over a two week period to determine a reference compound (R1). Once validated, the methods facilitated the collection of accurate PK and FF data for forty-four discovery compounds by two lab scientists in three days. Using the data from the cocktail studies, a short list of nine compounds was chosen and advanced for PK analysis following single dosing. The validation analysis, methods and conclusions, results of the cocktail approaches and comparison with the data derived from single analysis are presented below.

MATERIALS AND METHODS

Drugs and Reagents

All new chemical entities (NCEs) for the discovery program were synthesized at Pfizer Central Research (Groton, CT), ranged from molecular weights of 432 to 623 and possessed MlogP (5) values of 1.22 to 3.58 (structures of these investigational compounds cannot be disclosed at this time). HPLC-grade acetonitrile (CH_3CN) and reagent-grade dimethyl sulfoxide (DMSO), phosphoric acid (H_3PO_4) and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC-grade methyl *tert.*-butyl ether (MTBE) was purchased from Burdick and Jackson (Muskegon, MI). Ammonium acetate (CH_3COONH_4), acetic acid (CH_3COOH), and 37% hydrochloric acid (HCl) were purchased from J. T. Baker (Phillipsburg, NJ). All reagents were used without further purification. The water used for preparing aqueous solutions was obtained from a Millipore Milli-EQ System (Bedford, Massachusetts), 18.2 MΩ.

Experimental *In Vivo* Methods

Validation of Cocktail Dosing Approach

Four compounds (R1, A, B, C) were chosen to determine if cocktail dosing was feasible for this series. Comparisons of PK data were made between single versus cocktail dosing

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approaches described below. From these four compounds, R1 was chosen as a positive control for future cocktail studies.

Single Dose Preparation

The single oral dosing solutions were prepared with one of twelve selected NCEs for dosing at 1 mg/kg/NCE/animal. The NCEs selected for single PK profiling were identifications R1, A, B, C, 2, 7, 13, 15, 41, 42, 43, 44. Preparation of dosing solutions involved weighing 0.83 mg of each NCE in 1 ml of 2% DMSO and 98% of a 0.1% methyl cellulose:0.6% Tween 80 (v:v) solution. Using this dosing solution, rats were dosed with 6 ml/kg (i.e., a 200 g rat received 1.2 ml).

Cocktail Dose Preparation

To avoid issues related to dissolution, compounds R1, A, B, C, and 1 through 44 were dosed in cocktail in solution. Each oral cocktail dosing solution was prepared with four discovery compounds and R1 for dosing at 1 mg/kg/NCE for a total of 5 mg NCE/kg/animal. Preparation of dosing solutions involved weighing 0.83 mg of each of four NCEs plus 0.83 mg R1 in 1 ml of 2% DMSO and 98% of a 0.1% methyl cellulose:0.6% Tween 80 (v:v) solution. Using this dosing solution, rats were dosed with 6 ml/kg (i.e., a 200 g rat received 1.2 ml).

Animals

Male Sprague-Dawley rats (n=2/single or cocktail dose) were administered an oral dose of the dosing solutions described above for an oral dose of each NCE of 1 mg/kg. Rats were purchased from Charles River Laboratories (Kingston, New York) and ranged from 180 to 200 g.

Sample Collection

Blood samples (approximately 0.5 ml whole blood per sampling per animal) were collected in plasma collection tubes (part #365969, Becton Dickinson, Franklin Lakes, New Jersey) predose and at 0.5, 1, 2, 4 and 8 hours post dose. Plasma was isolated from whole blood by centrifugation and stored at −20°C for HPLC/APCI/MS analysis.

In Vivo *Sample Preparation*

A 100 μl aliquot of plasma was added to 0.5 ml of water and then fortified with 10 ng of an internal standard (IS, a structural analog of the NCEs examined). Samples were acidified with 0.5 N HCl, extracted with MTBE and evaporated to dryness in conical centrifuge tubes. Dried samples were then reconstituted with LC/MS mobile phase and transferred into injection vials for analysis. For analysis of single compound samples a standard curve of plasma containing one NCE and IS was prepared using the same procedure outlined above for the experimental samples. For analysis of cocktail samples, a standard curve of plasma containing four NCEs, R1 and IS was prepared using the same procedure outlined above. Standard curves for each compound in rat plasma consisted of solutions at concentrations (two replicates per concentration) of 0.01, 0.05, 0.1, 0.5, and 1 μg/NCE/ml.

Quantification of Plasma Concentration

A least-squares regression fit was made of known analyte concentrations to measured NCE/IS ratios with a weighting factor of 1/x applied to the regression analysis. Quantification of NCE in plasma was determined by comparing the NCE/IS mass ratio to that of the standard curve with the lower limit of quantification at 10 ng/ml. The dynamic range of the standard curve was from 10 to 1,000 ng/ml for all cocktail and single dose sample analysis.

PK Calculations

The area under the plasma concentration versus time curve from zero to either the last sampling time T [$AUC_{(0-T)}$], or the last time with a measurable concentration, was estimated using linear trapezoidal approximation. C_{max} values were obtained from direct examination of the plasma concentration versus time profile.

Ultrafiltration (UF) for Determination of Plasma Free Fraction (FF)

Procedure

Cocktail solution standards of NCEs 1 through 44 (100 μg/NCE/ml) were prepared which contained five NCEs, one of which was consistently R1. Rat plasma and buffer (100mM KH_2PO_4 (pH 7.4)) pools were fortified with these standard solutions for a final concentration of 1 μg/NCE/ml of plasma or buffer. The buffer samples were used to investigate nonspecific binding of each NCE to the UF device. The plasma and buffer pools were incubated 15 min at 37°C with gentle shaking. Following removal of 1 ml of plasma or buffer from each cocktail pool for initial plasma or buffer NCE concentration analysis, 1 ml samples (n=3/cocktail/matrix) were loaded into UF devices (part #4104, Centrifree, Amicon, Inc., Beverly, MA) and centrifuged 1 hr at 37°C, 1800 g. Initial plasma, initial buffer and ultrafiltrate were stored at −20°C for HPLC/APCI/MS analysis.

UF Sample Preparation

Initial plasma, initial buffer and ultrafiltrate samples were processed in the following manner: A 100 μl aliquot of initial plasma, initial buffer or processed ultrafiltrate was added to 0.4 ml of water and 100 μl buffer, control rat plasma or control rat plasma, respectively, and then fortified with 10 ng of IS. Samples were acidified with 0.5 N HCl, extracted with MTBE and evaporated to dryness in conical centrifuge tubes. Dried samples were then reconstituted with LC/MS mobile phase and transferred into injection vials for analysis. With this procedure all extracted samples contained plasma and buffer or ultrafiltrate matrix, ensuring the same assay procedure and recovery from all UF experimental samples.

Quantification of FF

The following equation was used to determine rat plasma free drug fraction (FF):

$$FF = [\text{ultrafiltrate NCE/IS mass ratio}] / [\text{initial plasma NCE/IS mass ratio}].$$

HPLC/APCI/MS Equipment

The HPLC system consisted of a Hewlett-Packard series 1100 injector and pump (Palo Alto, CA). The HPLC mobile phase consisted of 60% CH_3CN:40% 10 mM CH_3COONH_4 (v/v) with a pH adjustment to 4.5 with CH_3COOH. A flow rate of 1 ml/min was established and injections were made onto a C-18 Supelco (Bellefonte, PA) Supelcosil (3.3 cm × 4.6 mm, 3 μm) HPLC column. All LC analyses were performed at ambient temperature. The column effluent was introduced into the mass spectrometer via the heated nebulizer interface of a PE/Sciex API-100 (Perkin-Elmer, Norwalk, CT) single quadrupole mass spectrometer (nebulizer probe temp = 475°C). Experiments were performed in either the positive or negative ion mode, selectively monitoring the parent ions (m/z of 432.2 to 623.2) to achieve optimum signal to noise ratio. PE/Sciex software LC2Tune 1.3 was used to scan for the parent ions and MacQuan version 1.5 was used to calculate peak height ratios of NCE/IS. Under the above conditions, all NCEs examined eluted within a 2.5 minute run time.

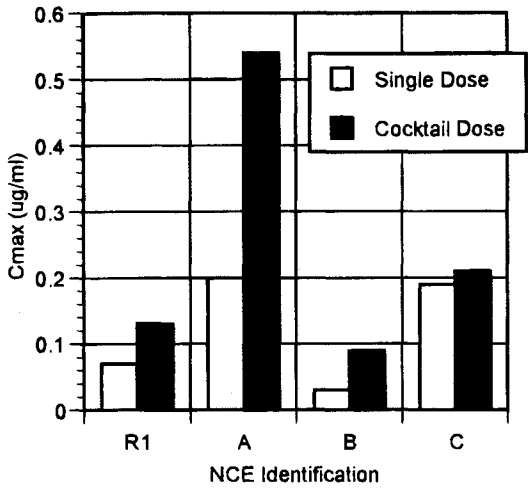

Fig. 2. Rat plasma C_{max} (μg/ml) of four NCEs following cocktail or single oral dosing. (Values are the mean of two determinations per compound).

RESULTS

$AUC_{(0-T)}$ and C_{max} data confirming cocktail procedure validation are presented in Figures 1 and 2, respectively. Data following FF determination of the 44 NCEs and the reference compound (R1) are presented in Figure 3 and Table I, respectively. Free AUC and C_{max} values for the 44 compounds examined are illustrated in Figures 4 and 5, respectively. Data describing the final comparison of nine compounds' AUC and C_{max}, single versus cocktail dosing, are presented in Figures 6 and 7, respectively.

Validation of Cocktail Methods

Approximately two weeks were invested to determine appropriate dose and method of preparation and to compare the PK of R1, A, B and C following single and cocktail dosing (Figs. 1 and 2). These four compounds had a 7-fold range in exposure following single dosing ($AUC_{(0-T)}$ 0.11 to 0.83 μg·hr/ml and C_{max} 0.03 to 0.2 μg/ml), providing representative examples of exposure. To avoid solubility issues and nonlinear PK, 1 mg/kg/compound was chosen for cocktail dosing. $AUC_{(0-T)}$ and C_{max} following cocktail dosing were 27% and 47% higher, respectively than following single dosing for these four compounds. The PK data suggested that cocktail dosing appeared to overestimate exposure of these four compounds, yet the rank order of exposure remained the same between compounds (i.e. $AUC_{(0-T)}$ and C_{max} of B<R1<C<A regardless of single or cocktail dosing). From this validation experiment, R1 was chosen as the reference compound for the subsequent cocktail experiments.

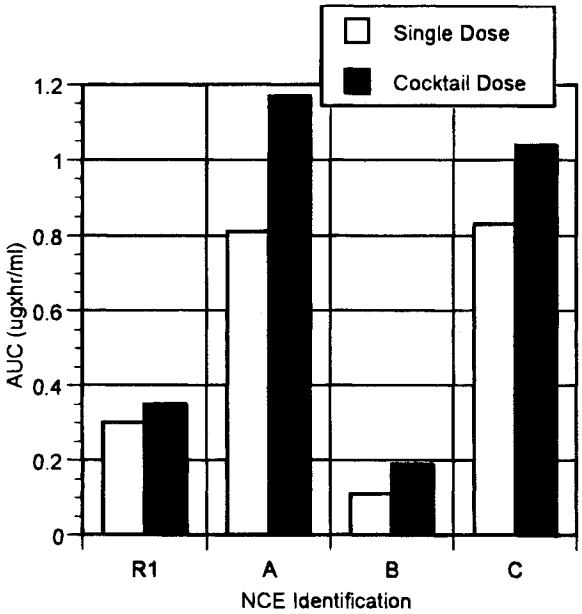

Fig. 1. Rat plasma AUC (μg·hr/ml) of four NCEs following cocktail or single oral dosing. (Values are the mean of two determinations per compound).

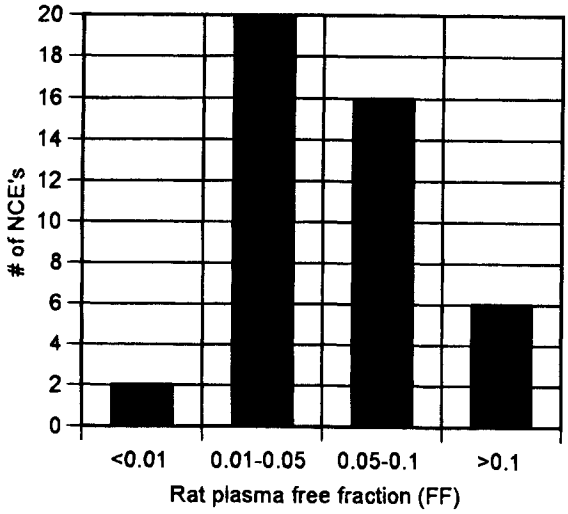

Fig. 3. Rat plasma free drug fraction (FF) of NCEs in a drug discovery program determined using cocktail method and analysis. (Values are the means of three determinations per compound. Nonspecific binding to the ultrafiltration device was not observed for any of the NCEs tested.)

Table 1. Accuracy and Precision of R1 Positive Control for Cocktail Methods and Analysis to Determine Rat Plasma Free Drug Fraction (FF) and the Pharmacokinetic Parameters (PK) AUC and C_{max}

Analysis Description	FF	$AUC_{(0-T)}$ (μg·hr/ml)	C_{max} (μg/ml)
Replicate 1	0.034	0.32	0.10
Replicate 2	0.069	0.36	0.15
Replicate 3	0.047	0.30	0.12
Replicate 4	0.052	0.42	0.19
Replicate 5	0.094	0.34	0.17
Replicate 6	0.050	0.36	0.19
Replicate 7	0.058	0.36	0.19
Replicate 8	0.036	0.36	0.19
Replicate 9	0.039	0.30	0.12
Replicate 10	0.042	0.32	0.10
Replicate 11	0.064	0.32	0.10
Mean Cocktail	**0.053**	**0.34**	**0.15**
Standard Deviation	0.018	0.04	0.04
Accuracy (%)	98	106	250
Precision (±%)	34	12	27
Single Determination	**0.054**	**0.32**	**0.06**

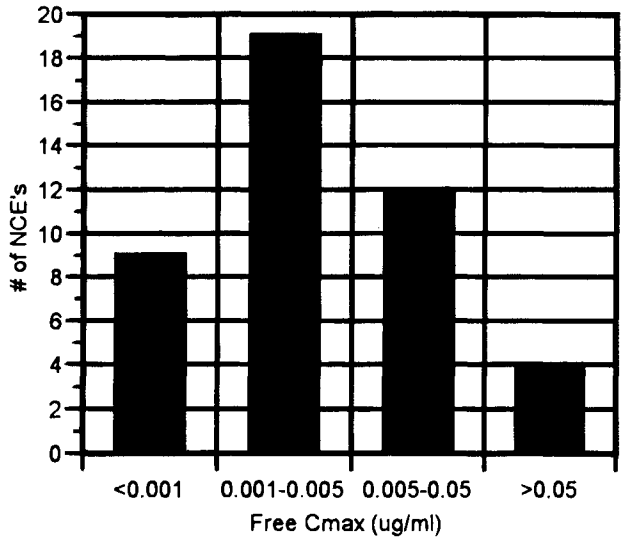

Fig. 5. Rat plasma free C_{max} of NCEs in a drug discovery program derived from plasma free fraction (FF) and oral C_{max}. (Values were obtained using the following formula: Free C_{max} = FF × C_{max}.)

Free Fraction

Rat plasma free fraction of the 44 NCE discovery compounds tested ranged from 0.006 to 0.271 (Figure 3). Nonspecific binding to the ultrafiltration devices was not observed for any of the NCEs studied (data not shown). Rat plasma free fraction of R1 determined singly was 0.054 (Table I). Using replicate cocktail method and analysis (n=11), R1 free fraction ranged from 0.034 to 0.094 with a mean and standard deviation of 0.053 and 0.018, respectively. Mean accuracy and precision of R1 FF was 98% and ±34%, respectively.

In Vivo Cocktail PK

Mean $AUC_{(0-T)}$ and C_{max} of R1 determined after 1 mg/kg single oral dosing was 0.32 μg·hr/ml and 0.06 μg/ml, respectively. Following eleven cocktail doses, R1 $AUC_{(0-T)}$ ranged from 0.30 to 0.42 μg·hr/ml with a mean and standard deviation of 0.34 and 0.04, respectively. C_{max} of R1 following cocktail dosing (n=11) ranged from 0.10 to 0.19 μg/ml with a mean and standard deviation of 0.15 μg/ml and 0.04, respectively. Mean % accuracy (±% precision) of R1 $AUC_{(0-T)}$ and C_{max} were 106 (±12) and 250 (±27), respectively. As in the validation experiment, R1 C_{max} appeared to be overestimated by cocktail dosing. Comparisons of cocktail data with single R1 data revealed no significant differences between the approaches with regard to FF and $AUC_{(0-T)}$ however, hence inter-validating the use of cocktail methods for this discovery project.

Mean plasma $AUC_{(0-T)}$ ranged from 0.01 to 9.28 μg·hr/ml and C_{max} ranged from 0.04 to 2.17 μg/ml for the 44 NCE discovery compounds dosed in cocktail. Using the plasma free

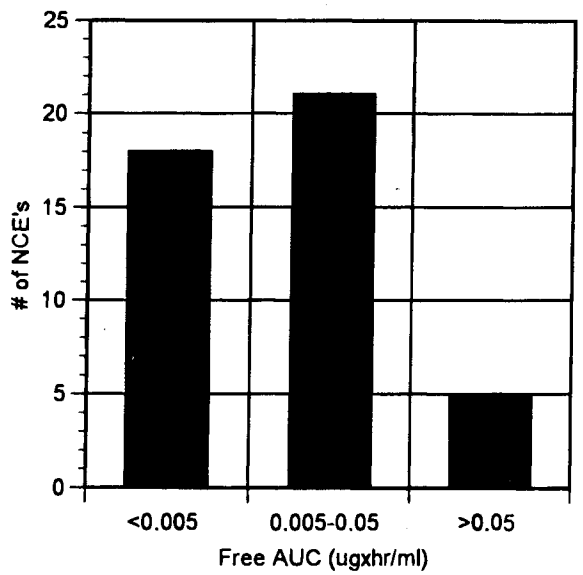

Fig. 4. Rat plasma free oral AUC of NCEs in a drug discovery program derived from plasma free fraction (FF) and oral AUC. (Values were obtained using the following formula: Free AUC = FF × AUC.)

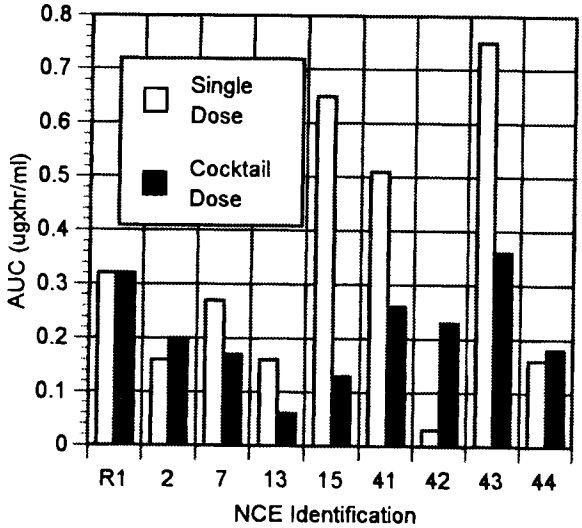

Fig. 6. Rat plasma AUC (μg·hr/ml) of nine NCEs following cocktail or single oral dosing. (Values are the mean of two determinations per compound).

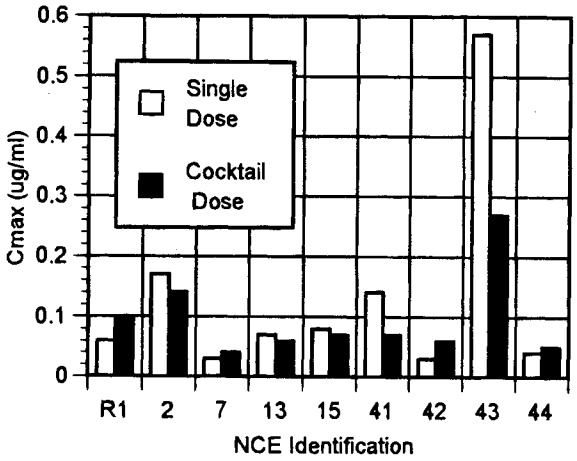

Fig. 7. Rat plasma C_{max} (μg/ml) of nine NCEs following cocktail or single oral dosing. (Values are the mean of two determinations per compound).

fraction in conjunction with the determined PK resulted in free $AUC_{(0-T)}$ and C_{max} values ranging from 0.001 to 0.473 μg·hr/ml and 0.001 to 0.119 μg/ml, respectively, for the 44 compounds examined in this drug discovery program (Figures 4 and 5).

Single vs Cocktail PK

Comparisons of PK following single and cocktail dosing of nine selected NCEs (ID #'s R1, 2, 7, 13, 15, 41, 42, 43, and 44) resulted in three compounds with similar AUC values regardless of dosing method (Figure 6), and six compounds with similar C_{max} values regardless of dosing method (Figure 7). Average C_{max} and AUC following cocktail dosing was 9 and 45% higher, respectively, than following single dosing. Of the NCEs that had AUC and C_{max} values >2-fold different when comparing single and cocktail doses, in all but one case the single doses were higher.

CONCLUSIONS

The results of this study in conjunction with the 3 available reports (3,4,6) provide positive evidence that the cocktail dosing/analysis method, incorporating a well characterized control, is effective *in vivo* with rats and dogs and *in vitro* with protein binding and metabolism studies. In addition, based on all available data, it appears that the cocktail methods are feasible with a broad range of chemical entities further supporting their application in the industrial setting.

Previously, others have reported the use of LC/MS/MS to screen for multiple compounds in animal matrices (6). In the present example, LC/MS was applied since it is the primary quantitation instrument for the Pfizer disposition scientist. As with the previous examples, reproducibility of data and sensitivity were driving forces in the decision to use 4 compounds and a control for all cocktail studies. Through use of LC/MS, sensitivity was such that a dose of 1 mg/kg/compound was possible. Through administration of this low dose and limitation to 5 total compounds, it was more likely that nonspecific effects related to compound interactions, metabolism and distribution could be avoided.

Preliminary evaluation of cocktail versus single dosing for this program with four NCEs revealed that cocktail dosing appeared to overestimate the exposure of these compounds. However, the rank order of exposure was similar between dose methods. Reference compound (R1) was chosen as a positive control and used to track inter-dose validity of the FF and *in vivo* cocktail methods. The pharmacokinetic results from cocktail studies with the 44 NCEs suggest that the range of exposure for AUC and C_{max} was 400 and 50-fold, respectively. When AUC and C_{max} were corrected for free fraction, a respective 400 and 100-fold range existed for the 44 compounds tested.

Following data collection of the forty-four cocktail dosed NCEs, nine compounds were chosen based on adequate pharmacologic activity (ED_{50} < 1 mg/kg) and low exposure, based on free AUC < 0.03 μg·hr/ml, for additional profiling. Examination of AUC and C_{max} following single or cocktail dosing suggests that cocktail dosing had less impact on rate than extent of absorption. On average, cocktail C_{max} was within 9% of single C_{max} and cocktail AUC was within 45% of single AUC. This example supports use of cocktail as a high-throughput tool and as an effective method of narrowing a large group of compounds into a manageable number for classical analysis. Based on single comparisons, the cocktail dosing method slightly overestimated oral exposure of a potential compound in this discovery project.

In summary, these methods provided timely data to a discovery program helping in the nomination of a compound which had good *in vivo* potency with low peripheral exposure. The development of these methods provided the ability to generate FF, AUC and C_{max} data for 44 compounds within three work days by 2 lab scientists.

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