Use of Entero-Test, a simple approach for non-invasive clinical evaluation of the biliary disposition of drugs

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Keywords

non-invasive bile sampling, simvastatin metabolism

Received

11 October 2010

Accepted

19 February 2011

Accepted Article

2 March 2011

WHAT IS ALREADY KNOWN ABOUT THE SUBJECT

- Biliary secretion is often a major route of elimination of drugs and metabolites. Knowledge of the biliary disposition of a drug in man is important in understanding the contribution of this route to overall clearance and the potential for drug–drug interactions which may have clinical consequences.
- Current methods for collecting human bile as part of drug disposition studies are invasive and complex, requiring intubation of the duodenum. As a result, limited data exist regarding the biliary elimination of many drugs and their metabolites.

WHAT THIS STUDY ADDS

- This proof of utility study clearly demonstrates that bile samples for drug and metabolite analysis can be collected simply, safely and cost-effectively from subjects using non-invasive technology.
- Using such technology, the biliary disposition of simvastatin was evaluated successfully in healthy subjects. Qualitative metabolite data compared favourably with reported biliary data derived from intubated patients dosed with radiolabelled simvastatin.
- This technique has substantial utility for early clinical evaluation of biliary disposition of drugs.

AIM

To evaluate the non-invasive collection of bile from healthy human subjects for the qualitative characterization of the biliary disposition of a drug, using spectrometric techniques.

METHODS

Twenty subjects underwent non-invasive bile capture using a peroral string test (Entero-Test) device prior to and following a single oral dose of simvastatin (80 mg). The device, consisting of a weighted gelatin capsule containing a highly absorbent nylon string, was swallowed by each subject with the proximal end of the string taped to the face. Once the weighted string was judged to have reached the duodenum, gallbladder contraction was stimulated in order to release bile. The string was then retrieved via the mouth, and bile samples were analysed for drug-related material using spectrometric and spectroscopic techniques following solvent extraction.

RESULTS

Numerous metabolites of simvastatin were detected, and the major metabolites were consistent with those reported from studies where bile was collected using invasive techniques from patients dosed with [¹⁴C]-simvastatin.

CONCLUSIONS

The results from this study demonstrate the utility of deploying the Entero-Test in human studies to provide structural information on biliary metabolites. This can be readily applied in drug development studies, including those in the target patient population and may eliminate the need for more invasive sampling techniques.

Introduction

Biliary secretion is often a major route of elimination of drugs and their metabolites from the body. As a result, elimination of compounds by this route can impact the systemic exposure, toxicity and pharmacological effects of drugs [1]. In the absence of knowledge around the biliary disposition of a drug, the relative importance of different routes of metabolism and their relationship to overall clearance may be misinterpreted.

Human metabolism studies are typically conducted as part of the drug development process. Information obtained on the biliary disposition of drug-related material can help to contextualize not only the absorption, distribution, metabolism and elimination (ADME) profile but also the pharmacokinetic profile of a drug. For example, identification of conjugated metabolites (e.g. glucuronides) that participate in entero-hepatic recycling can explain longer than expected exposure in man. Until recently collection of these samples has been a complex and invasive process.

A simple method for the collection of duodenal bile in human subjects has been reported [2, 3]. The method is based on administration of the Entero-Test, a commercially available device, originally used to sample upper gastrointestinal fluid for the diagnosis of enteric pathogens and parasites. These studies demonstrated that the device could be used reliably to collect human duodenal bile in order to determine bile acid profiles and cholesterol saturation. The work described herein builds upon this concept to establish that the technique can be used to enable the qualitative characterization of the biliary disposition of a drug using spectrometric and spectroscopic techniques.

Our group has recently characterized the biliary elimination of simvastatin (SV) in dogs using bile samples successfully captured using the Entero-Test device [4]. SV is an appropriate tool compound for the evaluation of the device in man, because SV has been reported to undergo extensive first-pass metabolism before being secreted into the bile [5, 6]. Our current work compares the biliary metabolites of SV captured non-invasively with those reported in bile samples collected from patients using a highly invasive sampling technique who had been dosed orally with [^{14}C]-SV [5, 7].

Methods

Materials

Entero-Test devices (adult version, 140 cm) were purchased from HDC Corporation (Milpitas, CA, USA). SV was purchased from Toronto Research Chemicals (Ontario, Canada). High-performance liquid chromatography (HPLC) grade acetonitrile and acetic acid were obtained from Fisher Scientific (Loughborough, UK). Analytical grade ammonium acetate was purchased from BDH (Poole, UK). Formic acid and leucine enkephalin were purchased from

Sigma-Aldrich (Gillingham, UK). De-ionized 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).

Entero-Test

The Entero-Test is a commercially available diagnostic tool for the recovery of upper gastrointestinal fluid in the clinic which can be used for the examination of 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 h, the string and any adsorbed gastrointestinal 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. Images of an Entero-Test device and the positioning of the proximal end of the string post swallowing are shown in Figure 1.

Study design

The clinical study was conducted at the GlaxoSmithKline Clinical Unit, Addenbrooke's Hospital, Cambridge (UK) in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. The protocol was reviewed and approved by the Essex Independent Ethics Committee. Written informed consent was obtained from all subjects prior to any protocol-specific procedures. A total of 20 subjects (15 male and five female) with a mean age of 36 years participated in this study.

The subjects were admitted to the study unit on the evening prior to dosing. Three and a half to four hours following an evening meal, each subject was given a food stimulus (each subject was allowed to visualize photographic images of different favourite food types and asked to imagine for approximately 60 s that they were eating the food; following this, each subject had orange peel squeezed under their nose which they inhaled for several seconds). The purpose of this was to cause gallbladder contraction and release stored bile before baseline sampling. About 0.5 h later each subject swallowed an Entero-Test capsule with the proximal string taped to the face. Ingestion was assisted by drinking approximately 200 ml of water. The subjects retired to bed with the devices left *in situ* in order to facilitate overnight collection of control bile. The following morning (approximately 12 h later), each subject was given the same food stimulus as previously about 45 min before the Entero-Test string was withdrawn through the mouth and processed as described below (see *Sample processing*). The purpose of this sample was to provide a baseline bile sample in the absence of any administered drug. Each subject was subsequently dosed

Figure 1

From left to right: Entero-Test device prior to swallowing, Entero-Test *in situ*

with a single oral administration of SV (80-mg tablet) with 100 ml of water. Two hours post dosing, when the oral dose was judged to have transitioned from the stomach to the gastrointestinal tract, each subject swallowed a second Entero-Test as described above and was permitted up to 500 ml of water over the subsequent period prior to string removal. Three and a half hours later (the manufacturer's recommended timing to allow the string to reach the duodenum) each subject was given a small, high-fat food morsel which comprised either a small cocktail sausage (12 subjects) or up to three French fries (eight subjects) in addition to the same food stimuli as described above, in order to stimulate bile release from the gallbladder. One hour later, the strings were withdrawn from each subject and processed as described below (see *Sample processing*). The timings described were designed to ensure sufficient time for transit of the string from the stomach into the duodenum and adequate exposure to released bile, prior to withdrawal. Following completion of all study procedures the subjects were offered a meal and allowed home.

Sample processing

Following removal from each subject, all Entero-Test strings (pre- and post-dose) were placed immediately into individually labelled 50-ml Falcon tubes containing 0.2-M ammonium acetate buffer (2 ml, pH 4 with acetic acid) with details of the subject number. A record was made of those strings which had obvious bile staining and those which had little or no obvious colouration. The strings were frozen over solid carbon dioxide then stored at -80°C until use. On the day of processing, the strings were allowed to defrost at room temperature. Each bile-soaked string and any residual buffer were individually placed into the barrel of a 10-ml hypodermic plastic syringe before the plunger was reinserted and hand pressure applied in order to squeeze the sample from the string into a labelled glass

vial. Acetonitrile (approximately 2 ml) was drawn into the syringe containing the string which was then agitated by hand before being eluted into the glass vial as above. The sample was eluted with two further washes of acetonitrile. The final string wash was used to rinse the appropriate 50-ml Falcon tube which had originally contained the string before being combined with the other washes as above.

The extracted samples were concentrated to near dryness using a Genevac DD4-X solvent evaporator (Genevac, Ipswich, Suffolk, UK) at 37°C . The concentrated extracts were individually reconstituted to a volume of 1 ml with 0.2-M ammonium acetate, pH 4 : acetonitrile (50:50, v : v). Aliquots (approximately 75 μl) were taken for analysis by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). The remaining extracts from strings with obvious bile staining (approximately 900 μl , subjects 3, 4, 5, 7, 9–12, 18 and 19) were pooled for fractionation by preparative HPLC prior to analysis by nuclear magnetic resonance spectroscopy (NMR). An aliquot (approximately 75 μl) was taken for analysis by UPLC-MS.

In addition, control (pre-dose) bile samples collected from each subject were processed as per the post-dose samples, pooled and analysed by UPLC-MS to assist with discrimination of drug-related material from endogenous components in the bile.

Ultra-performance liquid chromatography-mass spectrometry analysis

Ultra-performance liquid chromatography separation was carried out on a Waters Acquity system (Milford, CT, USA) using an Acquity BEH C18 column (100×2.1 mm, 3 μm ; Waters, Milford, CT, 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⁻¹ with an initial gradient of 5% B held for 0.2 min increasing linearly to 95% B at 10 min. Injections of individual and pooled bile samples (10 µl) together with an appropriate control sample were made.

The UPLC was coupled to a Waters Acquity photodiode array detector (Milford, CT, USA) and a Waters QToF Premier mass spectrometer controlled with MassLynx™ version 4.1 software (Manchester, UK) with electrospray ionization in positive and negative ionization modes. Capillary voltages of 0.8 and 2.7 kV (positive and negative ion respectively), a 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⁻¹) was introduced via the lock spray inlet at 10 µl min⁻¹ 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 and for taurocholic acid (a potential biliary biomarker) were automatically generated on the relevant ion traces using MassLynx integration tools in order to compare concentrations across the samples.

Preparative HPLC

The pooled bile extract (approximately 9 ml) 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⁻¹ 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 s per fraction. This resulted in 180 fractions, each containing 1 ml of column eluent. The flow was split 100:1 into a Micromass ZQ mass spectrometer (Waters, Manchester, UK) fitted with an electrospray source operated in both positive and negative ionization modes. System control was mediated through MassLynx™ and FractionLynx™ (Waters, Milford, CT, 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

Nuclear magnetic resonance spectroscopy experiments were performed on all 180 fractions using a Bruker AVII⁺ spectrometer equipped with an inverse 5-mm TXI Cryo-

Probe™ (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 12 019 Hz (20 ppm) with an inter-scan delay of 3 s giving a pulse repetition time of 5 s. 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 because of 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

The procedure was generally very well tolerated and no serious adverse events were reported. In order to gauge the volunteers' experiences regarding the Entero-Test procedure, a sample of eight subjects was asked their opinions regarding the procedure. The responses are detailed in Table 1. Nursing staff overseeing the procedure were asked to rate their experiences; each rated all aspects of the procedure straightforward and easier to perform than they had anticipated.

A small number of retrieved strings had evidence of minor blood staining on the proximal end of the string. The likelihood of this complication is noted on the device information pamphlet and is probably because of peristaltic movements pulling the string down into the oesophagus which in turn may result in a minor paper cut-like wound at the back of the mouth or upper oesophagus. This finding was evident in two of the three strings which had failed to unravel completely. Failure of the device to deploy correctly is probably caused by the string snagging as it emerges from the opening in the capsule.

Identification of metabolites

Twenty subjects underwent the bile sampling procedure twice. Based on colour, 10 out of the 20 post-dose Entero-Test strings appeared to be soaked in duodenal bile, four had limited bile colouration, while three strings had no obvious bile staining. The remaining three strings had failed to unravel by more than 15–25 cm and were also devoid of bile. Similar numbers of successful sampling occasions were noted for the pre-dose procedures.

Table 1

Subject ratings of their experience of the Entero-Test sampling procedure

	Number of subjects/rating		Negative	Comments
	Positive	Neutral		
Experience of sleeping overnight with device <i>in situ</i>	5	3	0	Those reporting neutral feelings reported tugging or tickling at back of throat or a dry mouth.
Willing to undergo procedure again?	6	2	0	Those reporting neutral feelings had experienced some gagging owing to a tickling sensation on swallowing the first capsule. Both agreed to undergo a second procedure.
	No discomfort	Slight discomfort	Very uncomfortable	
Experience of withdrawing the string	5	3	0	

A total of 12 metabolites were detected by UPLC-MS comprising multiple oxidations, hydrations and glucuronide conjugations of the prodrug, SV or simvastatin hydroxy acid (SVA) formed by the *in vivo* hydrolysis of SV. Where possible, a combination of ^1H NMR data, accurate mass MS on the molecular ions and MS/MS data were used for definitive structural assignment of individual metabolites. For other metabolites, comparison of retention time, MS and MS/MS data with those metabolites previously reported for dog bile [4] was used. In general, only the major metabolites will be discussed in this manuscript, namely those detected by NMR, because the remit of our investigations was to compare the biliary metabolites collected with the Entero-Test with the notable metabolites reported using more invasive collection techniques [5, 7].

Three drug-related components were detected by ^1H NMR: 3',5',6'-dihydrotriol-SVA (M1), 3'-hydroxy-SVA (M3) and SVA-6'-carboxylic acid (6'-COOH-SVA, M6) as shown in Figure 2. A glucuronidated hydroxy-dehydrogenated-SV metabolite (M5) was detected by MS only. SV and SVA were not detected in the pooled bile sample and in general, were only seen in those extracts originating from strings with no apparent bile staining. The summed reconstructed ion chromatogram for SV, SVA and the three major metabolites in the pooled bile extract is shown in Figure 3 together with the pre-dose bile for comparison.

Quantitative evaluation of major metabolites

Relative concentrations of the major metabolites in the pooled bile sample were estimated from the ^1H NMR data by integration of a common proton using similar methodology to that described by Dear *et al.* [8]. Absolute levels of observed drug-related material in bile were estimated by comparison of the ^1H NMR integrals with a 1-mg mL^{-1} standard solution of SVA. The total amount of observed drug-related material in the pooled sample (bile from 10 subjects) was estimated to be 10 μg , with the quantification limit for an individual component being approximately 0.5 μg by ^1H NMR.

The dihydrotriol (M1) was the major metabolite accounting for approximately 70% of the total drug-

related material detected by NMR. 6'-COOH-SVA (M6) accounted for approximately 20% with 3'-hydroxy-SVA (M3) accounting for the remainder.

Discussion

Knowledge of the elimination pathways of a drug in humans is essential to contextualize fully its pharmacokinetic and ADME properties, as inadequate understanding can lead to misinterpretation of clearance data and thus, e.g. the risk of drug-drug interactions. Information regarding the biliary disposition of a drug is important in providing an understanding of the contribution of individual routes of metabolism to the overall clearance of the drug, and this information is often requested by regulatory authorities during the development of novel pharmaceuticals. Indeed, the FDA in its Guidance to Industry document specifies that the underlying mechanisms for drug-drug interactions be explored [9]. Several methods exist for *in vivo* sampling of bile in man; however, these are often limited to studies in patients undergoing surgery, as historically there has been only limited success in the development of reliable techniques with which to quantify biliary elimination in healthy subjects [1]. In a recent review of current methods to evaluate biliary secretion of drugs in humans, the authors concluded that these invasive techniques, including T-tube drainage (which involves a temporary bile shunt from the liver for external collection) [10], collection of bile from patients undergoing cholecystectomy [5] and naso-biliary drainage from patients with bile duct stenosis [1] all have substantial drawbacks in that they are technically challenging to conduct and all involve patients with some underlying hepatobiliary disease. Underlying disease may influence bile flow as well as the expression and function of bile transporter proteins, all of which may modulate the disposition of drugs into the bile [11, 12]. It is therefore optimal to be able to collect bile samples from the target patient population. In early studies this may include healthy volunteers.

Figure 2

Structures of simvastatin (SV), simvastatin hydroxy acid (SVA) and notable metabolites identified in human bile captured using the Entero-Test

Several studies have recruited healthy volunteers who have been fitted with oroenteric tubes which facilitate the sampling of duodenal bile. The majority of these studies used an adaptation of the Loc-I-Gut technique as described by Lennernas *et al.* [13]. Nevertheless, the technical difficulty in sampling directly from the gallbladder or duodenum, not to mention high study running costs and the need for suitably qualified personnel, means that the studies are rarely conducted. It should also be considered that the information generated in these studies is often variable because of inter-subject differences with regard to bile flow [14] which are partly because of intra-subject variation of gallbladder emptying patterns [15] as well as incomplete recovery of compounds secreted into the bile with a limited sampling period. In addition, the uncomfortable nature of the procedures means subject withdrawals

are not uncommon [14–16]. Given the complexity, technical difficulty and invasive nature of the aforementioned methods, our group has evaluated the Entero-Test as an alternative non-invasive, simple and cost-effective device for collecting bile in order help characterize the biliary disposition of drugs.

A previous study in healthy human subjects described the collection of bile with the Entero-Test for the characterization of entero-hepatic recycling of duodenal bile acids [2]. The study found that there was very close agreement between bile acid profiles in samples captured using the device and bile acid profiles obtained from duodenal bile collected simultaneously by a standard intubation technique (correlation coefficient of 0.99). The study also concluded that the technique could replace the traditional intubation technique when measuring bile acids. Studies

Figure 3

Reconstructed ion chromatograms for major human metabolites in (A) bile from humans dosed orally with simvastatin (SV) at 80 mg and (B) pre-dose bile. SVA, simvastatin hydroxy acid

examining the use of the technique for analysis of drugs and their metabolites have not been conducted.

The disposition and biliary excretion of SV in cholecystectomy patients with T-tube drainage has been reported [5]. Each patient received a single oral 100-mg dose of [^{14}C]-SV following an overnight fast; 25% of the dose was excreted in the bile with four major and at least five minor radioactive peaks observed. Little or no SV or SVA were detected. However, two major metabolites showing pharmacological activity were identified as 6' β -COOH-SVA and 6'-hydroxy-SVA. The latter is known to rearrange under acidic conditions to produce the 3'-isomer [6]. In an earlier study by the same group [7], bile was collected by aspiration from hypercholesterolaemic patients participating in a long-term study of SV and who had received an oral dose of [^{14}C]-SV (40 mg) 4 h after feeding. Two major metabolites were identified as 6' β -COOH-SVA and 3'-hydroxy-SVA with a minor metabolite identified as 6' β -hydroxymethyl-SVA. Again, little or no SV or SVA were detected.

In our study employing the Entero-Test for the collection of human bile from healthy volunteers, the major metabolites were identified as the dihydrotriol, 3'-hydroxy-SVA [potentially formed by rearrangement of the 6'-isomer (M2) under the acidic conditions used] and 6' β -COOH-SVA.

The latter two are consistent with the major metabolites described by both Cheng [5] and Vickers [7]. It is probable that the dihydrotriol was also present at notable concentrations in the bile from these studies. There is a major early eluting unidentified radioactive peak in both chromatograms from the previously referenced studies which is devoid of UV absorbance at 238 nm (λ_{max} of SV) or 239 nm, consistent with the loss of the diene chromophore. In addition, no pharmacological activity was observed by Cheng [5] for this early eluting metabolite which again is consistent with the findings for the dihydrotriol [17]. The minor metabolite, 6' β -hydroxymethyl-SVA reported by Vickers [7] was not observed in our study but this metabolite may have been metabolized further in our subjects to 6' β -COOH-SVA [18]. Alternatively, as assignment of this minor metabolite by Vickers [7] was only inferred by retention time comparison with an authentic standard, an alternative structure for this metabolite may have been proposed had additional NMR or MS data been available. Nevertheless, in general, major metabolites identified using our methodology are consistent with those described for intubated subjects and also from previously reported *in vitro* investigations [19].

The minor peaks detected in bile collected with the Entero-Test also included a glucuronide conjugate (M5),

a component not reported previously in human bile, presumably because it is a minor component. This study represents the first time that this route has been reported in man *in vivo*, having previously only been postulated based on *in vitro* data [20]. This observation highlights the potential value of the technique, i.e. that labile biliary conjugates such as that described above can be captured in such a non-invasive manner, which if reproduced with other drugs will provide considerable confidence that this represents a viable alternative to invasive techniques.

One concern relating to the use of the Entero-Test is the possibility that the device may become contaminated by the oral dose leading to difficulties in differentiating unabsorbed drug-related material from that which had been absorbed and then secreted into the bile. The period between dosing SV and swallowing the Entero-Test capsule was 2 h which was anticipated to be sufficient time to allow the drug to empty from the stomach, thus minimizing the likelihood of the string being contaminated with the dose. Consistent with the findings of Cheng [5] and Vickers [7], neither SV nor SVA were observed in the pooled bile sample providing evidence that the string had not been contaminated with the oral dose. Nonetheless, SV and/or SVA were detected by MS in individual bile extracts from nine subjects although it was noted that, in general, they were only detected in extracts derived from those strings which had no obvious bile colouration. In these instances, the presence of SV and SVA could be indicative of contamination of the string with the oral dose (SV) and hydrolyzed drug (SVA). Contamination of the string therefore only appeared to be an issue when sub-optimal bile samples were collected. Colouration of the string together with relative MS responses for taurocholic acid (TCA) were used as a measure of the quality of the bile sample collected and low or negligible concentrations of TCA were apparent in the samples where there was little colouration of the string, thus indicating negligible or no bile collected.

A key factor in generating a successful bile sample using this technique is in the application of an appropriate food cue so that gallbladder contraction can be elicited, thereby releasing bile into the duodenum for adsorption onto the string. Following ingestion of a meal, fats and proteins reaching the duodenum trigger the release of the endogenous neurohormone, cholecystokinin (CCK), which in turn causes the gallbladder to contract. Other groups have used a wide range of food types containing a high-fat content when assessing gallbladder contractility, including egg yolks, French fries, milk-based products and even chocolate bars [21, 22]. It has been reported that at least 10 g of ingested fat are needed to produce gallbladder emptying [23]. Our subjects were fed either a single cocktail-type sausage (12 subjects) or several French fries (eight subjects), which resulted in bile being captured from 70% of subjects. Some subjects remarked that they did not find the food cue appetizing, and this may have contributed in part to a lack of bile sample for a number of sub-

jects. The food cue used in this study was applied 60 min prior to string withdrawal which has previously been determined to be sufficient time to allow maximal contraction of the gallbladder following ingestion of a fatty meal [21, 24]. Further work is being conducted to investigate whether other types of food cue would be more appropriate. Some groups have used CCK-like compounds (e.g. ceruletide) injected i.m. to elicit gallbladder contraction [25]. They report that the degree of contraction caused by such agents compares favourably with ingestion of a fatty meal [26]. However, sources of these compounds with approval for human administration are limited.

A plausible explanation for non-recovery of bile in some subjects is the inherent inter-subject variability in gallbladder contraction which is often spontaneous in nature and which has been described as problematic when collecting bile using invasive techniques [14]. Gallbladder physiology is complex; in the fasting state it continually undergoes partial emptying and refilling in conjunction with the migrating motor complex (MMC) of the small bowel. During the second half of the duodenal MMC, the gallbladder contracts approximately 40%. Post-prandial contraction has an immediate cephalic phase regulated by excitatory cholinergic vagal nerves and a more prolonged phase following CCK release in response to nutrients entering the duodenum. In addition, there appears to be wide 'normal' variation in gallbladder volumes (full and residual) and the percentage of gallbladder contraction between subjects and within an individual [26]. Thus, bile flow may display a high intra-individual variability, and a key factor contributing to this variability is the fact that bile is emptied into the duodenum as a pulse rather than as a constant flow of bile as described for the fasted state. Ghibellini and co-workers visualized biliary secretion and gallbladder contraction in four subjects using technetium coupled to gamma scintigraphy. They reported that each subject exhibited a different pattern of bile secretion into the intestine, attributing this to differing spontaneous contractions of the gallbladder. It is anticipated that inclusion of ultrasonography in future studies will allow imaging of gallbladder filling and emptying processes when deploying the Entero-Test to help better understand the timings and effectiveness of emptying, thereby improving the bile sampling technique.

In conclusion, the results from this work demonstrate, for the first time, the utility of the Entero-Test to assess the biliary disposition of drugs that undergo biliary secretion of drug and/or metabolites in humans. Using the device to establish, e.g. that glucuronidation occurs, when this route cannot be detected in plasma or excreta, demonstrates that the Entero-Test may offer a viable alternative to more invasive sampling techniques. Furthermore, if radio-labelled drug is dosed, metabolite standards are available for UPLC-MS quantification or alternatively, sufficient concentrations of individual metabolites are collected to facilitate NMR quantification (as described here), it may be

feasible to provide a quantitative 'snap-shot' of the relative concentrations of drug and metabolites present in bile. At present the technique does not readily facilitate quantitative estimation of the percentage of dose eliminated via the bile, although further work is planned to address this.

It is accepted that the Entero-Test may not be suitable or indeed successful in providing biliary data for all compounds under investigation; e.g. where biliary secretion of drug-related material is very low or following administration of very low doses which may preclude the characterization of metabolites. It is acknowledged that variations in gastric emptying may affect sampling using the Entero-Test. Specifically, a decrease in gastric emptying could slow absorption and hence appearance of metabolites in the bile, as well as increasing the risk of parent drug contamination of the string sample. Particular care should be taken with drugs that alter gastric emptying or which exhibit variable absorption kinetics. Therefore, knowledge of the plasma pharmacokinetic profile of the drug is important, as this will aid the selection of an appropriate time period in the study design for bile collection. Notwithstanding the present limitations, given the simplicity, low-cost and non-invasive nature of the technique, we believe the device is worthy of investigation ahead of invasive techniques in order to provide limited information on the biliary disposition of a drug and its metabolites in humans to complement metabolite information derived from other matrices, such as plasma and excreta. The simplicity of the technique favours its deployment in both healthy subjects and patients at any stage of the drug development process including the post-marketing phase, and it has now been deployed within our organization to collect biliary information from first administration to human, clinical drug-drug interaction and radiolabelled human ADME studies.

Competing Interests

There are no competing interests to declare.

The authors are grateful to Rita Tailor for providing technical assistance, as well as the nursing staff at Clinical Units Cambridge, GlaxoSmithKline, Addenbrooke's Hospital, Cambridge, UK.

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