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Use of trifluoroacetic acid to quantify small, polar compounds in rat plasma during discovery-phase pharmacokinetic evaluation

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Abstract

Although it is accepted that trifluoroacetic acid (TFA) can cause suppression of an analyte during LC/MS analysis, this paper presents a relatively sensitive gradient method that uses a TFA mobile phase for the improved quantification of small, polar drug-like compounds. The described method was developed in a discovery drug metabolism and pharmacokinetics (DMPK) laboratory for the screening measurement of compound concentrations to calculate PK parameters and CNS exposure of compounds from a chemical series that had poor chromatography under generic methods using formic acid mobile phase. The samples were collected by a Culex automated sampling unit, and the plasma proteins were precipitated by a Tecan robot in 96-well plates. After centrifugation, the supernatant was removed, dried down using a SPE-Dry unit, and the samples were reconstituted in aqueous buffer on the robot. The samples were analyzed on an Agilent LC/MSD using a 5-min gradient on a 5 cm phenyl column. No additional steps, such as the "TFA-fix", were necessary. Although sample batches were analyzed over 6 h, no drift or degradation of signal was observed. The improved chromatography resulted in a method that was selective, rugged, and had a dynamic range from 5 to 20,000 nM, which was sufficient to quantitate low volume, serial plasma samples collected out to 8 h postdose.

Keywords: Trifluoroacetic acid; Polar; Plasma; Mass selective detector; Automated; Pharmacokinetics

1. Introduction

Pharmacokinetic screening in drug discovery is an important tool in choosing compounds with good DMPK qualities, such as high bioavailability and low clearance, to progress to toxicological evaluations and eventually to the clinic. In the past, this has been time consuming, and DMPK labs are evaluating new automation techniques and generic LC/MS methods to keep turnaround in pace with requests [1–5]. Low molecular weight, polar compounds may be important in drug discovery because of the good physical properties they possess, including bioavailability [6–11]. However, these properties can make them difficult to chromatograph with standard generic methods used today in discovery-phase pharmacokinetic screening, including poor retention and peak shape. These types of compounds have required more complicated analytical techniques to chromatograph reliably well (i.e. derivitization) [12,13]. Specific physical properties have been investigated, to help predict whether a compound can be chromatographed well enough using a generic formic acid mobile phase or if a different modifier should be used (data not shown). These data suggest that compounds with $\log D_{3.5} < -2$ will have short retention times and poor peak shape when using a generic 0.1% formic acid gradient (this is based on *calculated* $\log D$ ($\mathrm{clog}\,D$) values from ACD software [Advanced Chemistry Development, Inc, Toronto, Canada]).

A chemical series with low $\mathrm{clog}\,D_{3.5}$ (see Table 1 and Fig. 1) was selected for pharmacokinetic testing *in vivo*, due to good efficacy in a behavioral model. After generic methods failed to provide a method for plasma sample analysis with a limit of quantification (LOQ) and dynamic range that allowed the analyst to quantify the samples and calculate pharmacokinetic parameters, an alternative method needed to be developed to improve retention, peak shape, and limit of quantitation. Ion-pairing was investigated to see if it would improve retention and peak shape sufficiently to provide an adequate LOQ [14]. Volatility was an important parameter to consider, since MS detection was going to be used, and better ionization could counter any suppression [15–17]. One concern was the established fact that TFA could

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Table 1
Physical properties of test compound

Molecular weight	284.32
pK_a acid	3
pK_a base	7
Clog P	−0.22
log $D_{3.5}$[a]	−3.89
# H-bond donors[b]	0
# H-bond acceptors[b]	6

[a] Approximate pH of mobile phase.
[b] Lipinski's method.

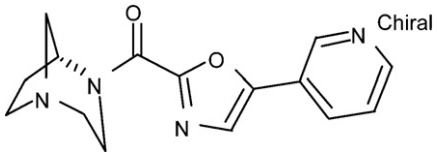

Fig. 1. Structure of test compound.

mask the protonated sample cations from the ESI-MS electric fields by rendering them neutral [18]. Previously, Agilent Technologies and others [19] have described methods to minimize the suppression caused by TFA, called the "TFA-fix", which returns the sample to its ionized state post-column by addition of an ionizing agent. However, it has been reported [18] that weakly basic molecules, such as the one pictured in Fig. 1 and described in Table 1, should not be suppressed by using TFA. The method worked as desired, producing sharper peaks and yielded an appropriate LOQ for discovery PK analysis. Another reason that suppression may have been minimized is the orthogonal design of the Agilent MSD, due to its improved nebulizer efficiency [20]. Evaluation of a 3 cm phenyl column yielded issues with matrix and formulation interferences, so a 5 cm column was used, in order to separate the compound from other underlying analytes [21,22].

2. Experimental

2.1. Chemicals and materials

The compound of interest was synthesized at AstraZeneca, Wilmington, DE. A 1 mM solution was prepared in acetonitrile/methanol (50:50). The methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). The TFA for the mobile phase was obtained from Sigma–Aldrich (St. Louis, MO, USA). The 2-mL 96-well plates were obtained from Phenomenex (Torrance, CA), and the 1.5 mL microcentrifuge tubes were obtained from Fisher Scientific (Pittsburgh, PA).

2.2. Equipment and conditions

2.2.1. Sample preparation

The method for sample preparation was protein precipitation, with supernatant transfer and evaporation, followed by dry down and reconstitution in mobile phase. The assay was processed on a TECAN (Maennedorf, Switzerland) Genesis Robot, using 1 mL syringes, the standard tubing set and non-disposable tips. To dry down the samples, a SPE-Dry (Jones Chromatography, Hengoed, UK) was used, with a nitrogen gas flow of 20 L/min, and drying gas temperature, above and below the plate, set at 40 °C.

Table 2
MS detector settings for test compound

Setting	Value
Ionization mode	API-ES
Polarity	Positive
SIM ion	285
Fragmentor (V)	160
Gas temperature (°C)	350
Drying gas (l/min)	13.0
Nebulizer pressure (psig)	50
V_{cap} (V)	4500

2.2.2. Analysis

The analyzer was an Agilent 1100 Series MSD (Agilent Technologies, Waldbronn, Germany), and was equipped with an Series D Mass Selective Detector, G1312A binary pump, G1379A degasser, G1367A thermostatted autosampler, G1316A thermostatted column compartment. 0.1% TFA was chosen over 0.1% formic acid as the mobile phase modifier, to improve peak shape, and thus, yield a lower LOQ. The column was Zorbax SB-phenyl (4.6 cm × 50 cm, 3.5 μm), and was controlled to 40 °C. The flow rate was 0.8 mL/min. The conditions for the MS are listed in Table 2.

2.3. Bioanalytical method development

2.3.1. Sample processing procedure

A Tecan Genesis method was written that prepared standard solutions, aliquotted unknowns and quality control samples, aliquotted and spiked control plasma for calibration standards, precipitated the plasma with acidified solvent, and transferred the supernatant into a clean plate. Flexibility was written into the method, which included variables to enter the number of samples, and the procedures, or subroutines, to be performed. Liquid sensing was active, so the user is notified if an insufficient volume of plasma is available to aliquot. An additional method reconstitutes the dried-down samples.

Because DMPK pharmacokinetic samples are usually collected by a Culex ABS system (BAS, West Lafayette, IN) over a 24-h period, the volume of blood, and therefore, plasma, are small, so the assay uses a 25 μL aliquot of plasma and 5 μL spike of standard solution. The volume of precipitation solution and reconstitution solution used for this assay is 100 μL, to insure consistent injection volumes and to dilute possible interferences, thereby producing a cleaner baseline.

2.3.2. Calibration solution preparation

A 1 mM stock solution was prepared manually in acetonitrile/methanol (50:50), and the robot prepared dilutions over the range of 500–0.001 μM. A blank solution was also prepared. Internal standard was not used.

2.3.3. Control plasma

Control rat (Sprague–Dawley) plasma was collected in-house using Sarstedt EDTA tubes, to minimize fibrinogen clot formation. Control plasma was stored at −70 °C until needed for the assay. A plasma blank was prepared along with the standards and samples, to check for specificity of the assay.

2.3.4. Calibration curve

Twenty-five microliters of control plasma and 5 μL of a standard solution was aspirated by the Tecan in tandem, and was dispensed together, to allow thorough mixing. The concentration range evaluated was 2–100,000 nM.

2.3.5. Robot precision and accuracy (knowns)

The purpose of the knowns was to evaluate the accuracy of the Tecan during the aliquotting of the unknowns. These knowns are not quality control samples, because, typically, they should be prepared with separated aliquots of undissolved compound. In discovery, the amount of compound may be small, so only one weighing is used for standards and knowns in screening studies. Known samples were prepared on the day of analysis. The spiking solutions were used to spike the plasma. One known was placed before the first sample in the rack, and a second was placed after the last sample. For method evaluation, three concentration levels were tested.

2.3.6. Ion suppression and recovery

Prior to sample analysis, evaluation of recovery and ion suppression from the matrix or dosing formulation is performed. Infusion methods described in the literature [23,24] are relatively time-consuming for a drug discovery laboratory, and more appropriate for methods used in support of regulated studies. Instead, two analytical standards were prepared and one was spiked with a biologically relevant concentration of the dose formulation, 20% hydroxypropyl-β-cyclodextrin. The peak areas were compared, and if they agreed within 20%, the effect is considered negligible.

Evaluation of recovery and matrix suppression was also conducted similarly, rather than by infusion, as described in the literature [25,26]. Plasma spiked at the same concentration as the analytical standard was prepared. These two samples were compared, in order to evaluate recovery and matrix-induced ion suppression. If the change in response was greater than 20%, the method was modified to remove the interference from the retention window. If this did not improve the difference, recovery was considered the issue, and the precipitation solvents were changed to try to improve recovery, if it would have negatively affected the limit of quantification. Previously, under full scan conditions, plasma and different formulations had been analyzed and it was noted that under these conditions, the retention time window where k' was ∼3, there were no stray unknown peaks in the chromatogram (not shown). Therefore, retention time of about 3–4 min was viewed to be most ideal.

2.3.7. Short-term stability

Short-term stability was needed for the time required to analyze the batch. Therefore, alternating standards were analyzed at the beginning and end of the run. If no trends in the responses of the two sets of standards were observed, and standards from both sets had errors relative to the calculated line of less that 20%, the compound is considered stable for batch analysis if prepared by this method.

3. Results and discussion

3.1. Method development

3.1.1. HPLC method development

A generic HPLC gradient method, using 0.1% formic acid and a Zorbax 3 cm SB-C8 column, was first evaluated, but the retention time was too short, relative to k', and the peak was split (see Fig. 2A). In an attempt to get better retention, the column was changed to a Zorbax 5 cm phenyl column and reinjected (B). Retention was better, but the peak was still split. A fresh standard prepared in MeOH:ACN was diluted in 0.1% formic water, to reduce solvent strength of the sample, and more closely match the mobile phase (C), but the peak shape was still poor, and it was not retained enough to avoid interferences. To improve retention further, as well as peak shape, ion-pairing was investigated. The formic acid mobile phase was replaced by 0.1% TFA in both the ACN and water. While the retention was improved, the peak was still split and broad (D). To correct for this, the neat standard was diluted in 0.1% TFA in water and injected onto the phenyl column. This produced sharper, symmetrical peaks, and the k' value was approximately 3 (E). The HPLC conditions are in Table 3.

3.1.2. Sample preparation evaluation

Some compounds chromatograph with better peak shape in the presence of plasma, probably due to the reduced solvent strength relative to neat injections, so a standard curve with a range from 2 to 20,000 nM was prepared by precipitating the plasma with 0.1% formic acid. However, in this case, there was no improvement in peak shape. 0.1% TFA in ACN was used

Table 3
Final HPLC conditions for analysis of test compound

Column packing material	Phenyl
Column length (cm)	5
Column temperature (°C)	40
Flow (mL/min)	0.8
Mobile phase A	0.1% TFA
Mobile phase B	0.1% TFA in ACN
	% B
Gradient time (m)	
0.00	7
2.00	7
2.01	7
4.00	70
4.01	100
5.01	100
5.02	7
Re-equilibration time (min)	3
Injection volume (μL)	5

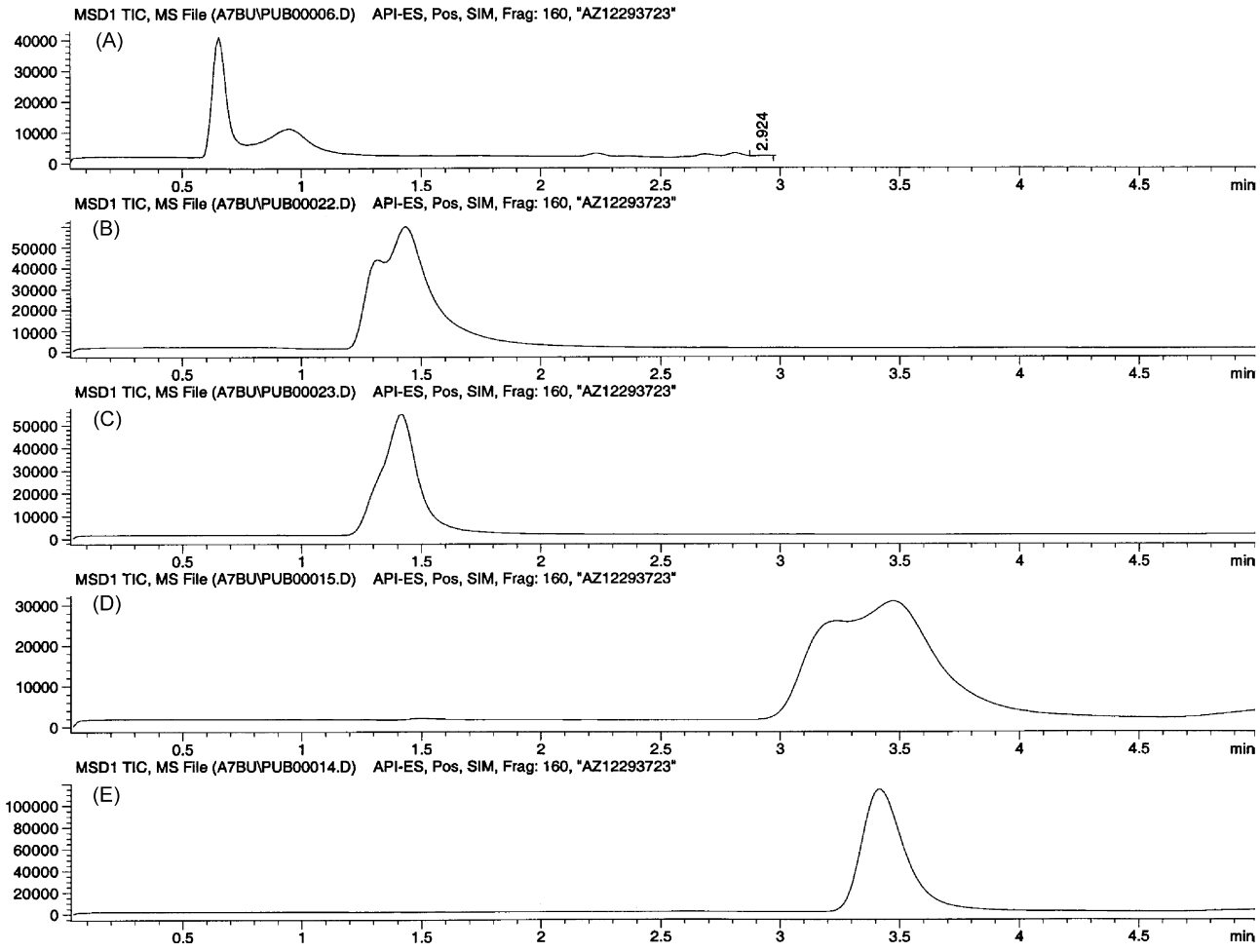

Fig. 2. Chromatogram of test compound injected onto C8 using formic acid mobile phase prepared in ACN (A) and water (B), onto phenyl using formic acid mobile phase prepared in water (C), onto phenyl using TFA mobile phase in ACN (D) and 0.1% TFA in water (E).

as the protein precipitation solvent, but after injection of the supernatant, the compound was not detected, probably due to the broadness of the peak (see Fig. 3). Since the peak shape improved when the neat solution was previously diluted in 0.1% TFA in water, standards were precipitated using 0.1% TFA in ACN, dried down under nitrogen using the SPE-dry with the temperature controls set to 40 °C, and then reconstituted in 0.1% TFA in water. This yielded an improved peak shape, and the LOQ was 5 nM, and the plasma blank had a clean baseline at the analyte's retention time (see Fig. 3). The dynamic range was 5–20,000 nM, with a quadratic fit with quadratic weighting. The need to dry the samples down and reconstitute added time to the sample preparation, but the use of automation in the method, including the reconstitution of the plate, kept the additional time to a minimum.

3.1.3. Specificity, ion suppression, and recovery

The chromatograms of the rat plasma blanks evaluated ($n = 3$) had no interferences within ±1 min of the retention time of the analyte. If possible, the same source of plasma was used throughout the experiment. A comparison of the peak areas of the analytical standards with and without formulation (20% hydroxypropyl-β-cyclodextrin, or HP-β-CD) showed a difference in peak areas to lower than 20%, so the formulation did not appear to affect the ionization of this compound under these conditions. After comparing the peak areas of the manually spiked plasma standard to the analytical standard without formulation, the difference was also lower than 20% (data not shown), so there appeared to no suppression of this compound under these conditions.

3.2. Calibration curve precision

The 2 nM standard was not detectable in any of the three runs. The dynamic range of the calibration curve was from 5 to 20,000 nM. The results of three curves analyzed on different days are presented in Table 4.

3.3. Precision and accuracy at known concentrations samples

Data from three levels of knowns are presented here, although only one level is typically run during practical use in the lab. The precision and accuracy results are summarized in Table 5.

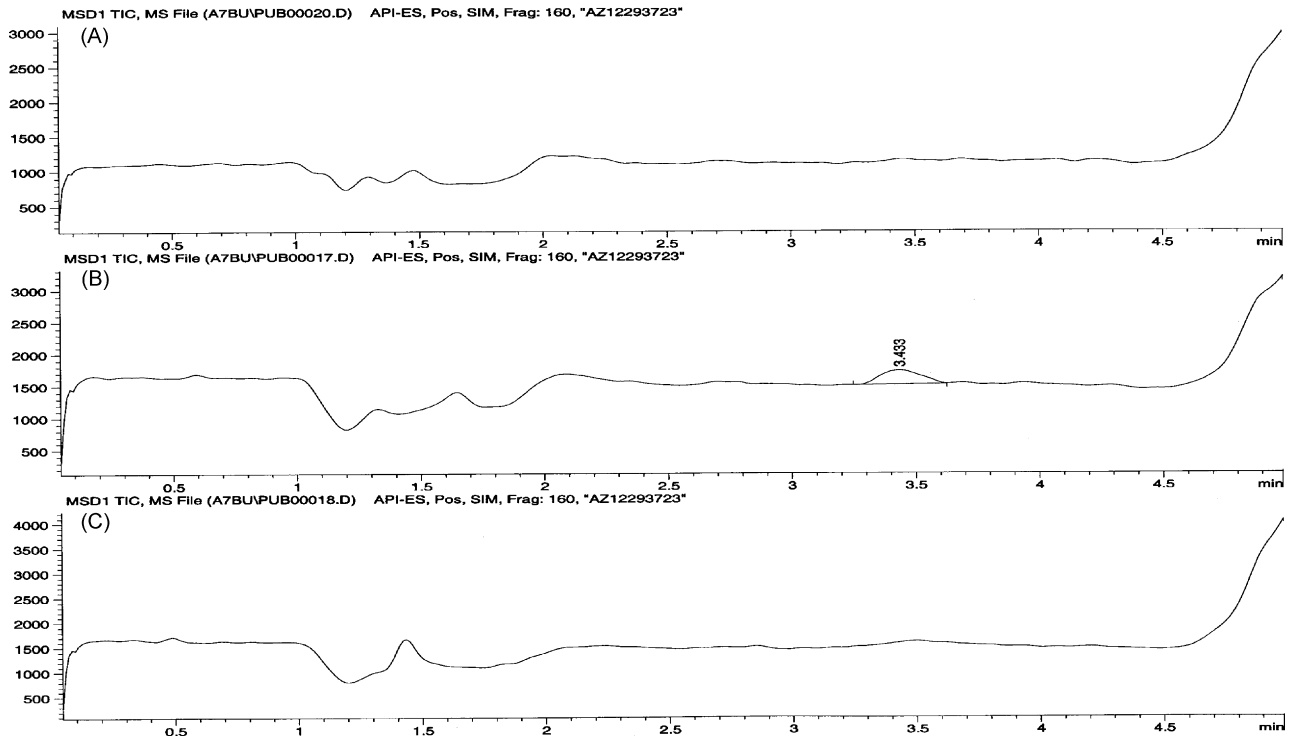

Fig. 3. Chromatograms of blank plasma (A), a 5 nM plasma standard which was precipitated, dried down, and reconstituted in 0.1% TFA in water (B), and a 5 nM plasma standard prepared with protein precipitation with direct injection of the supernatant.

Table 4
Calibration curve results and statistics over three runs

Theoretical concentration (nM)	Mean (nM)	S.D. (±)	Imprecision (S.D./mean%)	Accuracy (mean/theoretical concentration, %)
0	0	0	–	–
5	4.66	0.32	6.9	93
10	9.71	0.20	2.1	97
20	18.0	1.2	6.9	90
50	55.3	4.0	7.2	111
100	102	3	2.8	102
200	201	23	11.4	101
500	532	47	8.8	106
1,000	1,008	56	5.6	101
2,000	1,931	214	11.1	97
5,000	5,375	275	5.1	108
10,000	9,376	433	4.6	94
20,000	20,397	375	1.8	102

$n = 3$ for each concentration.

3.4. Short-term stability

Short-term stability of the processed samples was tested for the length of the run. Standards alternately analyzed at the beginning and end of the batch were included on the same calibration curve, and had a relative error of 0.99. Therefore, it was agreed by the analyst and study director that the compound is stable for the length of time required for the analysis of the batch.

Table 5
Precision and accuracy results of the known samples

Knowns theoretical concentration (nM)	Mean (nM)	Imprecision (% R.S.D.)	Accuracy (% of theoretical)
10	9.76	13	98
100	87.9	2	88
1000	839	2	84

4. Discussion

Ion-pairing has been shown in this paper to be a suitable method for the analysis of small, polar, weakly basic drug candidates. However the choice of which reagent to use can make the difference between rapid analysis and further method development. The analyte evaluated in this case was so polar that the generic 3 cm C8 column did not provide enough retention

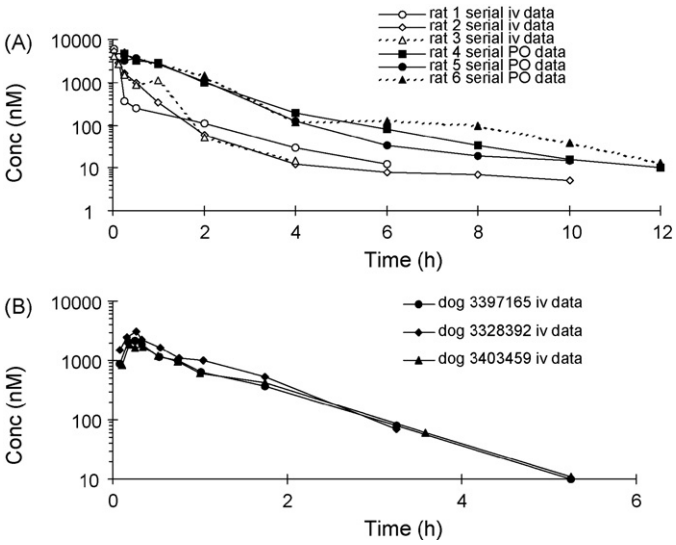

Fig. 4. Pharmacokinetic profiles for test compound. (A) Following a 10 μmol/kg i.v. dose or a 30 μmol/kg PO dose in fasted male Sprague–Dawley rats and (B) pharmacokinetic profile following a 5 μmol/kg 15 min i.v. infusion dose in fasted male Beagle dogs.

to quantify plasma samples, even at very low formic acid–ACN starting conditions. Switching to a phenyl column slightly improved the chromatography, but pairing that with TFA improved retention *and* peak shape adequately. Direct injection of a neat standard or the manually spiked plasma sample produced a chromatogram with a split peak. This was remedied by drying down the extracts under nitrogen, and reconstituting in 0.1% TFA in water. This method was used in rat pharmacokinetic screening studies for the quantification of samples out to 6 h following a 10 μmol/kg IV dose and 10 h following a 30 μmol/kg oral dose, and it was also used to quantify dog plasma samples out to 5 h following an IV infusion study (Fig. 4). Exposure parameters calculated from values measured with this method, along with other in-life and efficacy data, were used to make critical decisions on the future plans of the project team.

There are a variety of ion-pairing agents that could have been used, but important parameters such as volatility, surfactant properties, residue deposition on the detector interface (and possible signal degradation over time), and the possibility of adducts should be considered for quick analysis in drug discovery labs [15]. In this experiment, the analyte was analyzed over 3 days, and calculated concentrations of the standards and the knowns were found to be consistent, even without the use of internal standard. Analytical runs were analyzed on the same detector, and this consistency shows that a method could be developed for poorly retained analytes relatively quickly and be used to generate concentration data of study samples. This method also worked for other compounds in the same chemical series, and the concentrations measured were used to calculate pharmacokinetic parameters to rank and progress discovery compounds through milestone decisions. When screening compounds, the analyst should check the $\log D_{3.5}$, and if it is <-2, to forgo the generic formic acid methods, but move quickly to a robust method that will easily enable the analyst to quantify screening PK samples.

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