Use of the Entero-Test®, a novel approach for the non-invasive capture of biliary metabolites in dogs.

W J Guiney, C Beaumont and S R Thomas

Toxicokinetics and Biotransformation, PCD DMPK Department, GlaxoSmithKline Research & Development, Ware, UK, SG12 0DP.
Non-invasive sampling of biliary metabolites in dogs

Address correspondence to:

William J. Guiney, Toxicokinetics and Biotransformation, PCD DMPK Department, GlaxoSmithKline, Park Road, Ware, Hertfordshire, SG12 0DP.

Telephone: (0044) 1920 882618

Fax: (0044) 1920 884374

Email: william.guiney@gsk.com

Text pages: 24

Figures: 3

References: 12

Abstract: 248 words

Introduction: 649 words

Discussion: 1269 words

Abbreviations used are: SVA, simvastatin hydroxy acid; SV, simvastatin; UPLC, ultra performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; ADME, absorption, distribution, metabolism and elimination; NOESY, nuclear Overhauser effect spectroscopy; CCK, cholecystokinin; 3Rs, replacement, refinement and reduction.
ABSTRACT:

Pre-clinical information on the biliary metabolites of a drug candidate is typically obtained through the collection of bile following surgical cannulation of the bile duct. Here we describe a novel approach using the Entero-Test®, a simple device which facilitates the non-invasive sampling of duodenal bile. The Entero-Test was used to collect bile from six fasted dogs which had been dosed either orally with simvastatin (SV) or intravenously with simvastatin hydroxy acid (SVA), compounds which have been previously reported to undergo extensive metabolism and biliary secretion in the dog. The devices, consisting of a weighted gelatin capsule containing 90 cm of a highly absorbent nylon string, were swallowed by each dog with the proximal end of the string taped to the animal’s face. Once the weighted string had reached the duodenum, gallbladder contraction was stimulated in order to release bile. Each bile-stained string was then retrieved via the mouth and, following solvent extraction, samples were analysed for drug-related material by ultra performance liquid chromatography-mass spectrometry and NMR spectroscopy. Numerous metabolites of SV and SVA were observed, and in general, the major metabolites have been reported previously, from studies with bile duct-cannulated animals dosed with [14C]-SV or [14C]-SVA. The results from this study demonstrate the utility of deploying the Entero-Test in absorption, distribution, metabolism and elimination studies, to provide information on the nature of biliary metabolites which, on occasion, may be sufficient to negate the need for more invasive sampling techniques. The benefits and limitations of the technique are discussed.
Introduction:

Biliary excretion is often a major route of elimination of drugs and their metabolites from the body, and elimination of compounds via the bile can impact the systemic exposure, toxicity and pharmacological effects of drugs. It is important to characterise hepato-biliary elimination, since this is often the key site of drug-drug interactions which may facilitate significant alterations in either systemic or hepatic exposure (Ghibellini et al., 2006).

Pre-clinical drug metabolism studies are typically conducted as part of the drug development process and are used to quantify and structurally identify drug-related material in order to help understand the absorption, distribution, metabolism and elimination (ADME) characteristics of a drug. Coupled to having plasma, blood, faecal and urine metabolite data available from such studies, any additional information obtained on the biliary disposition of drug-related material will be of benefit, since a knowledge of the metabolic routes of elimination therein may help to contextualise not only the ADME profile but also the pharmacokinetic profile of the drug. Furthermore, information gathered pre-clinically on metabolites secreted in the bile is important, since it may help predict how the drug will behave in man. For example, certain conjugated metabolites (e.g. glucuronides) which are released into the bile are often subject to hydrolysis by the gut micro-flora; this phenomenon can lead to parent drug being made available for re-absorption, which in turn may lead to re-exposure to the drug (entero-hepatic cycling), thus prolonging the residence time of the drug in the body. Therefore, it is only by collecting bile before it is exposed to the gut micro-flora that these metabolites of interest can be captured for analysis.

As part of ADME studies in dogs, bile can be obtained following surgical cannulation of the bile duct. This method is usually deployed as part of a mass balance
investigation using radiolabelled drug. Nonetheless, the cannulation technique involves an invasive surgical procedure conducted under general anaesthesia and is normally conducted only by a veterinary surgeon. Subsequent to this, a long post-surgical recovery period is necessary prior to a dog being fit for study purposes, and there are often complications associated with such surgery, including peritonitis and infection.

A simple method for the collection of duodenal bile in human subjects has previously been reported. The method was based on administration to several human subjects of the Entero-Test, a commercially available device which is used to sample upper gastrointestinal fluid for diagnostic purposes. The aforementioned studies demonstrated that the device could be used reliably to collect bile in order to determine bile acid profiles (Vonk et al., 1986) and cholesterol saturation (Muraca et al., 1989) in human subjects. Despite these investigations, very little information exists on the use of the Entero-Test in dogs, with the exception that it has been shown to be a practicable method for the diagnosis of *Giardia* infections (Hall et al., 2008). Additionally, there are no reports in the literature for use of the device in dogs specifically for bile sampling.

The objectives of the work described here were firstly, to evaluate the safety and tolerability of the device in dogs, and secondly, to determine whether it could be used to non-invasively capture small bile samples from dogs which would facilitate the qualitative characterisation of the biliary disposition of a drug using spectrometric and spectroscopic techniques.

Non-radiolabelled simvastatin (SV) and simvastatin hydroxy acid (SVA) were selected as being appropriate tool compounds for the evaluation of the device, since in dogs these compounds are known to undergo extensive phase I and II metabolism.
(including the formation of glucuronide conjugates), before being secreted into the bile (Prueksaritanont et al., 2002). Our work attempted to compare the biliary metabolites of SVA and SV captured non-invasively using the device with those which had previously been described, from bile samples collected from bile duct-cannulated dogs dosed with \([^{14}C]-SVA\) or \([^{14}C]-SV\) (Prueksaritanont et al., (2002), Subramanian et al., (2002) and Vickers et al., (1990a)).
Materials and Methods:

Materials: Entero-Test® devices (paediatric version (90 cm) part no. 101-01) were purchased from HDC Corporation (Milpitas, USA). Simvastatin (SV) and simvastatin hydroxy acid (SVA) were purchased from Toronto Research Chemicals (Ontario, Canada). Ceruletide (caerulein, sulfated, synthetic) was purchased from Sigma-Aldrich Company Ltd. (Poole, UK). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK). Analytical grade ammonium acetate was purchased from BDH (Poole, UK). De-ionised water was generated in the laboratory using a Millipore Mill-Q water filter unit (Molsheim, France). Deuterium oxide was purchased from GOSS Scientific Ltd (Essex, UK). Certified Beefy Bites™ (dog treats) were purchased from Bio-serv (Frenchtown, New Jersey, USA).

Entero-Test intended use. The Entero-Test is a commercially-available diagnostic tool for the recovery of upper gastrointestinal fluid in the clinic which can be examined for fungi, parasites and other enteric pathogens. The device consists of a gelatin capsule containing either 90 cm (paediatric version) or 140 cm (adult version) of a highly absorbent nylon string. The capsule is swallowed and one end of the string is taped to the corner of the mouth. The capsule dissolves in the stomach and the string, which is weighted at its distal end, passes into the duodenum. Following a period of approximately 4 hours, the string and any adsorbed gastro-intestinal fluid is withdrawn through the mouth. During withdrawal, the small steel weight which is attached to the distal end of the string detaches and is eliminated in the stool. The sample is collected from the string for microbiological analysis.
In vivo metabolism studies: All procedures were carried out under the authority of a Project Licence granted by the United Kingdom Home Office issued under the Animals (Scientific Procedures) Act 1986. Six health-checked, male beagle dogs each weighing between 9-14 kg were used in this study.

Oral leg: Three dogs were fasted overnight. Each dog was dosed orally with SV formulated as a suspension in 0.5% hydroxypropylmethylcellulose at a dose level of 30 mg/kg and at a dose volume of 5 ml/kg. Approximately 1 hour post-dosing, each dog was given a single food pellet in order to stimulate the release of dilute bile from the gall bladder following overnight fasting. After 2 hours post-oral dosing, each dog swallowed an Entero-Test capsule with the proximal string taped to the face. The dogs were placed into restraining slings for the remainder of the procedure. Two and a half hours post-capsule swallowing, each dog was fed a single food pellet in order to stimulate gallbladder emptying. Forty-five minutes later the strings were withdrawn from each dog and the bile-soaked sections immediately eluted using the method described below (see Elution of bile).

Intravenous leg: Three dogs were fasted overnight before being placed into restraining slings. Each dog then swallowed an Entero-Test capsule, with the proximal string taped to the face. Approximately 1 hour post-capsule swallowing, each dog was given a single food pellet in order to stimulate the release of dilute bile from the gallbladder following overnight fasting. One hour later (by which time the device had been adjudged to have reached the duodenum) each dog was dosed intravenously with simvastatin hydroxy acid (SVA) formulated as a saline solution containing 20% (v/v) polyethylene glycol 200 at a dose level of 2 mg/kg and at a dose volume of 1 ml/kg. Fifteen minutes post-intravenous dosing, each dog was fed a single food pellet in order to stimulate gallbladder emptying and bile release.
Forty-five minutes later the strings were withdrawn from each dog and the bile-soaked sections immediately eluted using the method described below (see Elution of bile).

**Elution of bile:** Immediately following removal from each dog, the proximal end of each bile-stained string was taped to a retort stand such that it hung vertically with the distal end placed into a glass vial. The bile sample (obvious by its yellow colour) was eluted from the string by gently pipetting 2 ml of 0.2 M ammonium acetate buffer (pH 4 with acetic acid) : methanol (3:1, v/v) onto the string which was then collected into the glass vial. The string was eluted for a second time using 3 ml of methanol which was collected into the same vial. Any residual material was scraped from the string into the methanol sample using a gloved thumb and forefinger, slid down the length of the string. Samples were immediately stored at -20°C, pending further processing.

**Sample processing:** On the day of processing, the samples were allowed to warm to room temperature. The samples were then concentrated to a volume of approximately 1000 μl under nitrogen gas using a Genevac DD4-X solvent evaporator (Genevac, Ipswich, Suffolk, UK) at 37°C. Approximately 200 μl of each bile sample concentrate was taken for ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis whilst the remainder from each sample (approximately 800 μl per sample) was pooled for additional nuclear magnetic resonance spectroscopy (NMR) analysis. In addition, a control bile sample collected from an un-dosed dog was also processed and analysed by UPLC-MS to discriminate drug-related from endogenous components in the bile.

**UPLC-Mass Spectrometry Analysis:** UPLC separation was carried out on a Waters Acquity system (Milford, USA) using an Acquity BEH C18 column (100 x 2.1 mm, 3 μm; Waters Milford, USA). The column temperature was maintained at 50°C. The
mobile phase consisted of 0.1% aqueous formic acid (v/v, solvent A) and acetonitrile (solvent B) and was delivered at a constant flow of 0.3 ml/min with an initial gradient of 5% B held for 0.2 min increasing linearly to 95% B at 10 min. 15 µl injections of individual and pooled bile samples together with an appropriate control sample were made.

The UPLC was coupled to a Waters Acquity photodiode array detector (Milford, USA) and a Waters QTof Premier mass spectrometer controlled with MassLynx™ version 4.1 software (Manchester, UK) with electrospray ionisation in the negative ionisation mode. A capillary voltage of 2.7 kV, cone voltage of 35 V, collision energies of 5 and 20 eV, and source and desolvation temperatures of 150°C and 400°C, respectively, were employed. Leucine enkephalin (200 ng/ml) was introduced via the lock spray inlet at 10 µl/min using a Waters Reagent Manager pump to act as a lock mass. Mass resolution was set at 8000 full width at half maximum at 500 Da. MetaboLynx™ processing software (Waters Manchester, UK) was used to assist molecular ion detection and a combination of accurate mass and MS/MS data on the molecular ions was used for structural identification of individual metabolites.

Peak areas for metabolites of interest were automatically generated on the relevant ion traces using MassLynx integration tools in order to compare concentrations across the samples.

**Preparative HPLC:** An aliquot (ca. 3.5 ml) of pooled bile extracts was separated by preparative HPLC using an Agilent series 1100 Preparative-LC system (Waldbronn, Germany). Separations were carried out on an Xbridge Prep-Phenyl HPLC column (250 × 10 mm i.d., Waters, Manchester, UK) at ambient temperature with a mobile phase of 0.1% aqueous formic acid (v/v, solvent A) and acetonitrile (solvent B) at a constant flow rate of 4 ml/min with an initial gradient of 5% B held for 1 min
increasing linearly to 95% B at 42 min and held at 95% B for a further 3 min. HPLC eluent was collected into fractions, in a time-slice mode, into two 96 deep well plates using a frequency of 15 seconds per fraction. This resulted in 180 fractions, each containing 1 ml of column eluent. The flow was split 100:1 into a Waters Micromass ZQ mass spectrometer fitted with an electrospray source operated in both positive and negative ionisation modes. System control was mediated through MassLynx and FractionLynx™ (Waters, Milford, USA). The fractions were taken to dryness under nitrogen at 37°C within the 96 deep well plates using a Micro DS96 dry down station (Porvair Scientific Ltd, Shepperton, UK) and then reconstituted in approximately 0.6 ml of deuterium oxide:acetonitrile (1:1) before being transferred to 5 mm NMR tubes.

**Nuclear Magnetic Resonance Spectroscopy:** NMR experiments were performed on all 180 fractions using a Bruker AVII⁺ spectrometer equipped with an inverse 5 mm TXI CryoProbe™ (¹H/¹³C/¹⁵N) operating at 600.4 MHz under the control of TopSpin version 2.1 (Bruker, Rheinstetten, Germany). ¹H NMR spectra were acquired using a standard NOESY presaturation pulse sequence for solvent suppression with time shared double pre-saturation of the water and acetonitrile frequencies. In these experiments, 128 transients were acquired into 48 K data points over a spectral width of 12019 Hz (20 ppm) with an inter-scan delay of 3 seconds giving a pulse repetition time of 5 seconds. Fractions observed to contain drug-related components were subsequently re-acquired with 1200 transients to improve signal to noise. Routinely, the optimum receiver gain is determined solely by residual solvent signals due to the small amounts of material present in the isolated fractions, therefore, to improve inter-sample reproducibility an identical receiver gain was employed for all data acquisitions. Prior to Fourier transformation, an exponential line broadening function of 0.3 Hz was applied to each spectrum to improve the signal-to-noise ratio.
Appropriate peaks were quantified using the proton integration feature of TopSpin 2.1 software.
Results:

Safety and Tolerability of the Entero-Test in Dogs: Prior to this work, initial investigations were conducted to elucidate the safety and tolerability of the Entero-Test device in beagle dogs. During these investigations, which involved administering the device to 20 dogs on more than 50 occasions, no adverse clinical signs were noted. It was also determined that the 90 cm ( paediatric) version of the device was more successful in capturing bile than the 140 cm (adult) version, which would often be contaminated by what appeared to be faecal material at the distal end. It was noted that the device needed to be in place for at least 2 h to ensure it had reached the duodenum; removal of the device prior to this often led to incomplete capsule digestion and/or non-detachment of the steel weight. On two occasions, the whole string was ingested due to poor attachment of the strings to the face using adhesive tape, however, in both instances these were retrieved whole in the faeces within 24 h of swallowing. In addition, faecal samples collected from dogs for up to 24 h after they had swallowed the device were subject to X-ray in order to confirm passage of the 1 g steel weight. The dogs were held in slings following Entero-Test swallowing, primarily so that a single animal technician could readily watch over six dogs on a study at any one time. Subsequent studies have also employed unrestrained dogs housed in steel metabolism cages to facilitate the collection of urine and faeces (as well as bile) as part of a mass balance study using radio-labelled drug without issue. In later studies, it was also established that the levels of bile-associated, drug-related material could be increased by fitting the dogs with two Entero-Test devices simultaneously.

Identification of Metabolites: UPLC-MS data obtained following dosing by either route were qualitatively similar. A total of 31 metabolites were detected comprising
multiple oxidations, hydrations and glucuronide conjugations of SV or SVA. Only the major metabolites will be discussed in this manuscript because the remit of our investigations was to focus primarily on those components for which NMR data were obtainable, thereby allowing comparison of definitive structures with metabolites previously described in the literature.

Unequivocal structural assignments were made for SV, SVA and for three metabolites, namely, 3',5'β,6'β-dihydrotriol-SVA (M1), 6'-hydroxy-SVA (M2) and 3'-hydroxy-SVA (M3) (see Figure 1), all of which have been identified previously as notable metabolites in dog bile (Subramanian et al., 2002, Vickers et al., 1990a, b). An O-glucuronidated SVA metabolite (M4) was also identified but the $^1$H NMR chemical shifts indicated an ether glucuronide (chemical shift range typically 4.0 - 4.8 ppm) rather than the acyl glucuronide (typically 5.5 - 6.0 ppm) previously reported (Prueksaritanont et al., 2002). The anomeric proton of SVA O-glucuronide (M4) exhibited a $^1$H chemical shift of 4.50 ppm, appearing as a doublet with a scalar coupling constant of 7.9 Hz, consistent with the β-anomer (see Figure 2). Total correlation NMR spectroscopy showed couplings to other glucuronide resonances at 3.42 and 3.17 ppm. Slight downfield shifting of proton 3 supported glucuronidation at this position, however insufficient material for nuclear Overhauser enhancement experiments meant glucuronidation at the 5 position could not be totally discounted. Nonetheless, the data were consistent with an ether O-glucuronide conjugate and not an acyl O-glucuronide.

Limited NMR data were additionally obtained on another glucuronidated metabolite (M5) exhibiting a deprotonated molecular ion at 607.2764 Da which is consistent with glucuronidated hydroxy-dehydrogenated-SV (theoretical mass 607.2755 Da).

Relative to SVA, the proton chemical shifts of the dimethylbutyric acid side chain, 2'
and 6'-methyls and 3'-, 4'- and 5'-protons were barely altered indicating no metabolic changes to the decalin ring nor the dimethylbutyric acid side chain. No other $^1$H NMR signals were identified and a Markush structure for M5 is shown in Figure 2. It was noted that to date, this structure had not been reported in the literature.

**Quantitative Evaluation of Major Metabolites:** Relative concentrations of SV, SVA and the major metabolites in the pooled bile sample were estimated from the $^1$H NMR data by integration of a common proton using similar methodology to that described by Dear et al (2008). Absolute levels of observed drug-related material in bile were estimated by comparison of the $^1$H NMR integrals with a 1 mg/ml standard solution of SVA. The total amount of observed drug-related material in the pooled bile sample was estimated to be 30 μg, with individual components representing between 1 and 10 μg.

Subsequent UPLC-MS analyses of both pooled and individual samples were conducted. Comparison of MS responses for each metabolite in the pooled sample (where levels had been quantified by NMR) with those in individual bile samples, allowed an assessment of relative levels of each metabolite to be made after oral and intravenous dosing. After oral dosing with SV, the major biliary metabolites included 3’,5’β,6’β-dihydrotriol-SVA (M1) and 3’-hydroxylated-SVA (M3). After intravenous dosing with SVA, the major biliary metabolites included M1 and M3 together with SVA-O-glucuronide (M4). In general, low levels of SV and SVA were observed following both oral and intravenous dosing.

The UPLC-UV chromatograms at 240 nm (see Figure 3) are an indicator of the relative levels of drug and metabolites in the samples. However, a notable difference was observed in the UV response for M1 due to the loss of the diene chromophore.
Discussion:

This work describes the first time that the Entero-Test has been used to harvest bile from dogs with a view to structurally identifying drug-related material.

Prior to the experimental work conducted with SV and SVA, pilot experiments were conducted which confirmed that dogs would tolerate the device, and that by optimising the experimental procedure, consistent bile sampling was achievable. In addition, experiments were used to evaluate whether a food stimulus or a chemical stimulus were required in order to initiate gallbladder contraction, to increase the likelihood of bile being collected onto the string. During fasting, bile is stored in the gallbladder until the ingestion of food causes the release of cholecystokinin (CCK) from endocrine cells into the circulation, which subsequently induces gallbladder contraction (Bridger et al., 2008). More successful sampling occasions (i.e. bile soaked string retrievals) were generally achieved when the dogs were given a small food morsel to eat (i.e. a single dog treat) in preference to allowing them to simply smell or lick the same food source, with the optimal time of applying the food stimulus being 30-45 minutes prior to string withdrawal. Other studies have successfully employed a chemical stimulus using the CCK analogue ceruletide to induce gallbladder contraction in both dogs and human subjects (Bridger et al., 2008, Krishnamurthy et al., 1985). The utility of administering ceruletide to dogs was evaluated during our pilot investigations, with some dogs receiving an intramuscular injection of ceruletide (aqueous) at a dose level of 0.3 μg/kg bodyweight, instead of a food stimulus. Replacing the food stimulus with ceruletide administration produced a satisfactory bile sample in terms of volume on every occasion (typically producing 30 cm of bile soaked string, equivalent to approximately 500 μl of bile), whereas on a few occasions using the food stimulus resulted in poor sample collection if the dog
exhibited signs of nervousness, or when the food pellet was refused. A food morsel was used in preference to the chemical stimulus in the study described here, since we were developing the technique to be as non-invasive as possible from an animal welfare perspective. Nonetheless, should the chemical stimulus be utilised in future studies, this is unlikely to have any unwanted effects on the ADME or pharmacokinetic properties of the drug under investigation, due to the extremely low dose of ceruletide which only differs in structure by a single amino acid from CCK which is released naturally in response to eating (Krishnamurthy et al., 1985). Another potential benefit of a chemical stimulus would be that there could be no food contamination of the string which may cause interference with spectrometric analysis of drug-related material.

A primary concern surrounding the use of the Entero-Test was the possibility that the device may become contaminated by the oral dose which could lead to difficulty in differentiating unabsorbed drug-related material from that which had been absorbed and then secreted into the bile, together with any dose which may have undergone metabolism in the gut. Low levels of SV were observed after oral dosing (See Figure 3), providing evidence that the string had not been excessively contaminated with the oral dose. It was estimated, based on fasted dog stomach emptying times (Karali 1995), that the device should not be swallowed by the dogs for at least two hours post-oral compound administration in order to minimise the risk of contamination.

The metabolism of SVA in pre-clinical species is well documented. In a study with bile duct-cannulated dogs, within 24 h following intravenous administration of [14C]SVA dosed at 1.2 mg/kg, approximately 65% of total radioactivity was detected in the bile, with unchanged parent drug accounting for less than 5% of the total recovered, suggesting that SVA underwent extensive metabolism. These studies also
reported the presence of an acyl glucuronide of SVA (approximately 20% of the dose) and its lactone, SV (Prueksaritanont et al., 2002). Another study reported biliary secretion to be a major route of elimination following oral administration of $^{14}$C-SV (60 mg/kg) to dogs, with several metabolites secreted in both lactone and hydroxy acid forms, of which 3’- and a 6’-hydroxylated metabolites of SV were the major biliary components. In addition, the study reported that the 6’-isomer readily undergoes rearrangement to the 3’-isomer under acidic conditions and that SV, a lactone pro-drug, undergoes spontaneous, reversible metabolism to its hydroxy acid form in vivo (Vickers 1990b). A further study reported 3’,5’β,6’β-dihydrotriol-SVA in dog bile following intravenous administration of $^{14}$C-SVA (Subramanian et al., 2002).

Each of these reported metabolites were captured using the Entero-Test at sufficient levels to produce interpretable $^1$H NMR spectra. However, our $^1$H NMR data support the presence of an ether O-glucuronide (M4) in preference to an acyl glucuronide, contradicting reported data. Prueksaritanont et al., (2002) describe the $^1$H NMR of an acyl glucuronide obtained using metabolite isolated from dog liver microsomes which was subsequently compared using chromatographic retention time and MS data with the O-glucuronide metabolite observed in dog bile. It is feasible therefore, that these two metabolites gave comparable chromatographic and MS data, but in fact were different and would only have been distinguishable had NMR been performed on the latter bile sample. It should be noted that the detection of the O-glucuronide in bile following oral administration of SV represents the first time that glucuronidation of SV has been confirmed via this dosing route.
In addition, 26 components were detected by UPLC-MS only and insufficient data were obtained to enable unequivocal structural identification. These metabolites were formed via multiple oxidations, hydrations and glucuronide conjugations.

A methyl ester of M3 was also detected by UPLC-MS and NMR, however, it was concluded that this was an artefact and was probably formed during the processing of the strings; it is therefore recommended that methanol be replaced with acetonitrile during the extraction procedure.

In conclusion, the results from this work demonstrate the utility of the Entero-Test for the assessment of compounds that undergo hepatic metabolism and/or biliary secretion in the dog. Using the device to establish, for example, that glucuronidation occurs, when this route cannot be detected in plasma or excreta, demonstrates that the Entero-Test may occasionally offer a viable alternative to bile duct-cannulation. If radiolabelled drug is dosed, metabolite standards are available for UPLC-MS quantification or alternatively, sufficient bile volumes are collected which facilitate NMR quantification (as described here), then it may be feasible to provide a quantitative ‘snap-shot’ of the levels of metabolites present in bile. However, the Entero-Test does not facilitate quantitative evaluation of the percentage of the dose eliminated via the bile. Consequently, this limits the interpretation of the biliary data compared to that typically generated using radiolabelled drug administered to bile duct-cannulated animals. It is also anticipated that the device may not be suitable or indeed successful in providing biliary data for all compounds under investigation; for example due to inappropriate ADME or pharmacokinetic properties of the compound, or the use of very low doses which may preclude the characterisation of metabolites.

Notwithstanding, given the simplicity and non-invasive nature of the technique, we believe the device is worthy of investigation ahead of using surgical techniques,
particularly when quantitative information is not essential during the early drug development cycle. Additionally, from an ethical standpoint and within the spirit of the 3Rs (replacement, refinement and reduction in animal experimentation) we believe that the technique may, on occasion, provide sufficient metabolism data to negate further investigations using surgically prepared dogs.

Finally, given the encouraging results achieved in dogs, and the fact that the Entero-Test is routinely used in the clinic, we have now initiated studies to explore the utility of the device in capturing human biliary information for drug candidates.
Acknowledgements:

The authors are grateful to Rita Tailor, Rachel Collier, Brian Mardell and Rachael Lincoln for providing technical assistance.
References:


Legends for Figures:

Figure 1: Structures of SV, SVA and major metabolites identified in dog bile captured using the Entero-Test®.

Figure 2: Comparison of $^1$H NMR resonances to assign glucuronide regiochemistry in M4 and M5.

Figure 3: Representative UPLC-UV (240 nm) chromatograms of bile from (a) dogs dosed intravenously with SVA at 2 mg/kg, (b) dogs dosed orally with SV at 30 mg/kg and (c) control animal.
Figure 1
Figure 3