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LC fluorescence method for multiple synthetic compounds to rapidly create in vivo pharmacokinetic database utilizing 'N-in-One' dosing

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Abstract

This manuscript reports development and validation of an assay method in rat serum for the simultaneous estimation of C1, C2, and C3, in-house CDRI molecules, of the class of aryloxy-substituted aryl-piperazinyl derivatives. The assay was applied to determine pharmacokinetic data after simultaneous intravenous administration of these three compounds. A high-performance liquid chromatography assay method using isocratic elution and fluorescence (excitation, 250 nm; emission 350 nm) was developed for simultaneous estimation of all the three compounds in rat serum. Linearity was observed between 12.5 and 400 ng/ml for all the three compounds in serum. Recoveries were highly consistent over the concentration ranges for all the analytes. Variations in the intra- and inter-batch accuracy and precision were within the acceptable limits of $\pm 20\%$ at the limit of quantitation, whereas at higher concentrations it was $\pm 15\%$. A mixture of the three compounds was administered intravenously to rats. Blood samples were collected over a period of 6 h and analyzed to determine serum levels and pharmacokinetics of each compound. The pharmacokinetics of the aforementioned three compounds was also determined after individual administration. The results obtained in the N-in-One dosing correlated well with discrete dosing of compounds. Based on the results obtained, C2 emerges to be the compound with appropriate pharmacokinetic parameters. Thus, the N-in-One method is a useful method for increasing the throughput to obtain the pharmacokinetic information.
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Keywords: Liquid chromatography and fluorescence detection; N-in-One dosing; Cassette dosing; In vivo pharmacokinetics

1. Introduction

In the development of a new drug, pre-clinical trials are of immense importance, where a new chemical entity gains importance or is discarded. In the progression from drug discovery to development, the success rate increasingly relies on the ability to rapidly identify quality molecules that possess the desired attributes of bioavailability, chemical tractability, selectivity and potency [1]. Today, in the race to develop the next selling drug, the key tools for rapidly generating potent and selective compounds are combinatorial chem-

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istry and functional genomics [2]. These technological advances have now given drug discovery scientists the ability to deliver a large number of lead compounds for final optimization. Turning a chemical lead into a marketable drug requires a balance of potency, safety and pharmacokinetics, which are traditionally low-throughput processes [3]. Thus, combinatorial chemistry and high-throughput screening techniques have generated a potential bottleneck in the final optimization processes that seek to align potency with good pharmacokinetics in order to produce a drug [4]. In addition to the fundamental changes in the drug discovery technology, there are other important dynamics that have a major impact on the need for deriving information on pharmacokinetics; those of cost and value. One of the major reasons for the failure of a compound in the drug discovery program often involves inappropriate kinetics, and any means of uncovering this early will be highly valuable in reducing the fallout rate at the more costly late stage of the drug development cycle. Thus, there is a need to increase the throughput of the in vivo pharmacokinetic optimization studies, to aid selection and to add value to the development candidates [5,6].

Increased throughput in in vivo pharmacokinetic screening has recently been reported by: (a) dosing mixtures of compounds to a single animal, i.e. N-in-One dosing/cassette dosing; and (b) by pooling samples from singularly administered compounds prior to analysis. Both the methods capitalize on tandem liquid chromatography/mass spectrometry (LC/MS) as a sensitive and specific method for analysis [7–13]. As the availability of the LC/MS systems are limited, we have investigated an alternative increased throughput approach by simultaneously characterizing the in vivo pharmacokinetics of multiple compounds utilizing traditional high-performance liquid chromatography (HPLC) for illustrative purpose. In the present investigation, the basic concepts of cassette dosing was followed. Owing to the limitation of HPLC/fluorescence, a cassette of only three compounds, 1-(4-propionyl phenoxy)-3-[N^4-(3′,4′-dimethoxyphenyl)-piperazinyl]-N^1-propan-2-ol (C1), 1-(phenoxy)-3-[(N^4-phenyl-piperazinyl)]-N^1-propane (C2) and 1-(2-isopropyl phenoxy)-3-[N^4-(2′-methylphenyl)-piperazinyl)]-N^1-propan-2-ol (C3), which are molecules synthesized in this institute as a part of the drug discovery program, was formed. Renewed interest in these molecules owing to discovery of new activities demanded the evaluation and establishment of pharmacokinetic parameters in rats.

In the present study, we report for the first time an isocratic HPLC assay method with fluorescence detection for the estimation of C1, C2, and C3. The assay method was validated using accuracy and precision as the parameters so as to facilitate accurate collection of pharmokinetic (PK) data. The method was used for simultaneous determination of the pharmacokinetic parameters after a single intravenous dose of mg/kg/compound. Cassette dosing concept was applied to aid in the selection of the compound with the most optimum pharmacokinetic parameters. The pharmacokinetic parameters were also determined following single intravenous injection of 3 mg/kg/compound discretely so as to determine the feasibility of cassette dosing in increasing the throughput of the PK optimization using conventional analytical techniques.

2. Materials and methods

2.1. Chemicals and reagents

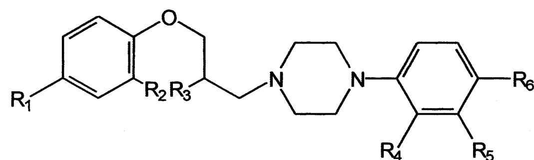
The chemical structures of C1, C2, and C3 are shown in Fig. 1. The compounds were synthesized in house. HPLC-grade acetonitrile was obtained from JT Baker (USA). *n*-Hexane was obtained from Mallinckrodt (USA). HPLC-grade isopropylalcohol IPA and dimethylsulphoxide (DMSO) were obtained from Spectrochem Pvt Ltd. (Mumbai, India). Diethyl ether (Solvent Ether) was purified before use by washing with potassium hydroxide followed by distillation. Triple-distilled water obtained from an all quartz distillation unit was used to prepare the mobile phase, buffers and reagents. All other chemicals were of analytical grade and procured from local sources unless mentioned.

2.2. Bioanalytical methods

An HPLC (Shimadzu, Japan) (LC-10ATvp) with CBM-10A (Communication Bus Module), FCV10ALvp (quaternary valve) and DGU-14 (Degasser) was used to pump the mobile phase (65:35% v/v acetonitrile:phosphate buffer (25 mM, adjusted to pH 4 with orthophosphoric acid)) at a flow rate of 1.5 ml/min. Chromatographic separations were performed on a C18 reversed-phase column (Spheri-5, 5 μm; 220 × 4.6 mm^2 I.D) preceded by a guard column (30 × 4.6 mm^2 I.D) (Perkin Elmer, Norwalk, CT, USA) of the same material. Mobile-phase solvents were filtered and degassed before use. Samples were injected by SIL-10ADvp autoinjector fitted with a 50 μl loop. The compounds were found to have native fluorescence in the mobile phase (excitation (Ex), 250 nm; emission (Em), 350 nm). After the elution, the compounds were monitored using a model RF-10Axl spectrofluorimeter detector set at 250 nm (Ex)/350 nm (Em). The chromatography was carried out at ambient temperature. Chromatographic peaks were integrated using Class LC10 work station (Shimadzu, Japan).

2.3. Stock and standard solution preparation

Individual stock solutions of C1, C2, and C3 (200 μg/ml) were prepared by dissolving 10 mg in 50 ml acetonitrile. Mixed stock solution of all the three compounds (16 μg/ml) was prepared by transferring 800 μl in 10 ml volumetric flasks and the volume made up with acetonitrile. Further dilutions were prepared by appropriate dilution in the range of 2000–62.5 ng/ml for the determination of recovery.



Compound	Substitution					
	R_1	R_2	R_3	R_4	R_5	R_6
C1	$COCH_2CH_3$	H	OH	H	OCH_3	OCH_3
C2	H	H	H	H	H	H
C3	H	$CH(CH_3)_2$	OH	CH_3	H	H

Fig. 1. Chemical structures of C1, C2, and C3.

2.4. Calibration curve

Calibration and quality control (QC) samples of all the analytes from 12.5 to 400 ng/ml in serum were prepared by adding various volumes of mixed stock solutions in appropriate volume of pooled drug-free rat serum so that the volume ratio of the organic phase added was less than 2.5%. Calibration and the QC standards were stored at −30 °C until analysis. Prior to HPLC analysis, these analytical standards and the QC samples were processed according to the method outlined in the following.

2.5. Sample preparation

To blank or spiked serum (0.5 ml), 1 ml acetonitrile was added. The tubes were vortex mixed for 15 s and then centrifuged at 1000 r.p.m. at 10 °C for 5 min. Then, 750 μl supernatant was transferred to a clean conical tube and evaporated to dryness under reduced pressure in speed vac concentrator (Savant Instrument, Farmingdale, NY, USA) below 40 °C. The residue was acidified with 200 μl of 0.5 N hydrochloric acid and washed with 2 × 2 ml *n*-hexane. The acid layer was basified with 50 μl of 2 M KOH and extracted with 4 ml of 2% isopropylalcohol in ether. The organic layer was transferred into another tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness in a speed vac concentrator. This whole process of extraction required 4–5 h for processing 30 samples. The residue was reconstituted in 0.1 ml acetonitrile and injected into HPLC. The calibration curve was obtained by linear regression ($y = mx$) of the peak heights of C1, C2, C3 versus concentration with Microsoft Excel version 5.0 on the IBM PC computer.

2.6. Stability in autoinjector

Replicates of the spiked samples at different concentrations of 16, 80, and 400 ng/ml were

processed and reconstituted at the same time. The reconstituted samples were placed in the autoinjector, and one set was injected immediately and the other after 12 h. The percent relative standard deviation (%RSD) for all the three concentration levels was calculated.

2.7. Method validation

The validation program for the HPLC method included within- and between-precision and accuracy studies on three different days. These studies were carried out in triplicates at three different concentrations: low, 16 ng/ml; medium, 80 ng/ml; and high, 400 ng/ml concentration levels.

2.8. Specificity

The specificity was defined as non-interference in the regions of compounds of interest with the endogenous substances, drug metabolites or other compounds of the cassette in the determination of the concentration.

2.9. Limits of detection and quantitation

The detection limit of the HPLC assay method (LOD) of C1, C2, C3 is the drug quantity in the serum after the sample clean-up corresponding to three times the baseline noise ($S/N > 3$). The limit of quantitation (LOQ) was defined as the concentration quantity of the sample, which was quantified with less than 20% deviation in precision.

2.10. Accuracy and precision

The accuracy of each sample preparation was determined by injection of calibration samples and three QC samples on three different days ($n = 27$; three each of low, medium and high concentration). The precision was determined by one-way analysis of variance as within and between %RSD. The accuracy was expressed as % bias:

$$\%\text{Bias} = \frac{(\text{observed concentration} - \text{nominal concentration})}{\text{nominal concentration}} \times 100$$

2.11. Pharmacokinetic studies

Young, healthy, male Sprague–Dawley rats (obtained from the animal house of the institute) were housed in well-ventilated cages and kept at room temperature (24 ± 2 °C) while on a regular 12-h light–dark cycle. Standard pellet rodent chow and tap water were freely available. Animals were cared for in accordance with principles of The Guide for the Care Use of Laboratory Animals (Department of Health, Education and Welfare, number [NIH] 85-23). At all times during the study, pain to the animals was minimized by the use of anesthesia.

The intravenous dosing formulations were prepared with the compounds for dosing 3 mg/kg per compound per animal. Preparation of dosing solutions involved weighing 45 mg each compound individually or all three (45 mg each compound) for cassette formulation in 2 ml DMSO:PEG600 (50%:50%). To 1 ml of this solution, 2 ml of 50% DMSO:PEG600 (50%:50%) was added, resulting in a final concentration of 7.5 mg/ml of each compound either individually or in the cassette. Rats were dosed with 0.4 ml/kg (i.e. a 250 g rat received 0.1 ml), with this dosing solution, using a tuberculin glass syringe fitted with a 26 G needle via the caudal vein, after the dilation of the tail with xylene in restrainer cages.

Blood samples for pharmacokinetic studies were collected at different time points up to 6 h post-dose. The samples (2.5–45 min) were collected by cardiac puncture under light ether anesthesia. Terminal samples (60–360 min) were collected from the inferior venacava. All blood samples were allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at $1000 \times g$ for 10 min at 4 °C and were stored at -60 °C.

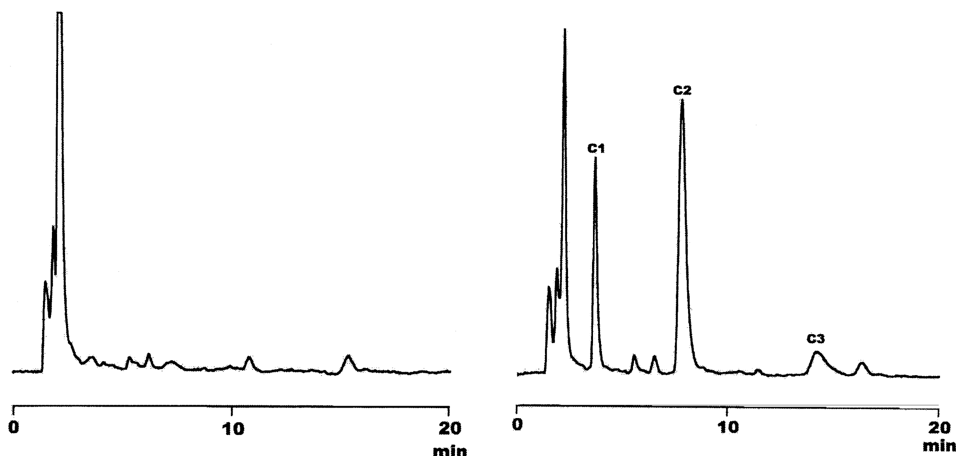


Fig. 2. Chromatogram of blank control serum and control rat serum spiked with all three compounds (80 ng/ml).

2.12. Pharmacokinetic and statistical analysis

The clearance (CL), steady-state volume of distribution (V_{ss}), mean residence time (MRT), elimination phase half-life ($t_{1/2}$), and area under curve (AUC) of each of the compounds were determined from respective serum concentrations versus time curves by applying non-compartmental methods using WIN NONLIN software (SCI consultants, USA). The model with the least residuals was selected for the estimation of the parameters. Overlapping confidence intervals and statistical significance tests were performed on the mean concentrations at each time point.

3. Results

3.1. Chromatographic results and stability

The new assay method developed for the compounds C1, C2 and C3 was found to be specific. The chromatogram of the blank control serum (500 μl) is given in Fig. 2, where it is shown that no endogenous peaks interfere with compound peaks. An HPLC chromatogram of the control rat serum spiked with all the three compounds is shown in the Fig. 2. The compounds were also found to be stable for at least 12 h in the autoinjector as the %RSD at all three concentration levels was less than 10%.

3.2. Linearity, LOD, and LOQ

The peak heights of C1, C2, and C3 in rat serum varied linearly with concentration over the range tested (12.5–400 ng/ml). An unweighted ($y = mx$) linear equation was used to perform standard calibration, and the %RSD obtained was 3.39, 1.60, and 5.87 for C1, C2, and C3, respectively. The complete results are summarized in Table 1. The LOD determination demonstrated that all the analytes gave a signal-to-noise ratio of 3 and above for 12.5 ng/ml extracted/injected level. The LOQ was established at the concentration of the low standard of 16 ng/ml for all the three analytes.

3.3. Assay validation

3.3.1. Recovery

The recoveries of C1, C2, and C3 from spiked serum samples were calculated by comparing the

Table 1
Calibration curve equations for C1, C2 and C3 during the assay validation

Compound	Equation	Slope	%RSD
C1	$y = mx$	822.89	3.39
C2	$y = mx$	1092.76	1.60
C3	$y = mx$	83.14	5.87

Table 2
Mean recoveries of C1, C2 and C3 from spiked rat serum

Concentration (ng/ml)	Absolute recovery (mean ± SD, $n=3$) (%)		
	C1	C2	C3
12.5	101.56 ± 4.70	98.28 ± 5.60	73.08 ± 1.41
25	102.18 ± 7.90	94.28 ± 1.81	62.76 ± 9.67
50	91.96 ± 10.22	92.98 ± 0.91	65.74 ± 0.34
100	96.68 ± 6.80	97.18 ± 0.89	66.55 ± 0.22
200	93.63 ± 7.36	94.23 ± 2.08	65.34 ± 0.78
400	97.54 ± 8.61	91.47 ± 0.52	73.95 ± 4.76

peak heights with those obtained from the analysis of the corresponding analytical standards. The mean recoveries from serum at all concentrations are summarized in Table 2.

3.3.2. Accuracy and precision

The overall percent bias and the precision at the three concentrations are presented in Table 3. The results show that the analytical method is accurate and the bias is within the acceptance limits of ±20% at low concentration and ±15% at all other concentrations. Similarly, the %RSD for the various analytes was within ±15% at all the concentration levels studied.

3.3.3. Comparison of the pharmacokinetics after individual and N-in-One dosing

Substantial amounts of PK information for all the three compounds are simultaneously available. The peak response at 2.5 min after N-in-One dosing is shown in Fig. 3. It is interesting to note the presence of metabolite at 2.5 min (Fig. 3). The chromatograms of all the three compounds when dosed individually are also shown in Fig. 3. The chromatograms clearly show that the metabolite is formed from C1.

The mean ($n=3$) serum concentration time profile of all the compounds when administered individually as well as in cassette is shown in Fig. 4. The overlapping confidence intervals and statistical significance tests performed on the mean concentrations at each time point showed no statistical difference except for one time point each in C1 and C3. Moreover, the regression analyses of the serum concentration–time data for all the three compounds when dosed individually as well as using N-in-One dosing followed linear trend. The regression analysis curves for C1, C2 and C3 were governed by the equations $y=0.73x+25.3$, $y=0.98x-17.08$ and $y=1.02x-7.79$, respectively. The R^2 values for C1, C2 and C3 are 0.97, 0.98 and 0.94, respectively, thus showing a good correlation. Therefore, pharmacokinetic parameters obtained from these concentration–time profiles are also not statistically different. The pharmacokinetic parameters of C1, C2, and C3 after cassette dosing as well as discrete dosing are presented in Table 4. There was a good correlation of the pharmacokinetic parame-

Table 3
Accuracy and precision of C1, C2 and C3 in spiked rat serum

Analyte	Concentration (ng/ml)	%Bias		%RSD	
		Intrabatch	Interbatch	Intrabatch	Interbatch
C1	16	3.38	3.21	6.58	3.82
	80	6.23	6.71	0.53	1.47
	400	1.66	0.72	6.42	3.89
C2	16	6.24	8.94	1.41	5.88
	80	3.43	3.43	1.56	3.45
	400	4.92	0.4	8.53	5.75
C3	16	6.42	6.62	1.05	0.44
	80	5.29	5.31	1.11	1.39
	400	−2.96	−6.16	6.47	12.89

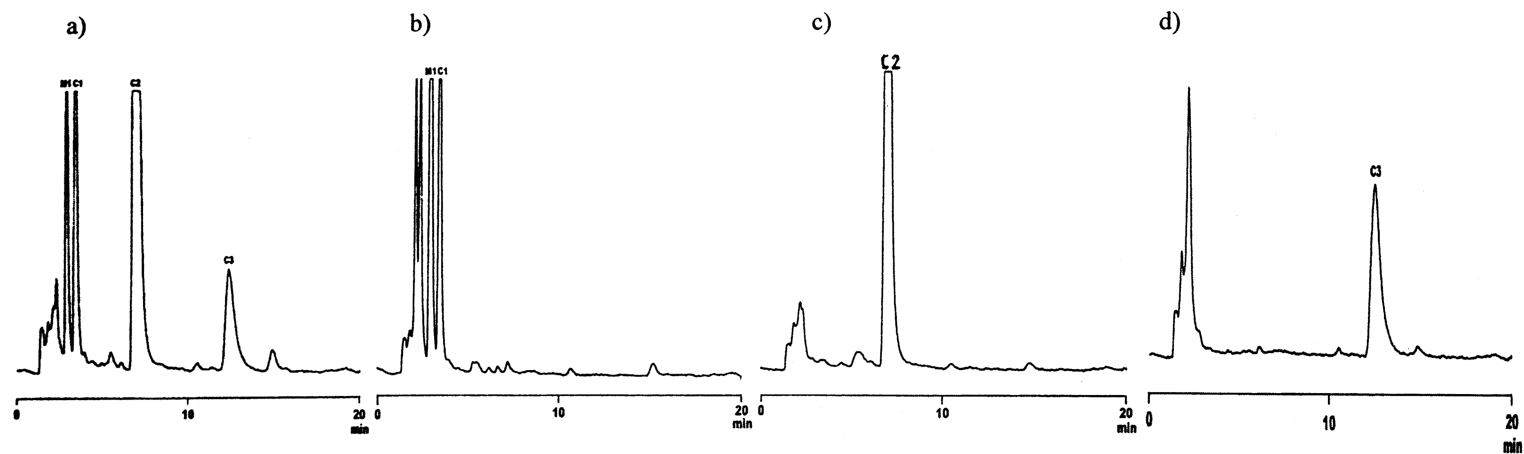


Fig. 3. Chromatogram of a 2.5 min post-dose sample of N-in-One dosing and discrete dosing of C1, C2, and C3.

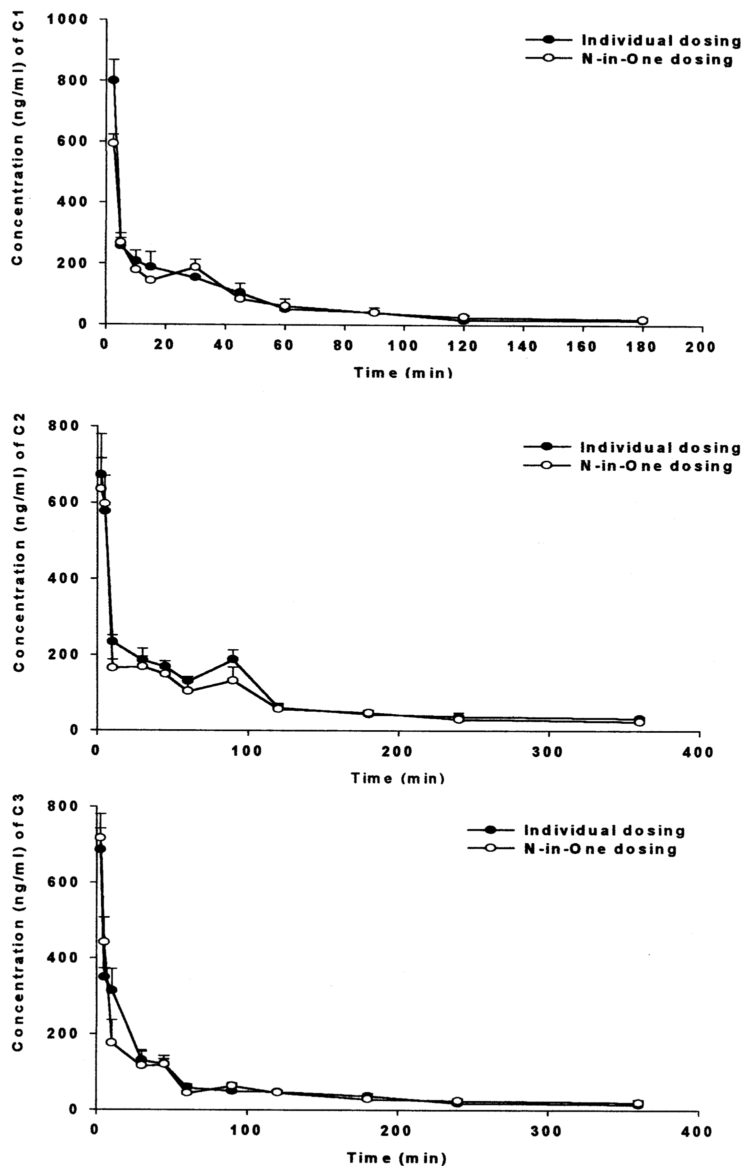


Fig. 4. Comparison of the mean serum concentration–time profile of C1, C2, and C3 when administered discretely and as N-in-One.

ters, particularly those of MRT, V_{ss} and clearance, obtained after N-in-One and individual dosing, while a modest correlation was obtained for $t_{1/2}$ and AUC_{all}, as shown in Fig. 5a–e. From this data, one can come to the conclusion that, of the three compounds, C2 is that with optimum PK parameters.

4. Discussion

The original goal of this effort was to determine whether N-in-One dosing could be used to rapidly determine the pharmacokinetic parameters. In essence, this goal was achieved by the fact that, of the three compounds, one compound, C2 emerged

out as that with optimum PK parameters, using conventional HPLC/fluorescence detection. While quite a few studies using LC/MS (LC/MS or LC/MS/MS) have been shown to hasten pharmacokinetic screening via administration of intravenous cocktail dosing of multiple compounds to experimental animals, no study has been reported to hasten PK screening in a broader sense using traditional HPLC [14] [15]. The availability of LC/MS systems is limited; we have investigated an alternative approach to enhance the throughput by reducing the number of compounds to be screened at a time so that the basic goal is achieved using traditional HPLC. It should be noted that although LC/MS is a powerful analytical tool, there are important issues that should addressed to appreciate the results. The qualitative analysis potential of LC/MS techniques is very high for the characterization of metabolites, degradants, and impurities. But the factors such as linearity, accuracy, precision, and repeatability sometimes differ from the performance characteristics provided from other detectors. Although LC/MS techniques provide acceptable results in each of these areas, one sometimes can obtain better precision and accuracy by using HPLC/UV or fluorescence techniques. There are also some restrictions with regard to the mobile phases, additives, and flow rates used in conventional HPLC when one couples HPLC with MS. The mass spectrometer depends on a reliable and stable flow of a clean mobile phase, requiring good maintenance and operational procedures for the HPLC equipment. It is also important to appreciate the vagaries of biological sample preparation and the issues relating to matrix suppression of the ionization and how excessive numbers of the biological extracts adversely affect the performance of the mass spectrometers. However, it has been recognized that traditional techniques can rarely compete with the sensitivity, selectivity and the throughput capability offered by LC/MS techniques [16].

The successful analysis of the drugs in the biological fluids using HPLC relies on the optimization of sample preparation, chromatographic separation and post-column detection. Each of these three steps were carefully optimized for developing a sensitive, selective, reproducible, and robust assay method for all the three compounds in 0.5 ml serum matrix. Various mobile phases with different composition were tried to elute the compounds on a C18 column, and the chromatograms were studied for the peak shape, sensitivity and selectivity. With the mobile phase containing 65% acetonitrile and 35% buffer (pH 4.0) at a flow rate of 1.5 ml/min, the compounds eluted as a sharp peaks. The sample cleaning of 0.5 ml serum was tested using various solvents. The chromatograms exhibited endogenous serum interference in either one of the regions of interest or low extracting efficiency. The endogenous serum interference was removed by back extraction with hexane owing to the low solubility of the compounds. The extraction efficiency was improved by initial extraction with acetonitrile, which also aided in the sample clean up, followed by back extraction with hexane after the acidification of the residue and, finally, re-extraction of the compounds from the basified aqueous phase with 2% IPA in ether. This method provided optimum extraction efficiency and reproducibility at a flow rate of 1.5 ml/min.

Table 4
Pharmacokinetics of C1, C2 and C3 in N-in-One and discrete dosing studies

Compound	Dosing	Half-life (min)	Clearance (ml/min/kg)	Steady-state volume of distribution (l/kg)	MRT (min)	AUC (ng min/ml)
C1	N-in-One	73.67	177.31	12.35	41.49	14 864
	Discrete	71.51	163.54	9.50	33.43	16 532
C2	N-in-One	148.00	94.73	16.83	97.90	26 368
	Discrete	163.01	79.86	15.89	98.73	29 976
C3	N-in-One	174.00	112.73	22.11	92	21 316
	Discrete	141.00	117.67	16.72	81.83	22 301

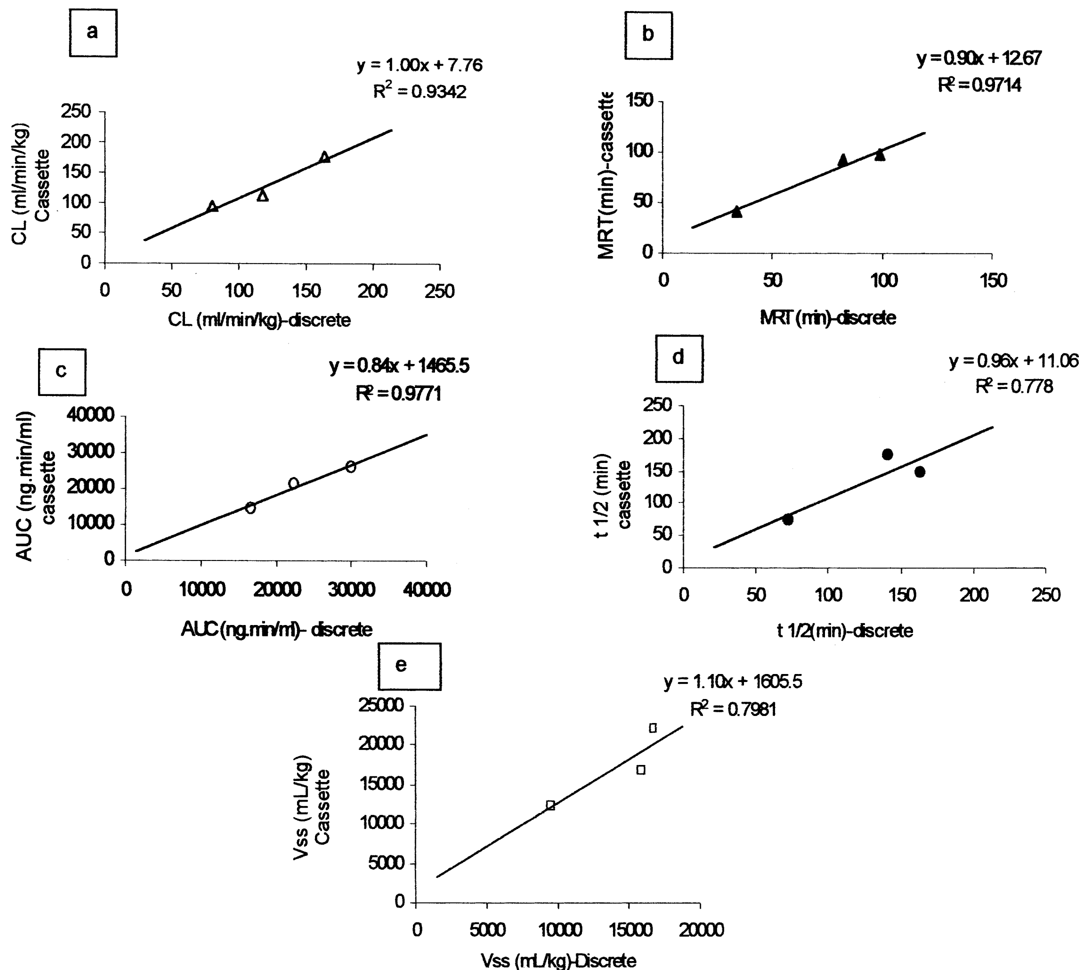


Fig. 5. Regression analysis when dosed discretely and as N-in-One of: (a) the serum elimination half-life for C1, C2, and C3; (b) the MRT for C1, C2, and C3; (c) the clearance for C1, C2, and C3; (d) the AUC for C1, C2, and C3; and (e) the steady-state volume of distribution for C1, C2, and C3.

The concentrations of the compounds in serum at various time points after intravenous administration obtained from the N-in-One dosing are in good correlation with those obtained from discrete dosing as evidenced by linear regression analyses. Furthermore, the estimation of overlapping confidence intervals and statistical significance tests on the mean concentrations showed $P > 0.05$, indicating the pharmacokinetic parameters obtained by cassette and discrete dosing are statistically not different. Table 4 compares the pharmacokinetic parameters of the compounds C1, C2 and C3 when dosed individually with those obtained by N-in-One dosing. There was no statistical difference for the parameters between two methods. Fig. 5a–e shows the linear regression analyses for the elimination half-life, clearance, steady-state volume of distribution, mean residence time and AUC of C1, C2 and C3 when measured by both individual and N-in-One methods. A very good correlation was obtained for the model-independent parameters MRT, V_{ss} and a modest correlation for $t_{1/2}$, CL, and AUC_{all}. These tests establish that drug–drug interactions usually associated with N-in-One are not evident in the present study. In the research program from

which these compounds were derived, the desired criteria for the PK variables are low clearance, high AUC and residence time. Based on the aforementioned criteria and data generated, C2 emerges to be the compound with the most favorable pharmacokinetic attributes. The rate of clearance of C2 is lowest (C1 = 177.31, C2 = 94.73, and C3 = 112.73 ml/min/kg) among all the three compounds, while the MRT (C1 = 41.49, C2 = 97.92, C3 = 92 min) is comparatively higher. V_{ss} and AUC_{all} of C2 are comparable with those of C3, while a good prediction of the $t_{1/2}$ value of the compounds by N-in-One dosing could not be obtained. The high clearance of C1 is owing to the rapid metabolism to M1, which was confirmed by administration of all the compounds individually in one rat each and analysis. This is supported by the fact that metabolite could be seen even 2.5 min after the administration. However, it should be noted that, to rank the compounds, information of the extent of the oral absorption is necessary. This pitfall can be overcome by a separate oral study, which is presently under investigation. Another possibility is the existence of conjugate metabolites that are not detectable by the present analytical method. This hurdle in the method development can be overcome by the use of LC/MS where the separation is based on the detection of molecular weight.

N-in-One dosing reduced the workload several-fold compared with discrete dosing. This could be achieved despite the fact that the time required for the development of the assay method and the run time of cassette was longer compared with that of individual compounds. However, the time spent in the validation of the assay method was directly reduced by three times as the analytical method was validated for all the three compounds simultaneously. Moreover, the number of unknown samples analyzed was reduced by 67%, i.e. from 108 samples to 36 samples (12 time points, $n = 3$ per time point per compound). N-in-One dosing also facilitates the overall reduction in the number of rats, from 54 rats (six rats per study, $n = 3$ per compound) for individual dosing to 20 rats used in the study. The end result of this reduced assay workload and number of animals used in the studies is enhanced and economic throughput of the PK characterization, which was achieved by traditional HPLC. However, it should be noted that the number of compounds that can be included in a cassette depends on the system capacity. It is recognized that application of LC/MS would further accelerate and economize the PK process, once they are more readily available, more user friendly, more efficient and reproducible in detection.

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