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In vivo Methods for Evaluation of Drugs for the Treatment of Gastrointestinal Motility Disorders

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Abstract
The gastrointestinal tract (GIT) performs multitude of essential functions after ingestion of food, finally leading to absorption of nutrients. These functions are directly influenced by the motility patterns of the gut and any aberration in such patterns can disturb functionality of the GIT. The evaluation of gastrointestinal (GI) motility with suitable a probe is helpful in determining the therapeutic potential of investigational drugs in various motility disorders. The clinical conditions of motility disorders such as achalasia, gastric stasis, outlet obstruction, etc., deserve treatment with safer drugs. In vivo methods exhibit true effects of investigational drugs in biological milieu. This paper describes various in vivo methods of assessment of GI motility from stomach to colon. Among all the methods the visible marker methods are known to be simple and inexpensive

INTRODUCTION

The motility function of gastrointestinal tract (GIT) is associated with (1) forward propulsion of ingested food (2) digestion (3) absorption of nutrients and (4) expulsion of unabsorbed food material. These functions are adequately supported by cyclic motor activity occurring in almost all parts of the GIT which is due to migrating myoelectrical complex (MMC) through electrical activity of the GIT. Any discrepancy in motility patter can affect functionality of the GIT. A decrease in motility can lead to the stasis of food / chime in the intestine which favours the increases in the quantum of bacterial growth and cause constipation. Sometimes such situation may cause medical emergency when the barrier is breached, leading to bacterial translocation to other organs of the body. On the other hand, increased motility interferes with the digestion and absorption process and can lead to diarrhea and the malabsorption syndrome. The clinically known conditions of motility disorders such as achalasia, gastric stasis, outlet obstruction, acute intestinal ileus, chronic intestinal pseudoobstruction, megacolon, and generalized disorders of motility deserve treatment with safer drugs.

The evaluation of gastrointestinal (GI) motility is helpful in (1) determining the therapeutic potential of new drugs in motility disorders, (2) determining alteration in motility secondary to physiological or pharmacological stimuli (3) evaluating the effect of pathological condition on GI transit.

In vivo methods in animals exhibit true effects of investigational drugs in biological milieu. Studies of motility of GIT are associated with observation of marker in immediately excised sections of GIT or observation of motility in conscious animals using electrical gadgets. The following are the most popular *in vivo* methods to study GI motility in experimental animals-

(1) Assessment of intestinal transit
(2) Assessment of gastric motility
(3) Measurement of gastrointestinal transit
(4) Measurement of colon motility
(5) Long term recording of intestinal mechanical and electrical activity
(6) Assessment of GIT motility in dogs

1. Assessment of intestinal transit

Measurement of intestinal transit is a widely used technique for assessing the actions and mechanisms by which compounds influence intestinal motility. Intestinal transit is usually quantified by measuring the movement of charcoal, dyes, radio-opaque pellets or radio active markers that have been instilled into the stomach or intestinal lumen of conscious animal (after overnight food deprivation), to travel along the length of small intestine. The movement of non-radioactive marker is quantified by measuring the distance traveled by the leading edge of marker in the immediately excised portion of intestine from the animal. This assumes that

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the pattern by which the marker is distributed within the intestine is identical in all animals. Small amounts of marker materials are ideally suited for the measurement of propulsion since they do not produce changes in motility themselves.

1.1. Measurement of small intestinal transit

1.1.1. Marker methods: Inert, non-digestible, distinct colour with suitable viscosity (eg. phenol red, charcoal meal, chromic acid) or radioactive markers are administered intragastrically (i.g.) to overnight food deprived animals (0.3ml for mice or 0.5ml for rats). The drug under investigation may be given 10-30 min prior to test meal, if the route of administration is i.p. or i.v. If the drug should be given orally, it must be given at least 8 hr prior to meal. Other groups should be treated with vehicle. Any other oral intake should not be allowed within this period as it may vitiate the results. After prescribed time period, animals are sacrificed and distance traveled by the marker front in intestine is noted and expressed as % small intestinal transit (SIT) with reference to the whole length of intestine. It gives quantitative information about propulsion by visualized interpretation. This is generally performed in smaller animals like mice or even in rats.

Use of marker can be traced back to 1931 by Macht and Barbara-Gose, their meal consist of aqueous (aq) suspension of purified animal charcoal (5%) and gum tragacanth (5%).[1] Their method is described as follows. Mice are given 0.2 ml of the meal by oral route (p.o.), 15 min later they are killed by cervical dislocation. The intestine is excised immediately; the distance traveled by the meal is measured. The method quoted by Janssen and Jageneau (1957) received the highest citations.[2] It involves 2-4 months old mice fasted for overnight, after one hour of drug administration (i.p.) their stomachs are instilled with 0.3 ml of an aq suspension containing 10% charcoal and 5% gum acacia by stomach tube. Two hours later, the mice are killed and intestine immediately excised from cardia to anus and measured the distance traveled by charcoal from pylorus to anus and pylorus to appendices.

1.1.1.1. Charcoal meal method: Appropriate number of animals are deprived from food for overnight period. Each mouse (0.3 ml) or rat (0.5 ml) in a group is given of suspension of charcoal meal (10% w/v wood charcoal in 5% w/v gum acacia aqueous suspension) i.g. by using oral feeding needle After 20 min, animals are sacrificed by cervical dislocation, abdomen is cut opened, the leading edge of marker is found. The intestine at the leading edge is tied with a cotton thread or after excising the whole intestine (from pyloric end of stomach to ileocaecal junction) is immediately immersed in 5% formalin solution to halt the peristalsis. The distance traveled by the leading edge of charcoal and the total length of intestine are measured. The SIT can be expressed as the % distance traveled by the charcoal of the total length of the intestine as follows-

$$\% \text{ SIT} = \frac{\text{Distance traveled by charcoal meal (cm)}}{\text{Total length of small intestine (cm)}} \times 100$$

The change in % SIT recorded under the influence of test drugs is compared to control values will give indication of prokinetic or antikinetic effect of the drugs. This can be further compared with agents having known action. Although SIT measurement with charcoal is not a quantitative method in the sense that of radioactive methods, it is still widely used as visible marker to estimate gastrointestinal motility *in vivo* because of its simplicity.[3,4]

1.1.1.2. Phenol red test meal: Fifty milligrams of phenol red is dissolved in 100 ml of distilled water and filtered. The filtrate is heated to 70 ºC, about 750 mg of methyl cellulose is added with continuous stirring. The solution is rapidly cooled to 37 ºC. Mice or rats are used after overnight food deprivation. After i.g., administration of marker (0.3ml for mouse or 0.5ml for rat), as it reaches the small intestine due to motility, the colour of the marker changes to red due to the alkaline nature of contents. % SIT is determined as stated above. *Precautions:* While preparing the phenol red test meal, methyl cellulose is added only when the temperature rose to 70 ºC. The solution should be constantly stirred to avoid clamp formation, also rapid cooling under flowing cold water is necessary to prevent the liquid becoming too viscous.

The marker assay measures the combination of gastric emptying and intestinal transit. Summers et al (1970) proposed surgically modified method to study effect of drugs on gastric emptying and small intestinal transit.[5] This model may eliminate the influence of the stomach emptying on small bowel transit. But this model has a factor of surgical stress and may interfere the results.

1.1.1.3. Radioactive markers: The methods for quantifying the movements of radioactive intestinal markers are handicapped by requirements for specific

distribution that may not always exist. Miller et al (1981) developed a radioactive marker method and tested through geometric centre (GC) method, which became a simple method for assessing intestinal transit that is free of distributional assumptions.[6] Adult Wistar rats are implanted with indwelling silastic cannula in the proximal duodenum. After 30 min of drug administration, approximately 0.2 ml of radiochromium (0.5 mic ci $Na^{51}CrO_4$ in saline) is instilled into the small intestine *via* the indwelling silastic cannula. Twenty five min later, the animals are sacrificed by a suitable method. The small intestine is carefully removed and divided into 10 equal segments. These organs are then placed on a ruled template and are consecutively placed into individual culture tubes and the radioactivity present in the each tube is determined by counting for 1 min using a tracer analytic automatic gamma counter. Transit along the intestine is calculated for each mouse, in which values ranges from a minimum of 1.0 (all radioactivity present in the first intestinal segment indicating complete inhibition of transit) to a maximum of 10 (complete transit of the marker to the most distal intestinal segment). Although the results are expressed as GI transit (because the influence of the pylorus can not be excluded), it measures only transit of marker along the small intestine.[7]

Quantitative differences in intestinal transit are assessed by three methods (1) the most distal intestinal segment containing radioactivity is used as an estimate of the leading edge of marker (2) slope calculated from linear regression on cumulative percent of radio chromium that has passed through each segment (3) calculating the GC of the distribution of radio chromium.

The slope method for quantifying intestinal transit involves transforming the percent radioactivity measured in each segment to cumulative percent radioactivity. Cumulative percent radioactivity for each segment is equal to the % radio activity in the segment plus the % radio activity in all segments distal to it. The linear regression analysis on the plot of cumulative percent radioactivity for each segment versus the segment number produces a line with negative slope. Inhibition of transit is characterized by an increase in the negativity of the slope. Statistical analysis is performed on the individual slope values obtained for the animals in each treatment group.

The GC of the distribution of radio chromium within the intestine is actually the centre of gravity for distribution of marker and is calculated by or represents a weighted mean of the distribution of marker within the intestine.

$$GC = \Sigma \text{ (fraction of } ^{51}Cr \text{ per segment X segment number)}$$

This method is influenced by both the distribution and distance traveled by marker, but assumes no specific underlying distribution. Therefore, unlike the most distal segment and slope methods, the GC method can be reliable, describes any distribution of marker, there by increasing versatility of the method. Thus the GC is more reliable measure of intestinal transit. The GC of each animal can be compared to the mean GC of the control group according to the following formula

Percentage inhibition = 100 (test GC – Control GC) / (1.0 – control GC)

2. Assessment of gastric motility

The function of the stomach includes initiation of digestion by exocrine secretions such as acid and pepsin, which are under the control of the endocrine secretion of hormones that also coordinate intestinal motility. Various techniques have been developed to assess gastric motility causing the gastric emptying (GE). The influence of drugs on gastric mechanical actions on the bioavailability of novel compounds is of critical importance in drug development. Disturbed gastric myoelectric activity leading to gastroparesis can cause delayed GE, often found in patients with diabetes mellitus. Electrogastrography (EGG) may be used to evaluate the influence of prokinetics and other drugs on this condition and aid in determining effective therapy.[8]

2.1. Evaluation of gastric emptying by phenol red: Animals (rats or mice) are deprived from food for overnight period. Phenol red test meal is administered i.g., (0.5 ml for a mouse or 1.5 ml for a rat). Immediately after administration of test meal (0 min), two animals are sacrificed from the group by cervical dislocation. Laparotomy is performed to locate the stomach and clamped / ligated at cardiac and pyloric ends. The clamped stomach is freed from the duodenum and washed its surface with normal saline. The stomach is transferred into 0.1 N NaOH (50 ml for mice or 100 ml for rats,) solution and homogenized. To 5 ml of the homogenate, 0.5 ml of trichloro acetic acid is added and centrifuged for 20 min at 300 rpm. The clear supernatant fluid is withdrawn from the centrifuge tube by aspiration with syringe needle then added 4 ml of 0.5 N NaOH. The absorbance of the resultant pink coloured liquid is measured spectrophotometrically at 560 nm. The result

correlates with the concentration of phenol red meal in the stomach which in turn depends upon the GE. The treated animals are sacrificed after 20 min and GE is determined.[9]

% GE = 1 (1-x/y) X 100

x = absorbance of phenol red recovered from the stomach of animals sacrificed 20 min after the test meal

y = mean absorbance of phenol read recovered from the stomach of control animals (sacrificed at 0 min)

The difference in the % GE recorded under the influence of test drug compared to control value will give an indication of prokinetic or antikinetic nature of the drug. Further, if the prokinetic nature is found, it may be compared with established prokinetic drug.

2.2. Evaluation of gastric emptying by weighing method: Droppleman et al (1980) described a simplified method for assessing drug effects on GE in rats.[10] Three millilitres of a semi-solid test meal, based on methylcellulose, are given i.g., to rats fasted overnight prior to the experiment. At a specified time following the test meal, the rats are sacrificed, laparotomized and the stomachs are removed. The full stomachs are weighed then they are opened and rinsed. Excess moisture is blotted and the empty stomachs are weighed again. The difference is subtracted from the weight of 3 ml of the test meal, indicating the quantity emptied from the stomach during the test period.[11] Gastric motor stimulants (metoclopramide) increase and anticholinergic compounds decrease the GE.

3. Measurement of gastrointestinal transit using fluorescent beads in mice

The mice are food deprived for overnight period. Each animal is individually housed in a wiremesh cage to prevent coprophagy during fasting. The microbeads (Flow Check High Intensity Alignment Grade Particles, 6 mm; Polysciences, Inc.,Warrington, PA, USA), used as markers. They are 6 mm in diameter, labelled with a fluorescent yellow–green dye, and emit yellow–green fluorescence when excited with a 488 nm argon laser. The quantity of the fluorescent microbeads in each sample was measured with a flow cytometer (FACScan; BD Biosciences, San Jose, CA, USA). The variability of GE and gastrointestinal transit measured using these microbeads is reasonably small. Saline 0.2 ml, containing the 6 mm fluorescent microbeads together with the 2.14 mm non-fluorescent microbeads (2 ml), is infused via a metal cannula into the stomach. Thirty minutes later, the animals are sacrificed with a suitable means. The oesophagus, just proximal to the gastric fundus, and the duodenum, just distal to the pylorus are cross clamped (to prevent spillage of contents from the stomach), and the stomach is removed. The small intestinal tract is also removed with clamping of the tract at several locations to minimize the movements of contents. The time interval is chosen to obtain the geometric centre (GC) of 6–7 and to prevent the leading edge of the test fluid from going beyond the ileocaecal junction. The intestinal tract is placed on a ruled template and divided into 10 equal segments. The stomach and each segment of the intestinal tract are placed into individual tubes containing 5 ml of phosphate buffered saline. Each tube is vortexed and the supernatant liquid is filtered through a strainer Cell-Strainer, and subjected to flow cytometry. Using flow cytometry, the 6 mm microbeads are selected (gated) by their distinct forward light scatter and by their side light scatter profiles. The gated particles are further analysed for the presence of intense fluorescence. The number of particles with high fluorescence intensity is counted for 30 sec at the high flow rate of the cytometer.[12] Gastrointestinal transit is assessed using the GC method.

3.1. Roentgenologic visualization for GI motility: Passage of barium meal has been used extensively in larger animals and is one of the oldest methods. The time taken for the barium meal to reach some identifiable anatomic point is measured. It has been used in dogs and cats but not been of much value in smaller laboratory animals.

4.1. Assessment of colon motility in anesthetized rats:

The influence of spasmolytic drugs on carbachol-induced increase of colonic motility can be measured in anaesthetized rats. This method has also been used to study the stimulation of colonic motility by the enkephalin analogue pentapeptide.[13] **Procedure:** Rats are anaesthetized with pentobarbital i.v. A pressure sensitive tip catheter is inserted into the colon ascendens and the signals of the intraluminal pressure changes are recorded. The colonic contractions are stimulated by i.v., injection of 3 mg/kg carbachol. The height and the duration of the contractions are recorded. Then the test compound is injected i.v. The decrease of contractions is measured and the duration of the spasmolytic activity determined by repeated administration of carbachol at 15 min intervals until the contractions are not significantly different from

the response obtained with carbachol alone.

Maggi and Meli (1984) used eserine-induced hypertonus of guinea pig distal colon *in vivo* as a pharmacological procedure for testing smooth muscle relaxants.[14] Male albino guinea pigs weighing 300 g are anaesthetized with 1.5 g/kg urethane s.c. Through a midline abdominal incision, the proximal part of the hypogastric loop of the distal colon is exposed and occluding silk ligature are applied at a distance of 2 cm from each other, taking care to avoid any lesion to the vascular and nervous supply. Through a small incision, the flanged tip of the polyethylene tube (1 cm internal diameter and 1.5 cm external diameter) is inserted into the lumen and secured by means of purse-sting ligature. The free end of the tube is connected to a pressure transducer and the whole system is filled with saline. Intraluminal pressure and its variations are recorded on a polygraph. The effect of drugs is assessed as inhibition of eserine-induced hypertonus.[11]

4.2. Measurement of colon transit time in rats: Rats are anaesthetized with 50 mg/kg pentobarbitone sodium A PVC catheter is implanted into the caecum with the distal end fixed on the animal's neck. The animals are allowed to recover and are placed individually in a wire meshed cages to enable the faeces to fall through onto blotting paper. Carmine red (10 mg in 0.4 ml of normal saline per animal) is injected through the catheter immediately after administration of the test substance. The time until appearance of the first coloured feces is noted. Laxatives of the sennoside type act mainly by acceleration of large intestine transit and inhibition of fluid absorption in the colon.[15,11]

4.3. Assessment of colon transit by bead expulsion in mice: Colonic bead expulsion (CBE) is a simple method that provides a measure of colonic propulsion of boluses through the intestinal and external anal sphincters.[16] Immediately after the injection of test compound, a glass bead (3 mm diameter) is inserted 2 cm retrograde into the rectum of the mouse. The time of expulsion of bead was noted with 100 min as the time limit. Mice which are not expelling the bead within that time limit are excluded from the study. Data expressed as % inhibition of CBE, calculated in the following manner-

Percentage inhibition = 100 (Test time – mean control time) / (100 - control time)

5. Long term recording of intestinal mechanical and electrical activity in un-restrained rats:

This model has been developed to study simultaneous mechanical and electrical activity of GIT in the conscious rats.[17] Cyclic motor activity occurs in almost all parts of the GIT which is due to migrating myoelectrical complex (MMC) through electrical activity of the GIT.[18] In the fasting state, the electrical activity of the duodenum shows 3 distinct phases. The Phase I (80% of cycle) consists of slow waves with frequency of 42 cpm resulting in complete rest of motor activity. Phase II (15%) is a period of regular intense electrical activity with bursts of spike activity occurring on every slow wave resulting in rhythmic ring contractions. Phase III (5%) consists of burst of regular activity resulting in ring contractions occurring at the maximum possible frequency and amplitude. These 3 phases of electrical activity constitute the migrating myoelectrical complex (MMC) that occurs in the rat duodenum at intervals of 10.53 min. Smooth muscle cells show periodic oscillations of membrane potentials called electrically controlled activity; they are also named slow waves, basic electrical rhythm; or pacesetter potentials. If the membrane potential deplorizes beyond a certain threshold during such an oscillation, the smooth muscle contracts. This is usually associated with a rapid burst of electrical oscillations, called electrical response activities or spikes. Muscular contractions as seen by strain gauge upward deflections correlates well with the electrical spike potentials. Electrical response activity is therefore associated with contractions on a 1:1 basis. Implantation of extraluminal force transducers allows the monitoring of contractile activity in conscious animals. Mechanical activity is detected using miniaturized half-bridge metal foil strain gauge force transducers connected to wheatstone bridge. The electrical activity is monitored by Ag/AgCl bipolar electrodes. The lead wires from the recording units are encased in metal compression spring and are permanently joined to ball connector positioned on top of modified cage. The data can be analyzed by computer. This allows the animal free access to all parts of the cage.

Rats are premedicated with atropine sulphate (24 mic g/kg) to prevent salivation and then fully anaesthetized using a pad soaked in ether. The abdomen and the area of the shoulder blades are shaved and cleaned with savlon. A midline incision on the abdominal skin is made

together with a longitudinal incision of the skin between the scapulae. A fine pair of artery forceps is inserted s.c., through the abdominal wound and brought out between the shoulder blades. The strain gauges and electrodes are grasped with the forceps and drawn back into the abdomen. The protective spring is sewn to the outer abdominal muscles and to the muscle covering of the scapula using 3/0 (2 metric) silk sutures. The skin incision over the shoulder blades is closed, again using 3/0 silk sutures. The peritoneum is opened with a longitudinal midline incision and the gastroduodenal area is exposed. Strain gauges and electrodes spaced at 1 cm intervals are sutured alternatively to the muscular layer of the duodenum with 6/0 (0.75 metric) mer silk sutures, the first strain gauge being adjacent to the pylorus. The Dacron weave reinforcement preventing the sutures from tearing out. The gauges are aligned to record transverse muscle activity, the electrodes are positioned longitudinally. The duodenum is replaced in the abdomen with strain gauges and electrodes lying as naturally as possible. The muscle layers are then closed with a continuous 4/0 (1.5 metric) chromic sutures and finally the skin with 3/0 (2 metric) mer silk. Recording from the rats are made at intervals from several weeks after implantation. Recording can be made immediately after surgery, allowing the return of normal electrical and mechanical GI activity to be studied. The rats are housed in wire-bottom cages to prevent coprophagy and are fasted 8 hr before study recordings are commenced.

This method not only helps in recording mechanical / electrical activity of intestine in conscious un-restrained animals but also enables recording to be made in the recovery period because all the connections are made out side the rat cage. This method has been successfully applied to study the effect of inhalational anesthetic agents on small intestinal motility.

6.1. Assessment of GIT motility in dogs

Intraluminal pressure and motility of the small intestine can be measured in unanaesthetized dogs with balloon catheter systems *via* a duodenal Mann and Bollman (1931) fistula according to Tasaka and Farrar (1976) or in the loop of a Thiry-Vella fistula.[19,20] **Procedure:** Fistula of the small intestine is created in male Beagle dogs weighing 20 g. The animals are anesthetized with 40 mg/kg pentobarbital i.v., and fixed on an operation table. After shaving and careful disinfection of the skin a midline incision is made. A 10-15 cm length of ileum, approximately 15 cm proximal to the caecum, is excised. The remaining ileum is anastomosed end-to-end. The excised ileum is anastomosed, end to side, to the proximal or middle jejunum. Radioopaque tantalum markers are sutured to the serosa distal to this anastomosis in order to guide the direction for subsequent intubation. The other end is sutured to the skin. To create a skin ileostomy which does not shrink rapidly, small amount of muscle, fascia and subcutaneous tissue are excised from the abdominal wall.

For the measurement of the pressure inside the intestine, an air-filled system is used. Air-filled latex balloons, 5 cm in diameter, are attached to air-filled 190 polyethyle catheter (I.D. 1.19 mm) with a length of 120 cm. Three balloon-catheter pressure assemblies are tied together with the balloons 5 cm apart. The catheters are connected to the pressure transducers and to a polygraph. The transducers are rendered airtight by repeated application of latex to all the connections. The dogs are withheld from food, but not from water 18 h prior to the experiment. The balloon catheters assemblies are introduced through the fistula and secured in an appropriate position. The system is filled with air to a pressure of 10 mm Hg. Similarly, balloon-catheter assemblies can be introduced into a Thiry-Vella fistula. Intraluminal pressure is measured continuously and amplitude of pressure waves is recorded. After a period of 1 hr, the test drug is administered orally or s.c., and the above mentioned parameters recorded for 10 min intervals. Goldenberg and Burns (1973) reported on a technique using rubber balloon catheters inserted in the duodenum, ileum or colon of dogs, secured by purse-string sutures and filled with water to record intraluminal pressure monitored by Statham pressure transducers connected to a polygraph.[21] Furthermore, an antispasmodic agent can be tested for relaxation of morphine sulphate (0.3 mg/kg i.v.) induced spasms of he intestine.[11]

6.2. Thiry-Vella loop preparation in dogs: As described first by Thiry (1864), a part of the jejunum is isolated and the ends are exteriorized through the abdominal wall allowing to measure *in vivo* motility and function of the intestine in dogs and other species.[11] Male Beagle dogs weighing 20 kg are used. Dogs are fasted 12 h prior to the experiment. Pentobarbital sodium (30 mg/kg; i.v.) provides satisfactory anesthesia. The

abdominal part is shaved with electric clipper, then with a razor. The skin is disinfected. A midline linea alba incision is made. A loop of the jejunum, about 70 cm in length, is separated leaving the blood supply through the mesenterium intact. Both distal and proximal ends are exteriorized through the abdominal wall and provided with stomata. An end-to-endjejuno-jejunal anastomosis is performed. A latex balloon connected via polyethylene catheter to a pressure transducer is introduced through the proximal ostium. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of intestinal motor activity. A simple method can be done as follows. A loop of intestine with its mesentery intact is isolated in anaesthetized dogs. It is brought to the body surface in the form of proximal and distal fistula and intestinal transit is determined from the time taken for unindigestible bolus placed through the proximal fistula to reach its distal counterpart. Secretin inhibits motility of the small intestine dose-dependently.

6.3. Radiotelemetry: An encapsulated radio transmitter small enough to be passed through the digestive tube and the sphincter is introduced and its signals are monitored.

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