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Development of a discriminating *in vitro* dissolution method for a poorly soluble NO-donating selective cyclooxygenase-2 inhibitor

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Abstract

A discriminating dissolution method using a USP apparatus 2 dissolution tester was developed for a nitric oxide donating selective COX-2 inhibitor to support phase I and II formulation development, clinical supplies release and stability testing of an immediate release oral tablet. The BCS class II compound showed very low aqueous solubility and required the use of surfactant-containing (sodium lauryl sulfate (SLS)) dissolution medium in order to achieve an appropriate release profile. The dissolution method utilized 900 mL of 2% SLS (w/v). Samples were withdrawn at five specified time-points over 60 min, at a paddle speed of 75 rpm. Analysis of samples was performed using a validated HPLC method. Despite the use of high levels of SLS, the ability to discriminate variations in physical properties such as drug particle size, granule particle size and tablet compression force was demonstrated. In order to confirm the relationship between these physical parameters and the tablet *in vivo* release profile, oral dosing of the formulations in fasted beagle dogs was performed to determine if the changes observed in the dissolution profiles were biorelevant. The results of the dissolution and corresponding *in vivo* experiments helped identify the critical processing parameters likely to influence product bioavailability.

Keywords: Dissolution; COX-2; Rofecoxib; HPLC; Formulation development

1. Introduction

Dissolution testing has been demonstrated as an important tool to evaluate performance of solid dosage form pharmaceuticals [1,2]. Developing an appropriate *in vitro* dissolution test can be particularly challenging for highly lipophilic compounds which demonstrate very low aqueous solubility, since the use of surfactant/solubilizer systems are almost always required. These types of drugs are classified by the biopharmaceutical classification system (BCS) as either class II or IV, depending on their apparent permeability (P_{app} values); as a result, dissolution testing is quite critical since oral absorption will be limited to a large extent by the dissolution rate [3,4].

During formulation development the ideal dissolution test should provide product quality information as well as some preliminary *in vivo*/*in vitro* correlation (IVIVC) or biorelevance [5]. This is not always feasible if an artificial medium containing high levels of surfactant is used. Typically for insoluble compounds, dissolution methods are developed to support manufacturing, filing and stability studies and may employ surfactants such as sodium lauryl sulfate (SLS) or polysorbate 80 (e.g. Tween® 80) to assist drug dissolution and solubilization [6]. Usually, biorelevant dissolution testing is then performed separately using media such as simulated gastric fluid (SGF), simulated intestinal fluid (SIF), milk, fasted-state or fed-state simulated intestinal fluid (FaSSIF or FeSSIF) [7,8] and possibly an alternate apparatus such as flow-through systems (USP apparatus 4) [1]. This type of approach may be effective but it is somewhat inefficient and

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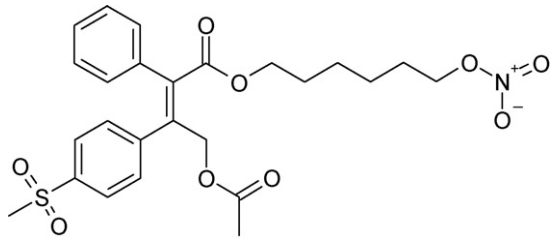

Fig. 1. Chemical structure of NO-donating COX-2 inhibitor.

consumes additional dosage forms/drug supply, which can be limited in early formulation work. In addition, use of biorelevant dissolution media can add additional chromatographic challenges. Ideally, a single *in vitro* test would provide both product quality and bio-performance information, thereby reducing formulation development and optimization time [9].

A dissolution method using a USP apparatus 2 was developed for a BCS class II nitric oxide donating selective cyclooxygenase-2 (COX-2) inhibitor to support phase I and II formulation development, clinical supplies release and stability testing of an immediate release oral tablet. This novel pro-drug contains rofecoxib and an NO-donating linker (Fig. 1). Nitric oxide has been demonstrated to act as a potent vasodilator and was designated as the molecule of the year in 1992 due to its important biological function [10]. In addition, nitric oxide donating COX-2 inhibitors have been shown to reduce gastrointestinal injury in animals [11]. If this mechanism were demonstrated in humans, the potential therapeutic benefit would allow co-administration of the NO-donating COX-2 inhibitors with low-dose aspirin [12,13]. Further development of this program was suspended after the voluntary withdrawal of rofecoxib.

The dissolution profiles obtained were able to discriminate various formulation and process changes. The drug substance particle size was found to have the greatest impact on release profiles. In addition, dissolution results were compared to pharmacokinetic results obtained after oral dosing in beagle dogs. A simple level C *in vivo*/*in vitro* correlation (IVIVC) was demonstrated between dissolution time and C_{max} in a fasted dog model using tablets prepared with three different particle sizes of drug substance. Although level C correlations are insufficient for regulatory filing, they are generally considered acceptable during formulation/product development [9,14]. Additionally, level B and C IVIVCs are recommended for studying dissolution of immediate release dosage forms [15].

2. Experimental

2.1. Reagents and materials

Trifluoroacetic acid was purchased from Pierce (Rockford, IL, USA). Water was obtained from a Millipore Milli-Q water filtration system (Bedford, MA, USA). HPLC-grade acetonitrile was purchased from EM Science (Darmstadt, Germany). Sodium-lauryl sulfate (SLS) and Tween® 80 were obtained from Sigma (Oakville, Ont., Canada). All HPLC measurements were made using an Agilent HP1100 (Palo Alto, CA, USA) consisting of a G1322A degasser, G1312A binary pump, G1313A autosampler, G1316A thermostated column compartment and a G1314A variable wavelength detector. The active pharmaceutical ingredient (API) was supplied by the Merck Chemical Repository (Rahway, NJ, USA). Twenty-five millimeter syringe filters containing 1 μm GxF glass fiber were purchased from Gellman-Pall (Ann Arbor, MI, USA).

2.2. Methodology

Dissolution testing was performed using a calibrated Distek 2100B (North New Brunswick, NJ, USA) dissolution tester equipped with USP apparatus 2 paddles operated at 75 rpm. The media consisted of 900 mL of 2% (w/v) SLS in water maintained at 37 °C. Each dissolution test was performed with six tablets. For the 4 mg tablets, a volume of 500 mL media was used. Sampling time-points were 10, 20, 30, 45 and 60 min. At each time-point, a volume of 5 mL was withdrawn and filtered through the 1 μm glass fiber syringe filter and the last 1 mL was subsequently collected for analysis. Standard solutions were also prepared in 2% (w/v) SLS in water. Analysis of dissolution samples was performed by HPLC using a 6 min isocratic elution through an Advanced Chromatography Technologies ACE5 C18 4.6 mm × 75 mm ID, 5 μm column (Aberdeen, Scotland) maintained at 30 °C. The mobile phase consisted of water–acetonitrile–trifluoroacetic acid (40:60:0.1, v/v/v) operated at a flow-rate of 1 mL/min with an injection volume of 25 μL and detection at 250 nm. Tablet hardness measurements were made with a Key PTB-301 hardness tester (Englishtown, NJ, USA) while disintegration time was determined using a Vankel reciprocating basket apparatus model number 10-911-71 (Edison, NJ, USA), a Haake DC1 circulating bath (Loughborough, UK) and a calibrated timer.

2.3. Pharmacokinetic analysis

Twenty-milligram tablets containing nitric oxide donating COX-2 inhibitor were administered orally to beagle dogs after an overnight fast followed by a 50 mL water rinse. Blood was drawn into heparinized tubes at 0, 15, 30 min, 1, 2, 4, 6, 8 and 24 h time-points. Plasma samples were extracted using protein precipitation with an equal volume of acetonitrile, vortexed and centrifuged at 20,000 × *g* for 15 min. The analysis of rofecoxib in extracted plasma samples was performed by HPLC with UV detection at 275 nm, using an Agilent Eclipse XDB-C18, 4.6 mm × 75 mm, 3.5 μm particle size column (Palo Alto, CA, USA) operated at 45 °C. Twenty-five microliters of the supernatant was injected. The mobile phase consisted of water–acetonitrile–trifluoroacetic acid (65:35:0.1, v/v/v) at flow-rate of 1 mL/min. The total run-time was 7.5 min. Pharmacokinetic calculations were performed using Pharsight's WinNonlin ver 5.0.1 (Mountain View, CA, USA). All procedures for *in vivo* experiments were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research (Kirkland, Quebec, Canada) and were performed according to guidelines established by the Canadian Council on Animal Care [16].

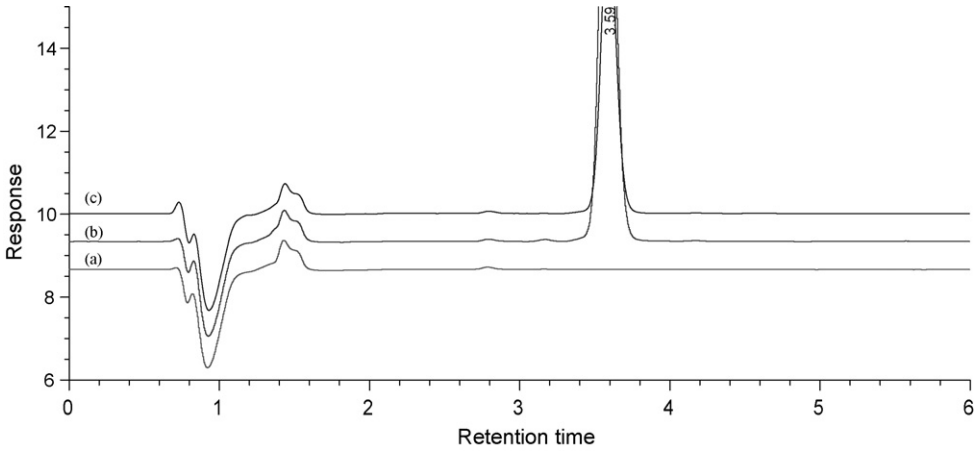

Fig. 2. Overlaid chromatograms of: (a) placebo formulation in dissolution medium, (b) 4 mg tablet in dissolution medium and (c) 25% standard solution (4 mg dose) in dissolution medium, using the dissolution HPLC method.

2.4. Formulation

Compressed tablets of the NO-donating COX-2 inhibitor were made at three dose strengths of 4, 20 and 80 mg. The formulation was comprised of microcrystalline cellulose, lactose monohydrate, croscarmellose sodium, magnesium stearate and red ferric oxide. A high shear wet granulation process followed by fluidized bed drying, milling and lubrication was developed. Sieved fractions of the granulation were obtained by sieving through standardized mesh screens and subsequently extracted and analyzed for potency by HPLC using the same chromatography conditions as the dissolution method.

3. Results and discussion

3.1. Dissolution method validation

An original analytical method was developed to analyze the dissolution of immediate release tablets. In order to determine the suitability of the dissolution method for evaluating tablets, validation of the HPLC method for specificity, precision, linearity, solution stability, accuracy and recovery was performed. A short isocratic method using a C18 column was used to ensure adequate separation from the dissolution medium and the excipient components. A detection wavelength of 250 nm corresponded to a peak maximum and provided enough sensitivity for all dose strengths. A pH controlled mobile phase maintained an adequate peak shape and a stable retention time of approximately 4 min.

3.1.1. Specificity

Overlaid HPLC chromatograms of a placebo formulation, sample and standard in the dissolution medium are shown in Fig. 2. The lowest dose strength and standard concentration are shown. No interferences from excipients with the peak of interest were observed, confirming the specificity of the method.

3.1.2. Precision

Measurement precision was evaluated by injecting sample and standard solutions prepared at all three dose strengths. The relative standard deviation of six replicate injections was $\leq$0.1% in all precision tests, indicating acceptable precision.

3.1.3. Linearity

Linearity of the method was evaluated with a seven-point calibration curve spanning a concentration range of 2–130 μg/mL drug substance. This linear range approximates 25% of the low dose strength and up to 150% of the highest dose strength formulation. The slope of the regression line was 13.56x with a y-intercept of −0.82 and a determination coefficient of 1.0000. The results were deemed acceptable.

3.1.4. Accuracy and recovery

Accuracy of the method was determined in duplicate by spiking the dissolution media containing the appropriate placebo formulation with active drug at 50, 100, 125% of the target dose strength. The mean recovery was found to be within 98.1–98.7% at all levels and dose strengths (Table 1). Furthermore, filter compatibility was assessed by determining the recovery from

Table 1
Recovery of NO-donating COX-2 inhibitor from spiked placebo solutions

Spike level (%)	Recovered 4 mg dose-strength (%)	Recovered 20 mg dose-strength (%)	Recovered 80 mg dose-strength (%)
50	98.1	98.5	99.0
100	97.8	98.2	98.1
125	98.3	98.2	98.9
Total mean (n = 3)	98.1	98.3	98.7
Total R.S.D. (n = 3)	0.3	0.2	0.5

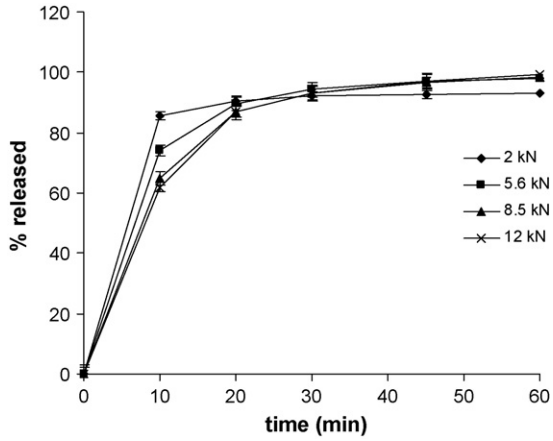

Fig. 3. Dissolution rate of 20 mg tablets as a function of tablet compression force. USP apparatus 2 with 900 mL of 2% SLS, paddle rotation set to 75 rpm.

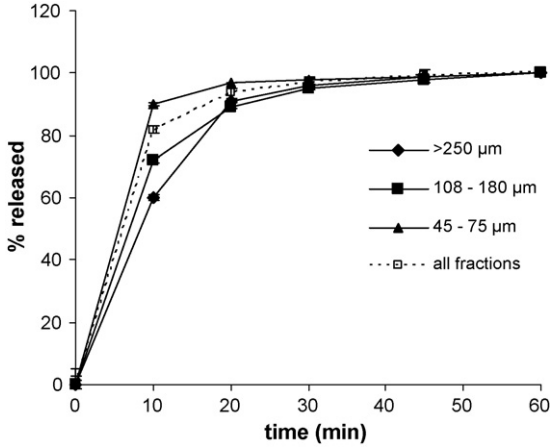

Fig. 4. Dissolution rate of 20 mg tablets as a function of granule size. USP apparatus 2 with 900 mL of 2% SLS, paddle rotation set to 75 rpm.

successive aliquots of a spiked placebo solution representing the lowest dose strength. Twenty milliliters of the solution were filtered through the 1 μm glass fiber filter and 1 mL aliquots were collected for HPLC analysis. Recovery ranged from 99.0 to 99.4% and therefore full recovery was achieved after filtering the first 1 mL of the solution, indicating no issues with filter absorption.

3.1.5. Solution stability

Solution stability was demonstrated for at least 5 days for all standard and sample solution concentrations. Analyte recovery was observed between 98 and 102% of the original value over the 5-day test period.

3.2. Dissolution method development

Initial selection of the dissolution media was based on equilibrium solubility data. The implementation of a surfactant system was decided after aqueous solubility across the pH range 2–8 was found to be less than 2 μg/mL. The equilibrium solubility in 2% SLS (w/v) showed 2.3 times the solubility required (∼200 μg/mL) for the highest tablet dose strength, 80 mg, assuming a 900 mL media volume. Alternatively, solubility in a non-ionic surfactant Tween® 80 (polysorbate 80) was evaluated at levels up to 5% (v/v); however, less than 80 μg/mL was dissolved. The surfactant levels evaluated are well above the reported critical micelle concentrations [6]. Further dissolution testing of 80 mg tablets showed only 75% release after 60 min in 5% Tween® 80 (v/v). As a result of the initial screening, 2% SLS (w/v) was selected as the dissolution medium for all dose strengths. Apart from choosing the dissolution medium, the paddle speed is a critical parameter to select for the USP apparatus 2 dissolution tester in order to eliminate artifacts such as coning. An evaluation of paddle speed indicated 75 rpm was able to dissolve at least 85% of the 80 mg tablets in 30 min and eliminated slight coning observed at 50 rpm. The dissolution profiles of 4, 20 and 80 mg tablets also indicate the release profile was solubility limited with the slowest release occurring at the highest dose strength.

To further understand the mechanism of the dissolution of tablets, 20 mg tablets were prepared using four different compression forces. Using a 900 mL volume of 2% (w/v) SLS, approximately 10× sink conditions were maintained during these tests to adequately evaluate the effect of tablet compression on the disintegration and subsequent dissolution profile. The dissolution profiles, shown in Fig. 3, showed a slower release during the first 10 min as the compression force was increased. The corresponding hardness and disintegration results are summarized in Table 2. It is evident that the harder tablets resulted in longer disintegration times. Therefore, in the early stages of dissolution, e.g. during the first 10 min, tablet disintegration is the rate-limiting step for solubilization. This was also confirmed by comparing the dissolution of granules and tablets. Tablet dissolution was 12% and 4% lower than granules at the 10 and 20 min time points, respectively (data not shown). Tablets in subsequent experiments were compressed to a hardness of 4–5 kPa, which was found to be the minimum force needed to achieve a robust, scalable tablet, while providing a fast release profile.

In order to investigate the effect of granule size on dissolution, 20 mg tablets were compressed from granules collected from small (45–75 μm), medium (106–180 μm) and large (>250 μm) mesh-size sieve fractions. Data in Fig. 4 were normalized to

Table 2
Hardness and disintegration results for 20 mg tablets prepared using increasing compression forces

Compression force (kN)	Hardness, kPa (R.S.D.) [range]	Disintegration time, min mean [range]
2.0	1.9 (5.8) [1.7–2.0]	1 [1–1]
5.6	6.1 (2.4) [5.9–6.3]	4 [3–4]
8.5	7.0 (9.3) [5.9–7.5]	5 [4–5]
12.0	7.0 (4.5) [6.5–7.3]	4 [4–5]

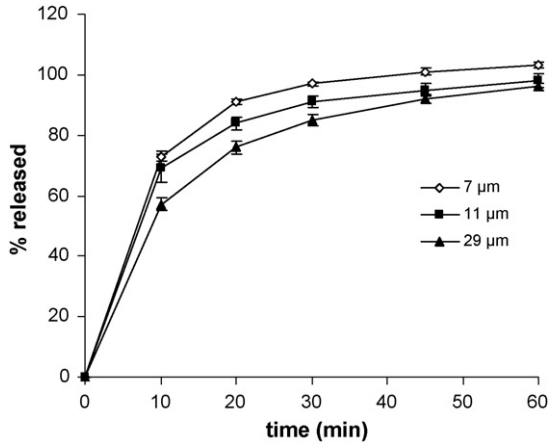

Fig. 5. Dissolution rate of 20 mg tablets as a function of API median particle size. USP apparatus 2 with 900 mL of 2% SLS, paddle rotation set to 75 rpm.

100% at the final time-point to account for uneven drug distribution in the different fractions. The dissolution method was able to discriminate this change in particle size, demonstrating its usefulness as a product quality test; however, the dissolution profile was found to be dependent on granule size at early time-points only. The release of the active from larger granules was slower while smaller granules showed a rapid initial dissolution rate. Finally, changes in the particle size of the API (active pharmaceutical ingredient) were also evaluated using the dissolution method (Fig. 5). The results indicate that decreasing the API particle size through milling serves to improve solubilization throughout the entire dissolution test. Correspondingly, the surface area of the API available for dissolution will impact its ability to be wetted by lumenal fluids [17]. Therefore, identification of an appropriate particle size was important in setting specifications for subsequent API deliveries. Additional *in vivo* testing was then pursued to determine if this method provided any information regarding bio-performance. For BCS class II compounds, dissolution testing can be predictive of performance since absorption is solubility limited [1].

3.3. Pharmacokinetic results

3.3.1. Bioanalytical method

An original HPLC–UV bioanalytical method was developed to evaluate extracted dog plasma samples for rofecoxib. Overlaid representative HPLC chromatograms of blank plasma, LOQ standard (0.1 μg/mL), spiked standard and an unknown sample are shown in Fig. 6. The main peak was well separated from the extracted plasma components confirming the specificity of the bioanalytical method. The chromatograms show no interference and no co-eluting peaks. The nitric oxide donating COX-2 inhibitor was not detectable in plasma samples due to the rapid cleavage of the ester in the pro-drug to yield the NO-linker and rofecoxib (Fig. 1). The NO-linker was not measured using this method. For our studies, only rofecoxib plasma levels were monitored to assess the effect of formulations on bioavailability. As a consequence, a separate bioanalytical HPLC method was developed and partially validated for this purpose. Method precision was evaluated over the standard range of 0.1–10 μg/mL, the coefficient of variation ($n = 9$) was less than 2% at all levels. The slope of the regression line was 3.23x with a y-intercept of −0.023 and a determination coefficient of 1.0000. Spiked QC samples were prepared at 0.2, 2 and 8 μg/mL. Mean recovery of triplicate spiked QC samples from three separate calibration curves ranged from 94.3 to 103.5%. Spiked QC samples subjected to three freeze-thaw cycles followed by 3 h at room temperature, recovered between 90.9 and 97.4%. The working standards were stable for at least one week in neat acetonitrile. The LOQ was determined in triplicate to be 0.1 μg/mL, with a mean signal-to-noise ratio of 12. Extracted blank plasma from six different canine donors showed no interference with the main peak, further demonstrating specificity.

3.3.2. In vitro/in vivo interpretation

In vivo performance in large animals such as dogs and monkeys is commonly used in the pharmaceutical industry to rank order formulations and drug products. However, it is costly and requires skilled animal handling, bioanalytical capabilities and

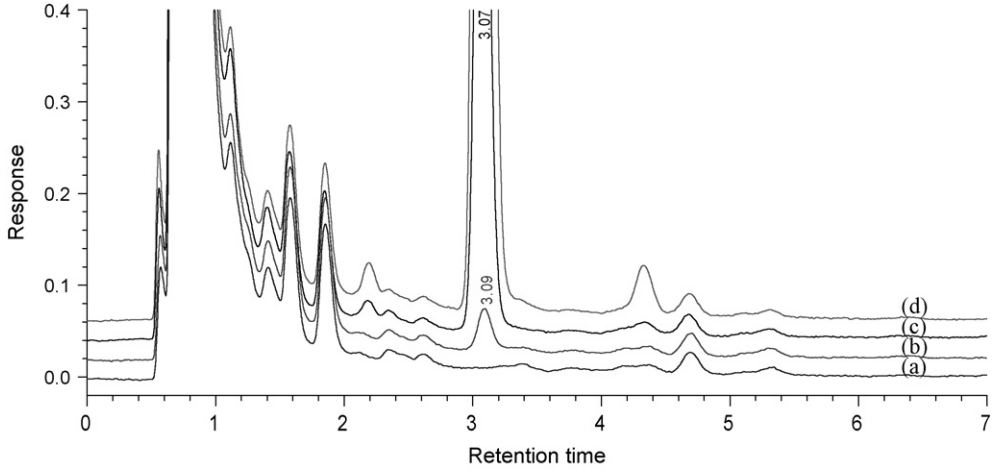

Fig. 6. Overlaid chromatograms of: (a) extracted blank dog plasma, (b) LOQ standard, (c) extracted sample plasma and (d) extracted spiked standard, using the bioanalytical HPLC method.

Table 3
Bioavailability results for three different 20 mg tablets dosed orally in fasted dogs

API median particle size (μm) [surface area] (m^2/g)	AUC (μm h) ± S.E.	C_{max} (μm) ± S.E.	T_{max} (h)
7 [2.45]	0.94 ± 0.43	0.33 ± 0.16	1
11 [2.13]	1.04 ± 0.17	0.26 ± 0.07	1
29 [1.38]	0.58 ± 0.13	0.11 ± 0.02	2

should only be used after thorough *in vitro* evaluations have been performed. Consequently, bioavailability studies in fasted dogs were used to evaluate the effect of API particle size on the rate (C_{max}/T_{max}) and extent of absorption (AUC). As shown in Figs. 3 and 4, changes to compression force and granule size only affected the early dissolution time points and tablet disintegration. Therefore, in order to minimize the number of animal studies, only API particle size, which varied between 7 and 29 μm for three batches, was evaluated *in vivo* as a critical parameter to optimize, since its effects were observed throughout the dissolution test. Table 3 summarizes the pharmacokinetic results after oral dosing of 20 mg tablets (equivalent to approximately 2 mg/kg) compressed with a range of API particle sizes. A significant decrease in C_{max} and AUC is observed at the largest particle size (29 μm) (Table 3). In addition, the T_{max} shifted from 1 to 2 h. A level C correlation between C_{max} and dissolution time is shown in Fig. 7. The correlation coefficient, *r*, of 0.9975 indicates a strong correlation between the C_{max} obtained in beagle dogs and the time required to release 90% label claim in dissolution testing ($T_{90\%}$). C_{max} was found to be a more relevant parameter to correlate than AUC for an immediate release dosage form since release is typically completed within 60 min. Changes in the dissolution rate were not reflected in AUC measurements. In addition, a rapid onset, as indicated by the short T_{max}, was potentially an ideal property to optimize for a pain relief indication.

As expected for a BCS class II compound, the bioavailability is therefore dissolution-rate limited and the larger API particle size and corresponding decreased surface area results in a larger undissolved fraction which is unavailable for absorption during GI transit [18]. The rank order performance obtained from *in vitro*/*in vivo* studies was similar and indicated a smaller particle size would provide faster exposure. Ideally, performance of subsequent tablet batches could be evaluated using only *in vitro* testing to minimize animal resources and costs associated with *in vivo* studies.

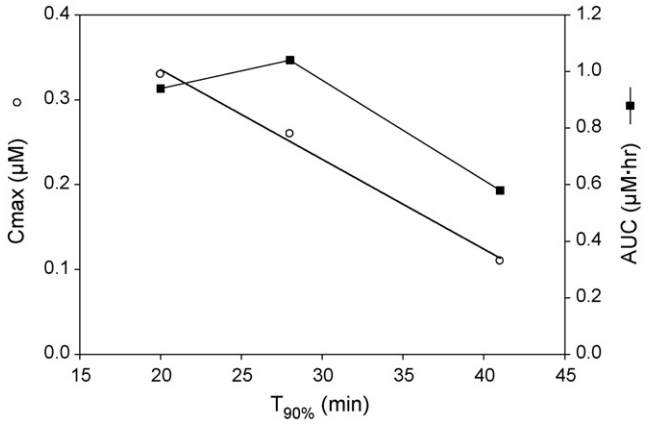

Fig. 7. Level C IVIVC between C_{max} (open circles) and AUC (solid squares) in beagle dogs and $T_{90\%}$ for three formulations of different particle size API.

4. Conclusions

A novel dissolution test and HPLC methods were developed and validated to support formulation development. The *in vitro* method demonstrated discriminating power and the ability to rank order changes in API particle size in a similar manner to *in vivo* studies despite the use of an artificial medium containing a high level of surfactant. In addition, changes to the granulation particle size and tablet compression force were detected using this method. While a strict IVIVC was outside the scope of this work, the dissolution performance of tablets prepared with varying particle size drug substance correlated to the C_{max} results obtained after oral dosing in beagle dogs. In comparison to more complex approaches using simulated intestinal fluids, the use of this simple dissolution method is advantageous since it has the potential to serve as both a robust quality control method and a biorelevant method with discrimination power. To further evaluate biorelevance, additional testing could include comparing *in vitro*/*in vivo* results to discriminate changes to excipient composition, particularly solubility enhancers.

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