UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

GALLBLADDER CONTRACTILITY: *IN VITRO* AND *IN VIVO* STUDIES.

By

Neil William Pearce FRCS

Thesis for the degree of Doctor of Medicine

April 2004

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

Doctor of Medicine

GALLBLADDER CONTRACTILITY: *IN VITRO* AND *IN VIVO* STUDIES.

By Neil William Pearce FRCS

Cholecystectomy is a commonly performed operation, however the physiology of gallbladder motility and in particular the relationship between *in vivo* emptying and *in vitro* contraction are only partly understood. The large overlap between the symptoms of patients with gallstones and irritable bowel syndrome (IBS) has stimulated investigation of gallbladder emptying in these two groups.

Following a review of the current knowledge of gallbladder motility an *in vitro* model for studying gallbladder contractility is described. The *in vitro* response of gallbladder muscle strips to bradykinin is described and the receptor subgroups investigated. Contraction in response to bradykinin is dose dependent, in normal gallbladders this is mediated via the B1 receptor and via both B1 and B2 receptors in stone bearing gallbladders. This raises the possibility of a relationship to gallstone pathogenesis.

Sonographic estimation of *in vivo* gallbladder emptying is described and used to measure gallbladder emptying in normal individuals and gallstone and IBS patients in response to a standard fatty meal. Gallstone patients demonstrate low levels of emptying, suggesting that this may be a contributory factor in gallstone pathogenesis. A very high level of emptying is seen in patients with IBS.

Serum CCK-8 levels in response to a standard fatty meal are measured using a radio immuno assay in IBS patients and the normal group. IBS patients have higher fasting and peak levels of CCK-8. This suggests a possible role for cholecystokinin in the heightened visceral smooth muscle sensitivity seen in IBS.

In vivo gallbladder emptying and *in vitro* gallbladder strip contraction are compared in cholecystectomy patients. The results show weak correlation between gallbladder emptying and response to CCK *in vitro* and no correlation with *in vitro* response to bradykinin. This sheds doubt on the relevance of this *in vitro* model to the *in vivo* state. Further investigation of these findings is merited and future work is suggested.

2

List of Contents

Title

Abstract

List of Contents

List of Tables and Illustrations

Author's Declaration

Acknowledgements

3

List of Tables and Figures

9

11

ACKNOWLEDGEMENTS

ABBREVIATIONS

B1, bradykinin 1 receptor

B2, bradykinin 2 receptor

Bk, bradykinin

CCK, cholecystokinin

CCK-8, cholecystokinin octapeptide

CRC, concentration response curve

dAL-Bk, des-Arg9-(Leu8)-bradykinin

ej. Fn., ejection fraction (peak gallbladder emptying)

ERCP, endoscopic retrograde cholangio pancreatography

IBS, irritable bowel syndrome

NSAIDs, non-steroidal anti inflammatory drugs

PBRG, Southampton pancreatico biliary research group

PR, per rectum

RIA, radio immuno assay

s.d., standard deviation

S.E.M., standard error of the mean

Chapter I.

INTRODUCTION

1.1 Background

Gallstones are a common cause of surgical disease in the western world. The incidence of gallstones in the United Kingdom is approximately 7% of the population whilst 12% of Americans are similarly affected. In one autopsy study in the United States, 20% of women and 8% of men over the age of 40 had gallstones. It is estimated that approximately one million new cases of cholelithiasis develop in the U.S.A. each year whilst half a million cholecystectomies are performed during the same period.

The development of minimal access surgery during the last decade and the particular explosion of interest in laparoscopic cholecystectomy have changed the face of gallstone management in an unprecedented fashion. For the majority of patients with symptomatic gallstones a relatively safe fast and effective surgical cure can now be offered which enables a rapid return to full physical activity within a fortnight of the operation. However despite technically successful removal of the gallbladder up to a third of patients will continue to display some abdominal symptoms. This is probably a reflection of the key role that a normally functioning gallbladder has in the complex interplay of neural and hormonal factors which regulate gastrointestinal motility and function.

The delivery of bile into the duodenum following a meal is the predominant function of the gallbladder; however this simple step is set in the middle of a chain of both negative and positive stimuli and responses that also coordinate gastric emptying, pancreatic secretion, Sphincter of Oddi relaxation, duodenal motility and small and large bowel contraction. Knowledge of the roles of the vagus nerve, cholecystokinin and the other regulatory hormones in controlling gallbladder contraction is important in understanding both the pathogenesis of gallstones and their symptoms.

16

A strong overlap is seen between the symptoms of cholelithiasis and those of functional gastrointestinal disorders such as irritable bowel syndrome and functional dyspepsia; this raises the possibility that a proportion of the symptoms suffered by gallstone patients may be due to disturbance of the normal gut regulatory mechanisms rather than to direct mechanical or inflammatory factors within the gallbladder.

There are three factors which are widely considered to underlie gallstone formation in humans; bile super saturation, kinetic or nucleating factors and gallbladder stasis. All three have been extensively studied to determine their influence on the pathogenesis of gallstones and also to see if they present an axis along which a non surgical treatment might be attempted.

Gallstones can be divided into three major types depending on their composition; cholesterol stones, pigment stones and mixed stones'. Most mixed stones have a greater than 70% composition of cholesterol and their formation relies on the same pathogenic factors as pure cholesterol stones. Combined together cholesterol and mixed stones account for 80% of all gallstones in the United Kingdom.

The epidemiology of gallstones reflects the influence of their pathogenic factors, with the old description of the typical western gallstone patient as being fat, female and forty still holding partly true for the patient with cholesterol stones.

Cholesterol and mixed stones are common in northern Europe and both North and South America, probably because of familial and hereditary aspects. Obesity is a significant risk factor, reflecting a high calorie diet with its associated high biliary cholesterol output. Other factors which increase biliary cholesterol include pregnancy, drugs (e.g. clofibrate) and diabetes mellitus. Similarly decreased bile salts can produce a relative increase in the concentration of biliary cholesterol. This can occur because of malabsorption of bile acids (e.g. ileal resection or Crohn's disease) or decreased bile

salt secretion. Factors which impair gallbladder motility are also associated with cholesterol gallstones, notably pregnancy, drugs (oral contraceptives, calcium channel antagonists) and prolonged parenteral nutrition. Increasing age is another independent risk factor.

Pigment stones are more common among Orientals, Asians and Africans again probably reflecting genetic characteristics as well as the influence of other risk factors more common in their geographic area which predispose to stone formation. Chronic haemolysis (e.g. sickle cell disease) raises biliary levels of unconjugated, insoluble bilirubin which results in the precipitation of bilirubin and aggregation to produce stones. Chronic biliary tract bacterial infection promotes stone formation by encouraging deconjugation of soluble bilirubin mediated by the enzyme β-glucuronidase. Parasitic infestation, old age and alcoholic cirrhosis are also predisposing factors.

1.2 Historical Background

Since the earliest recorded days of medical practice the gallbladder has exercised the minds of physicians and surgeons. The ancient Greek physicians propounded the theory of the four humours blood, yellow bile, phlegm and black bile whereby an imbalance of these was at the root of all disease. The writings of the 14th century Venetians compiled in the Fasciculus Medicinae of Johannes De Ketham, the first medical textbook, reveal a number of cures for jaundice and gallstones; the patient should be "flebotomised from the basilic vein of the right arm at the ninth hour for liver ailments and an excess of bile" according to Galen, whilst those with stones are recommended to drink a preparation of "ginger, balsam, zedoary and muscat, long pepper and white pebbles of a crab". The results of these therapies are not recorded.

18

This theory of the four humours found favour until the eighteenth century when the role of bile and the gallbladder in the digestion of food became apparent.

The gallbladder defied scientific investigation other than anatomical dissection and descriptive studies of gallstone composition (Thudichum 1863), until the advent of general anaesthesia in the latter half of the 19th century This not only allowed Langenbuch (1882) to perform the first cholecystectomy, but it also permitted in vivo experiments to be carried out on anaesthetised animals to record the activity of the gallbladder in response to stimuli. The first such work was carried out by Doyon in France in 1893; he described a series of experiments in which he passed a cannula attached to a simple manometer into the fundus of the gallbladder in anaesthetised dogs. He thus recorded pressure changes which he interpreted as spontaneous activity of the gallbladder.

This theory was given weight in 1905 by the experiments of Bainbridge and Dale who demonstrated motor activity of the gallbladder by stimulating the vagus nerve at laparotomy as well as recording spontaneous motor activity.

The work of Mann in 1924 took the understanding of gallbladder physiology to a new level by examining the response of the gallbladder to a meal in dogs. Under anaesthetic he positioned permanent indwelling biliary catheters connecting the gallbladder fundus to a manometer. After recovery from the operation he observed that pressure in the gallbladder fell during fasting and rose following a meal, particularly so after milk.

The next significant advance in the understanding of gallbladder motility came with the development of cholecystography by Graham and Cole; in 1926 they reported opacification of the human gallbladder on X-ray after intravenous injection of calcium tetrabromophenolphthalein. This led Boyden to perform serial cholecystography in humans following a variety of meals. He calculated volume using a primitive sum of

19

cylinders method taking an image along the longitudinal axis of the gallbladder and assuming that it was circular on cross section. He demonstrated marked gallbladder emptying in response to an egg based meal. In a further extension of his work he performed transfusion experiments in cats and demonstrated gallbladder contraction in a fasting cat given the blood of a cat that had just had an egg based meal. From this he deduced that a hormonal mechanism could be involved in gallbladder contraction.

Firm evidence supporting this hormonal theory came in 1928 with the cross circulation experiments of Ivy and Oldberg. They anaesthetised two similar dogs of compatible blood type and performed laparotomies to connect their gallbladders to manometers. The dogs medial carotids were then connected via cannulas to create a cross circulation. In three out of four experiments they found that instillation of hydrochloric acid into the first dog's duodenum caused its gallbladder to contract followed 6 to 10 minutes later by that of the second dog. As a result they inferred the existence of a hormone that they termed cholecystokinin.

Despite this body of evidence, debate still continued as to the relative contribution of the gallbladder muscle layer to gallbladder emptying. Meltzer in 1917 had proposed that the muscle fibres of the gallbladder and sphincter of Oddi behave in an opposite and complementary fashion with contraction of the gallbladder and simultaneous relaxation of the sphincter facilitating bile ejection into the duodenum. Halpert and Lewis in 1930 suggested that the muscle layer acted purely to maintain tone in the wall of the gallbladder and prevent excessive distension with it acting purely as a passive reservoir. They based this statement on the observation that an extract of duodenal mucosa which produced gallbladder contraction when given intravenously failed to produce contraction in dog gallbladder strips at the same concentration in vitro.

The discrepancy between these findings was overcome by Jung and Greengard in 1933 who demonstrated that contraction of guinea pig gallbladder strips in vitro could be

20

achieved using a fifty times greater concentration of duodenal extract than was required in vivo, they attributed this requirement to the large size of the molecule and the subsequent reduced rate of diffusion within the organ bath compared with diffusion from the blood stream.

The first recorded in vitro experimental work on gallbladder tissue from humans was performed by Ravdin and Morrison in 1931 who recorded spontaneous muscular activity in strips taken from a single human gallbladder which had contained multiple stones and whose wall was thickened by inflammatory changes; however they made no recording of response to any added substances.

1.3 Anatomy

The gallbladder is a piriform sac lying in a groove on the inferior surface of the right lobe of the liver to which its upper surface is attached by connective tissue; its other surfaces are invested with peritoneum continued from the liver. Typically the gallbladder is from 7 to 10 cm long, 3cm broad at its widest part and has a volume from 20 to 50 ml. It has a fundus, body and neck, the neck connects to the cystic duct which is usually 2 to 4 cm long and passes back, down and left to join the common hepatic duct to form the common bile duct.

Anatomical variants

A large number of anatomical variants are described; in rare cases the gallbladder may be completely duplicated (Mincsev 1967) or absent, more commonly there may be a partial septum and bilobular appearance (Phyrgian cap) to the gallbladder. The arrangement of the cystic and hepatic ducts is widely variable; the cystic duct may be short or long, it may join the right hepatic duct or it may be fused with the common

21

hepatic duct. Accessory ducts may lie between the gallbladder and the right lobe of the liver.

Hartmann's pouch is a dilatation of the wall of the neck of the gallbladder, it has been widely regarded as a constant feature of the gallbladder however it has been shown to be an acquired appearance always associated with pathological conditions (Davies and Harding 1942)

Embryology

Embryologically the gallbladder is a foregut derivative that arises as a ventral outgrowth from the hepatic diverticulum or liver bud which is of endodermal origin.

Histology

Histologically the gallbladder is arranged in three layers; serous, fibromuscular and mucous. The fibromuscular layer consists of bundles of smooth muscle fibres lying in longitudinal, circular and oblique directions and separated by loose fibrous tissue. The mucosa consists of a single layer of columnar epithelium thrown into folds and rugae. The surface of the cells is lined with microvilli, reflecting their role in water and electrolyte absorption, tubuloalveolar glands extend into the lamina propria and secrete mucous.

Vasculature

Arterial supply is from the cystic artery which usually arises from the right branch of the hepatic artery. Venous drainage is via the cystic veins, which are numerous short vessels that drain into the hepatic veins through the liver. Lymphatic drainage is to the cystic and hepatic nodes.

Innervation

Innervation of the gallbladder is purely autonomic; the anterior trunk of the vagus supplies motor fibres via its hepatic branches and the coeliac plexus provides sympathetic innervation derived from the thoracic sympathetic chain, these are both sensory and vasomotor fibres. Pain from the gallbladder is typically felt in the shoulder tip region which has led to the assumption that there is a contribution from the right phrenic nerve to it's sensory innervation; fibres from the right phrenic nerve do communicate with branches of the coeliac plexus at the phrenic plexus and phrenic ganglion which in turn give branches to the hepatic plexus, however there is no evidence that any fibres extend to the gallbladder.

1.4 Gallbladder Motility

1.41 Control of Gallbladder Motility

The gallbladder exhibits two forms of activity; in the fasting state it maintains a resting tone and demonstrates spontaneous partial emptying and refilling of up to 10% of its volume, in response to a meal a prolonged and pronounced contraction of approximately 75% of its volume occurs.

23

Spontaneous Activity

Spontaneous emptying and refilling is thought to occur under the influence of coordinated motility patterns arising in the gastric antrum and duodenum (Toouli et al 1986). Long neural projections between the duodenum, sphincter of Oddi and gallbladder have been demonstrated in dogs (Furness et al, 1990). This activity is thought to aid mixing of hepatic and gallbladder bile and reduce stratification of bile within the gallbladder.

Digestive Phase

The gallbladder responds to a meal by producing a triphasic pattern of emptying which is divided into cephalic gastric and intestinal phases. During the cephalic phase sight, smell, taste and anticipation of food produce gallbladder emptying. Sham feeding can produce gallbladder emptying up to 65% of volume (Fisher et al 1986). This response is independent of a rise in plasma CCK levels (Ellenbogen et al 1988) and is blocked by the M1 muscarinic antagonist pirenzipine (Tankurt et al 1992) suggesting that the response is vagally mediated. The gastric phase of gallbladder emptying is also predominantly under vagal control, it is triggered by gastric distension stimulating autonomic mechanoreceptors in the stomach wall.

The intestinal phase starts when gastric contents enter the duodenum, the presence of polyunsaturated fat and to a lesser extent protein and carbohydrate stimulate the release of cholecystokinin (CCK) from the I cells in the duodenal mucosa (Solcia et al 1973) which produces gallbladder emptying (Everson et al 1983). After a liquid, fatty meal 75% of gallbladder volume is ejected over 30-45 minutes (Fisher et al 1987). The response to a solid meal is characterised by an initial contraction followed by a period of relaxation and refilling before a second prolonged contraction (Howard et al 1991). It has been observed on ultrasound studies that the gallbladder appears to exhibit

24

minute to minute fluctuations in volume (Lanzini et al 1987) with both refilling and emptying occurring during the period of post prandial emptying (Howard et al 1991). This has been confirmed by the use of dual markers for hepatic and gallbladder bile which have shown that mixing of hepatic and gallbladder bile occurs within the gallbladder according to the "bellows" model (Lanzini et al 1987). The control of this mixing is thought to be the periodic contraction and relaxation of the sphincter of Oddi releasing bile into the duodenum This mixing of bile is thought to be of importance in the prevention of stone formation by flushing out cholesterol crystals and preventing them forming into stones.

1.42 The Sphincter of Oddi

The sphincter of Oddi has a coordinated role with gallbladder contraction in facilitating gallbladder emptying. For many years the debate has continued as to what extent sphincter of Oddi contraction and relaxation affects gallbladder volume. In the extreme model the gallbladder is viewed as a low tone reservoir which fills passively when the sphincter of Oddi is contracted and pressure in the common hepatic and common bile ducts exceeds the cystic duct opening pressure of 8-10 cm of water (Everson 1991) and empties passively when the sphincter is relaxed and common duct pressure falls below cystic duct pressure. The cystic duct has also been postulated to possess a sphincter like action based on the observation that there is a small condensation of circular muscle fibres at its junction with the gallbladder (Lutkens, 1926). Although studies have confirmed the existence of a pressure gradient across the cystic duct (Doyle and Farrar, 1969) and demonstrated that resistance to flow along the duct increases with locally administered CCK (Scott et al, 1979) the significance of these findings and their role in normal gallbladder emptying and filling is not clear.

The sphincter of Oddi demonstrates relaxation in response to both CCK (Tokunaga et al, 1993) and vagal stimulation (Toouli et al 1984) confirming the law of reciprocal innervation proposed by Meltzer (1917). The vagally induced relaxation of the sphincter of Oddi appears to be regulated via a non adrenergic non cholinergic pathway; Sand et al (1997) demonstrated that sodium nitroprusside, a nitric oxide donor, abolished electrical activity in the porcine sphincter of Oddi in vivo. Similarly NO synthase inhibition in vitro increased KCl and acetyl choline induced contraction in human and pig sphincter of Oddi. In vivo nitric oxide inhibition enhances the contractile response to bethanacol in guinea pigs and in vitro abolishes the relaxation of the sphincter of Oddi in response to electric field stimulation (Mourelle et al 1993). Immunohisto chemical studies have identified NO synthase within the rich plexi of nerve fibres in the mucosa and muscle layers of pig and human sphincter of Oddi (Sand et al 1997).

Endoscopic Sphincterotomy

The advent of endoscopic sphincterotomy for removal of common bile duct stones has given new information on these matters since the procedure destroys the sphincter's function and the pressure gradient between the duodenum and common bile duct almost completely disappears (Funch-Jensen et al 1979). Despite these changes the gallbladder still fills and empties, although its maximum volume decreases by approximately 25% and its maximum emptying increases by 10-15% (Sugiyama et al 1996), these changes are apparent three months after sphincterotomy and appear to persist in the long term. Experiments on sphincterotomised dogs have confirmed these findings and also demonstrated an increased capacity for the clearance of glass beads introduced into the gallbladder as artificial calculi (Hutton et al 1988). This suggests a mechanism for the clearance of gallbladder stones which is occasionally observed in patients after endoscopic sphincterotomy (Cotton 1984).

26

1.43 Neural Control

Neural regulation plays a central role in maintaining resting gallbladder tone and also mediating contraction and relaxation via the autonomic fibres from the anterior trunk of the vagus and the 7th -10th thoracic spinal segments of the sympathetic chain (Ryan 1981). These form autonomic plexuses within all three layers of the gallbladder wall.

The vagus

The vagus contributes to fasting gallbladder tone via cholinergic pathways. Both vagotomy (Patankar et al 1993) and cholinergic blockade (Marzio et al 1987) have been demonstrated to increase fasting gallbladder volume and decrease emptying in response to a meal (Gullo et al 1984). Vagotomised patients have a higher incidence of gallstones which may be as a result of this decreased emptying (Clave and Gaspar 1969). Cholinergic blockade also decreases CCK induced gallbladder contraction suggesting that part of the action of this hormone is mediated via cholinergic pathways.

Sympathetic innervation

The precise physiological action of the sympathetic adrenergic innervation is unclear. Overall the action appears to be inhibitory although relaxation can only be demonstrated *in vitro* in response to adrenergic agents after pre-treatment with CCK (Amer and McKinney 1972).

Many other neuropeptides have been isolated within the intramural neural plexus including CCK, VIP, gastrin releasing peptide and neuropeptide Y. There is also evidence to suggest a role for nitric oxide (McKirdy et al 1994) in the mediation of non adrenergic non cholinergic gallbladder relaxation *in vitro*.

1.44 Cholecystokinin

Background

Cholecystokinin is the most important and well studied of the hormones known to affect gallbladder motility. Attempts were first made to identify it after the work of Ivy and Oldberg suggested the existence of a prokinetic gallbladder hormone (cholecystokinin) when they demonstrated that an extract of duodenal mucosa given intravenously produced gallbladder contraction in dogs. No further progress was made until 1943 when Harper and Raper identified a small bowel extract with stimulant effects on both pancreatic exocrine secretion and gallbladder contraction that they termed pancreozymin. Jorpes and Mutt (1964) identified that pancreozymin and cholecystokinin were identical compounds and they were the first to extract a pure CCK extract (1968) from pigs and describe its amino acid sequence.

Structure

Cholecystokinin is a peptide hormone with a 33 amino acid sequence (figure 1). Its carboxy terminal is the biologically active part of the molecule, with the last 8 amino acids (CCK-8) demonstrating full levels of activity. It is closely related to gastrin with both sharing the same first 5 amino acid sequence at the C terminal, gastrin has weak CCK like activity.
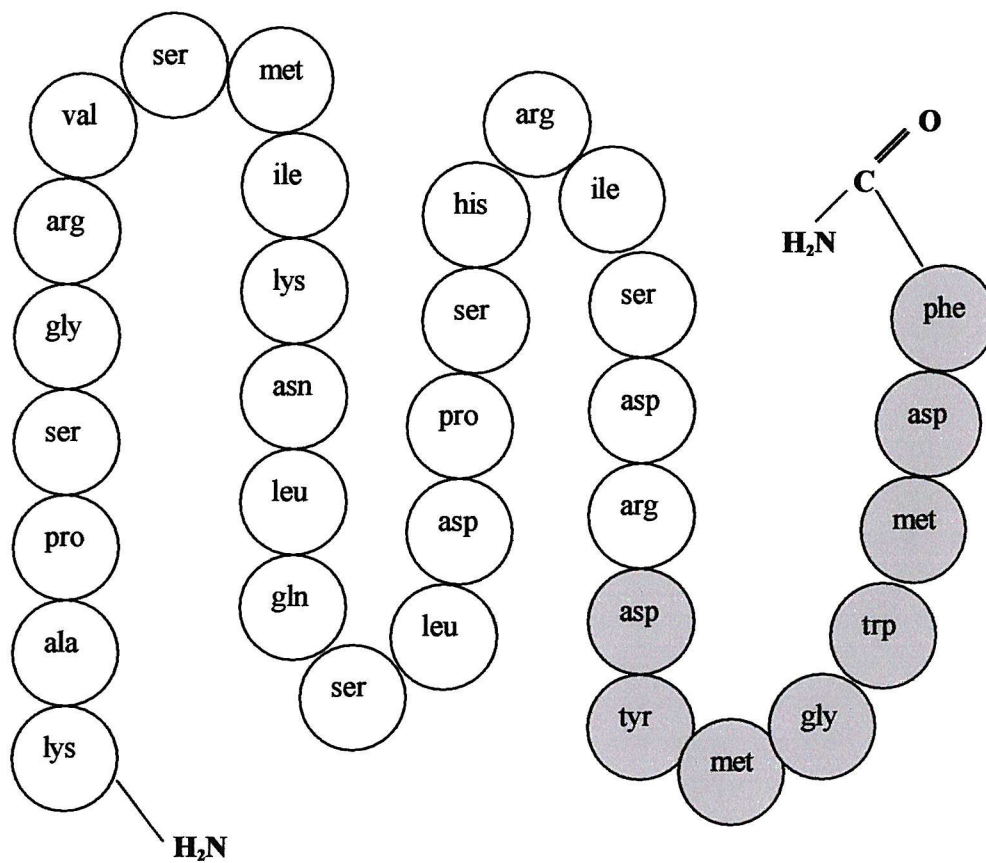
Figure 1.

The primary structure of Cholecystokinin-33. The shaded amino acids at the carboxy terminal end demonstrate the sequence of CCK-8.

Synthesis

CCK is synthesised within the duodenal I cells from a DNA template which translates into preprocholecystokinin, a 115 amino acid inactive precursor (Kato et al 1984). Enzymatic cleavage results in the formation of a number of active fragments as well as CCK-33, including CCK-8, CCK-12, CCK-22, CCK-39, and CCK-58 (Rehfeld 1978; Cantor and Rehfeld 1989).

Metabolism

CCK metabolism is rapid; larger fragments are cleared by the kidney whilst smaller fragments are metabolised by the liver, with 50% of CCK-8 undergoing first pass extraction in the liver (Sakamoto et al 1985). Overall plasma half life is 5-7 minutes (Harvey et al 1973).

CCK release

CCK release occurs in response to the presence of gastric contents in the duodenum. The breakdown products of fats (medium and long chain fatty acids) (Mclaughlin et al 1995) and protein (phenyl alanine, tryptophan) trigger a rise in plasma CCK (Singer et al 1985).

CCK release and the vagus

CCK release appears to be affected by the influence of the vagus; after highly selective vagotomy CCK levels in response to a meal are increased (Patankar et al 1994). This appears to be independent of the changes in gastric emptying which are produced by truncal vagotomy. Conflicting reports on the results of truncal vagotomy on peak plasma CCK levels probably reflect the effects of gastric drainage procedures

performed in conjunction with vagotomy. Also the reduced gallbladder contraction

seen in truncal vagotomy may contribute to a reduction in bile passage to the

duodenum with consequent impairment of negative feedback mechanisms. Electric

field stimulation of the vagus has been reported to increase plasma CCK levels (Kim et

al 1989).

Negative feedback

The mechanism for negative feed back control of CCK release in humans has not been

fully established. In rats there is strong evidence to suggest that the presence of

intraluminal trypsin in the duodenum and upper small intestine, secreted from the

pancreas in response to CCK, acts to inhibit further CCK release (Green and Lyman

1972). This has been further confirmed by studies of pancreatico biliary diversion in

which CCK levels and pancreatic protein output were elevated following a meal,

intraduodenal perfusion of trypsin returned both to normal levels.

In humans there is conflicting evidence for the role of **proteases** in negative feedback

inhibition. Owyang (1986) demonstrated a dose related suppression of CCK in

response to intraduodenal trypsin, however studies by Hotz (1983) and Dlugosz

(1983) using an oral trypsin inhibitor failed to demonstrate any resultant increase in

CCK levels. These conflicting results may indicate that negative feedback inhibition is a

multifactorial mechanism in humans with trypsin only playing a partial role. Further

study of this relationship is merited.

Bile salts also contribute significantly to negative feedback inhibition; Gomez (1989)

demonstrated that reduction in intraluminal bile salts produced an increased CCK

response to a standard fatty meal, by using cholestyramine a polymeric cationic

exchange resin that binds bile acids in the intestinal lumen. This mechanism has been supported by the finding that orally administered bile acids reduce CCK release in response to a meal (Koop et al 1989).

Other Inhibitory factors

Somatostatin has been shown to decrease CCK release and thus inhibit gallbladder contraction in vivo (Kanayama et al 1985). **Peptide YY** is released by the distal small intestine in response to intraluminal fat and is an inhibitor of CCK release (Adrian et al 1985).

CCK receptors

Two receptor subtypes have been identified, CCK-A and CCK-B. CCK-B receptors appear to be identical to the gastrin receptor, CCK-A is widely distributed through the GI tract from oesophagus to colon (Wank 1992). CCK-B receptors are expressed in neural tissue and are also demonstrated on the pancreas (Tang et al 1996), although the specific CCK-A receptor antagonist MK-329 has been shown to produce dose dependent inhibition of CCK-8 stimulated pancreatic enzyme secretion (Candor et al 1991).

The CCK A receptor will bind to any molecule displaying the minimum of the first seven amino acids of the C terminal end of the CCK chain. The type B receptor requires demonstration of only the first 4 of these residues for binding to occur (Jensen et al 1989).

Mechanism of action

The mechanism by which CCK receptor activation produces contraction of gallbladder smooth muscle is via a calcium dependent rise in cyclic adenosine mono phosphate (cAMP), (Davison et al 1980). It appears that intracellular sources of calcium are primarily required for contraction to occur. Lee (1989) demonstrated that feline gallbladder strips incubated in a calcium free solution contract normally in response to CCK suggesting a reliance on intracellular reserves of calcium. This work has since been repeated and the findings confirmed for human gallbladder muscle cells in vitro, by Behar (1993), who also identified that the release of intracellular calcium from endoplasmic reticulum is in turn dependent on generation of inositol triphosphate in response to stimulation of the CCK receptor. However, nifedipine the calcium channel blocker produces reduced gallbladder emptying in humans in response to a meal (Jonderko et al 1991), which implies a reliance on an extracellular calcium source. The significance of this finding is difficult to interpret since it takes no account of the action of nifedipine on the sphincter of Oddi, although it may be that in the prolonged gallbladder contraction seen in vivo, intracellular calcium reserves become depleted and calcium channel blockade may impair their replenishment thus ultimately reducing gallbladder emptying.

Actions of cholecystokinin

The wide distribution of CCK receptors within the gastrointestinal tract and the central and peripheral nervous systems reflects the diverse array of interactions which are mediated by this hormone.

Motility is affected by CCK throughout the GI tract; lower oesophageal sphincter tone is reduced, gastric emptying is slowed, small and large intestinal transit time is decreased, the gallbladder contracts and the sphincter of Oddi relaxes.

33

The **secretory** actions of CCK are also significant; pancreatic and hepatic exocrine secretion are stimulated, gastric pepsinogen secretion is increased, whilst gastric acid production is inhibited and alkaline mucus secretion from the duodenal Brunner's glands is stimulated.

There are several **neurological** actions including; reduced appetite, anxiety, drowsiness and in high levels an analgesic effect.

CCK is also involved in the control of **hormone release**, from other areas of the GI tract; pancreatic polypeptide, somatostatin, insulin, glucagon and GIP are all released directly or indirectly in response to CCK.

The effects of CCK on gastric emptying and the sensation of satiety appear to be mediated via stimulation of vagal afferent fibres in the gastric wall (Dockray et al 1989 and 1991).

1.45) Bradykinin

Bradykinin is a widespread nonapeptide member of the kinin family; it is increasingly recognised as having a major role in the regulation of many physiological processes on top of its well documented actions as an inflammatory mediator. Bradykinin is generated in plasma from high and low molecular weight kininogen precursors which are converted to the active molecule by the action of tissue and plasma kallikreins these proteolytic enzymes are in turn produced in response to pathophysiological stimuli such as trauma, inflammation, anoxia and low pH (Regoli and Barabe, 1980). Bradykinin acts in many ways; it promotes relaxation in vascular smooth muscle, contraction in non vascular smooth muscle, glandular secretion, immune cell

stimulation and sensitisation and activation of sympathetic and sensory neurones. Many actions of bradykinin are mediated indirectly via the activation of the arachidonic acid pathway and prostaglandin production (Gaginella and Kachur 1991).

The direct actions of bradykinin are mediated via a number of receptors located on cell membranes. B1 and B2 receptors were the first to be proposed (Regoli and Barabi 1980) and both have since been cloned as distinct molecular entities (Hess et al 1992, Menke et al 1994). The majority of physiological actions of bradykinin are mediated via the B2 receptor, whilst B1 receptors appear to be expressed more in tissues which have been subject to inflammation (Pruneau et al 1994, Roslan et al 1995), the B1 receptor has also been implicated in the pathways controlling hyperalgesia (Perkins and Kelly, 1993). Recent work has suggested the existence of B3 receptors in lung tissue (ref) and B4 and B5 receptors in the oesophagus (ref).

Work on the guinea pig has demonstrated that bradykinin is a potent stimulator of gallbladder smooth muscle contraction. Using the selective B2 antagonist HOE 140, it was demonstrated that this contraction was mediated via the B2 receptor. B1 blockade with des-Arg9-(Leu8)-bradykinin did not alter the contractile response (Cabrini et al 1995). Preliminary work from this unit by Johnson, Hillier and Rushton (1997) has supported these findings in human gallbladder tissue *in vitro* by demonstrating gallbladder strip contraction in response to bradykinin.

1.46) Other Hormones

A large number of other peptides and hormones have been shown to affect gallbladder motility,

Progesterone impairs gallbladder emptying; the high levels of this hormone seen in pregnancy are associated with impaired gallbladder emptying and increased fasting volume which probably accounts for the high incidence of biliary disease seen during this condition (Everson et al 1982). This reduced contractile response is also seen in vitro (Davis and Ryan, 1986).

Pancreatic polypeptide (PP) is released by the pancreas in response to cholinergic and CCK stimulation as well as in response to hypoglycaemia and protein. It produces in vivo gallbladder relaxation in humans (Adrian et al 1982), although it appears to have no effect on the gallbladder in vitro (Pomeranz 1983).

Histamine produces gallbladder contraction in vitro via an H2 receptor mediated response (Lennon et al 1984). **Substance P** has been demonstrated to produce contraction of the dog gallbladder in vivo and in vitro (Mate et al 1989), but has not been evaluated in human tissue.

The actions of **somatostatin** and **peptide YY** in negative feedback inhibition of CCK release have already been discussed

1.47) Drugs

A large number of drugs affect gallbladder emptying in vivo and contraction in vitro.

Erythromycin, a macrolide antibiotic, also binds to motilin receptors and acts as a motilin receptor agonist. Oral erythromycin reduces fasting and postprandial gallbladder volume and increases the rate of gallbladder emptying after a meal (Catnach et al 1992). It is thought that motilin is important in the regulation of fasting

gallbladder motility since gallbladder contractions observed during the interdigestive period are synchronous with phase III of the antroduodenal migrating motor complex and with peaks in plasma motilin levels. Administration of the selective 5-HT3 antagonist ondansetron (Fiorucci et al 1993) or atropine (Jebbink et al 1992) both negated this effect, suggesting that the effect is mediated by both cholinergic and serotonergic pathways.

Indomethacin a non steroidal anti-inflammatory agent decreases resting tone and spontaneous activity of gallbladder strips *in vitro* (Kotwall et al 1984) and *in vivo* has been demonstrated to reduce resting gallbladder pressure (Thornell et al 1985). There is also seemingly contradictory evidence of it increasing gallbladder emptying *in vivo*, an effect which may be explained by its actions on the sphincter of Oddi (O'Donnell et al 1992). The effects of indomethacin are thought to be mediated via inhibition of **prostaglandin** synthesis, since administration of PGF2, PGB2, PGD2, PGE1 and PGE2 have all been shown to produce gallbladder contraction *in vitro* (Kotwall et al, 1984).

Treatment with the serotonin analogue **octreotide** has the predictable effect of increasing gallbladder volume and reducing post prandial emptying (Fisher et al 1987), since serotonin is a potent inhibitor of CCK release (Kanayama et al 1985).

Cholestyramine increases gallbladder emptying. This is presumed to be because of its impairment of negative feedback inhibition of CCK release produced as a result of bile salt binding in the small intestine (Gomez et al 1989).

Women taking the combined progesterone and oestrogen **oral contraceptive pill** have increased gallbladder fasting volumes, but apparently normal emptying in response to a meal (Everson et al 1982), presumably due to the effect of the progesterone component.

37

Cisapride has been extensively investigated as a prokinetic agent throughout the gastro-intestinal tract (Lee et al 1984, Edwards et al 1987). It is a substituted piperidinyl benzamide which appears to act either through facilitation of acetylcholine release within the myenteric plexus (Schuurkes et al 1988), or through an agonist action on the 5-HT4 receptor (Craig and Clarke 1990). Marzio (1986) demonstrated reduced gallbladder fasting volume following administration of cisapride although no change in emptying in response to a meal. *In vitro* cisapride has been shown to increase contraction of gallbladder strips to electric field stimulation. Studies by Ziegenhagen (1992, 1993) have produced results demonstrating increased fasting gallbladder volume and reduced emptying after a meal. Subsequent studies (Patankar et al 1996) have supported the results of Ziegenhagen and demonstrated impaired gallbladder emptying after the administration of cisapride. Some of these seemingly contradictory results are probably explained via the actions of cisapride on sphincter of Oddi function an area which has not been extensively studied.

1.48) Gallbladder relaxation

The concept of gallbladder relaxation as an actively controlled process is relatively new. Davison et al (1980) demonstrated that cyclic guanosine monophosphate (cGMP) inhibited CCK induced gallbladder contraction. It has since been established that the widespread non adrenergic non cholinergic neurotransmitter nitric oxide (NO) acts through stimulation of cytosolic guanylate cyclase with a resultant rise in intracellular cGMP (Rapoport et al 1983) (Moncada et al 1988). Mourelle (1993) investigated these two findings and identified that the NO donor sodium nitroprusside abolished the *in vivo* contraction to CCK in guinea pig gallbladder, whilst conversely NO synthase inhibition *in vitro* and *in vivo* produced a significant enhancement of the response to CCK. They also identified NO synthase activity in homogenates of gallbladder tissue

suggesting that it is produced locally. From this the concept of a nitric oxide pathway regulating gallbladder relaxation was developed.

McKirdy et al (1994) were the first to demonstrate non adrenergic non cholinergic mediated relaxation of gallbladder strips in response to electric field stimulation. This effect was abolished by NO synthase inhibition, strongly suggesting that the neurotransmitter involved is nitric oxide.

Chen et al (1997) investigated the relaxant response of human gallbladder strips from patients with cholesterol and pigment stones. Strips from patients with cholesterol stones demonstrated impaired relaxation to the membrane receptor mediated stimuli isoproterenol, VIP and forskolin (a stimulant of membrane bound adenylate cyclase). However both groups showed similar relaxation in response to NO and 8-bromo-cAMP, which bypass cell membrane receptors to directly activate intracellular mechanisms. These findings suggest that the abnormal gallbladder motility seen in patients with cholesterol stones involves impairment of smooth muscle relaxation as well as contraction, all contributing to an immotile gallbladder, biliary stasis and subsequent cholesterol stone formation. The site for this defect appears to lie in the cell membrane, it has been suggested that it may be caused by excess cholesterol incorporation into the membrane reducing its fluidity and possibly interfering with receptor function (Chen et al 1997). This is similar to the theory developed to explain the finding that in prairie dogs and ground squirrels fed on a high cholesterol diet subsequent stone formation relies in part on poor gallbladder muscle contraction induced by excessive incorporation of cholesterol into the plasma membrane which occurs before stone formation (Fridhandler et al 1983) and appears to alter membrane receptor function (Yu et al 1996).

1.5 Kinetic factors

Cholesterol is secreted by the liver in conjunction with phospholipids arranged together to form vesicles, bile salts tend to form micelles predominantly with the phospholipid component leaving the vesicles increasingly cholesterol rich. Precipitation of crystalline cholesterol in bile occurs when vesicles become supersaturated with cholesterol relative to the corresponding concentrations of bile salts and lecithin (Admirand and Small, 1968). This precipitation appears to be accelerated by a number of kinetic factors which are present in bile (Burnstein 1983). A huge range of **non mucus biliary proteins** have been identified which speed up cholesterol crystal formation in vitro including IgM, IgA, IgG (Harvey et al 1993), fibronectin (Chijiiwa et al 1991), phospholipase C (Pattinso et al 1991) aminopeptidase N (Nunez et al 1995) and biliary haptoglobin (Yamashita et al 1995).

Biliary mucin itself increases cholesterol crystallisation in a prairie dog model (Lee et al 1981) and patients with cholesterol stones have been shown to have higher levels of biliary mucins than those with pigment stones as well as faster nucleating times (van Erpicum et al, 1996).

Deoxycholate, the most hydrophobic of the bile salts has been implicated in the formation of cholesterol gallstones. High levels of deoxycholate are hypothesised to disrupt the hydrophilic-hydrophobic balance of bile with consequent disruption of vesicles and resultant cholesterol crystallisation (Stolk et al 1994). Patients with cholesterol stones have raised levels of deoxycholate in their bile (Shoda et al 1995) which correlate with increased rates of cholesterol crystallisation (Portincasa et al 1996).

Bile samples from patients with multiple cholesterol stones have faster nucleating times than those with solitary cholesterol stones despite similar cholesterol saturations which

40

may account in part for the very high failure rate for cholesterol dissolution therapy in this group of patients (Jungst et al 1992).

It is exceedingly difficult to extrapolate all of these results regarding kinetic factors to the *in vivo* situation and judge what influence any one of these many factors may have on gallstone formation.

1.6) Gallbladder Imaging

The first successful imaging of the gallbladder was performed by Graham and Cole in 1926 when they used intravenous tetraiodophenolphthalein to produce a **cholecystogram** on X-ray films of a dog. However this was too toxic for human use and tetrabromophenolphthalein was used subsequently. Oral and intravenous cholecystography became the standard research tools for investigating the in vivo contractile responses of the gallbladder for the next fifty years with the sum of cylinders method becoming the accepted method of measuring gallbladder volume and emptying (Silva 1949). However its use became increasingly restricted with increased appreciation of the risks of radiation exposure.

Cholescintigraphy which uses radio-isotope labelling of substances secreted into bile was first demonstrated in 1966 by Englebert and Chiu who used it for quantitative analysis of human biliary evacuation. At this stage it was not appreciated that the technique could be applied to measuring gallbladder volume. Krishnamurthy (1981) demonstrated using an *in vitro* model that a linear relationship exists between counts of radioactivity and gallbladder volume. The advantage of this technique for measuring gallbladder emptying is that it is independent of the shape of the gallbladder thus bypassing some of the inherent errors encountered with the sum of cylinders methods

41

which assume that the gallbladder is always circular in cross section. The technique has been shown to be reproducible and accurate. Disadvantages of the technique as a research tool are that it involves radiation exposure, it requires parenteral administration of the cholescintigraphic agents, the facilities are relatively expensive and it measures gallbladder emptying in terms of flow of radioactive labelled bile through the organ rather than direct change in gallbladder size. It takes no account of gallbladder refilling with unlabelled bile, this is therefore a potential source of error in estimating gallbladder emptying.

Ultrasound has become the tool of choice for investigation of gallbladder emptying for many researchers since advances in the development of real time ultrasonography led Everson (1980) to compare volumes calculated from ultrasound images with those from cholecystograms both using the sum of cylinders technique. He found good correlation between the results *in vivo* and also using an *in vitro* model comparing true volumes of bile instilled into a human gallbladder in a bath he identified good correlation between real and calculated volumes. This method was used extensively by Everson and Braverman to compare gallbladder emptying between patients with gallstones and healthy controls. Although some ultrasound machines are capable of performing a sum of cylinders calculation directly on the image, many are not programmed for this function, which makes calculation unwieldy.

The **Ellipsoid** method which uses a simple calculation of gallbladder volume from measurements of maximum gallbladder length, height and width obtained from transverse and longitudinal images of the gallbladder has been validated *in vitro* and *in vivo* (Dodds et al, 1985) and gives values that correspond very closely to those obtained with the sum of cylinders method.

$$Volume = Length \times Width \times Height \times 0.52$$

The disadvantages of ultrasound are that obese patients can be very difficult to scan accurately and that whichever method of volume calculation is used assumptions are made about the shape of the gallbladder which may not hold true for all individuals. However the simplicity, availability, broad accuracy and low risk of the technique make it the method of choice for most investigators.

1.7) Symptoms

The principal indications for cholecystectomy are symptomatic gallstones and the complications of gallstones. Despite these well established and longstanding criteria the specific symptoms of gallstones are not as well established as might be anticipated.

The largest study of biliary symptoms and gallstones (GREPCO 1988), has shown that using a definition of biliary pain as abdominal pain lasting more than half an hour and situated in the right hypochondrium or epigastrium there is almost as high a prevalence of biliary pain in patients without gallstones as in those who have them. Wegge et al (1985) studied patients admitted to a surgical unit with upper abdominal pain, there was little difference in description of the pain between patients with gallstones and those without, except patients with gallstones described more attacks of similar pain, more fatty food intolerance and on examination were more likely to be tender over the gallbladder. Further population screening studies by Glambek et al (1989) and Jorgensen (1989) both observed similar levels of abdominal symptoms in individuals irrespective of the presence of gallstones.

Gracie and Ranshoff (1982) followed up 123 patients with asymptomatic gallstones and identified that the 15 year cumulative probability of development of biliary symptoms or complications was only 18%.

The description of the symptoms of functional intestinal disorders such as irritable

bowel syndrome (IBS) shows a marked overlap with the symptoms of gallstone

disease. The close integration of the physiological regulatory mechanisms already

outlined for the gallbladder and intestine make this unsurprising. Patients with IBS

demonstrate visceral hypersensitivity (Ritchie et al, 1973) and hypermotility; there is

some evidence to suggest that this extends to the gallbladder (Braverman 1987). A

study by Kellow (1987) demonstrated abnormal sensitivity of the gallbladder to

exogenous CCK-8 in patients with IBS. Previous work by Harvey and Read

demonstrated that intravenous CCK reproduced the abdominal pain of a proportion (8

out of 20) of patients with IBS particularly those who related their attacks of pain to

food. They also identified increased colonic motor activity in response to the CCK.

Chapter II.

HYPOTHESES AND AIMS

HYPOTHESES

1) Bradykinin is an *in vitro* stimulant of human gallbladder contractility via the B1 and B2 receptors.

2) Gallbladder emptying is lower in gallstone patients than in normal individuals.

3) Patients with irritable bowel syndrome have high levels of CCK-8 and abnormal *in vivo* gallbladder emptying.

4) *In vitro* gallbladder strip contraction is a reflection of *in vivo* gallbladder emptying.

AIMS

The aims of this thesis were to test the above hypotheses as follows:

1) Investigate the *in vitro* contractility of human gallbladder tissue to bradykinin and the relative effects of known B1 and B2 receptor antagonists on this response.

2) Evaluate *in vivo* gallbladder emptying sonographically in response to a standard fatty meal and compare the responses of normal individuals and gallstone patients.

3) Measure and compare gallbladder emptying in patients with irritable bowel syndrome and normal controls.

4) Measure and compare plasma CCK-8 levels in patients with irritable bowel syndrome and normal controls.

5) Measure and compare *in vivo* gallbladder emptying and *in vitro* gallbladder strip contraction in gallstone patients.

47

Chapter III.

METHODS

3.1) *In vitro* experiments

The following methods in this section outline the general techniques used throughout this period of research for the *in vitro* measurement of gallbladder contractility and the production of concentration response curves in response to a variety of chemical and hormonal stimuli.

The general principles for the *in vitro* assessment of human gallbladder contractility are well established and have been outlined in chapter one.

3.11) Patients

Human gallbladder tissue was obtained from two groups of patients; firstly, those with diseased gallbladders undergoing elective cholecystectomy for gallstone disease and secondly from patients with normal, healthy gallbladders undergoing cholecystectomy as part of major hepatic or pancreatic resections for malignant disease.

3.12) Exclusion Criteria

Several exclusion criteria were used to exclude gallbladder tissue which was damaged or likely to have impaired contractility for non physiological reasons.

Patients undergoing cholecystectomy for acute cholecystitis were excluded, as were any gallbladders that appeared macroscopically acutely inflamed at the time of surgery.

Gallbladders that when dissected into strips were grossly fibrotic were excluded.

Gallbladders that were severely damaged during operation were excluded.

Gallbladders from the normal group which contained stones as an incidental finding when opened, were transferred into the gallstone group.

Complete obstruction of the cystic duct (mucocoele) or common bile duct (obstructive jaundice) was also exclusion criteria in view of their potential to distort subsequent contractility *in vitro*.

3.13) Preparation of Krebs Bicarbonate Solution

Each day 5 litres of Krebs solution were made using 118.4 mM NaCl, 25 mM $NaHCO_3$, 11.7 mM glucose 4.7 mM KCL, 1.9 mM $CaCl_2$, 1.2 mM $KHPO_4$ and 1.2 mM $MgSO_4$ in distilled water. The solution was stored at 4^oC until used and any remaining solution was discarded at the end of the day...

3.14) Preparation of CCK-8 and Other Peptides

Sulphated cholecystokinin octapeptide (CCK-8, MW 1143.3, Sigma, Poole, UK) was prepared as a stock solution with distilled water to a concentration of 100μmolar. One millilitre aliquots of this solution were frozen at -20^oC. An aliquot of this solution was then thawed for each set of experiments and diluted to a concentration of 10 μmolar with Krebs solution. Dilute solution was then stored at 4^oC during the course of the experiment. Unused thawed reagents were discarded at the end of each series of experiments.

The other peptides used during these experiments were bradykinin, des-Arg[9]-Leu[8]-bradykinin, des-Arg[9]-bradykinin, carbachol and HOE-140 (all obtained from Sigma, Poole, UK). These were all made up individually as 10 mM stock solutions with distilled water and 1ml aliquots were frozen at -20^oC and thawed as required. After thawing, 100 μL each of the bradykinin and des-Arg[9]-bradykinin stock solutions were diluted with Krebs solution to give a concentration of 100μM, for preliminary experiments to establish the range of action of bradykinin 100μL of this dilute solution was further diluted with Krebs to give a concentration of 1μM. Adding sequential volumes of these dilute and concentrated stock solutions with a micropipette between

50

10-1000μL gave concentrations of reagents from 10^{-9}-10^{-3}M in the 10ml organ bath. All dilute solutions were kept at 4°C during the course of the experiments.

3.15) Tissue Preparation

Fresh human gallbladder tissue was obtained from the operating theatres and placed immediately into cold Krebs solution. When available, the time of ligation of the cystic artery, the time of removal of the gallbladder from the patient and the time of its immersion in Krebs solution were recorded. The tissue was transported to the laboratory and from 3 to 6, full thickness, longitudinal strips each approximately 15 x 2 mm, were cut from the anterior aspect of the fundus of the gallbladder.

Each strip was attached by size 20 fishing hooks and cotton thread at either end, to an isotonic transducer (Harvard Instruments, UK) with a 1 gram preload and suspended in a 10 ml organ bath (6 bay organ bath, Linton, UK) containing aerated (95% O_2, 5% CO_2) Krebs solution at 37°C (Figure 3.1). The transducers were connected to a custom made amplifier interface which was linked to a Maclab/8e Macintosh computer loaded with Scope and Chart (Figure 3.2). This gave a continuous recording of spontaneous isotonic activity and response to stimuli measured as millivolts in the range -200 to +200m
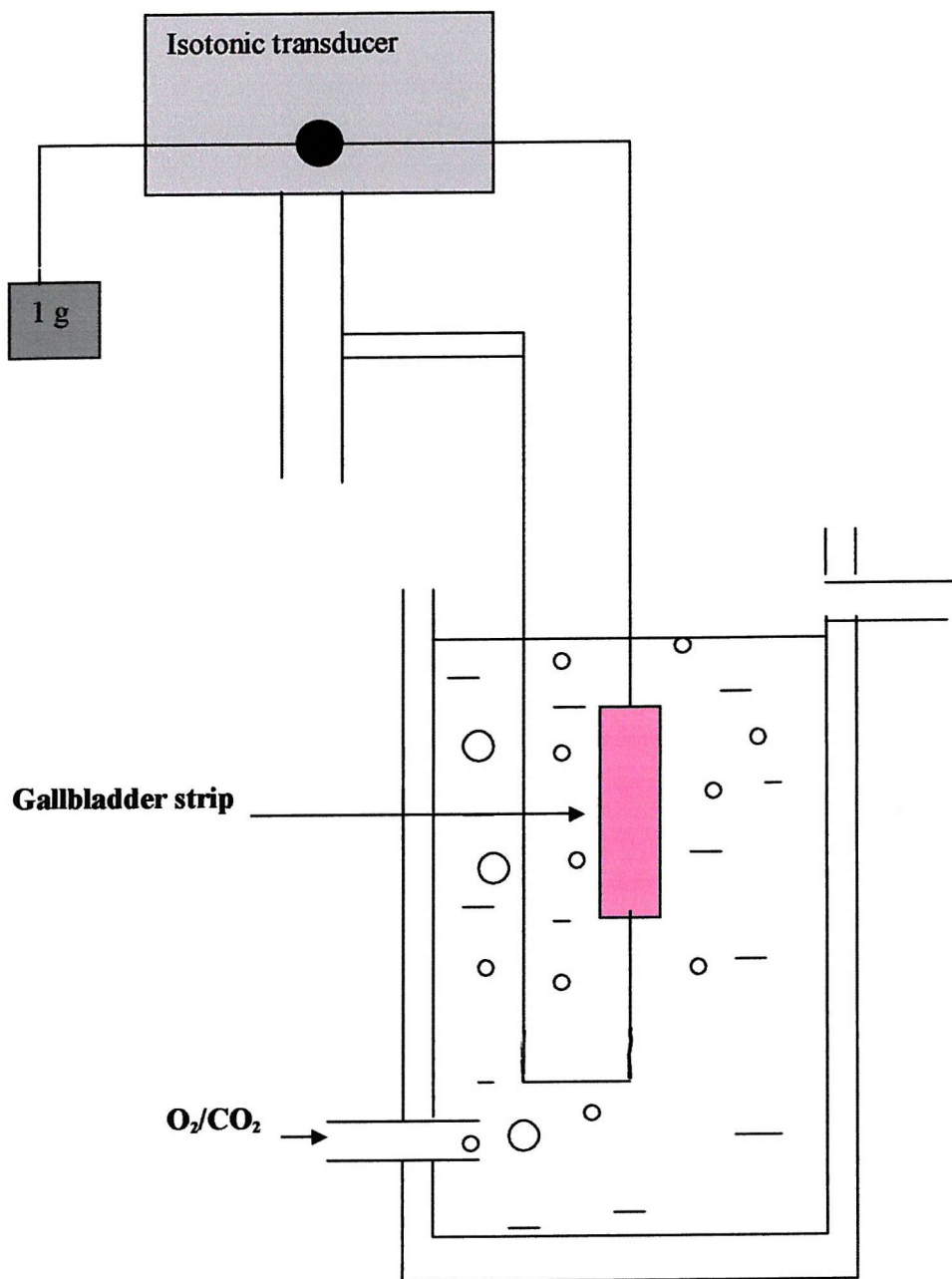
Figure 3.1

Organ bath and isotonic transducer, set up for *in vitro* measurement of gallbladder strip contractility.

3.16) Viability Testing

The time of placement in the organ bath was recorded and the strips were left to equilibrate for two hours. after which viability was assessed with carbachol 100μmol. Those strips which did not contract in response to this standard stimulus were discarded. Viable strips were then washed out and irrigated with Krebs solution and allowed to equilibrate until a steady baseline was achieved, before proceeding with the experiment. Strips which failed to return to a steady baseline were also discarded

3.17) Method for Generation of a Concentration Response Curve to CCK-8 or Other Peptides

The concentration response curve (CRC) is the standard method of assessing the contractile response of human tissue to hormonal or chemical stimuli *in vitro*. Throughout these experiments a standard technique has been used for producing CRCs.

CCK-8 produces a dose dependent contraction in human gallbladder tissue. To generate a CRC, CCK-8 was added incrementally, starting with 10μl of the dilute solution and going up to a cumulative total of 1ml, giving a concentration in the organ bath between 10-1000nM. At each concentration the contraction was allowed to reach a plateau before the level of contraction was recorded and prior to addition of further CCK-8. If no contraction was observed at a particular concentration within 3 minutes, then the next increment of CCK-8 was added. After achieving the maximum concentration within the organ bath and recording the peak contraction the strips were washed out and irrigated with Krebs solution until a steady baseline had returned prior to proceeding with any further part of the experiment.

The results were expressed as a percentage of the maximum observed contraction which was recorded as millivolts.

53

3.18) Baseline Activity

The majority of viable strips exhibit one of two typical baseline patterns prior to stimulation. Either a low frequency relatively high amplitude contraction or a fine high frequency contraction. These contractions were usually abolished by the addition of CCK-8 or other stimuli. For consistency and simplicity of calculation the mid point of these small contractions was taken as the baseline value.

3.19) Tissue Death

Viable strips would produce a consistent repeatable CRC for CCK up to 4 times over a period of 6 hours whilst in the organ bath. Tissue death was marked by any one of 3 patterns after washout and irrigation;

Persistent contraction.

Loss of normal tone and subsequent relaxation markedly below the previous baseline.

Bizarre irregular high amplitude contractions.

Strips displaying any of any of these patterns were deemed non viable and were discarded.

3.10) Analysis of Data

Further statistical and graphical analysis was performed using SPSS for Windows, Harvard Graphics and Excel (Microsoft Corporation, USA).
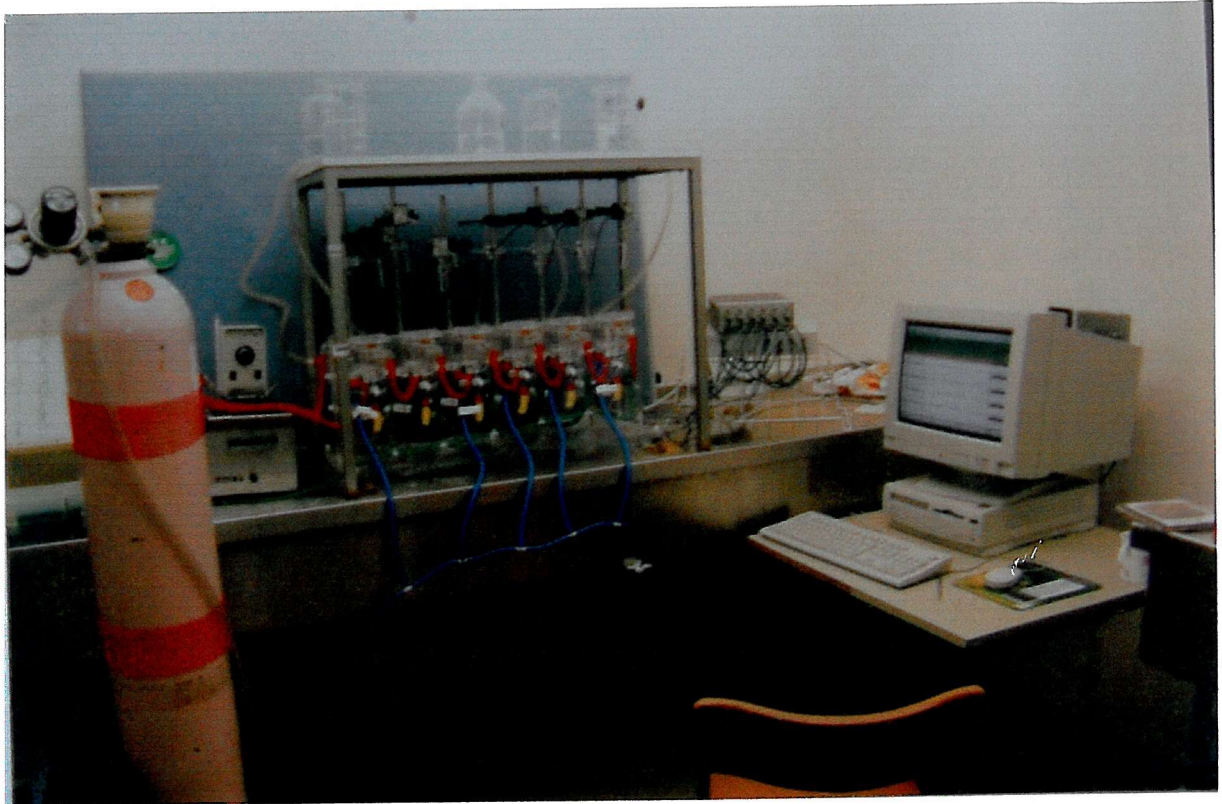
Figure 3.2 *In vitro* model laboratory set up.

3.2) Patients and Materials for *in vitro* analysis

Tissue from 83 gallbladders was obtained, 9 of these were discarded because of gross fibrosis (5), acute inflammation (3) or severe operative trauma (1).

287 strips from the remaining 74 gallbladders were set up in the organ bath for experimentation.

57 of the 74 gallbladders were removed for cholelithiasis, the remaining 17 normal gallbladders were removed as part of wider resections or bypass procedures for malignancy (pancreatic=9, hepatobiliary=8).

The age range of the gallstone patients was 16 - 83 years, mean 55 yrs, median 59 yrs, and the range for the normal group was 28 - 83 yrs, mean 61 yrs, median 67 yrs.

The normal group had a strong male bias with a male to female ratio of 15:2. The gallstone group contained 43 women and 14 men reflecting the relative increased prevalence of cholesterol gallstones in women.

Macroscopic appearances at the time of operation suggested that of the 57 gallstone patients 9 had pigment stones and 48 had cholesterol or mixed stones.

The majority of cholecystectomies for cholelithiasis were performed laparoscopically (45), the remainder were removed via the traditional open (8) or "mini" (4) cholecystectomy.

When possible the time from ligation of the cystic artery to placement of the strips in the organ bath was recorded, data was obtained for 48 of the first 55 gallbladders obtained and demonstrated a range of 15 to 55 minutes, mean 28 minutes, median 25 minutes. Similarly the time from putting the gallbladder into cold Krebs solution until final placement in the organ bath was measured, range 8 to 25 minutes, mean 16 minutes, median 15 minutes.

After equilibration and viability testing only 92 strips returned to a stable baseline and were deemed suitable for further experimentation.

3.3) *In vivo* Assessment of Gallbladder Emptying

3.31) Techniques Used for the *in vivo* Assessment of Gallbladder Emptying

Many methods exist for the measurement of gallbladder emptying, including oral or intravenous cholecystography, cineradiography, manometry, ERCP, cholescintigraphy and ultrasonography. Of these ultrasonography and cholescintigraphy are the most frequently used, however they measure different aspects of gallbladder emptying. Cholescintigraphy measures counts of radioactivity coming from labelled compounds excreted in bile; as a result it is a very accurate indicator of flow of labelled bile into and out of the gallbladder. The change in count in response to a stimulus has been shown to be very closely related to measurements of gallbladder emptying (Patankar et al 1994). Although this gives a very accurate picture of true gallbladder emptying in terms of bile ejection it cannot give you a value for gallbladder volume, hence for the purpose of comparisons with *in vitro* contractility it is better to use ultrasonography since it calculates a true anatomical volume for the gallbladder at an instant in time, comparing serial readings gives a net change in volume. This change in anatomical volume is a better reflection of changes in muscle fibre length than measurement of bile ejection from the gallbladder which is more correctly a measure of bile flow through the gallbladder which may not necessarily relate to change in muscle fibre length within the wall of the gallbladder.

Cholescintigraphy has the added drawbacks of being expensive when compared with ultrasonography and radiation exposure to the patient which makes it difficult to repeat studies in a short period of time as well as making the experiment more difficult to recruit volunteers for because of the perceived health risk.

57

3.32) Patients

Subjects were identified from three sources:

1) Patients awaiting elective cholecystectomy for gallstones.

2) Patients with irritable bowel syndrome attending surgical outpatient clinics. IBS was defined according to the Rome criteria (Thompson et al 1989) after organic pathology had been excluded by appropriate colonic and pelvic investigation. Patients subsequently shown to have gallstones on ultrasound were excluded from the study.

3) Normal controls were recruited from hospital staff.

3.33) Exclusion Criteria

Truncal or selective vagotomy.

Endoscopic sphincterotomy or stenting of the common bile duct.

History of common bile duct stones or obstructive jaundice.

Drugs with a known effect on gallbladder motility; anticholinergics, tricyclic antidepressants, nitrates, cisapride, antiemetics and prokinetic agents, NSAIDs, oestrogens and calcium channel blockers.

Diabetes or other causes of autonomic neuropathy.

Ultrasound evidence of stones filling greater than fifty per cent of the volume of the gallbladder hence impairing the potential for contraction.

3.34) Method

Subjects were examined using a Siemens sonoline SL-1 ultrasound machine (figure 3.3) with 3.5 and 5 MHz linear array probes. Subjects attended having fasted for six hours to ensure a full gallbladder on arrival. A series of initial scans were performed to confirm the presence or absence of stones and ensure that good views of the gallbladder could be obtained and consistent measurements made.

Volume was measured using the ellipsoid method which calculates volume from the following formula

$$\text{Volume} = \text{Length x Width x Height x } 0.52$$

This is a well established method for measuring the volume of the gallbladder; it has been shown to give accurate results which correlate well with *in vitro* volumes, cholescintigraphy, cholecystography and more laborious forms of calculation such as the sum of cylinders method (Wedmann et al 1991, Everson et al 1980).

Length was measured along the long axis of the gallbladder from the origin of the cystic duct to the tip of the fundus. Width and height were calculated from a cross sectional image at the widest point of the body of the gallbladder (figure 3.4). All measurements were taken as internal dimensions measured from the mucosa, where this could be identified.

Figure 3.3, Siemens sonoline SL-1 ultrasound machine used for *in vivo* measurement
of gallbladder volume.
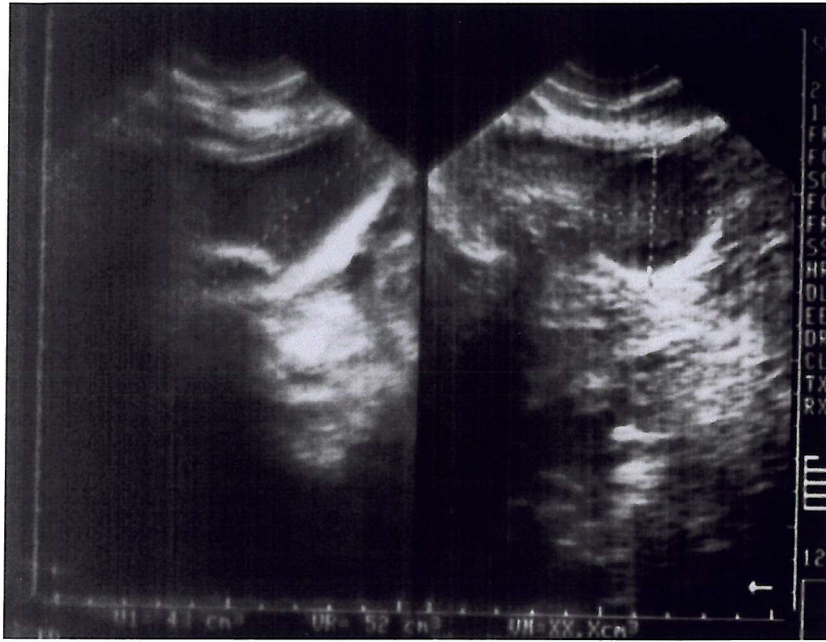
Figure 3.4 Sonographic estimation of gallbladder volume.

Longitudinal and cross sectional images of a gallbladder demonstrating measurement of height, length and width for volume estimation.

Measurement was accepted as accurate if three consecutive volumes were recorded which were within a range of 10% of their mean value, as minute to minute fluctuations in volume up to this level are known to occur.

A standard fatty meal was provided consisting of a Yorkie bar (Nestle, UK) which contains 29g of saturated fat in a 52g bar and 150ml of water. Patients were asked to consume this as quickly as possible.

Patients took between 30 seconds and four minutes to consume the meal. Time was recorded from completion of the meal and serial measurements of gallbladder volume were made every 5 minutes up to half an hour and every 10 minutes for the next half hour.

From these measurements ejection fraction was calculated as follows

Ejection fraction = $\underline{\text{fasting volume - minimum volume}}$ x 100
fasting volume

3.4) CCK-8 Analysis

Subjects undergoing *in vivo* assessment of gallbladder emptying were also asked to give blood for serial CCK-8 analysis.

Six millilitres of blood were taken in an EDTA tube (Vacutainer UK) immediately prior to the meal and then repeated at 15, 30, 45 and 60 minutes from completion of the meal. Blood was stored on ice until the sonographic measurements were complete before being centrifuged (at 2500 RPM for 10 minutes at 4°C) and plasma sent for CCK-8 analysis using a radio immuno assay which was developed in house and performed by Mr H.Brennan according to the following protocol.

Plasma samples were stored at -20°C until extraction. Samples were defrosted and 1ml of plasma was mixed with 2ml 98% ethanol, vortexed for 10 seconds and centrifuged

at 3000rpm for 15 minutes. The supernatant was poured into a polystyrene tube and the sample dried overnight in a rotary evaporator and stored at -20^{0}C.

The total tracer activity of I^{125}CCK-8 was 1000-1500 cpm per tube. Dried ethanol extracts of 1ml plasma were reconstituted in 0.5ml assay buffer. Antiserum stock (1ml of 1 in 10^{4} was diluted to 10ml in assay buffer. 100μl of this dilution was added to each tube. Reagents were added at room temperature to labelled polystyrene tubes. The total assay volume came to 1ml per tube, comprising 500μl reconstituted plasma extract, 100μl of antiserum and 400μl of I^{125}CCK-8. The tubes were capped and incubated at 4^{0}C for 72 hours.

After incubation free and bound tracer were separated. NORIT charcoal (0.8g) and Dextran grade C (0.08g) were added to 50ml of cold assay buffer and mixed in an ice bath for 20 minutes. 300μl of charcoal mixture was added to each tube with all tubes in a cold water tray at 4^{0}C. After addition of charcoal the tubes were centrifuged for 10 minutes (3000 rpm at 4^{0}C) and the supernatant separated from the pellet. The supernatant (bound) was counted in an automated gamma counter.

Plasma concentration of CCK was expressed as the CCK-8 like immunoactivity (pM) of each plasma extract.

Chapter IV.

RESULTS

BRADYKININ *IN VITRO* STUDIES

4.1) Introduction

Relatively little work has been performed on the action of bradykinin on the human gallbladder, the only data that exists is from Rushton et al (1995) who demonstrated that it caused gallbladder strips to contract *in vitro*, an effect which appeared to be controlled via the B2 receptor. This supported the work of Cabrini (1995) who had demonstrated similar responses in the guinea pig gallbladder. As so little published experimental evidence exists concerning these areas our initial experiments have set out to confirm the response of human gallbladder to bradykinin *in vitro* and establish a protocol for further work on this peptide.

4.2) Preliminary experiments

4.21) Background

The work by Rushton on human gallbladder strips *in vitro* identified that contraction started with a bradykinin concentration of 10^{-7}M and maximal contraction occurred at 10^{-4}M. He also demonstrated that strips appeared to be sensitised after the first bradykinin CRC so that subsequent CRCs generated higher levels of contraction than the initial maximum response. Studies on the guinea pig gallbladder obtained a broadly similar concentration range for the response to bradykinin, although they recorded very small but measurable contractions with concentrations of bradykinin as low as 10^{-12}M and they did not use concentrations above 10^{-5}M, nor did they demonstrate sensitisation of the strips (Cabrini et al 1995).

4.22) Aims

To determine the range of concentrations of bradykinin required to obtain a concentration response curve in human gallbladder strips *in vitro* and to identify if this response is repeatable.

4.23) Methods

Strips of gallbladder were set up in the organ baths (see general methods ch 3.) under physiological conditions. Viable strips were subjected to stimulation by bradykinin in increasing concentrations within the organ bath from 10^{-9}-10^{-3}M, as the work by Rushton had suggested that the most rapid changes in contractility occurred in the range of 10^{-6} - 10^{-4} M, proportionally smaller increases in bradykinin concentration were used in this region to give more detailed information on this important area of the curve. After identification of the range for the CRC further strips were subjected to successive CRCs to demonstrate whether or not a repeatable response was reproduced. Between each CRC the strips were thoroughly irrigated with fresh Krebs solution to washout the bradykinin from the organ bath and allowed to return to a stable baseline before the CRC was repeated.

4.24) Results

The contractions of 5 strips from 3 gallbladders were measured in response to an increasing concentration of bradykinin. No strip demonstrated a contraction of greater than 1% of maximum at a concentration below 10^{-7}M and a contraction within 2% of the recorded maximum was reached in all strips between 5×10^{-5} and 10^{-4}M (table 4.21, figure 4.21). Therefore all subsequent bradykinin CRCs used the range 10^{-7} to 10^{-4}M.

Table 4.21

Contraction of human gallbladder strips (A1-A5) *in vitro* in response to bradykinin in the range 10^{-9}-10^{-3} M, expressed as % of maximum contraction.

Bk conc. (M)	10^{-9}	10^{-8}	10^{-7}	10^{-6}	5×10^{-6}	10^{-5}	2×10^{-5}	5×10^{-5}	10^{-4}	10^{-3}
A1	0	0	1.1	4.5	4.5	23.7	45.1	73.8	99.9	100
A2	0	0	0.8	2.5	9.2	17.3	28.5	62.7	100	98
A3	0.4	0.1	9.3	61.9	81.2	91	93.9	99.7	98.8	100
A4	0	0	6.5	35.3	47	56	70	82.5	98.7	100
A5	1	1	6.8	24.8	45.3	60.8	73	82.3	100	99.1
Mean	0.3	0.2	4.9	25.8	37.4	49.8	62.1	80.2	99.5	99.4
S.E.M.	0.2	0.2	1.7	10.8	14	13.4	11.4	6.1	0.2	0.4

Four strips from 2 gallbladders were subjected to repeated bradykinin CRCs to identify the repeatability of the bradykinin CRC. Three of the 4 strips were still viable after 4 CRCs and 2 strips were still viable after 5 CRCs, the bradykinin CRC was found to be consistently repeatable with no evidence of sensitisation after the initial response curve (Table 4.22 and figure 4.22)(Appendix A)).

Table 4.22

Repeat bradykinin concentration response curves (runs 1-4, mean of 4 strips, run 5 mean of 3 strips)

Bk (μM)	0	0.1	1	5	10	20	50	100
Run 1	0	4.6	10.4	26.9	46.8	62.1	83.6	100
Run 2	0	3.4	13.8	29.5	43.3	63.2	86.1	106.9
Run 3	0	1.5	6.3	15.5	34.2	54	83.9	103
Run 4	0	2.3	9.5	21.7	38.3	55.8	85.6	104.3
Run 5	0	1.2	7.8	18.3	36.8	59.7	86.7	110.9

4.25) Summary

These results confirm that bradykinin concentrations in the range $10^{-7}M$ to $10^{-4}M$ stimulate gallbladder smooth muscle contraction *in vitro*. This response is repeatable without evidence of sensitisation of the strips to bradykinin.

Figure 4.21 Bradykinin concentration response curve (mean +/- s.e.m.)
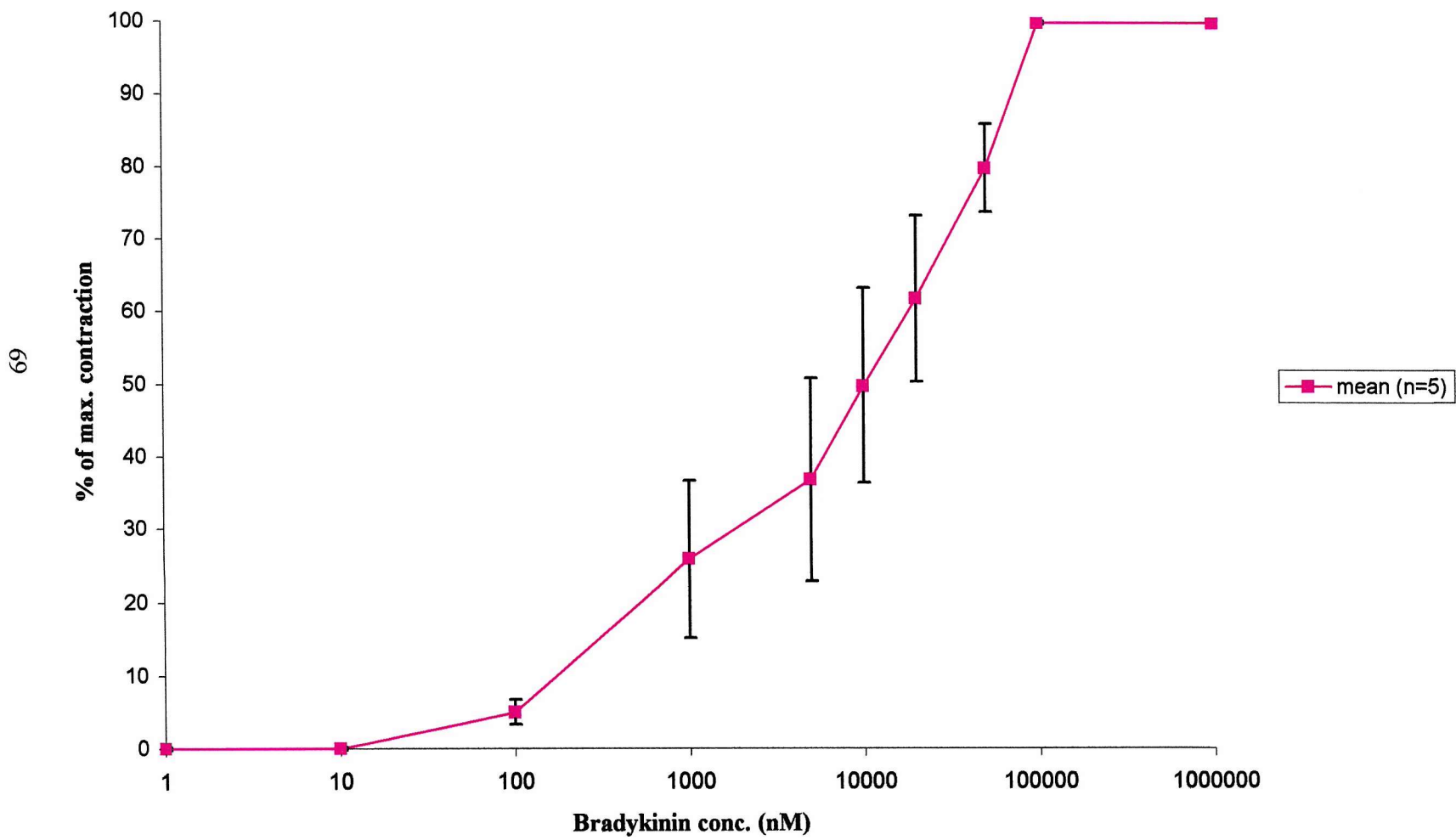
Figure 4.22 Repeat bradykinin concentration response curves
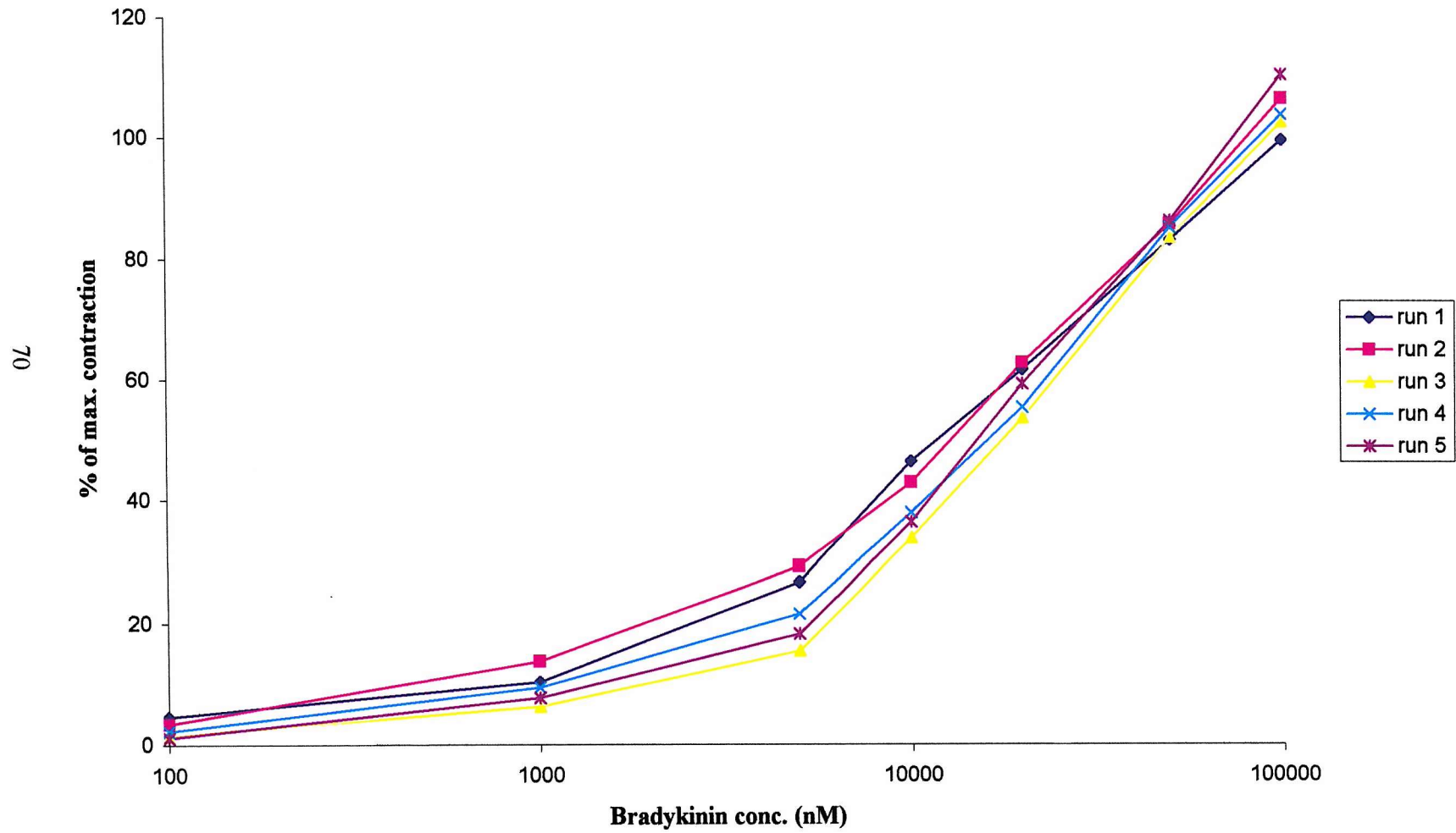
4.3) Bradykinin and des-Arg^9-Leu^8-Bk

4.31) Background

The selective B1 receptor antagonist des-Arg^9-Leu^8-Bk was developed following work by Regoli and co-workers (1977) who identified the B1 receptor and found that it was activated by the Bk-(1-8) fragment of the Bk molecule and selectively antagonised by Leu^8-Bk-(1-8). It is a competitive antagonist with similar affinity for the B1 receptor compared with the bradykinin metabolite Bk-(1-8), it does not bind with the B2 receptor. Bk-(1-8) is a normal by product of Bk breakdown by the plasma enzyme carboxypeptidase N, which cleaves the carboxy-terminal arginine residue from the Bk molecule. It has no affinity for the B2 receptor. The B1 receptor appears to be predominantly expressed in chronically inflamed tissues (Perkins et al 1993). The work of both Cabrini and Rushton suggests that in human and guinea pig gallbladder the contractile response is mediated via the B2 receptor and B1 receptor blockade makes no difference to the bradykinin CRC.

4.32) Aims

To identify the effect of B1 receptor blockade on bradykinin induced gallbladder smooth muscle contraction in vitro in gallbladder strips from both normal and stone bearing gallbladders.

4.33) Methods

Twenty six viable strips were obtained from 12 stone bearing gallbladders and 14 strips from 5 normal gallbladders. After equilibration in the organ bath a bradykinin CRC was generated for each strip. The strips were washed out and allowed to return to baseline, before addition of 100μL of 100μM des-Arg^9-Leu^8-Bk to achieve a concentration of

71

1 μM in the organ bath. After 15 minutes equilibration the bradykinin CRC was repeated, the strips were then washed out again and allowed to return to baseline.

4.34) Results

A similar bradykinin CRC was obtained for strips from both stone bearing and normal gallbladders (mean $E.D._{50}$ = 5.1 and 4.95 μM respectively). B1 receptor blockade did not affect the bradykinin CRC for strips from stone bearing gallbladders (mean $E.D._{50}$ = 8.5μM), however a significant (p<0.05, two tailed t-test) rightward shift was seen in the curve for strips from normal gallbladders (mean ED_{50} = 26.6μM). (Table 3 and 4, figure 4.3)(Appendix B).

Table 4.31 Mean bradykinin concentration response curve values for strips from normal and stone bearing gallbladders.

BK (μM)	0	0.1	1	5	10	20	50	100
Normal (14)	0	6.7	29.3	50.7	64.6	75	86.3	100
s.e.m.	0	1.5	5.1	6.4	6.7	6	3.5	0
Stones (26)	0	12	28	49.4	58	71	83	100
s.e.m.	0	2.1	4.3	5.2	5.4	3.7	4.3	0

Table 4.32 Repeat mean bradykinin concentration response curve values for strips from normal and stone bearing gallbladders in the presence of des-Arg^9-Leu^8-Bk (1μM).

des-Arg^9-Leu^8-Bk (1 μM)								
Bk (μM)	0	0.1	1	5	10	20	50	100
Normal (14)	0	1.7	4.1	17.3	25.6	38.5	69.5	79.1
s.e.m.	0	1.2	2.5	4.2	5.2	3.9	3.6	4.5
Stones (26)	0	6	19	38	51	69	77	101

4.35) Summary

The response of human gallbladder strips *in vitro* to bradykinin appears to be mediated via the B1 receptor in normal but not in stone bearing gallbladders.

Figure 4.31 Bradykinin CRCs for strips from normal gallbladders and repeat CRCs after addition of dAL-Bk
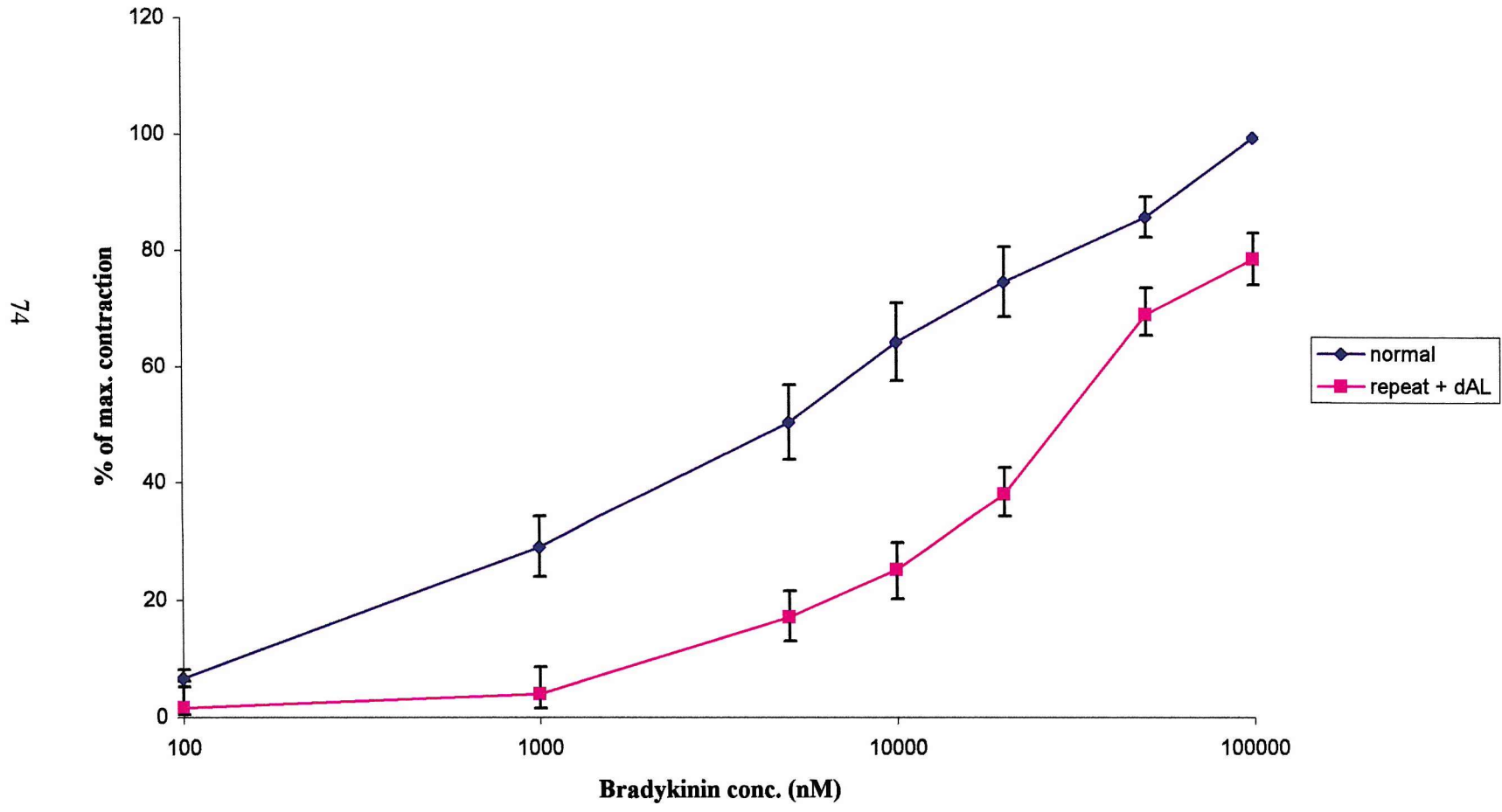
Figure 4.32 Bradykinin CRCs for strips from stone bearing gallbladders and repeat CRCs after addition of dAL-Bk
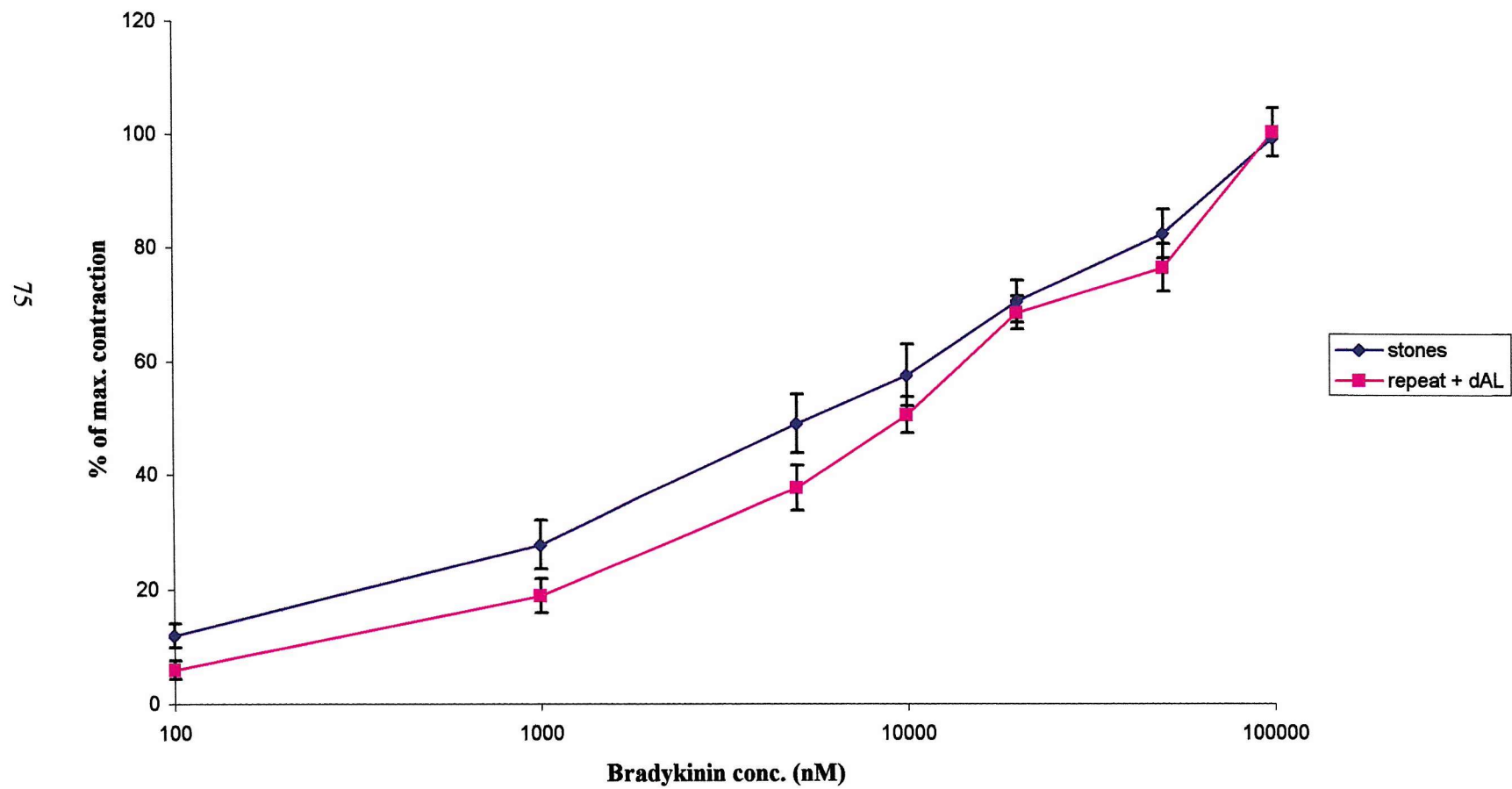
4.4) Bradykinin and HOE-140

4.41) Background

The work of Cabrini and Rushton suggests that in both guinea pig and human gallbladder contraction is stimulated via the B2 receptor which appears to be involved in the regulation of the majority of bradykinins physiological functions identified thus far. Both have demonstrated a rightward shift of the bradykinin CRC in the presence of the selective B2 competitive antagonist HOE-140. HOE-140 was developed by researchers at Hoechst as the first of the second generation of B2 receptor antagonists (Lembeck et al, 1991, Hock et al 1991) characterised by the substitution of unnatural peptides into the Bk molecule these antagonists have greater potency and stability than the first generation of peptide based antagonists which substituted aromatic amino acid residues at the 5 and 8 positions and a D-aromatic residue at position 7. HOE-140 incorporates the D-(1,2,3,4-tetrahydroisoquinolin-3-yl-carbonyl) molecule at the 7 position. HOE-140 has a 40 times greater affinity for the B2 receptor compared with first generation antagonists as well as a prolonged half life in serum which further adds to its potency. HOE-140 does not bind to the B1 receptor at all.

4.42) Aims

The aim of this experiment was to identify the interaction of bradykinin and HOE-140 on gallbladder smooth muscle strips in vitro and compare the responses of strips from patients both with and without gallstone disease.

4.43) Methods

The same 40 strips used in experiments described in section 4.3 were irrigated with Krebs' solution and allowed to return to a stable baseline before addition of 100μL of 100μM HOE-140 to give a concentration of 1 μM in the organ bath. The strips were allowed to equilibrate for a minimum of 15 minutes prior to repeating the bradykinin concentration response curve (range 10^{-7}-10^{-4}M). Following completion of the CRC

the strips were repeatedly washed out again with fresh Krebs' solution and allowed to return to baseline.

4.44) Results

Strips from both normal and stone bearing gallbladders demonstrated a significant (p<0.01, two tailed t-test) rightward shift of the bradykinin CRC in the presence of HOE-140 (E.D.$_{50}$ = 37.1 and 27.5 µM, respectively) (table 4.4, figure 4.4)(Appendix C).

Table 4.4 Mean bradykinin concentration response curve values for strips from normal and stone bearing gallbladders in the presence of HOE-140 (1µM).

HOE-140 (1µM)								
BK (µM)	0	0.1	1	5	10	20	50	100
Normal (14)	0	1.2	1.5	5.2	14.6	34.6	61.3	71.8
s.e.m.	0	0.7	0.7	2.2	4.8	7.6	11.8	13.4
Stones (26)	0	0.1	0.5	3.6	16	39	73	97.5
s.e.m.	0							

4.45) Summary

The response of human gallbladder strips *in vitro* to bradykinin is mediated via the B2 receptor in both normal and stone bearing gallbladders.

77

Figure 4.4 Bradykinin concentration response curves in the presence of HOE-140
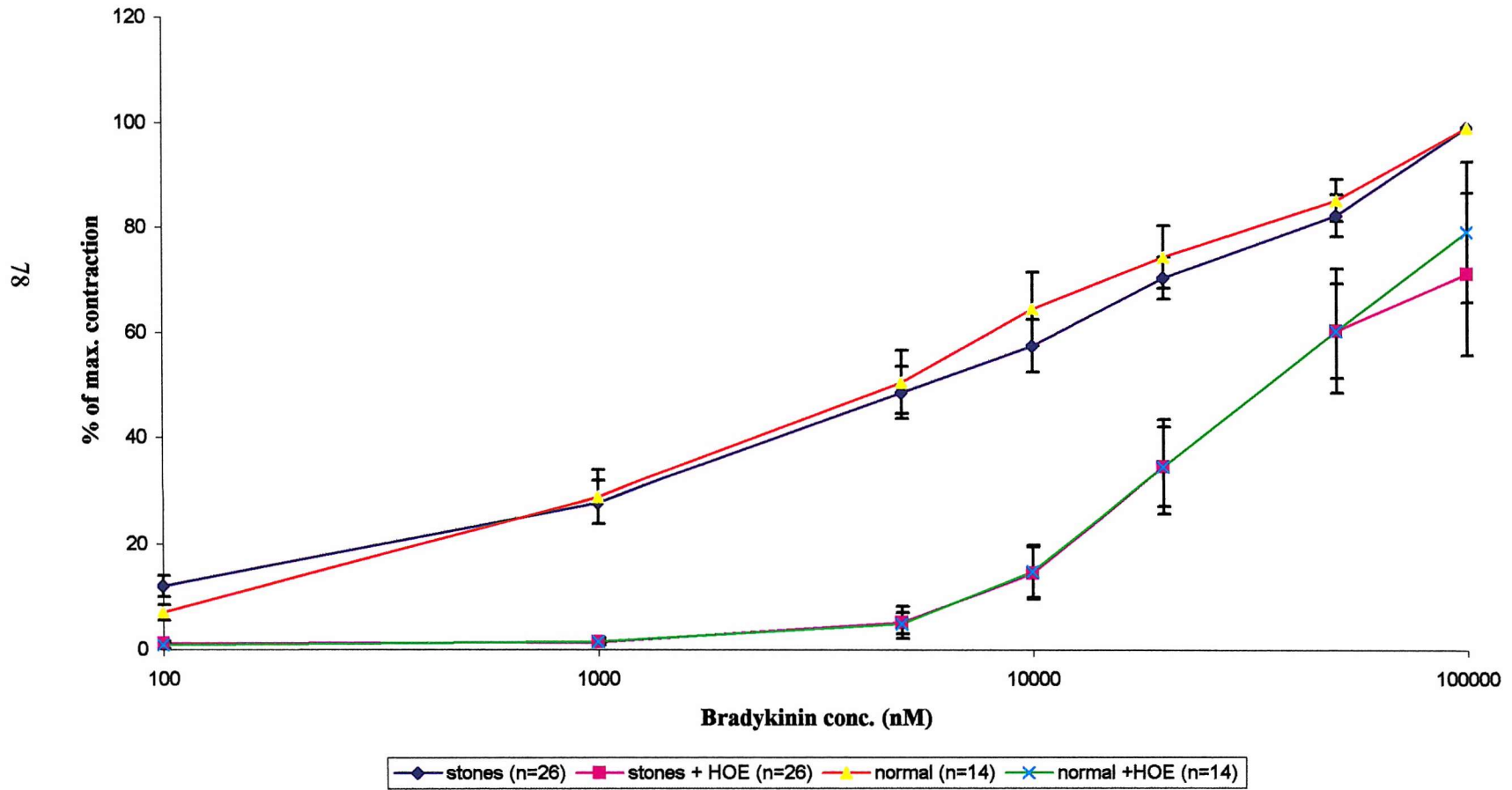
4.5) Combined B1 and B2 Receptor Blockade

4.51) Background

As a logical progression of the work of the experiments with separate B1 and B2 receptor blockade described above, we investigated the response of combined B1 and B2 blockade to see if there was any evidence of an additive effect in strips from normal gallbladders when compared with those from stone bearing gallbladders.

4.52) Method

The same strips used in the last two experiments were repeatedly washed out with fresh Krebs' bicarbonate solution and allowed to re-equilibrate and return to baseline. Those strips which returned to a stable baseline were deemed viable. HOE-140 and des-Arg^9-Leu^8-bradykinin were both added to the organ baths at a concentration of 1 μM. The strips were again allowed to equilibrate for fifteen minutes prior to repetition of the bradykinin concentration response curve in the range 10^{-7} - 10^{-4} M.

4.53) Results

Ten normal strips and 16 stone bearing strips were viable after the previous experiments. There was no evidence of an additive effect with combined B1 and B2 blockade on the bradykinin concentration response in either set of strips when compared with the rightward shift of the bradykinin concentration response curve previously obtained with B2 blockade (HOE-140) alone (table 4.5, figure 4.5) (Appendix D).

HOE-140 (1 μM) + des-Arg^9-Leu^8-Bradykinin (1 μM)								
BK (μM)	0	0.1	1	5	10	20	50	100
Normal (10)	0	0	1.3	6.8	13	39.8	78.9	92.1
s.e.m	0	0	0.8	4	7.2	10.7	9.1	12.1
Stones (16)	0	0.1	0.6	8.3	20.6	44	69	109.4
s.e.m	0	0.1	0.3	3.2	4.9	11.1	12.3	15.6

Table 4.5. Values for Bk CRC's in the presence of HOE-140 and dAL-bk

4.54) Summary

There is no evidence of an additive effect of combined B1 and B2 receptor blockade on the bradykinin concentration response curve when compared with B2 blockade alone, in strips from either normal or stone bearing gallbladders.

Figure 4.5 Bradykinin concentration response curves for strips from normal and stone bearing gallbladders in the presence of both dAL Bk and HOE-140

81
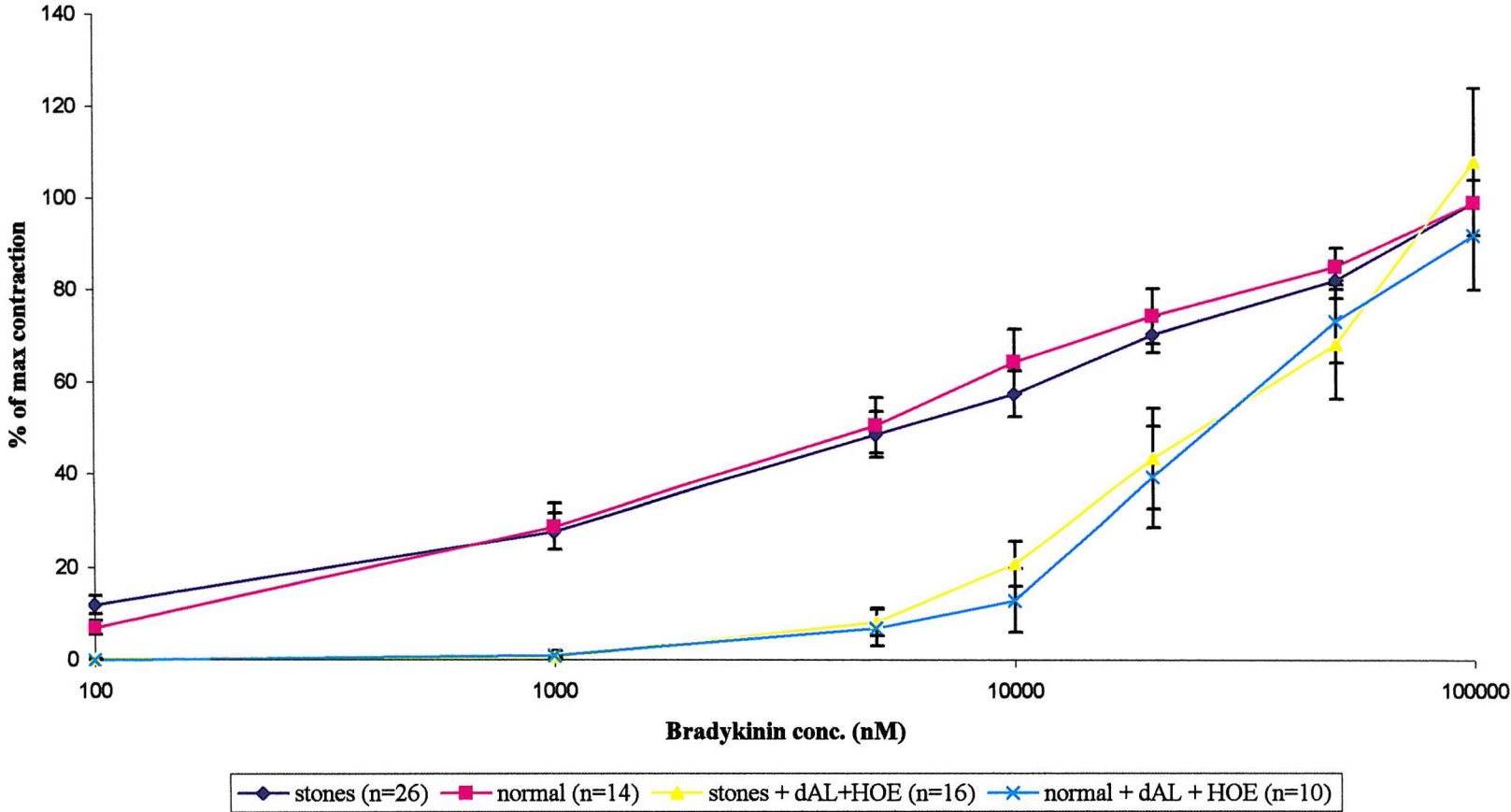
4.6) Repeat bradykinin CRC

4.61) Background

To confirm the viability of the strips and repeatability of the Bk curve after completion of the run of experiments strips which were still deemed viable were subjected to a further bradykinin CRC.

4.62) Method

Viable strips were repeatedly washed out and allowed to return to a stable baseline. After 30 minutes of equilibration viable strips were subjected to a repeat bradykinin CRC.

4.63) Results

14 viable strips remained from the original 40. Repeat bradykinin CRC demonstrated a marked leftward shift of the curve when compared with those generated earlier in the presence of B1 and B2 antagonists (Table 4.6) (Appendix E).

Table 4.6. Mean Bk CRC after washout of antagonists.

Bk	0	0.1	1	5	10	20	50	100
Mean	0	2.7	11.9	27	43	68	96	121

4.64) Summary

Viable strips demonstrate a return towards normal contractility after extensive washout of bradykinin 1 and 2 antagonists even after a prolonged period of experimentation involving repeated bradykinin CRCs.

Figure 4.6 Bradykinin concentration response curves for strips from normal and stone bearing gallbladders in the presence of both dAL-Bk and HOE-140 and then repeated after the washout of antagonists
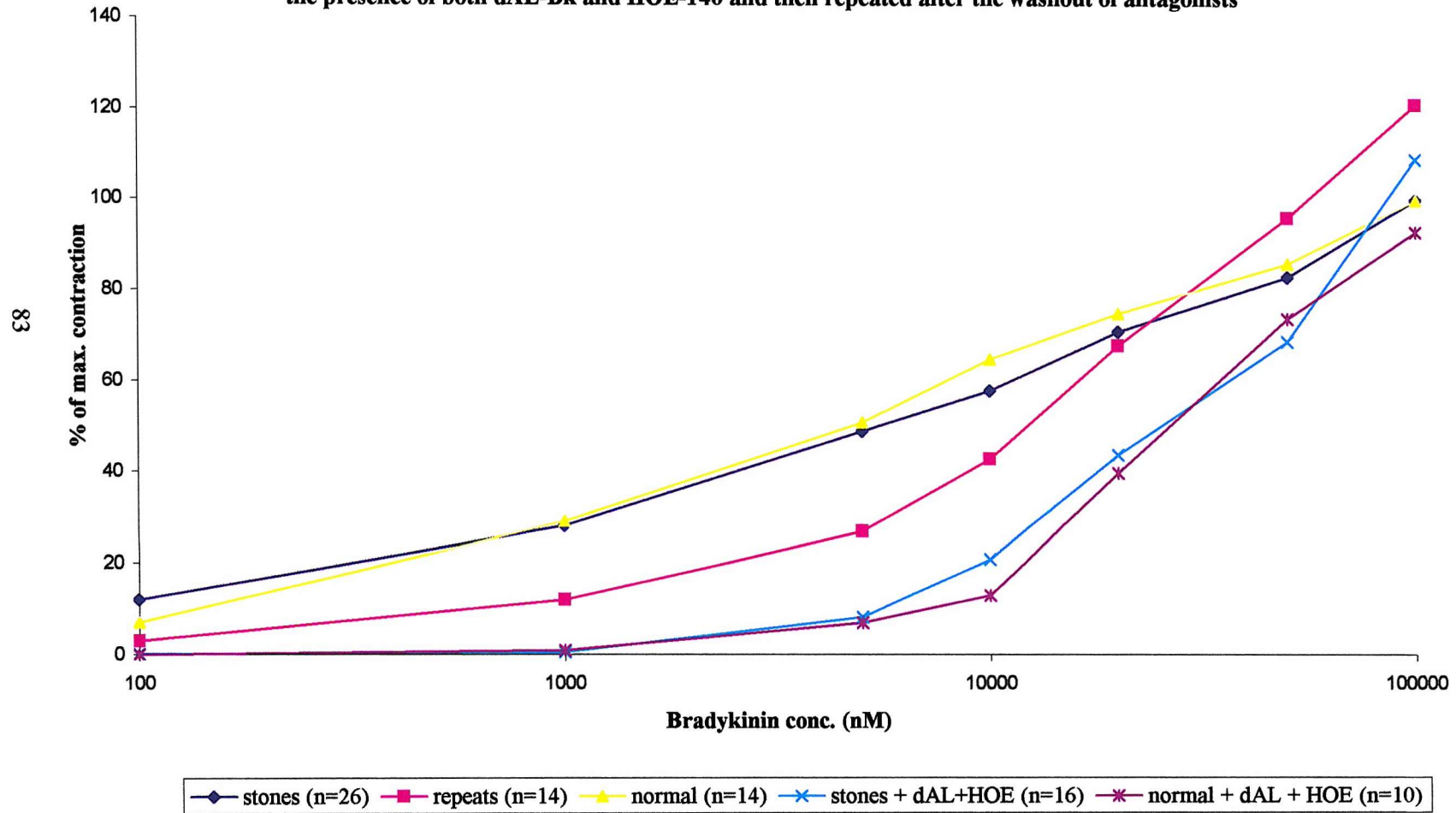
4.7) Conclusions

Bradykinin produces a repeatable dose dependent contraction of human gallbladder strips in vitro in the range 10^{-7}-10^{-4}M.

Strips from stone bearing gallbladders demonstrate a contraction mediated via the B2 receptor, whilst strips from normal healthy gallbladders demonstrate a contraction via both the B1 and the B2 receptors.

Chapter V.

IN VIVO STUDIES OF HUMAN GALLBLADDER

EMPTYING

5.1 Introduction

Many methods exist for the measurement of gallbladder emptying, including oral or intravenous cholecystography, cineradiography, manometry, ERCP, cholescintigraphy and ultrasound. Of these ultrasound and cholescintigraphy are the most frequently used, however they measure different aspects of gallbladder emptying. Cholescintigraphy measures counts of radioactivity coming from labelled compounds excreted in bile; as a result it is a very accurate indicator of bile flow into and out of the gallbladder. Counts have been shown to be very closely related to measurements of gallbladder volume (Masclee et al 1985). Although this gives a very accurate picture of true gallbladder emptying in terms of bile ejection, for the purpose of comparisons with in vitro contractility it is better to use ultrasound since this calculates a true anatomical volume for the gallbladder at an instant in time. Thus comparing serial readings gives a net change in volume. This change in anatomical volume is a closer reflection of changes in muscle fibre length than measurement of bile ejection from the gallbladder which is more correctly a measure of net bile flow out of the gallbladder which may not necessarily relate to change in muscle fibre length within the wall of the gallbladder since it takes no account of any refilling which may occur. As part of the aim of this work is to compare *in vivo* and *in vitro* contractility ultrasound has therefore been preferred as the tool for measuring gallbladder emptying.

The methods used for measurement of gallbladder emptying using the ellipsoid formula have been outlined in chapter 3.

5.2 Validation of results

5.21 Inter observer variability

Ultrasound measurement of gallbladder volume is a relatively easy skill to learn. After an initial period of tuition with the ultrasound scanner, gallbladder emptying in response to a standard fatty meal was assessed in twelve patients with gallstones by both the investigator and also an experienced radiologist, according to the protocol in chapter 3. Scans were performed alternately by the radiologist and the investigator to give two full sets of volume recordings for each patient for the one hour post prandial period (figure 5.21 and appendix F).

Mean change in volume with time expressed as a percentage of fasting volume was similar between the two observers for the group of 12.

Table 5.21 Mean change in volume with time expressed as a percentage of fasting volume for the investigator and a trained radiologist

Time (mins)	0	5	10	15	20	25	30	40	50	60
Inv. Mean	100	87	73	76	74	72	68	67	69	66
S.E.M.	0	4.3	6.0	5.2	6.8	6.8	7.0	7.1	7.1	8.9
Rad. mean	100	88	81	80	78	78	74	73	66	64
S.E.M.	0	3.5	3.9	4.4	6.2	6.8	6.6	7.0	8.3	9.1

(Rad. = radiologist, Inv = investigator).

Recordings of peak gallbladder emptying were also similar between the two observers, particularly in the latter half of the study as the investigators technique improved (figure 5.22).

Subject	1	2	3	4	5	6	7	8	9	10	11	12	Mean	SEM
Investigator	61	49	79	35	64	48	13	28	99	80	26	60	54	7.0
Radiologist	63	62	66	72	85	41	15	21	83	65	22	57	54	6.6
Difference	2	13	13	37	21	7	2	7	17	15	4	3		

Table 5.22

Inter observer variation in peak gallbladder emptying, expressed as a percentage of fasting volume.

Figure 5.21 Comparison of Gallbladder Emptying Measured by the Investigator and a Radiologist
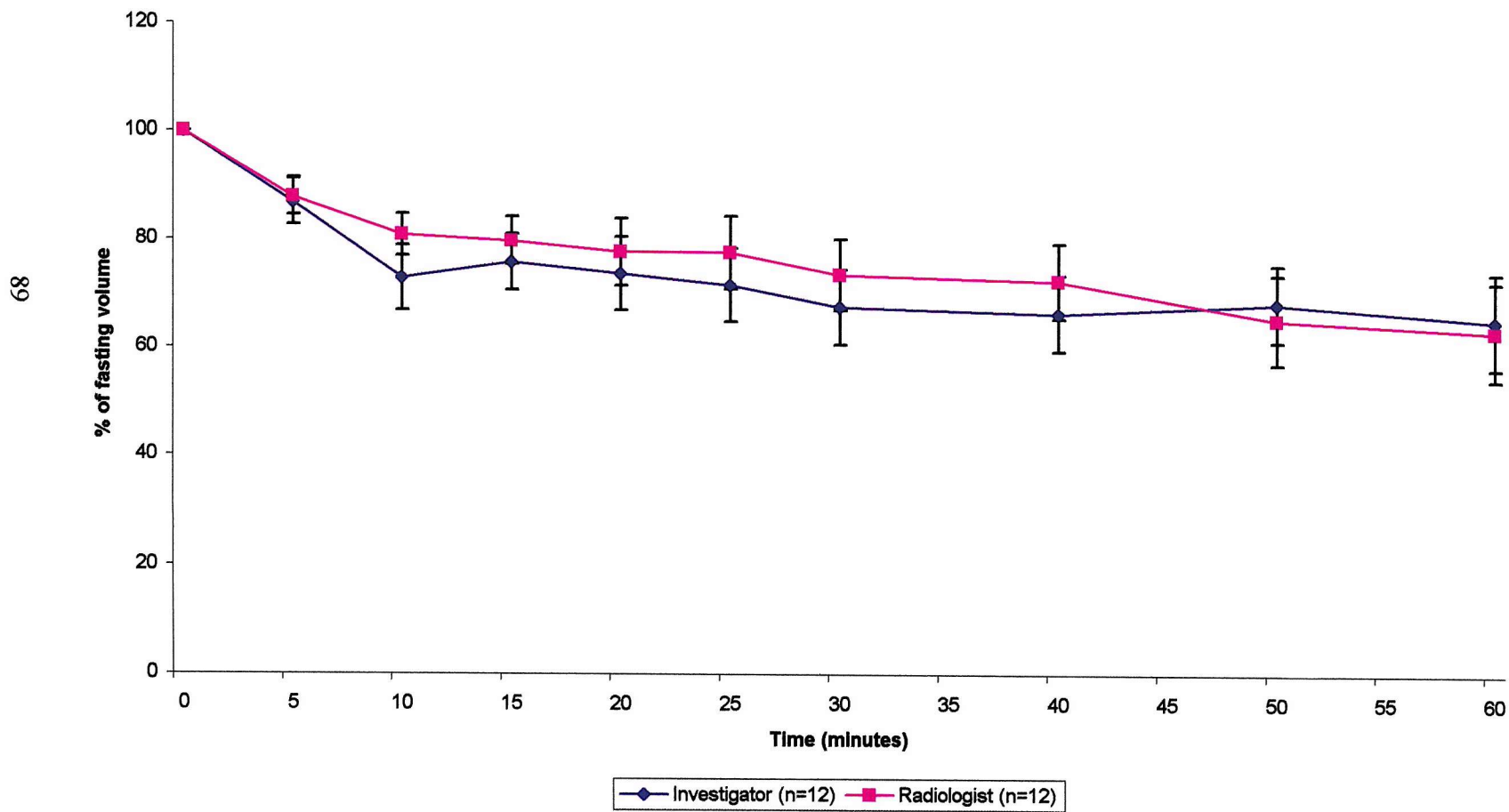
Figure 5.22 Peak gallbladder emptying measured by the investigator and a radiologist

$y = 0.9729x$
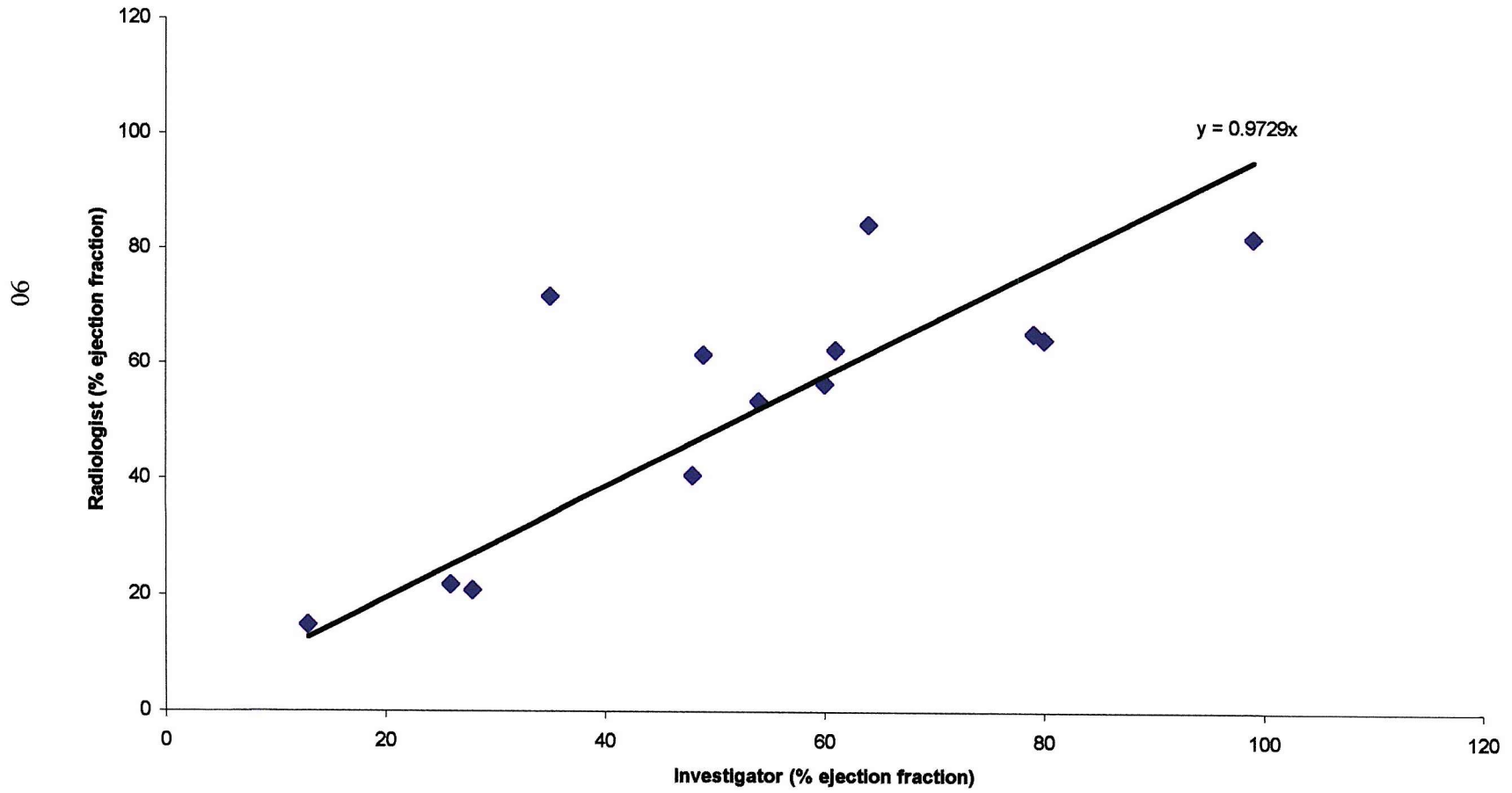
5.22 Observation Period

The initial period of scanning was for sixty minutes following the test meal. During the study it became evident that some patients' gallbladders did not contract significantly during this time, therefore a group of nine patients with gallstones were scanned for a period of ninety minutes following the meal to ensure that there was not a delayed gallbladder contraction that was being missed during the initial sixty minute study period (figure 5.23, Appendix G).

Table 5.23

Change in gallbladder volume in nine patients over a ninety minute period following a standard fatty meal, expressed as a percentage of fasting volume.

Minutes	0	5	10	15	20	25	30	40	50	60	70	80	90
Mean	100	92	82	79	77	69	67	72	72	80	77	81	89
SEM	0	2.2	3.5	5.3	6	7.5	6.4	5.5	5.6	5.9	5.8	4.4	2.7

There was no evidence that a significant delayed gallbladder contraction occurred in any of the nine patients studied, maximum contraction was measured between ten and fifty minutes after the test meal in all of the cases.

Table 5.24

Time, in minutes, to maximum measured gallbladder emptying (T max.) during the ninety minute observation period.

Subject	B1	B2	B3	B4	B5	B6	B7	B8	B9
T max.	25	25	10	50	40	25	25	30	30

Figure 5.23 Evaluation of 90 Minute Observation Period for Gallbladder Emptying
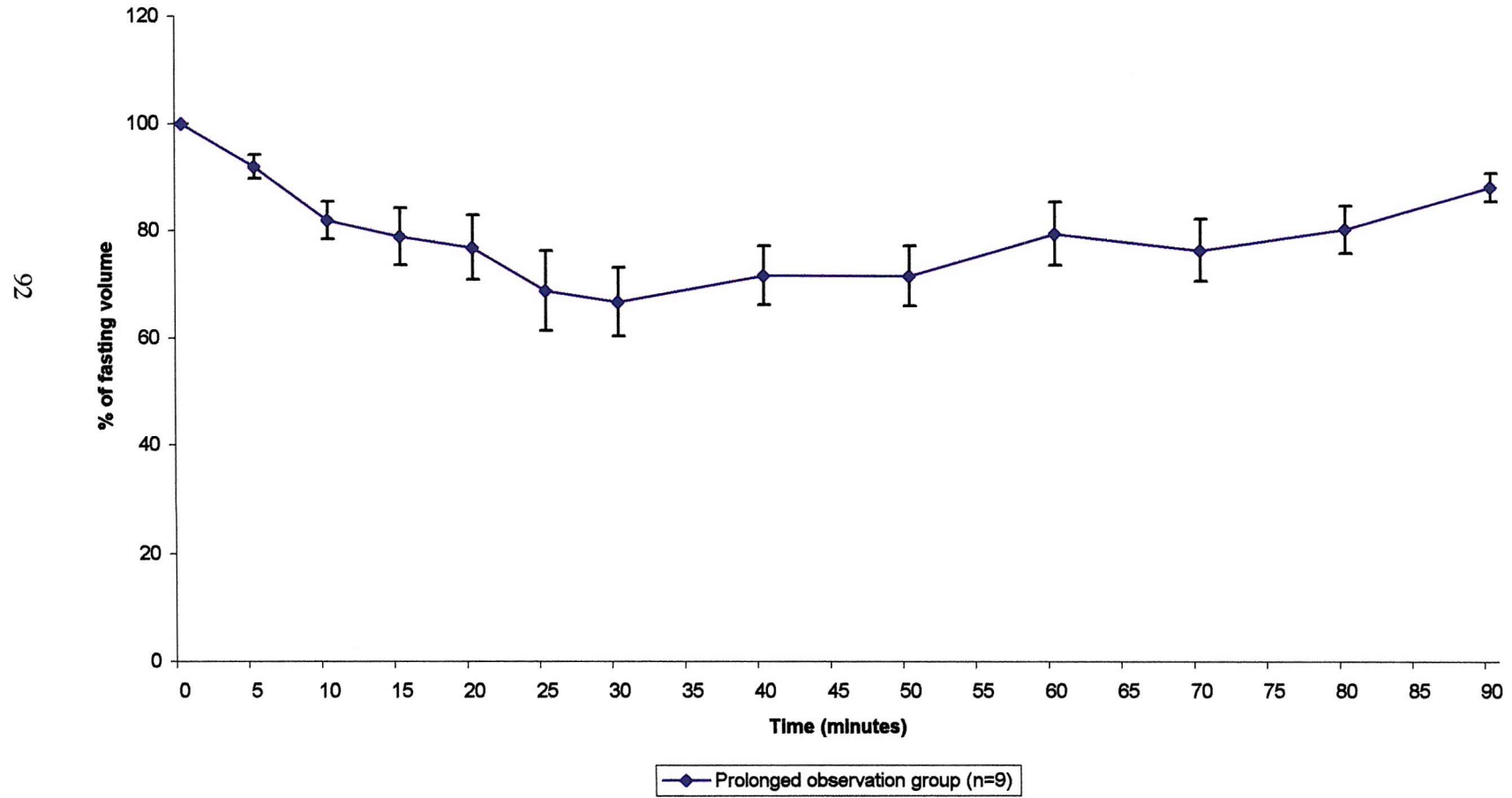
5.23 Repeatability

In order to assess whether findings concerning gallbladder emptying measured on ultrasound were repeatable and consistent, three individuals were each scanned on two separate occasions between three and twelve months apart (figure 5.3, table 5.25).

Table 5.25

Repeat gallbladder emptying profiles in three individuals in response to a standard test meal.

Time (mins)	0	5	10	15	20	25	30	40	50	60
C1	100	96	85	85	58	54	54	42	**38**	38
C2	100	64	75	79	70	33	58	**28**	42	42
C3	100	72	45	43	**25**	30	35	40	47	53
Mean	100	77	68	69	51	39	49	**37**	42	44
Repeats										
C1	100	90	100	76	62	67	57	**39**	47	45
C2	100	92	67	41	58	62	41	**28**	34	41
C3	100	82	51	43	28	26	**25**	41	49	56
Mean	100	88	73	53	49	52	41	**36**	43	47

Similar levels of peak emptying occurring after a similar post-prandial time interval were observed between the paired sets of readings (highlighted in bold on table 5.24) demonstrating the very consistent and repeatable nature of these findings.

Figure 5.24 Assessment of Repeatability of measurement of gallbladder emptying in three subjects
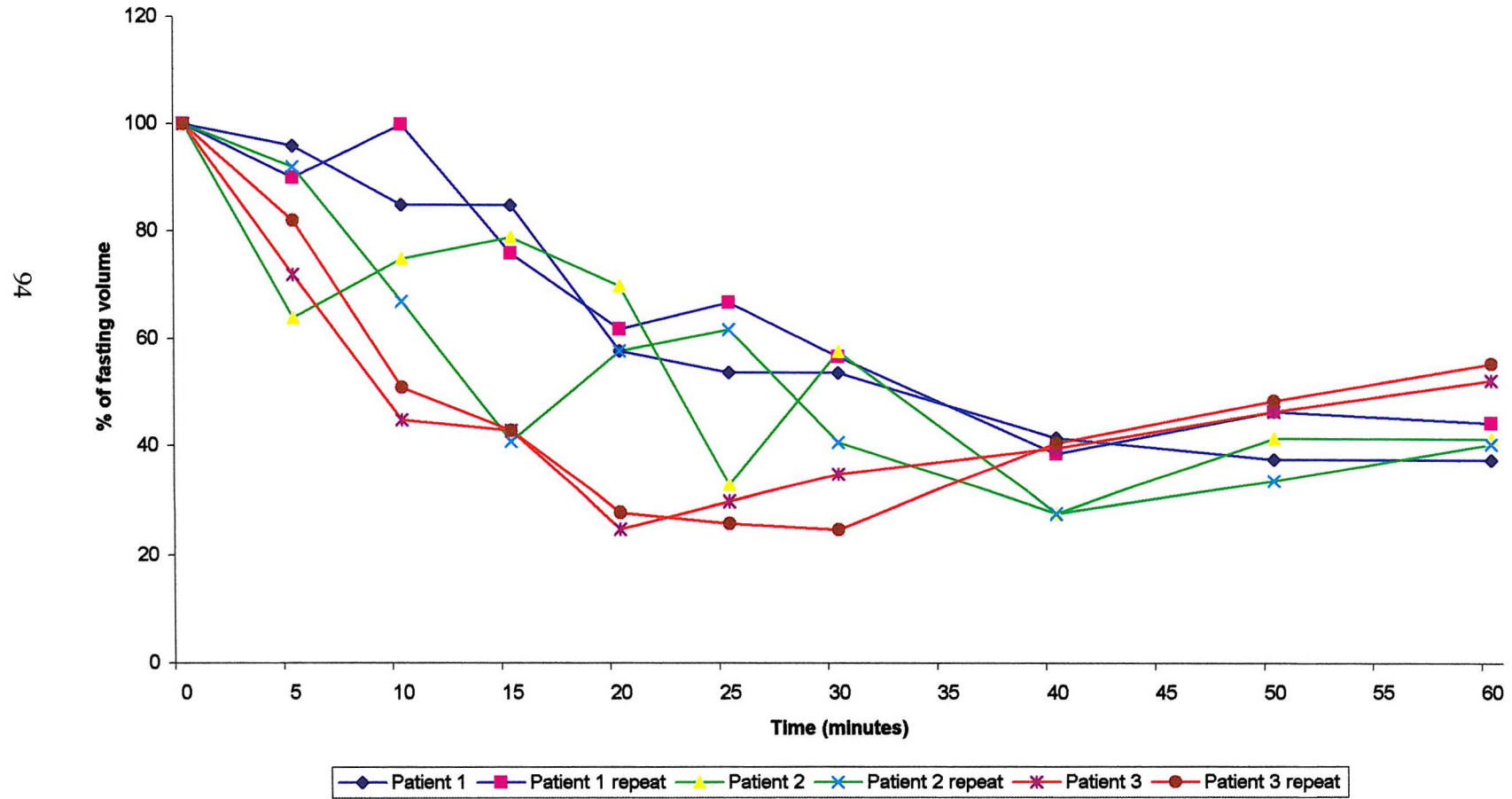
5.24 Conclusions

Ultrasound estimation of gallbladder volume and emptying is a relatively simple technique to learn and perform accurately.

Peak gallbladder emptying occurs within sixty minutes of a standard test meal.

Measurements of gallbladder volume and emptying are consistent and repeatable at least up to twelve months apart.

5.3 Gallbladder emptying in normal individuals

5.31 Background

Gallbladder emptying in normal subjects has been extensively studied (see chapter 1). In order to be able to make comparisons with other patient groups we have studied a group of normal individuals, thus generating a mean profile of gallbladder emptying for use as a control group in further studies.

5.32 Patients

Sixteen healthy subjects were recruited, predominantly staff from within the Southampton University Hospitals NHS Trust. Eight females (age range 17 to 51 years, mean 31 years, median 30 yrs) and eight males (age range 25 to 52 years, mean 34 years, median 31 years) were scanned following ingestion of a standard fatty meal (see protocol in chapter 3).

5.33 Results

Mean fasting gallbladder volume was 20 ml, range 6.9 - 33 ml, median 22ml.

Mean peak gallbladder emptying was 60% of fasting volume (S.E.M. 6.4%, range 32-75 %). (Appendix H, table 5.31, figure 5.31).

Table 5.31

Mean gallbladder emptying profile in 16 normal individuals after a standard test meal.

Time (mins)	0	5	10	15	20	25	30	40	50	60
Mean n=16	100	78	69	60	56	53	52	51	52	58
S.E.M.	0	3.2	4.2	4.6	4.2	3.2	3.4	3.6	3	3.8

5.34 Discussion

These results are consistent with those reported by other authors (Everson et al 1980, Portincasa et al 1994) and provide a normal control population for comparison with gallstone and IBS patients. Portincasa had previously used an ejection fraction of 46% as his cut off point for normal gallbladder contractility, with those individuals with an ejection fraction below this level termed as "hypocontractors". In this study 14 out of 16 of the normal individuals demonstrated gallbladder emptying at least equal to this level and 13 out of 16 achieved 50% or greater emptying. This latter figure was subsequently used for categorisation of gallbladder emptying as good or poor (Ch 5.4).

Figure 5.31 Gallbladder emptying in normal individuals (mean +/- SEM)
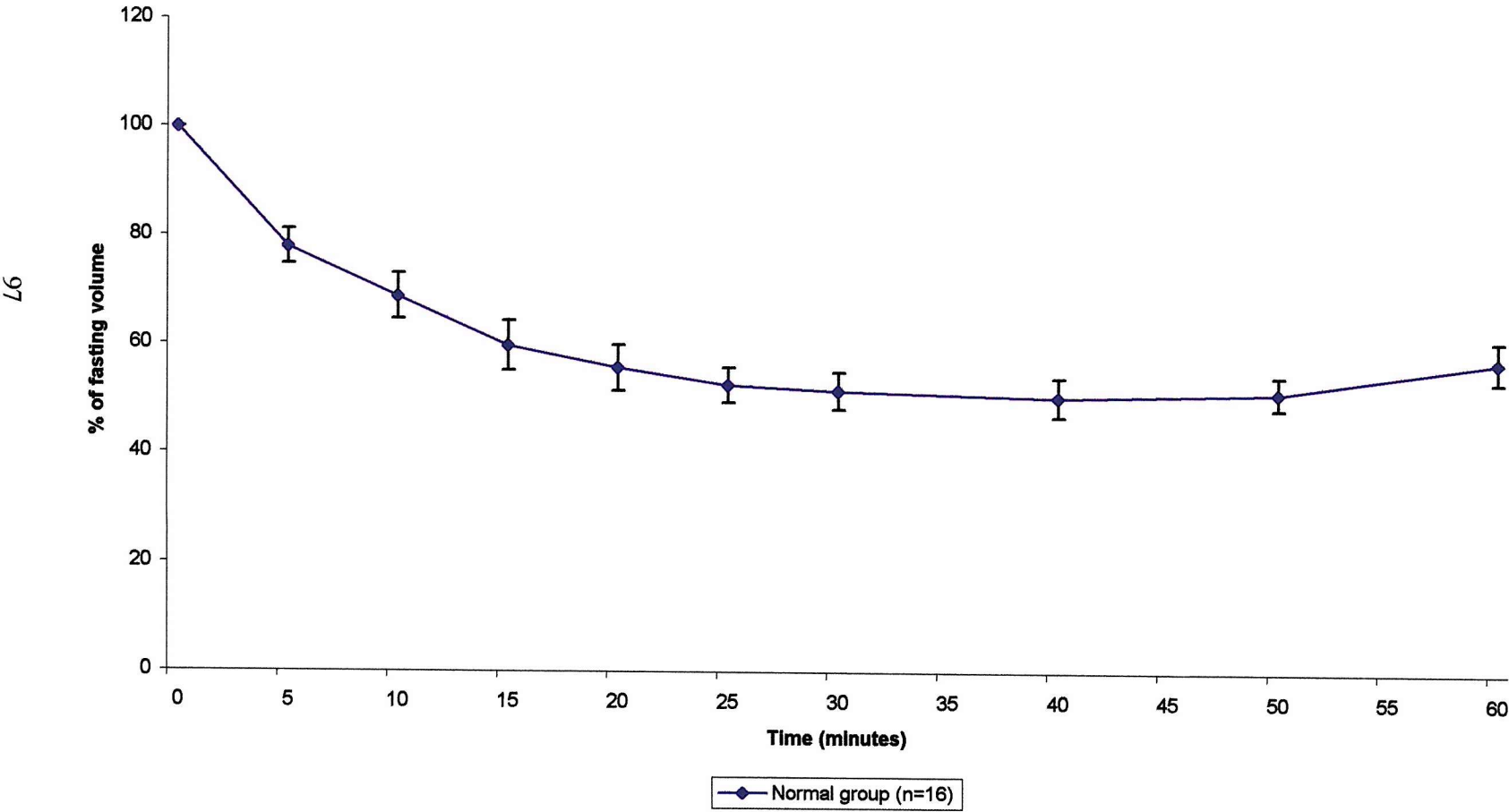
5.4 Gallbladder emptying in patients with gallstones

5.41 Background

A large subgroup of patients with gallstones has reduced gallbladder emptying in response to a standard fatty meal. The majority of these patients have cholesterol or mixed stones. Within this group there is a further proportion that appears to have little or no demonstrable gallbladder emptying in response to a meal. This part of the study aims to describe these three groups of patients (good contractors, poor contractors and non contractors) within the population of patients with symptomatic gallstones awaiting elective cholecystectomy in Southampton.

5.42 Patients

One hundred and twenty three patients were contacted, 71 patients were scanned, six failed to meet the inclusion criteria outlined in chapter three (1 post ERCP and sphincterotomy, 1 unable to visualise the gallbladder satisfactorily and 4 where the gallbladder was too full of stones) leaving 65 who completed the protocol (51 female age range 26-76 years median 55yrs, 14 male age range 40-76 yrs, median 65).

5.43 Results

Fasting gallbladder volumes were recorded with a mean of 27 ml, median 23 ml and range 9.2 to 90 ml.

A wide range of gallbladder emptying was observed (0-80%), mean peak emptying was 37% (median 36%, S.E.M. 2.56).

The patients were divided into three groups according to the degree of emptying of their gallbladders in response to the test meal. Patients whose gallbladder volume fell by at least 50% were termed good contractors (n=18), patients whose gallbladders

98

emptied by between 15 and 50% were termed poor contractors (n=36) and those whose gallbladders emptied by less than 15% were termed non contractors (n=11). (Table 5.4, Figure 5.4, Appendix I)

Table 5.4

Mean gallbladder emptying profiles for gallstone patients.

Time (mins)	0	5	10	15	20	25	30	40	50	60
Good con (n=18).	100	84	76	64	55	47	48	46	51	53
S.E.M.	0	3.2	3.9	3.2	2.9	3.0	3.1	2.8	3.6	4.7
Poor con. (n=36)	100	90	85	84	80	82	79	78	80	81
S.E.M.	0	1.2	1.8	1.7	2.1	2.3	2.0	2.1	2.3	2.9
Non con. (n=11)	100	97	98	97	97	98	98	100	99	98
S.E.M.	0	0.8	1.1	1.1	0.9	0.7	1.0	0	0.8	0.8

Figure 5.4 Gallbladder emptying in gallstone patients (mean +/- SEM)
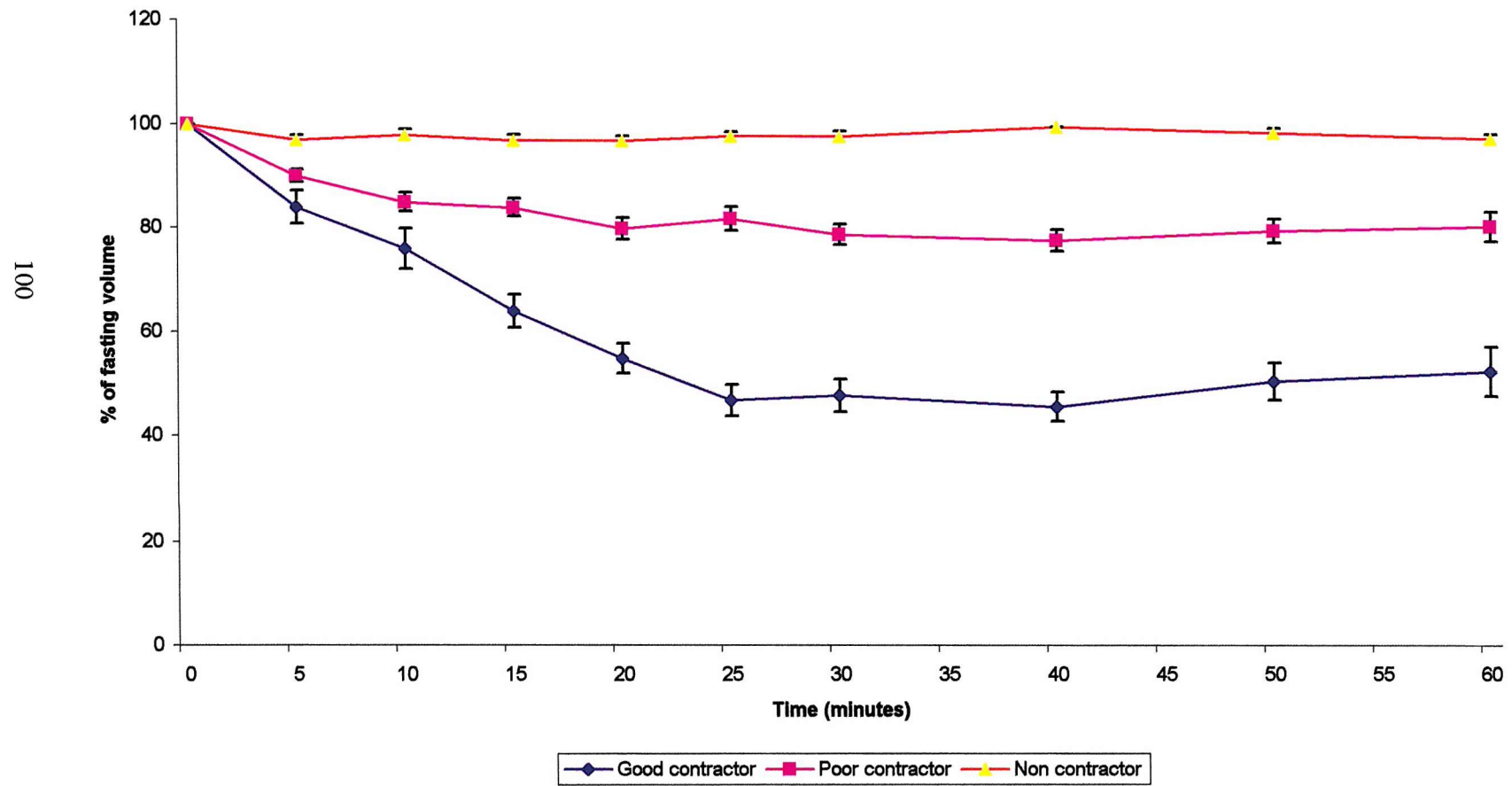
5.44) Discussion

These results confirm the findings of many previous authors (Patankar, Portincasa, Everson) that patients with gallstones have lower levels of emptying in response to a standard fatty meal than normal individuals. The question remains unanswered from this experiment as to whether this is an underlying abnormality that predisposes to stone formation or a consequence of the presence of gallstones.

5.45) Conclusion

The majority of gallstone patients have decreased levels of gallbladder emptying in response to a standard fatty meal when compared with the normal population.

Chapter VI.

GALLBLADDER EMPTYING IN IBS PATIENTS

6.1 Background

The description of the symptoms of functional intestinal disorders such as irritable bowel syndrome (IBS) shows a marked overlap with the symptoms of gallstone disease. The largest study of biliary symptoms and gallstones (GREPCO 1988), has shown that using a definition of biliary pain as abdominal pain lasting more than half an hour and situated in the right hypochondrium or epigastrium there is almost as high a prevalence of biliary pain in patients without gallstones as in those who have them. Wegge et al (1985) studied patients admitted to a surgical unit with upper abdominal pain, there was little difference in description of the pain between patients with gallstones and those without, except patients with gallstones described more attacks of similar pain, more fatty food intolerance and on examination were more likely to be tender over the gallbladder. Further population screening studies by Glambek et al (1989) and Jorgensen (1989) both observed similar levels of abdominal symptoms in individuals irrespective of the presence of gallstones.

The close integration of the physiological regulatory mechanisms for the gallbladder and intestine make the overlap of biliary and IBS symptoms unsurprising. Patients with IBS demonstrate visceral hypersensitivity (Ritchie et al, 1973) and hypermotility; there is some evidence to suggest that this extends to the gallbladder (Braverman 1987). A study by Kellow (1987) demonstrated abnormal sensitivity of the gallbladder to exogenous CCK-8 in patients with IBS. Previous work by Harvey and Read demonstrated that intravenous CCK reproduced the abdominal pain of a proportion (8 out of 20) of patients with IBS particularly those who related their attacks of pain to food. They also identified increased colonic motor activity in response to the CCK.

Braverman (1987) suggested that a proportion of patients with irritable bowel syndrome have an increased level of gallbladder emptying in response to a test meal, possibly reflecting the generalised increase in gastro intestinal smooth muscle tone seen in this condition. This part of the study aims to document this in a larger group of patients some of whom have also undergone simultaneous analysis of plasma cholecystokinin levels, to assess whether any observed change in gallbladder emptying

103

is a reflection of an altered level of this hormone when compared with either the normal population or gallstone patients.

6.2 Patients and Methods

Fifteen patients with irritable bowel syndrome defined according to the Rome criteria (Thompson et al 1989) were identified and entered into the study; there were 6 males (age range 28 to 58 years, mean 48 yrs) and 9 females (age range 22 to 67 years, mean 43 yrs). They reported a variety of symptoms typical of irritable bowel syndrome (Table 6.2)

Table 6.2

Irritable bowel syndrome symptoms

Symptoms	Number of patients reporting symptom (n=15)
Diarrhoea	10
Constipation	4
Alternating diarrhoea / constipation	1
Bloating	11
Lower abdominal pain	13
Upper abdominal pain	2
PR mucus	6

Each individual underwent sonographic estimation of gallbladder emptying in response to a standard test meal according to the protocol outlined in chapter 3. Blood samples were taken for plasma CCK-8 analysis at 0, 15, 30, 45 and 60 minutes from eleven of the patients.

The normal group of sixteen healthy individuals assessed in the previous chapter were used as a control group (8 males, 8 females, age range 17-52 years, mean 33 years, median 31 years). For comparative CCK-8 analysis blood was taken from all sixteen

members of the healthy control groups according to the protocol outlined in chapter three and plasma CCK-8 assay was performed.

6.3 Results of gallbladder emptying

The IBS patients had a mean fasting gallbladder volume of 20ml (S.E.M. 1.9ml, range 8.9 to 36.5 ml, median 18ml) and a mean peak emptying of 72% (S.E.M. 6.0%, range 17 to 94%, median 80%). figure 6.1, Appendix J, table 6.2.

Table 6.3

Mean gallbladder emptying profile for 15 irritable bowel syndrome patients.

Time mins.	0	5	10	15	20	25	30	40	50	60
Mean	100	84	67	55	46	39	40	39	41	48
S.E.M.	0	3.2	5.4	6.9	7.0	6.9	8.1	8.1	7.9	7.7

The results for IBS patients were compared against values for gallbladder emptying recorded in chapter 5 for normal individuals and gallstone patients, looking specifically at the levels of peak emptying recorded for individual patients in the three groups (figure 6.32) and also comparing the emptying profiles of normal and IBS patients (figure 6.33).

6.4) CCK-8 levels in IBS patients and normal individuals

Patients with IBS had higher fasting levels of CCK-8 (mean 2.14 pM, S.E.M. 0.28pM) than normal control patients (1.25 +/- 0.31 pM) and also exhibited higher peak levels (7.11 +/- 0.69pM) of CCK-8 in response to a standard fatty meal than normal controls (3.58 +/- 0.29pM) (figure 6.4).

6.5) Discussion

Irritable bowel syndrome patients recorded the eight highest peak emptying values out of the whole of the study (figure 6.32), although the presence of two poor contractors within the group of fifteen reduced the overall mean peak emptying values for the sample as a whole. The difference between the two mean peak emptying values for the IBS and normal samples has a p value between 0.1 and 0.05 on a two tailed, unpaired t-test thus failing to achieve significance at the 95% level.

The polarisation of extremes of gallbladder emptying seen in the IBS patients appears similar to that seen in bowel function in IBS. However there was no evidence of correlation between any particular IBS symptom and particularly high or low levels of gallbladder emptying, in particular there was no relationship apparent between either constipation predominant IBS and low emptying or between diarrhoea predominant IBS and high levels of peak gallbladder emptying.

The high levels of CCK-8 recorded in some IBS patients in this study have not previously been reported and add weight to the theory that in a subgroup of patient's cholecystokinin plays a role in the pathogenesis of this condition. These observations raise the question of whether this is a primary cause of the observed symptoms or whether it is merely a secondary incidental finding. Harvey and Read (1973) have previously documented increased colonic motor activity and reproduction of abdominal symptoms in IBS patients after intravenous CCK administration which lends weight to the proposition that it has a causative role. The next issue is what triggers the elevated levels, is it simply excess secretion, or is it a failure of the normal negative feedback inhibition or finally is it a defect in the metabolism of cholecystokinin which normally undergoes extensive (>50%) first pass breakdown in the liver before reaching the systemic circulation.

IBS patients attained peak gallbladder emptying faster than normal individuals (25 vs 40 minutes), this may be related to the rapid gastric emptying seen in this group, however this finding has not been previously documented and merits further study.

6.4 Conclusions

Patients with IBS exhibit a wide range of gallbladder emptying, however a large subgroup demonstrate levels of emptying higher than that found within the normal population. This may reflect the generalised increase in gastro intestinal smooth muscle tone seen in this condition.

High levels of peak and fasting CCK-8 demonstrated in some IBS patients raise the possibility of a contribution by this hormone to the pathogenesis of this condition.

Figure 6.31 Gallbladder emptying in IBS
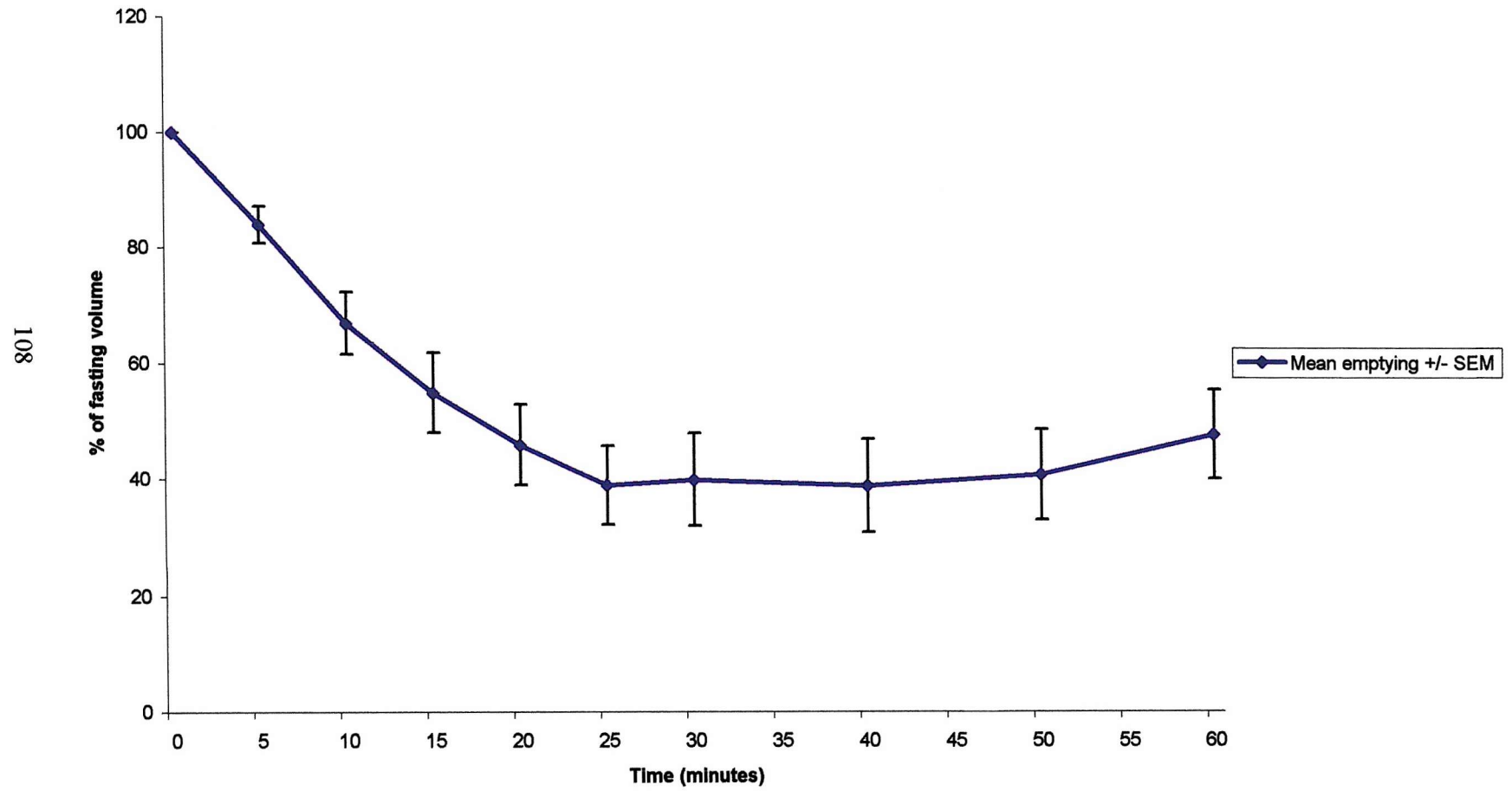
Figure 6.32 Peak Gallbladder emptying in Normal, IBS and Gallstone Patients
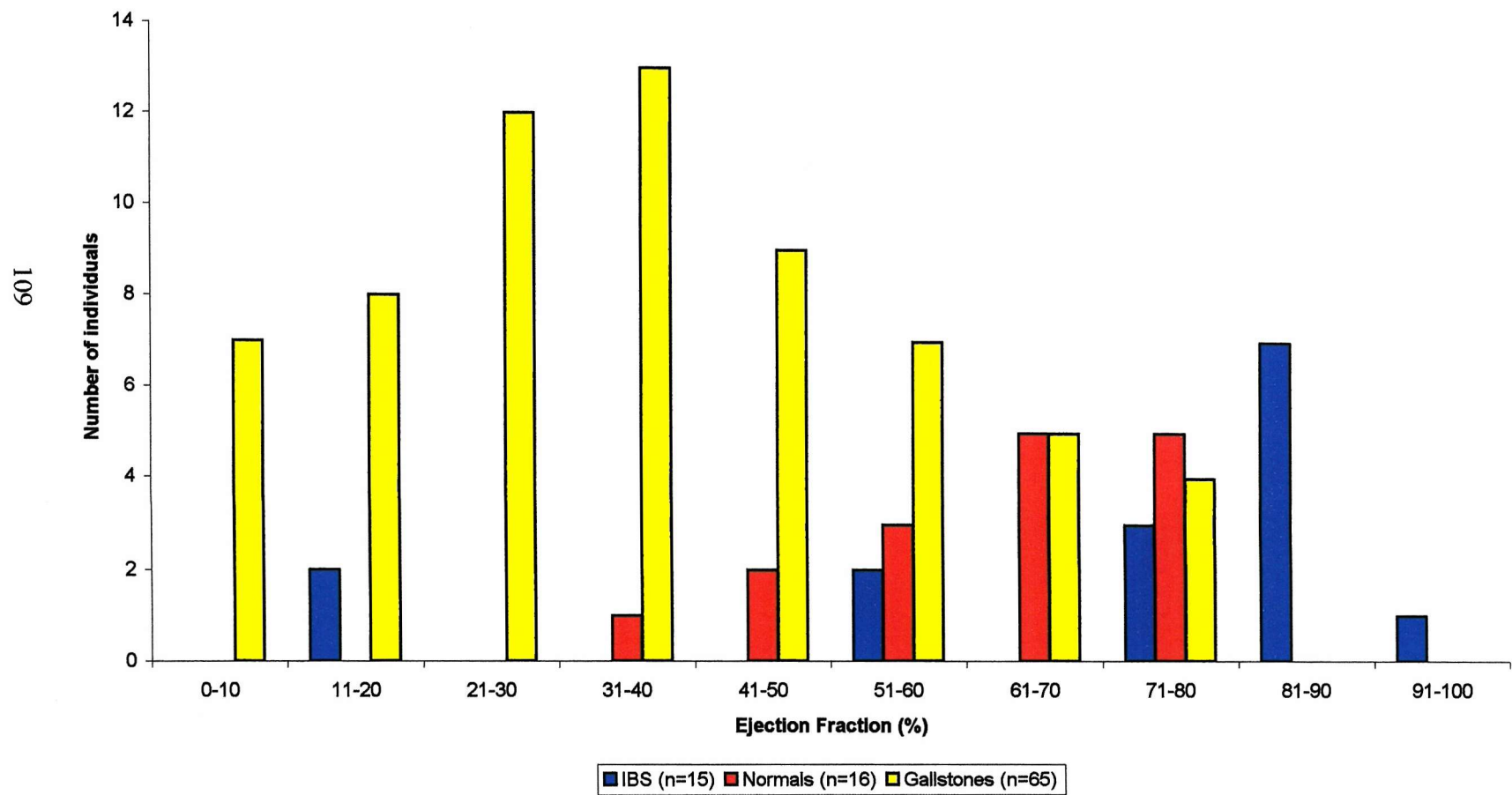
Figure 6.33 Gallbladder emptying in normal and IBS patients (mean +/- SEM)
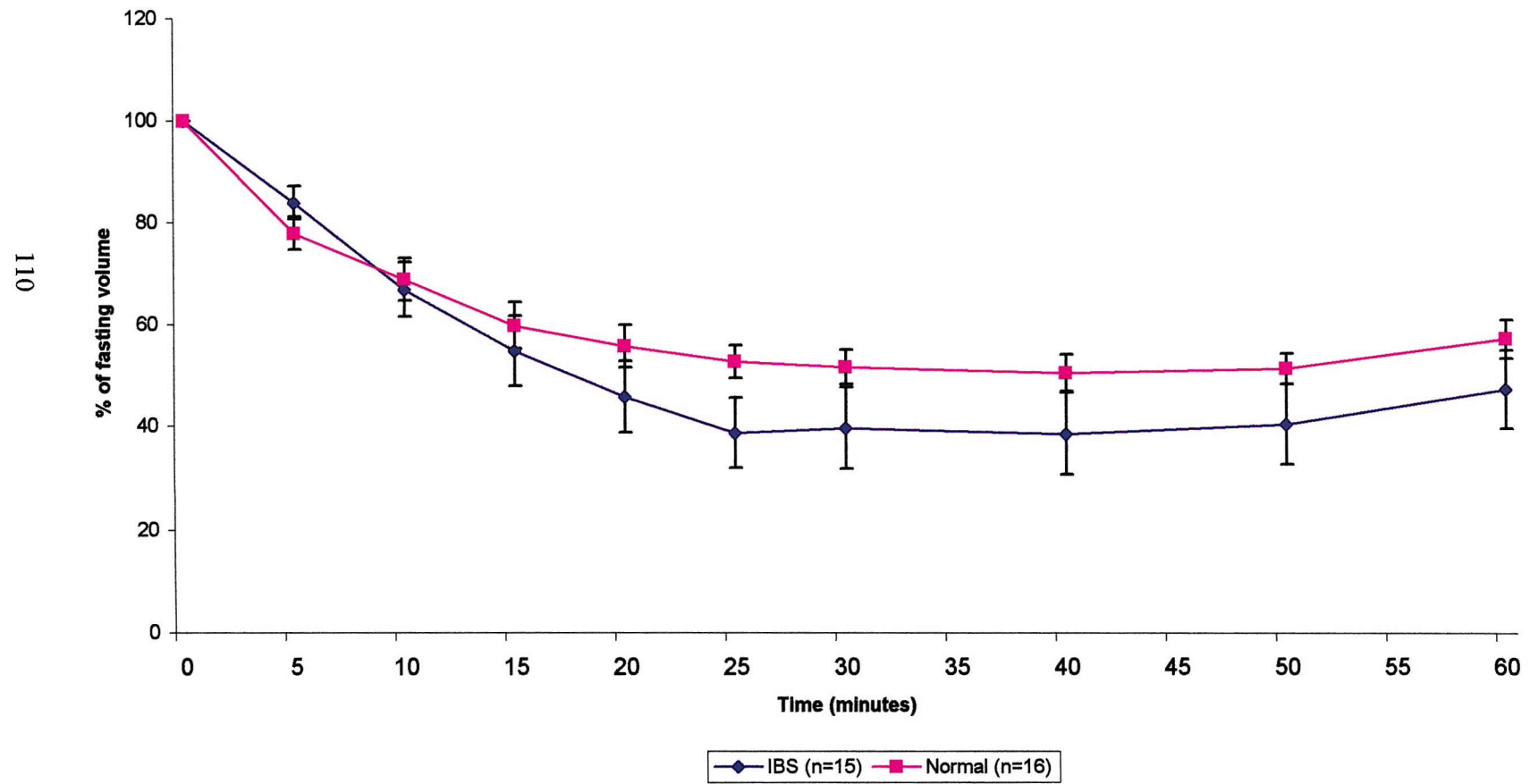
Figure 6.4 Plasma CCK levels in IBS patients and normal controls in response to a standard fatty meal

111
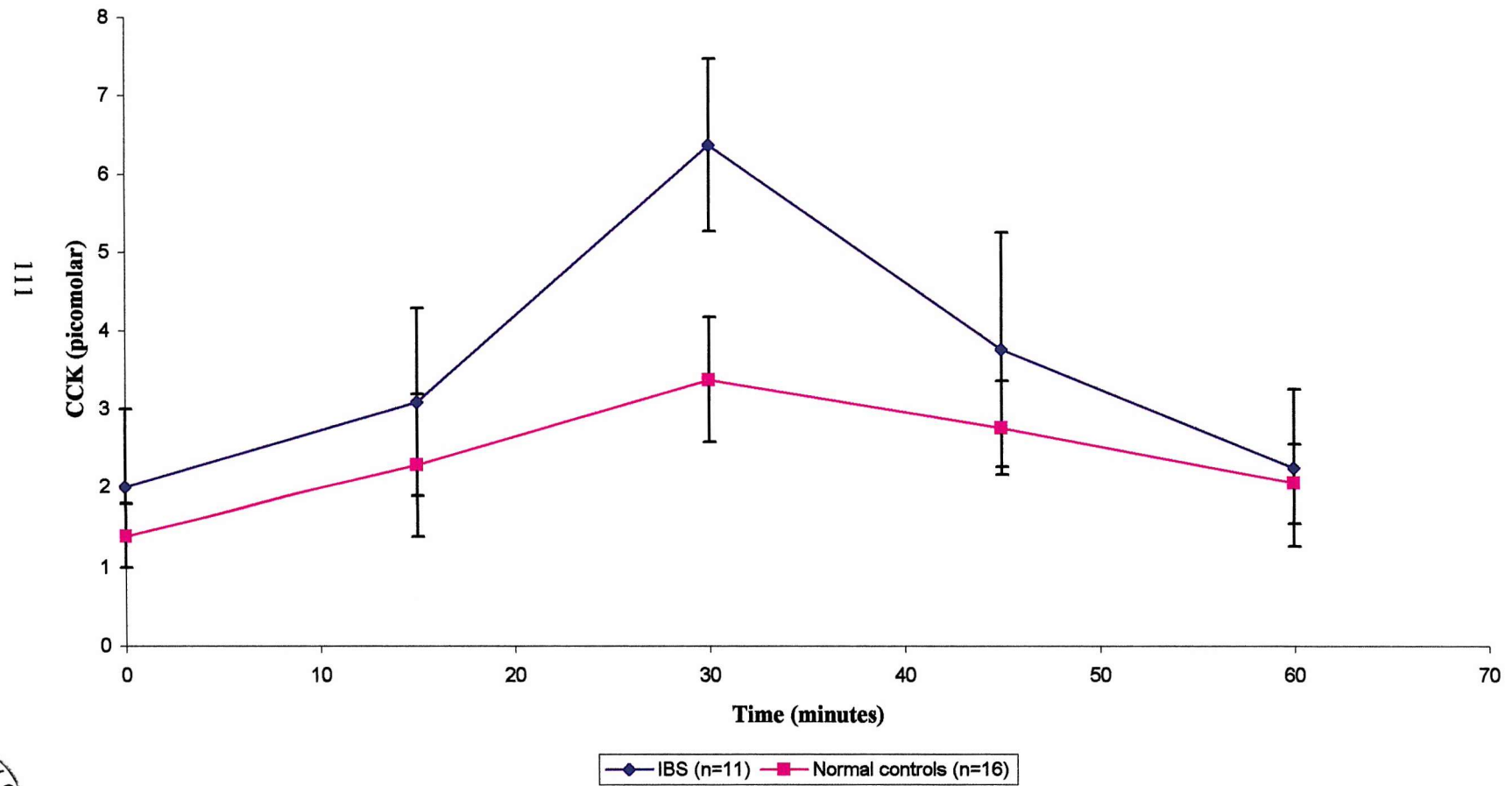
Chapter VII.

A COMPARISON OF *IN VIVO* AND *IN VITRO* GALLBLADDER CONTRACTILITY

7.1) Background

In vivo and *in vitro* gallbladder contractility have been studied individually in great detail over the last thirty years, however there is remarkably little work in the medical literature comparing the *in vivo* emptying and *in vitro* contractility of the human gallbladder. It is clearly important to demonstrate that results obtained *in vitro* can be applied to the *in vivo* state and hence to clinical situations. The most substantial comparison of *in vivo* and *in vitro* contractility has been carried out by Portincasa (1994) who demonstrated a strong relationship between gallbladder emptying measured on ultrasound in response to a standard fatty meal and subsequent *in vitro* contractility of gallbladder strips, measured using isometric equipment in response to CCK. Portincasa identified two groups of patients whom he termed contractors and hypocontractors according to whether *in vivo* peak gallbladder emptying in response to a meal was greater or less than 50% of fasting volume. Strips from patients who were hypocontractors required a significantly higher dose of CCK-8 *in vitro* to achieve half maximal response (ED$_{50}$) than strips from contractors and at each agonist concentration strips from contractors generated greater force than did strips from non contractors. Further work by Wegstapel (1998) has also demonstrated a correlation between *in vivo* contractility measured on ultrasound in response to a standard fatty meal and *in vitro* contractility in response to CCK measured using isometric apparatus. Although a significant criticism of Wegstapels work is that he did not have very stringent exclusion criteria. He recorded patients with gallbladders completely filled with stones as non contractors and also recorded patients whose volume measurements were incomplete as non contractors, whilst in his *in vitro* work he included strips from a gallbladder that showed no response to KCl as well as strips from gallbladders that were very small, shrunken and fibrous.

7.2) Patients and Methods

Patients awaiting elective cholecystectomy for cholelithiasis were recruited into the study (see chapter 5). 68 patients had an ultrasound assessment of *in vivo* gallbladder emptying in response to a standard fatty meal, measured using the ellipsoid method on

113

inbuilt computer software within the scanner, as outlined in chapter 5. 42 of these patients subsequently underwent cholecystectomy during the study period and from these tissue from 30 gallbladders was obtained for *in vitro* analysis of contractility (12 were missed through a variety of reasons, predominantly concerning timing of operation and availability of equipment). A segment of each gallbladder fundus was transported in cold Krebs solution to the laboratory and 10mm by 2mm strips were dissected out and set up in an organ bath under physiological conditions for a period of equilibration and viability testing (chapter 3). Forty viable strips were obtained from 18 gallbladders. Tissue from twelve gallbladders failed viability testing. On the basis of ultrasound assessment of gallbladder emptying patients were divided into three categories; good contractors (peak ejection fraction of 50% or greater), poor contractors (peak ejection fraction between 10% and 50%) and non contractors (peak ejection fraction of 10% or less).

The 18 viable gallbladders came from 16 female and 2 male patients, age range 26 to 76 years (mean 53 years, median 54 years). Sixteen of the patients underwent laparoscopic cholecystectomy and two had an open cholecystectomy. On macroscopic appearance three patients had pigment stones and the remainder had cholesterol or mixed stones.

Viable strips of gallbladder from patients from each of these groups were then used to generate *in vitro* concentration response curves for bradykinin range 10^{-7}-10^{-4}M (-chapter 4) and then after washout and a further period of equilibration for cholecystokinin octapeptide in the range 10^{-9}-10^{-6}M. The responses at each level were recorded in millivolts using isotonic transducers (chapter 3). The transducers were calibrated at a level of 50mV:1mm contraction.

7.3) Results

7.31) Sonography

Full ultrasound data was obtained on all 18 patients (table 7.31). Three patients had gallbladder emptying of 50% or greater and were termed good contractors, eleven patients displayed peak gallbladder emptying between 10 and 50% and were termed poor contractors, the remaining four patients had emptying of less than 10% and comprised the non contractor group.

The mean fasting gallbladder volume was 27.4 ml (s.d. 7.7ml, median 28ml, range 12-42 ml).

The mean peak gallbladder emptying was 32% (s.d. 16.6%, median 31.5%, range 4-59%).

The mean change in gallbladder volume was 8.2ml (s.d. 5.8ml, median 7.1ml, range 1-20ml).

The mean time taken to attain peak emptying following ingestion of the standard fatty - meal was 28 minutes (s.d. 13.7 minutes, median 20 minutes, range 10-50 minutes).

Table 7.31 *In vivo* ultrasound measurements of gallbladder emptying (Ej. fn.) in response to a standard fatty meal, in eighteen patients with gallstones

Patient	Age	Sex	T. max minutes	Ej. fn. %	Fasting vol. ml	Residual vol. ml	Ejection Vol. ml
1	28	f	50	24	31.6	24	7.6
2	46	f	40	25	24	18	6
3	37	f	25	44	25	14	11
4	72	f	10	9	23	21	2
5	60	f	20	4	28	27	1
6	74	f	15	50	32	16	16
7	70	m	10	19	14	11.4	2.6
8	34	f	20	41	12	7.1	4.9
9	57	f	20	28	23.5	17	6.5
10	26	f	20	11	19	17	2
11	64	f	30	29	35	25	10
12	58	m	40	59	26.7	11	15.7
13	49	f	40	41	32	19	13
14	34	f	25	34	35	23	12
15	26	f	20	4	27	26	1
16	50	f	50	56	36	16	20
17	64	f	15	10	42	38	4
18	48	f	50	44	28.3	16	12.3

T max. = the time at which peak gallbladder emptying was recorded.

Resid. Vol. = residual volume at time of peak contraction.

7.32) *In vitro* contractility

Forty viable strips were obtained from 18 gallbladders, all of these strips were subjected to increasing concentrations of bradykinin to generate concentration response curves. After this the strips were washed out and allowed to return to baseline, 27 strips remained viable and were subsequently used to produce concentration response curves for CCK-8.

There were 6 viable strips from the 3 good contractors, 26 strips from the 11 poor contractors and 8 strips from the 4 non contractors.

Bradykinin

Concentration response curves for bradykinin for the three groups showed similar mean responses when expressed as a percentage of maximum contraction (Appendix K) Figure 7.32.

Table 7.32 Mean % (+S.E.M.) contraction of strips from non, poor and good contracting gallbladders in response to increasing concentrations of bradykinin.

Bk conc. μM	0	0.1	1	5	10	20	50	100
non (n=8)	0	7.8 (11.1)	18.3 (14.1)	42.8 (19.7)	52 (20)	61 (19.6)	87 (9.8)	100
poor (n=26)	0	9.4 (11.7)	22.3 (20.8)	38.7 (20.9)	53.3 (18)	69.7 (15.9)	83.2 (15)	100
good (n=6)	0	6.3 (5.3)	18.7 (11.3)	42.5 (22.8)	59.3 (28.8)	77	85 (16.8)	100

There were no significant statistical differences between the levels of mean contraction for the three groups at any given concentration of bradykinin.

Figure 7.31 Bradykinin Concentration Response Curves for strips from good, poor and non contractors
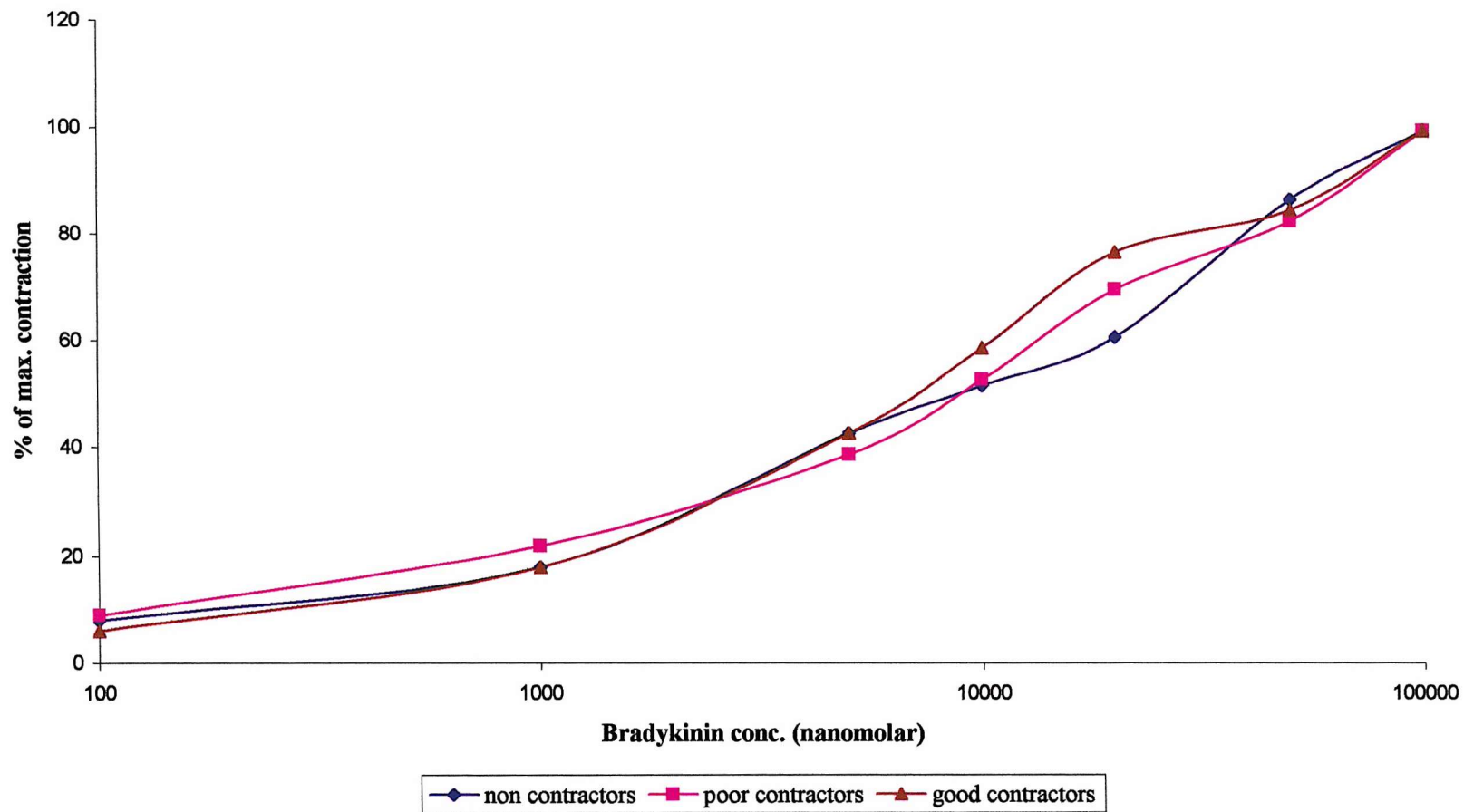
Cholecystokinin

Twenty seven strips remained viable after washout following the bradykinin concentration response curves, 5 good contractors, 16 poor contractors and 6 non contractors (Appendix L).

Table 7.33 Concentration response curves for CCK-8 for gallbladder strips from non, poor and good contractors. Expressed as mean (S.E.M.)

CCK conc. nM	0	10	20	50	100	200	500	1000
Non (n=6)	0	10.5 (15.4)	16.7 (23)	23 (28)	32 (31.3)	52 (21.8)	83 (18.7)	100
Poor (n=16)	0	13.8 (14.7	25 (19.3)	35.8 (23.3)	49.5 (26.9)	65 (26.9)	79 (20.1)	100
good (n=5)	0	3.6 (4.9)	8.4 (11.6)	10 (12.7)	11.6 (15.4)	25.6 (14.1)	55.4 (19.4)	100

Figure 7.32 CCK CRCs for strips from good, poor and non contracting gallbladders (mean +/- s.e.m.)
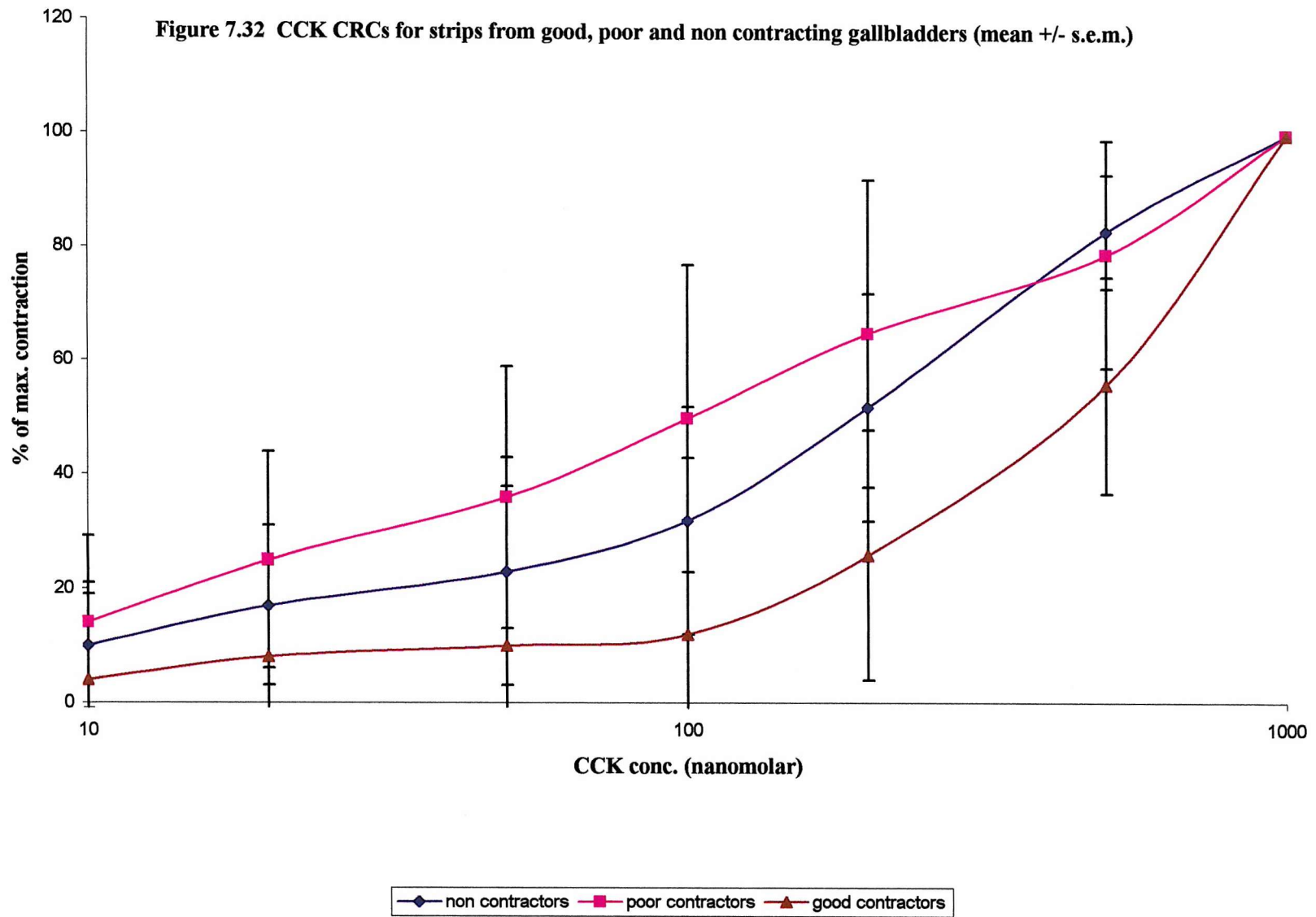
Although none of the differences between the groups were statistically significant, superficially the good contractors appear to have much lower levels of response to CCK-8 *in vitro* than did strips from either of the other two groups. One reason for this may be that 4 of the strips in the good contractor group came from a single gallbladder and all four showed a very low level of sensitivity to CCK particularly at concentrations below 500nM. Thus the results for this group are biased by the particular characteristics of one individual and may not necessarily reflect the performance of good contractors as a whole.

7.33) Comparison of peak contraction *in vitro* and *in vivo* emptying

A comparison was made between the peak level of emptying seen *in vivo* and the mean peak absolute level of contraction seen *in vitro* in response to both CCK-8 and bradykinin. Patients were ranked according to peak *in vivo* emptying (subject rank) which was expressed as the ejection fraction and also as the absolute volume ejected in ml (table 7.34).

Table 7.34

Ranking of *in vivo* emptying and *in vitro* contraction.

Subject rank	Emptying % (Vol. ml)	Peak Bk (mV)	BK rank	Peak CCK (mV)	CCK rank
1	59 (16)	27.5	15	-	-
2	56 (20)	46.1	11	61.4	4
3	50 (16)	50.7	9	35.8	8
4	44 (11)	131.9	3	161.9	2
5	44 (12)	35.2	14	49	6
6	41 (5)	70.3	6	35	9
7	41 (13)	65.7	7	-	-
8	34 (12)	23.8	16	45.5	7
9	29 (10)	95.5	4	60	5
10	28 (7)	272.5	1	242.3	1
11	25 (5)	22.8	17	19.2	11
12	24 (8)	77.1	5	-	-
13	19 (3)	56.7	8	-	-
14	11 (4)	9	18	-	-
15	10 (4)	141	2	152	3
16	8.7 (2)	46.9	10	-	-
17	4 (1)	36.9	13	1.2	12
18	4 (1)	39.9	12	21.4	10

Figure 7.33 Peak gallbladder emptying versus *in vitro* strip contraction in response to bradykinin
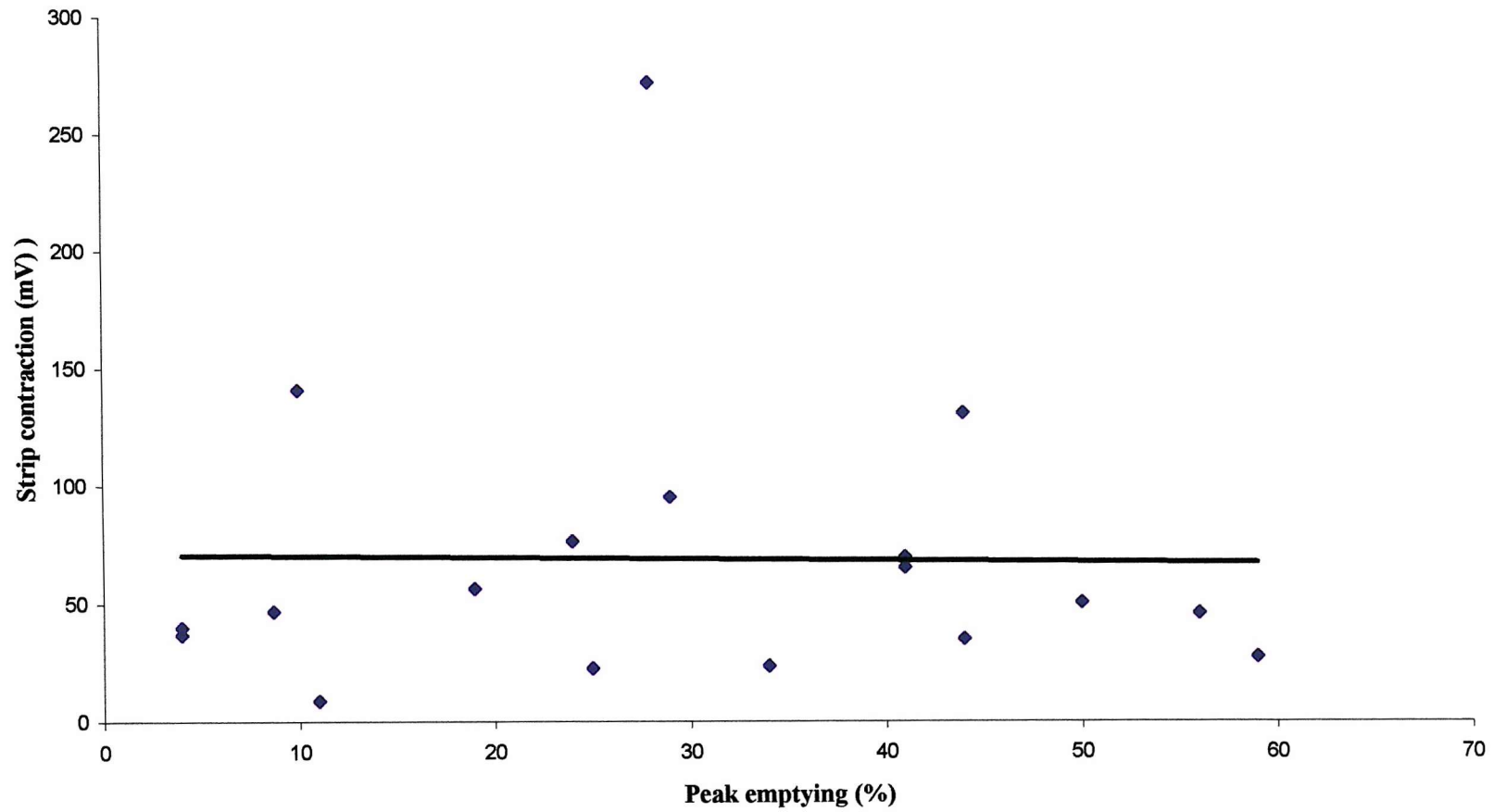
There was no evidence of correlation between peak gallbladder emptying (%) *in vivo* and absolute levels of gallbladder contraction *in vitro* (mV) in response to bradykinin (figure 7.33).

Correlation coefficient = -0.017, linear regression coefficient = -0.005.

Analysis of correlation between maximum change in gallbladder volume (ml) during emptying and *in vitro* contraction also showed no correlation (coefficient -0.024).

For CCK-8 a very weak positive correlation between *in vitro* contraction and *in vivo* emptying (ejection fraction) is apparent (Figure 7.34).

Correlation coefficient 0.16, linear regression coefficient 0.06.

Figure 7.34 gallbladder emptying and strip contraction in response to CCK-8
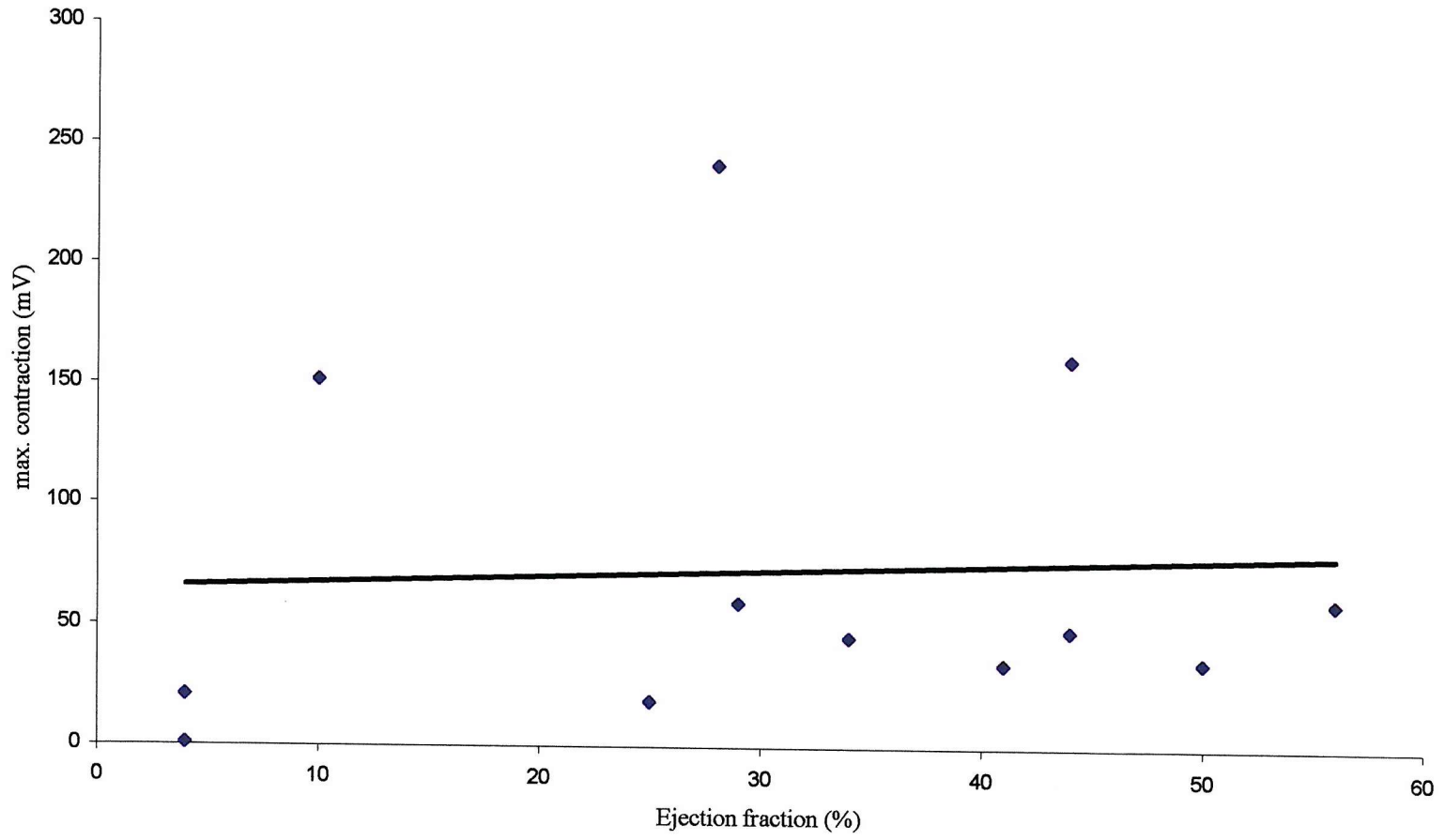
7.4) Discussion

The results for absolute levels of peak contraction are difficult to interpret for several reasons. Firstly the muscle strips were all cut to approximately the same size as judged by the naked eye, however this does not take account of the thickness of the strips or the thickness of the muscle layer within individual strips or the degree of fibrosis that may be apparent, thus the strips are not strictly standardised. Secondly, use of an isotonic transducer ensures that contraction of the strips is directly measured, however to compare this with an *in vivo* situation is difficult because pressure studies have clearly demonstrated the human biliary tract is not an isotonic situation as gallbladder emptying is greatly influenced by sphincter of Oddi tone and subsequent fluctuations in common bile duct and cystic duct pressure.

Although CCK-8 is the most biologically active form of cholecystokinin there are many other larger molecular forms of CCK-8 present *in vivo* all of which may have had a confounding effect on results. As previously described there are also neural and other non-hormonal causes of gallbladder contraction whose effect cannot be taken into account within the limits of this study.

Finally the mechanics of gallbladder emptying are dependent on the mathematical rules concerning spherical bodies, which dictate that volume is proportional to strip length to the power of three, hence for a large gallbladder a very small change in strip length may result in a large change in volume.

All of these factors contribute to make it difficult to assign significance to the poor correlation seen between the *in vivo* and *in vitro* results.

7.5) Conclusions

Bradykinin concentration response curves *in vitro* are similar for patients who demonstrate good, poor or no gallbladder emptying *in vivo* in response to a standard fatty meal.

CCK-8 concentration response curves *in vitro* are similar for patients who demonstrate good, poor or no gallbladder emptying *in vivo* in response to a standard fatty meal.

Peak levels of gallbladder emptying *in vivo* do not correlate with absolute peak levels of isotonic gallbladder contraction in response to bradykinin *in vitro* and show only weak correlation with response to CCK-8 *in vitro*.

Chapter VIII.

DISCUSSION

128

DISCUSSION

Cholecystectomy provides a ready source of material for *in vitro* analysis of gallbladder function. An ever increasing body of work has demonstrated a wide variety of neural and hormonal stimuli which affect gallbladder contraction *in vitro*. The initial simple single hormone system described by Ivy and Oldberg in 1928 when they discovered cholecystokinin has been modified as more factors influencing gallbladder emptying are described. However, despite the increasing knowledge regarding the actions of individual stimuli on gallbladder contraction, the significance of many of these various influences under physiological and pathological conditions remain unclear.

The first part of this study has established an isotonic method of measuring human gallbladder contractility *in vitro*, characterised the response to bradykinin and identified a difference in the bradykinin receptors controlling contraction in stone bearing and normal gallbladders. The second part of the study demonstrated the differing *in vivo* gallbladder emptying profiles of individuals with gallstones, compared against patients with irritable bowel syndrome and normal controls, plasma CCK assay was also performed and showed high levels of peak CCK in poor contracting gallstone patients and in the IBS group. The final part of the study endeavoured to compare *in vivo* gallbladder emptying and *in vitro* gallbladder contraction, however little positive correlation could be seen between the two responses.

In vitro

Gallbladder smooth muscle responds to a wide range of stimuli *in vitro*, however there are several factors which add to the difficulty of working with this tissue. Cholecystectomy is often a traumatic procedure which may involve considerable blunt trauma to the gallbladder fundus and Hartmann's pouch from tissue holding forceps used during open or laparoscopic gallbladder mobilisation and during laparoscopic delivery of the gallbladder from the abdomen. Thermal injury from diathermy dissection or coagulation to the wall of the hepatic aspect of the gallbladder during dissection from the piriform fossa also contributes to tissue damage. Handling of the

gallbladder during strip dissection prior to placement within the organ bath adds further to the physical trauma sustained by this relatively delicate tissue.

The gallbladder is very sensitive to adverse environmental factors encountered during transport from the operating theatre to the organ bath. Careful attention to the duration and conditions of transport is essential. Previous work within the Southampton PBRG had revealed that transporting strips in Krebs' surrounded by ice could lead to cooling of the Krebs' to well below $0^{\circ}C$ with the consequent possibility of low temperature thermal injury. In order to avoid this problem we transported the Krebs surrounded by iced water with a temperature of $0\text{-}4^{\circ}C$. Prolonged duration of transport also adds to the likelihood of impaired viability. The vast majority of experimental work described in this thesis was performed on the hospital site within the university surgical unit scientific laboratories, however during the initial phase of this study some preliminary experimental work was carried out at the biomedical sciences building located on the Southampton University campus using similar apparatus to our subsequent organ bath model to gain familiarity with the techniques required. It was found that the long duration of transit resulted in a much lower yield of viable gallbladder tissue than had been anticipated. This can presumably be attributed to anoxic damage resulting from the lack of oxygenation of the Krebs' solution during transit.

The difficulties encountered during the initial set up phase of the *in vitro* work are reflected in the very low viability rate encountered during the first few months of the study compared with the much higher success rate achieved subsequently. Only 7 of the 52 (13%) strips from the first 13 gallbladders were found to be viable compared with 19 viable strips of 45 (42%) from the last 12 gallbladders. Despite the improvement in survival rates over the course of the study the overall viability rates remained low compared with those recorded in other *in vitro* studies of gallbladder contractility. The reasons for this are not clear as the organ bath apparatus was set up in a similar manner to that described in other series and as much as possible was done to reduce transit times and gallbladder injury during strip dissection and placement in the organ bath. It is possible that the criteria that we used to define initial strip viability and subsequent strip death (chapter 3) were more stringent than those used by some

130

other authors (Wegstapel, Chen, Behar), in particular our requirement that after an initial response to the carbachol viability test, followed by washout, a steady baseline trace was achieved. Most strips exhibited some response to carbachol, but many failed to return to a stable baseline length after repeated washouts and were thus discarded since it was considered that it would be invalid to use data which relied on a best guess estimate of where the baseline lay. Only a relatively small proportion of strips demonstrated regular spontaneous activity during the initial equilibration phase, compared with other studies (Portincasa, McKirdy) and not all of these strips subsequently passed viability testing.

The term viability may not be strictly correct in this context since it would appear that some of these strips were exhibiting signs of life but were spontaneously contracting or relaxing in an erratic manner which made it impossible to base any observations upon them. This erratic behaviour was different from the regular spontaneous activity seen in some strips. This rhythm was easy to identify and usually did not make it difficult to calculate baseline length since it presented either as regularly spaced, low amplitude contractions or as a continuous sinusoidal type wave form of successive contraction and relaxation.

The high proportion of strips that failed the "viability" test may be due in part to the experimental set up. Almost all other researchers in this field have used isometric transducers to record changes in gallbladder strip tension as opposed to the isotonic arrangement that we used. It was thought that this arrangement would be appropriate for making comparisons with the *in vivo* results since change in gallbladder volume and change in strip length should be closely related. In theory this should not affect the nature of the dose response curve, however this method does make it hard to record a stable baseline since there is a non-linear relationship between strip length and developed tension. All visceral smooth muscle demonstrates a property known as plasticity which means that the relationship between fibre length and tension is not constant, thus the same tension may be developed for a range of fibre lengths, it is this property which allows hollow viscera such as the bladder and gallbladder to distend considerably with only slight variation in wall tension and internal pressure (Ryan and Cohen 1976). It is arguable which is the more valid experimental technique, but

131

certainly the isotonic method appears to make it hard to achieve a stable baseline and get consistent results and hence may in part account for the large number of strips that were deemed to have failed our viability test.

It is possible that using a higher preload may have reduced or overcome the effect of plasticity, however this would have reduced the sensitivity of our equipment to pick up small changes in strip length in response to low concentrations of bradykinin or CCK. Other researchers have used a preload of between 0.1g and 1g, for similar sized strips although with an isometric arrangement (McKirdy 1994, Portincasa 1994).

There is no record within the scientific literature of any previous work comparing the responses of strips from normal human gallbladders with strips from stone bearing gallbladders. The work of Behar (1989) does record that 14 "normal" gallbladders were obtained during his study, however they all came from patients having surgery for morbid obesity and on closer examination all displayed either cholesterol stones, macroscopic cholesterolosis or cholesterol crystals and thus cannot be considered as normal. This lack of experimental work on normal gallbladders can no doubt be attributed to the relative scarcity of such tissue which is seldom removed during routine general surgical practice. The specialist hepatobiliary and pancreatic surgeons within the Southampton University Surgical Unit provided this researcher with the opportunity to study healthy gallbladder tissue removed as part of the frequent major hepatic or pancreatic resections carried out within this regional unit.

Bradykinin

Recent evidence implicating bradykinin as a major regulatory factor in a variety of gastrointestinal motility pathways stimulated our interest in the role of this nonapeptide kinin which is classically recognised for it's actions as an inflammatory mediator. Bhoola (1992) has demonstrated that bradykinin produces contraction of ileal tissue, whilst within the duodenum it results in a biphasic response of relaxation and contraction. Cabrini (1995) demonstrated contraction of guinea pig gallbladder in response to bradykinin. Rushton (1996) demonstrated a similar effect on human

132

gallbladder tissue. Our results add to the body of evidence regarding the physiological actions of bradykinin.

The preliminary work on bradykinin in chapter 4 confirms the results previously achieved by Rushton within our laboratory. It confirms the same range of bradykinin concentrations over which the gallbladder strip contractile response is exhibited. Interestingly we did not observe the same effect of significant sensitisation of the strips that he noted, where after an initial bradykinin CRC the subsequent repetition of the CRC produced a greater contractile response (Ch 4.24). In this study repeated CRCs produced no evidence of strip sensitisation at any level of bradykinin concentration. Sensitisation of gallbladder tissue has not previously been reported for any other hormonal stimulus and would be difficult to explain at a physiological level, although it is a well recognised phenomenon in skeletal muscle in response to repeated neural stimulation. It may simply be a reflection of differing experimental practice, in that we allowed a greater time for a stable baseline to return, or performed a more thorough washout of reagents prior to repeating stimulation. Repeatability is an essential prerequisite for our subsequent work on B1 and B2 receptor blockade.

The finding that the contractile response to bradykinin is mediated via both B1 and B2 receptors in normal gallbladder strips and only the B2 receptor in stone bearing strips is surprising since the B1 receptor is usually only expressed in inflamed tissue whilst most of the other physiological actions of bradykinin are mediated via the B2 receptor (Pruneau et al 1994, Roslan et al 1995). The fact that there is a difference between normal and stone bearing gallbladders suggests that the B1 receptor action must be down graded in the stone bearing gallbladders; it is not clear whether this a secondary effect of the inflammatory process or a primary defect which contributes to the pathogenesis of the stones.

A further possibility that should be considered is whether or not our normal gallbladder group were truly representative of the normal population. The normal gallbladders all came from patients with hepatic or pancreatic tumours, which one can postulate may have had an effect promoting B1 receptor expression. However, they were a very heterogeneous group of benign and malignant conditions and those cases with bile duct

133

obstruction were excluded. Secondly there is no reported evidence of tumour related B1 over expression in any tissue, so it seems very unlikely that the underlying pathology would have had any uniform effect on gallbladder function, compared with disease free individuals. A final consideration is the operative procedure; laparoscopic and open cholecystectomy are relatively rapid operations compared with major hepatic or pancreatic resections. The B1 receptor can be induced within 3-6 hours in mouse trachea under experimental conditions *in vitro* (Trevisani et al 1999), this raises the possibility that intraoperative trauma or ischaemia produced stimulation of the bradykinin axis and subsequent induction of the B1 receptor. We do not have sufficient data with regard to the operations to completely exclude this factor.

The question arises as to what is the significance of these findings under physiological and pathophysiological conditions?

The presence of a B2 mediated contraction in both normal and stone bearing gallbladders suggests that there may be a physiological role for bradykinin in the regulation of gallbladder motor function, since this receptor has been identified as having a similar role in other parts of the gastrointestinal tract (Manning 1982, Roslan 1995). The significance of the absence of a B1 mediated response in stone bearing gallbladders is hard to determine since one would not normally expect this receptor to mediate a physiological response (Marceau 1995).

Bradykinin receptors are widely distributed throughout the human body where they play a major role in the pathophysiological response to the inflammatory cascade elicited by a wide variety of noxious stimuli. It is possible that the bradykinin elicited contraction that our study bears witness to is only produced during gallbladder inflammation and is not involved in the normal physiological regulation of gallbladder emptying. If this is the case then it may be that a bradykinin mediated contraction in response to inflammation has the beneficial action of expelling the toxic contents of the gallbladder.

Further study is required to determine whether bradykinin is a physiological or pathophysiological regulator of gallbladder motor function. If there is a role for

bradykinin in the normal regulation of gallbladder emptying then the next step is to identify if it acts via any of the established pathways, in particular either via the CCK or cholinergic axis. There is no chemical structural evidence to suggest that bradykinin should cross react with either cholinergic or CCK receptors and the rightwards shift of the CRC in response to B1 and B2 receptor antagonists suggests that the predominant response is via the bradykinin receptors.

Experiments to confirm the mode of action of bradykinin are merited, they would involve sequential repetition of the bradykinin CRC in the presence of cholinergic blockade (atropine), CCK antagonists (loxiglumide), arachidonic acid pathway inhibition (e.g. indomethacin), sympathetic blockade (guanethidine) and the neurotoxin tetrodotoxin. This series of experiments should serve to identify whether the mode of action of bradykinin involves either of the major neural or hormonal pathways of control of gallbladder motility. The use of the selective B1 agonist des-Arg^9-bradykinin, would be helpful in confirming the absence of B1 receptor expression in stone bearing gallbladders. If further evidence was required histochemical techniques could be used to identify if any site of bradykinin production or storage exists adjacent to the neuromuscular junction.

The presence of a residual contraction in response to bradykinin despite both B1 and B2 receptor blockade raises several possibilities. The simplest and most likely explanation is that dAL-bk and HOE-140 are both competitive antagonists with relative affinities of 5 and 6 for the B1 and B2 receptors respectively (Hock et al 1991, Regoli 1977, Regoli 1998)), hence at high concentrations bradykinin simply overcame their effect. The other possibilities are that there may be either a third bradykinin receptor in the gallbladder or that at high concentrations of bradykinin, contraction also occurs via a receptor independent mechanism. Although Farmer (1989) has presented some evidence for a third bradykinin receptor in the lung, current literature does not support this, nor is there any reported evidence of a direct receptor independent action (Regoli 1998, Paquet 1999). Repeat experiments with higher concentrations of antagonists should clear this issue.

The relative concentration levels of bradykinin required to produce a contraction in human gallbladder tissue appear to be quite high when compared with the levels of CCK. It is not known what concentration of bradykinin is generated locally in the extra cellular fluid surrounding the gallbladder smooth muscle cells during physiological or pathological circumstances, thus it is difficult to extrapolate our *in vitro* findings into the *in vivo* state with any degree of confidence.

Measurement of bradykinin levels around the gallbladder *in vivo* represents a near impossible task since it would require selective sampling of the venous drainage of the gallbladder. Unfortunately this occurs via a venous complex that drains into the liver and is thus too difficult and hazardous to perform for research purposes. More generalised venous sampling would be meaningless since bradykinin is produced from a wide variety of other viscera, as well as from vascular endothelium and circulating kininogens found in plasma. Equally bradykinin is short lived within the circulation, undergoing rapid first pass breakdown by angiotensin converting enzyme in the pulmonary capillary bed as well as inactivation by plasma kininases, notably carboxypeptidase N (Regoli et al 1994), all of which contribute to give it a half life of only 15 seconds.

Despite these various reservations regarding the limitations of our knowledge about the role of bradykinin in gallbladder emptying, it is still an area that at the least deserves further research. The identification of bradykinin as an *in vitro* stimulant of gallbladder contraction and the discovery of a difference in receptor expression between normal and stone bearing gallbladders, suggests that it may yet shed further light on the complex physiology of gallbladder emptying and the pathogenesis of gallstone disease.

In vivo

The *in vivo* studies of gallbladder emptying demonstrated firstly that the highly useful research tool of gallbladder sonography is a relatively simple skill to learn. Despite the highly dynamic nature of gallbladder emptying inter observer and intra observer

136

variations were small. In keeping with the results of other investigators (Fisher et al 1987) subjects were found to reach peak gallbladder emptying within sixty minutes of ingesting a standard test meal. Within the small group of subjects who underwent repeat scanning several months apart a high degree of reproducibility was found for measurements of fasting volume, peak emptying and time to peak emptying. This finding does not appear to have been previously reported and does add validity to the use of sonography as a research tool in this setting.

The standard test meal used in this work was a chocolate bar with a high saturated fat content and 150ml of water. Most other studies of gallbladder emptying have used test meals made from combinations of raw eggs and corn oil, or commercially available lipid suspensions. Our results were comparable with those obtained by other authors using traditional standardised fatty meals (Portincasa 1994, Dodds 1985) and the meal was cheap, hygienic, consistent, easy to prepare and universally palatable.

Since Everson (1980) first demonstrated impaired gallbladder emptying sonographically *in vivo* in gallstone patients, numerous other researchers have repeated his work (Stolk et al 1990, Dodds et al 1985, Pauletzki et al 1996, Portincasa et al 1994) with similar results. The present findings for fasting gallbladder volume and emptying in both normal controls and subjects with gallstones correlate well with these previous reports. This work again demonstrates that in a significant subgroup of patients with gallstones there is impairment of the normal pattern of emptying in response to a standard fatty meal when compared with a normal control population. The subdivision of the gallstone patients into three groups; good contractors, poor contractors and non contractors depending on levels of peak emptying is a rational method of breaking down a very broad range of different responses into three coherent groups. Similar groupings were used by Portincasa (1994). The good contractors had a similar emptying profile to the normal control group, the non contractors demonstrated peak emptying with a variation from fasting volume of ten per cent or less which is within the limits of experimental accuracy for sonographic estimation of gallbladder volume and also within established levels of fluctuation in fasting gallbladder volume as described by other researchers. The remaining patients with peak gallbladder emptying of between ten and fifty per cent were described as poor contractors and represent the

137

majority of gallstone patients. This finding supports the hypothesis that impaired gallbladder emptying with subsequent bile stasis within the gallbladder is a factor in the pathogenesis of gallstones.

Previous reports on gallbladder emptying in patients with irritable bowel syndrome have suggested that abnormal gallbladder emptying may be a feature of some subjects with this condition (Braverman 1987). The present results demonstrate that although IBS patients as a group have statistically similar mean fasting volumes and peak emptying to normal controls they are a very heterogeneous population with some (2/15) demonstrating very poor contraction (<20% peak emptying) which was lower than any recorded value in the normal group and many (8/15) demonstrating extreme contraction (>80% emptying) which was greater than that seen amongst any of the individuals within the normal control group.

There are a number of possible explanations behind these observations, the most likely being that this represents evidence of extension of the established picture of intestinal motor dysfunction to the gallbladder in patients with irritable bowel syndrome. Patients with IBS may display several different patterns of symptoms, in some diarrhoea is predominant and in others constipation (Thompson 1989). The former group show the paradox of decreased colonic contractions and the latter group increased colonic contractions (Connell 1962). Similarly diverse patterns of jejunal and ileal contraction have been observed (Gorard 1994, Whitehead 1980). The relatively extreme patterns of gallbladder emptying that were observed, with values outside the range established by normal controls suggests that there may be groups of hyper and hypo gallbladder contractors within the IBS population. This study did not identify any correlation between symptoms of diarrhoea or constipation and levels of peak emptying, although as numbers of poor contractors and patients reporting constipation were both very low the possibility of a relationship cannot be completely discounted.

Whether or not there is a contribution to the symptoms of IBS from the gallbladder dysfunction seen in some of these patients is not clear, again there was no correlation between the levels of emptying and the symptoms that are normally attributed to the biliary system, notably bloating and upper abdominal pain. Jones (1993), reported a

138

25% incidence of IBS type symptoms in patients awaiting cholecystectomy for gallstones and resolution of symptoms in the majority 1 year after operation.

It is possible that the gallbladder contributes to the symptoms of IBS indirectly via its interaction with the hormone cholecystokinin. The abnormal gallbladder emptying seen in some patients with IBS, may be due to abnormal release of, or sensitivity to CCK. The widespread effects of CCK on small and large intestinal motor function are well described and appear to arise at normal concentrations of the hormone which arise physiologically in response to a meal (Snape, 1978). Many patients with IBS report post prandial triggering or exacerbation of their symptoms. Previous work (Harvey and Read, 1973) has identified that a subgroup of patients with IBS have abnormally high levels of colonic motor activity in response to exogenous CCK-8, administration of which also reproduced their symptoms of IBS.

The heightened visceral sensitivity to CCK may be exacerbated by the finding in this study of increased mean peak CCK-8 levels in IBS patients in response to a standard fatty meal. The elevated CCK-8 levels were seen in subjects with high, normal and low levels of peak gallbladder emptying, so it is not clear whether the high concentrations of this hormone are a result of impaired negative feedback from poor emptying gallbladders as is the case with poor contracting gallstone patients, or if instead the high CCK-8 levels are a primary cause of the raised peak emptying levels seen in a proportion of the subjects. If high CCK levels persist for a long period of time then it is possible to hypothesise that this prolonged exposure might result in a down regulation of the CCK receptors within the gallbladder in some patients with a consequent reduction in motor response and hence account for the poor emptying seen in some patients.

These results add strongly to the evidence suggesting a role for the gallbladder / CCK axis in the pathophysiology of abnormal gastrointestinal visceral motility seen in a large subgroup of subjects with the irritable bowel syndrome.

Comparison of *in vivo* emptying and *in vitro* contractility

Results for the comparative study of in vivo emptying and in vitro contractility reveal a lower yield of in vitro results than had been anticipated at the start of the study. This was dependent on a number of factors

Subjects for comparative *in vivo* and *in vitro* assessment of gallbladder emptying and contraction were identified from the hospital waiting list, however position on the routine waiting list does not always reflect the duration of time likely to elapse prior to admission for cholecystectomy. Patients on the urgent waiting list are predominantly those who have had a recent bout of acute cholecystitis or are having severe symptoms from chronic cholecystitis. In both of these groups tissue subsequently obtained would be unlikely to be suitable for *in vitro* analysis because of the resultant inflammatory or fibrotic changes.

In order to try and overcome these recruitment problems efforts were targeted at those routine patients who had been provisionally booked onto operating lists, a process which usually occurs a fortnight prior to the anticipated date of surgery. Attempts to recruit patients who had received this notification of operation were hampered by several logistical factors. Firstly, availability of access to the ultrasound equipment, all of which is predominantly used for NHS clinical work and hence was seldom available for more than two half day research sessions each week and some weeks was not available at all. This restricted access meant that a mutually acceptable appointment time could not always be arranged within the limited time frame available. NHS bed shortages often meant that patients were cancelled from their allocated operation date, which sometimes resulted in them being rebooked on a date when other research or clinical commitments meant that it was not possible to perform any *in vitro* analysis on their gallbladder tissue. A further constraint on generation of *in vitro* results for this comparative study was the limited size of the available organ bath which had six chambers and was shared with another researcher, hence there was only the capacity to deal with strips from two gallbladders on any one day, on several occasions this compromised the ability to perform full *in vitro* experimentation on gallbladder tissue obtained from previously scanned individuals.

140

It is important to demonstrate that results obtained *in vitro* can be applied to the *in vivo* state and hence to clinical situations, however little previous work has been carried out in this field (Portincasa 1994, Wegstapel 1996). This study has assessed peak gallbladder emptying *in vivo* in response to a standard fatty meal and compared it with *in vitro* contractility in response to either CCK-8 or bradykinin There was no direct statistical correlation between the degree of gallbladder emptying *in vivo* and the response to bradykinin *in vitro*, there was however a weak correlation between emptying and response to CCK-8 *in vitro*. Previous authors have demonstrated correlation between CCK-8 induced contraction *in vitro* and *in vivo* peak emptying but have not assessed response to other *in vitro* stimuli. The less impressive results in this study raise the possibility that there may be a fundamental difference between the results generated in an isometric set up when compared with an isotonic arrangement. The majority of other researchers in this field have used isometric apparatus as opposed to the isotonic method outlined in this study. The previously mentioned effect of smooth muscle plasticity and its resultant non linear relationship between length and tension has the potential to significantly distort the results. Further study is required to determine whether results obtained under isometric conditions are comparable to those obtained with isotonic apparatus.

The mathematical relationship between strip contractility and gallbladder emptying is more complex than it might seem The ellipsoid formula is an approximate derivation from the rules governing circular bodies.

Volume = $4/3 \ \pi r^3$

and

Circumference = $2\pi r$

(r=radius)

Thus volume is proportional to the radius cubed for a sphere or height x width x length for an ellipse. For a sphere circumference is directly proportional to strip length. Hence for a sphere there is a simple relationship between volume and strip length.

Circumference = n(strip length)   therefore, strip length = $2\pi r/n$

and r = n (strip length)/2π

Substituting to give Volume = n^3(strip length)^3/ ($6\pi^2$)

Therefore, for a sphere, a change in volume is related to strip length by this equation.

Volume change = n^3(initial strip length)^3/ ($6\pi^2$) - n^3(final strip length)^3/ ($6\pi^2$)

Thus for a large sphere a small change in strip length can produce a large change in volume, effectively the same should be true for an ellipsoid shape such as the gallbladder. Hence for a small gallbladder a much greater magnitude of strip length change is required to deliver a set volume of bile, than in a large gallbladder.

The gallbladder is elliptical in shape and thus could be assumed to follow an approximation of these rules. However it is not a simple ellipse, it is a complex dynamic elliptical structure which does not maintain a uniform shape during contraction. It is fixed to the liver by peritoneal attachments, attached at one pole by cystic duct and artery and subject to the effects of gravity and external pressures from adjacent viscera. Hence in some gallbladders the initial ellipsoid shape may be near spherical in the fasting state but appear as a narrow, almost cylindrical, ellipse after stimulation, as the gallbladder demonstrates differential triplanar contraction. The complex mathematical equation required to describe the mechanics of this relationship, is different for each gallbladder and outside the scope of this thesis. Although the ellipsoid formula is perfectly satisfactory for estimating gallbladder volume, from a mathematical perspective it is difficult to accurately identify the precise relationship between change in *in vitro* gallbladder strip length and *in vivo* gallbladder emptying, for a strip from any given part of the gallbladder. This all adds to the difficulty in interpreting the comparative results.

The human biliary tract is subject to a number of factors which determine pressure within the gallbladder and hence tension within the wall of the gallbladder. The most

important of these are sphincter of Oddi tone and gallbladder muscle contraction. The combination of these two factors determines gallbladder emptying and distension. These factors also influence comparison of *in vivo* and *in vitro* contractility since increased gallbladder wall contraction does not necessarily produce gallbladder emptying. The gallbladder *in vivo* does not follow either an isometric or isotonic pathway which contributes significantly to the difficulty of comparing *in vitro* and *in vivo* results.

It is interesting to compare our results with those of Portincasa (1994), who identified good positive correlation between isometric *in vitro* strip contraction and sonographically measured gallbladder emptying *in vivo*. It may be that the difference between our methodologies and subsequent sets of results identifies a fundamental fact about the mechanism of gallbladder emptying. Good gallbladder emptying is more dependent on achieving high gallbladder wall tone than it is upon the absolute length of gallbladder strip contraction. Thus a high level of strip contraction under the lightly loaded isotonic set up we worked with is not necessarily associated with the ability to achieve high strip tone or by inference, good gallbladder wall tone and emptying.

The difficulty in ensuring uniform strip size, adds a further variable to the long list of confounding factors.

In view of these many confounding factors it is extremely difficult to interpret the poor correlation seen between *in vivo* emptying and *in vitro* contractility observed in this set of experiments. The absence of strong correlation means that we have failed to prove that the two are related, rather than disproving the hypothesis. However in the light of this result one must consider the possibility that the relative contribution of gallbladder wall contraction to gallbladder emptying may be less significant than previously thought, suggesting that the role of the sphincter of Oddi may be correspondingly greater.

143

In conclusion; human gallbladder strips respond to high doses of bradykinin *in vitro*, this effect appears to be predominantly via the B1 and B2 receptors in normal gallbladders and the B2 receptor in stone bearing gallbladders. The implications of this for human pathophysiology are not clear but merit further attention.

In vivo studies demonstrated lower levels of gallbladder emptying in patients with gallstones compared with normal controls.

High levels of peak plasma CCK levels were recorded in patients with IBS some of whom demonstrated extreme levels of gallbladder emptying, adding weight to the theory that this hormone may contribute to the visceral hypersensitivity seen in this condition.

Weak correlation was seen between *in vivo* gallbladder emptying and *in vitro* contraction in response to CCK-8. This result is difficult to interpret, but suggests that human gallbladder contraction may be less significant a factor in gallbladder emptying than previously thought, raising the possibility of a greater role for sphincter of Oddi contraction and relaxation in the control of gallbladder filling and emptying.

APPENDICES

Bk (μM)	0	0.1	1	5	10	20	50	100
B1 run 1	0	11.2	22.9	37.6	50.1	59.2	81.9	100
B1 run 2	0	3.2	23.1	36.3	45.9	63.7	91.1	110
B1 run 3	0	3.9	4.9	13.3	28.5	51.9	96.4	123
B1 run 4	0	1.2	7.9	22.1	33.2	63	106	134
B1 run 5	0	1.3	5.9	23.8	31.9	61.7	101	137
B2 run 1	0	1	4	14.5	33.3	46.7	76.3	100
B2 run 2	0	0.1	4	17.3	25.8	42.5	77.4	100.2
B2 run 3	0	0	3.3	5.5	9.9	26.9	62.8	90.1
B2 run 4	0	1.7	2.4	6.1	17.2	25.7	63.8	88.7
B2 run 5	0	0.8	2.4	8.6	16.2	26.7	58.9	93.8
B3 run 1	0	6.1	12.1	39.6	62	84.6	96.6	100
B3 run 2	0	7.2	18.3	45.7	70.2	99.5	105.9	115.2
B3 run 3	0	2.1	10.5	24.6	59.2	86.3	101.9	106.7
B3 run 4	0	5.2	13.8	24.4	62.6	79.4	90.8	96.2
B3 run 5	0	1.5	15	22.4	62.4	90.7	100.3	102
B4 run 1	0	0	2.7	15.7	41.9	57.7	79.5	100
B4 run 2	0	3.2	9.8	18.6	31.1	47	69.8	102
B4 run 3	0	0	6.3	18.4	23.2	50.9	74.3	92.1
B4 run 4	0	0.9	13.8	34.1	45	55	81.8	98.4

Repeat bradykinin runs to assess sensitisation of human gallbladder strips and repeatability of the bradykinin concentration response curve (contraction expressed as percentage of maximum).

Appendix B

Bk (μM)	0	0.1	1	5	10	20	50	100
C1	0	3.5	57.1	68.3	71	-	-	100
C2	0	14.5	60.3	75	79.8	-	-	100
C3	0	6.4	21.2	40.5	33.3	-	-	100
C4	0	14.3	41.6	60.3	77.9	85.1	94.2	100
C5	0	0	0	0	31.2	54.9	70.3	100
C6	0	10.5	39.3	53.1	65.5	90.1	96.5	100
C7	0	6.1	22.9	45.7	62.9	70.3	84	100
C8	0	6.1	28.2	49.1	65	74.3	86.1	100
C9	0	0	18.6	39.8	47	59	76	100
C10	0	6	25.5	60	75	75	81	100
C11	0	0	0	3.7	10	20	53	100
C12	0	0	0	1	17	36	76	100
C13	0	5.6	16	39	71	73	94	100
C14	0	6.9	23	49	92	93	94	100

Values for Bradykinin CRCs in normal gallbladder strips, expressed as percentage of maximum contraction (- denotes missing value).

dAL-Bk (1μM)								
Bk(μM)	0	0.1	1	5	10	20	50	100
C1	0	0	0	0	0	-	-	100
C2	0	0	0	3	6	-	-	44
C3	0	0	0	41	54	-	-	67
C4	0	0	0	20	34	37	82	87
C5	0	0	0	19	34	41	52	57
C6	0	0	0	26	35	39	73	76
C7	0	4	7	31	41	56	81	86
C8	0	3	10	29	31	41	70	72
C9	0	7	7	13	13	20	46	76
C10	0	10	34	47	65	68	86	109
C11	0	0	0	5	11	18	55	81
C12	0	0	0	3	18	38	71	77
C13	0	0	0	1	6	26	66	80
C14	0	0	0	3	11	41	80	96

Values for repeat bradykinin runs after addition of dAL-Bk

147

Bk(μM)	0	0.1	1	5	10	20	50	100
D1	0	21	36	61	70	-	99	100
D2	39	75	98	100	100	100	100	100
D3	0	4	12	23	28	-	37	100
D4	0	0	0	33	48	58	100	100
D5	0	22	36	59	68	83	92	100
D6	0	27	40	72	80	92	99	100
D7	0	2	6	18	31	-	69	100
D8	0	0	3	17	33	-	81	100
D9	0	0	0	8	9	-	32	100
D10	0	3	4	4	4	-	18	100
D11	0	13	34	62	73	83	97	100
D12	0	7	15	34	46	61	84	100
D13	0	6	15	65	97	97	97	100
D14	0	1	4	15	25	90	91	100
D15	0	1	1	7	14	76	78	100
D16	0	1	1	3	12	48	53	100
D17	0	0	4	31	52	71	91	100
D18	0	0	0	14	20	41	86	100
D19	0	5	40	46	55	66	80	100
D20	0	1	11	43	65	68	77	100
D21	0	24	74	93	96	98	100	100
D22	0	27	49	64	68	76	80	100
D23	0	8	20	41	52	60	70	100
D24	0	7	46	59	74	88	91	100
D25	0	11	19	31	53	75	92	100
D26	0	15	25	41	48	67	88	100

Values for bradykinin runs in strips from stone bearing gallbladders.

| DAL-bk (1μM) | | | | | | | | |
Bk (μM)	0	0.1	1	5	10	20	50	100
D1	0	0	10	15	53	-	80	82
D2	0	0	36	61	70	-	84	85
D3	0	5	21	29	35	-	110	114
D4	0	0	0	0	40	56	64	75
D5	0	31	36	58	64	82	103	103
D6	0	18	45	74	80	92	98	102
D7	0	0	6	21	36	-	62	99
D8	0	0	3	19	34	-	71	105
D9	0	1	32	44	55	-	61	147
D10	0	6	8	18	21	-	26	100
D11	0	0	4	21	28	48	53	64
D12	0	2	6	23	32	63	70	91
D13	0	0	8	35	50	62	69	82
D14	0	11	32	56	63	87	89	113
D15	0	1	9	27	43	74	74	102
D16	0	6	13	31	48	80	82	88
D17	0	2	2	31	46	67	74	94
D18	0	0	1	21	40	68	136	171
D19	0	10	38	47	63	75	85	109
D20	0	24	50	90	93	96	99	100
D21	0	4	7	37	53	56	75	80
D22	0	15	25	63	67	67	96	100
D23	0	10	29	48	50	59	103	120
D24	0	5	16	39	45	70	72	99
D25	0	0	2	30	47	72	72	95
D26	0	1	31	34	41	60	61	84

Values for bradykinin CRC after incubation of strips from stone bearing gallbladders with dAL-bk.

APPENDIX C

HOE-140								
Bk (μM)	0	0.1	1	5	10	20	50	100
C1	0	0	4	4	4	-	-	170
C2	0	0	0	0	0	-	-	38
C3	0	0	0	3	48	-	-	140
C4	0	0	0	0	0	0	1	4
C5	0	0	0	0	0	2	4	5
C6	0	0	0	0	0	19	25	28
C7	0	1	2	2	12	64	94	97
C8	0	0	1	8	18	58	84	84
C9	0	0	0	31	44	57	77	112
C10	0	10	10	13	42	47	60	99
C11	0	3	3	8	23	60	119	119
C12	0	0	0	2	6	38	97	101
C13	0	2	2	2	7	33	40	40
C14	0	0	0	0	0	2	68	85

Values for normal strips undergoing a bradykinin CRC after incubation with HOE-140.

HOE-140 (1 μM)								
Bk (μM)	0	0.1	1	5	10	20	50	100
D1	0	0	0	0	0	-	39	70
D2	0	0	0	22	36	-	87	87
D3	0	0	0	0	0	-	42	44
D4	0	0	0	2	24	45	82	98
D5	0	0	3	16	29	50	63	82
D6	0	0	0	3	11	21	61	92
D7	0	0	0	0	3	-	56	88
D8	0	0	2	9	26	-	93	112
D9	0	1	4	14	14	-	36	52
D10	0	0	0	4	12	40	65	88
D11	0	0	0	2	6	24	39	55
D12	0	0	0	0	1	12	38	69
D13	0	0	0	0	7	36	62	74
D14	0	1	2	6	36	64	82	109
D15	0	0	0	2	8	37	68	103
D16	0	0	0	0	6	39	69	74
D17	0	0	0	6	18	60	81	123
D18	0	0	0	0	13	39	82	189
D19	0	0	0	2	2	50	56	81
D20	0	0	0	0	67	88	149	157
D21	0	0	0	0	3	6	120	136
D22	0	0	0	0	23	38	157	199
D23	0	0	0	0	7	24	127	147
D24	0	0	1	5	29	68	89	114
D25	0	0	0	0	0	18	36	62
D26	0	0	0	0	0	10	22	39

Values for strips from stone bearing gallbladders undergoing Bk CRCs after incubation with HOE-140.

APPENDIX D

HOE-140 + dAL-bk								
Bk (µM)	0	0.1	1	5	10	20	50	100
C1	0	0	0	0	0	-	-	142
C2	0	0	0	0	0	-	-	76
C3	0	0	0	0	0	-	-	102
C4	0	0	0	0	0	43	69	69
C5	0	0	0	0	0	16	33	35
C7	0	0	1	7	14	61	101	103
C10	0	0	7	32	61	86	104	168
C11	0	0	5	29	49	49	68	91
C13	0	0	0	1	2	8	66	68
C14	0	0	0	0	5	16	78	79

Values for normal strips bradykinin CRCs with dAL-bk and HOE-140.

HOE-140 + dAL-bk								
Bk (µM)	0	0.1	1	5	10	20	50	100
D1	0	0	0	0	0	-	43	63
D2	0	0	0	50	52	-	97	97
D3	0	0	2	4	4	-	52	89
D5	0	0	5	17	27	36	47	87
D6	0	0	0	7	18	30	65	89
D9	0	0	0	0	0	-	0	42
D11	0	0	0	0	13	33	52	74
D12	0	0	0	0	5	39	49	83
D13	0	2	2	13	37	81	86	109
D18	0	0	0	18	45	53	134	280
D20	0	0	0	12	61	84	145	165
D21	0	0	0	2	10	24	119	138
D22	0	0	0	1	22	25	124	193
D23	0	0	0	10	29	33	77	136
D25	0	0	0	0	6	65	66	75
D26	0	0	0	0	1	14	14	32

Values for bradykinin CRCs for strips from stone bearing gallbladders after incubation with dAL-bk and HOE-140.

152

APPENDIX E

Bk (μM)	0	0.1	1	5	10	20	50	100
C9	0	0	0	6	16	33	88	167
C10	0	0	0	2	18	39	83	109
C13	0	2	11	38	63	71	91	96
C14	0	0	6	43	74	87	90	95
D2	0	0	0	0	29	-	73	83
D5	0	2	8	24	39	-	87	102
D6	0	0	4	25	47	-	115	141
D9	0	4	7	11	18	-	68	95
D11	0	1	4	15	27	-	95	104
D12	0	1	2	8	17	-	92	117
D13	0	0	5	39	63	-	111	119
D18	0	15	115	132	158	192	232	291
D25	0	0	1	2	18	-	61	103
D26	0	1	2	3	9	-	45	81

Values for repeat bradykinin CRCs in fourteen viable strips remaining after washout of all antagonists.

Appendix F

Inter observer variation in measured gallbladder volume in response to a standard fatty meal.

Investigator

	0	5	10	15	20	25	30	40	50	60
E1	100	78	61	94	100	94	100	88	100	100
E2	100	92	49	79	71	71	57	53	70	79
E3	100	86	79	90	100	96	87	94	96	100
E4	100	53	42	47	53	35	52	41	69	54
E5	100	64	93	90	90	77	77	64	71	79
E6	100	87	64	68	68	60	61	86	64	48
E7	100	100	41	46	39	64	59	45	27	13
E8	100	100	91	63	56	60	42	36	42	28
E9	100	100	100	99	100	100	100	100	100	100
E10	100	92	84	90	100	100	97	90	89	80
E11	100	100	89	65	43	35	24	32	37	26
E12	100	89	85	74	66	60	63	73	62	83

Radiologist

	0	5	10	15	20	25	30	40	50	60
E1	100	84	100	100	100	100	100	100	100	63
E2	100	93	87	73	87	80	80	73	62	66
E3	100	66	66	93	80	80	87	87	80	73
E4	100	100	73	100	83	100	92	100	100	100
E5	100	100	92	85	100	100	100	92	100	92
E6	100	65	72	79	80	90	65	83	48	41
E7	100	83	60	57	66	91	71	34	20	15
E8	100	94	75	63	42	53	41	40	27	21
E9	100	100	97	100	100	89	88	97	83	100
E10	100	83	72	69	100	65	67	67	69	86
E11	100	100	72	100	83	100	92	100	100	100
E12	100	93	93	71	57	64	78	71	71	86

Appendix G

Gallbladder emptying over a period of ninety minutes in response to a standard fatty meal.

	0	5	10	15	20	25	30	40	50	60	70	80	90
E1	100	90	66	48	38	31	33	38	48	49	44	55	72
E2	100	100	100	96	100	93	100	100	96	93	100	100	100
E3	100	96	70	78	87	74	70	78	91	100	91	87	83
E4	100	100	95	98	89	95	79	82	76	89	79	89	95
E5	100	88	83	96	92	96	79	75	84	88	79	92	88
E6	100	89	78	70	63	39	76	76	62	89	63	81	97
E7	100	88	76	64	68	56	64	64	72	60	76	72	84
E8	100	100	78	86	86	75	61	78	78	94	100	86	94
E9	100	80	88	76	68	63	41	56	45	58	63	68	88

Appendix H

Gallbladder emptying in normal subjects in response to a standard fatty meal.

Time	0	5	10	15	20	25	30	40	50	60
F1	100	87	64	68	68	60	61	86	64	48
F2	100	73	46	27	50	39	32	55	52	60
F3	100	100	41	46	39	64	59	45	27	87
F4	100	66	66	50	50	59	50	50	66	69
F5	100	67	89	48	41	48	44	33	56	36
F6	100	65	82	75	61	51	48	48	48	48
F7	100	64	75	79	70	33	58	28	42	42
F8	100	64	92	90	90	77	77	64	71	79
F9	100	72	45	43	25	30	35	40	47	53
F10	100	96	85	85	58	54	54	42	38	38
F11	100	91	70	36	42	39	31	48	43	61
F12	100	73	69	61	69	50	42	46	38	54
F13	100	82	77	55	44	40	26	29	35	38
F14	100	68	44	45	35	36	44	50	50	68
F15	100	95	100	92	95	98	100	86	80	68
F16	100	82	55	63	68	64	73	77	86	73

Appendix I

Gallbladder emptying profiles in gallstone patients.

N.C.										
Time	0	5	10	15	20	25	30	40	50	60
G1	100	100	100	96	100	93	100	100	96	93
G2	100	100	91	96	91	100	91	100	100	100
G3	100	100	100	100	96	100	100	100	96	100
G4	100	89	100	100	100	100	100	100	100	100
G5	100	87	100	100	100	100	100	100	100	100
D6	100	100	93	89	96	100	100	100	100	100
G7	100	95	100	100	89	89	95	100	100	95
G8	100	100	100	100	100	100	100	100	100	100
G9	100	100	100	100	100	100	100	100	100	100
G10	100	100	100	100	96	100	96	100	100	96
G11	100	100	93	90	98	100	95	100	93	100
P.C.										
G12	100	95	98	75	62	69	79	56	72	75
G13	100	89	85	74	66	60	63	73	62	83
G14	100	100	96	100	75	78	70	80	86	83
G15	100	96	70	78	87	74	70	78	91	100
G16	100	78	68	86	82	54	60	68	50	71
G17	100	98	71	85	76	83	68	73	76	61
G18	100	81	63	95	100	91	100	90	100	100
G19	100	100	95	98	89	95	79	82	76	89
G20	100	87	83	96	92	96	79	75	84	87
G21	100	88	76	64	68	56	64	64	73	60
G22	100	100	78	86	86	75	61	78	78	94
G23	100	84	91	87	99	91	84	91	99	95
G24	100	79	79	89	79	100	79	100	100	95
G25	100	96	83	64	58	64	67	73	70	77
G26	100	82	76	82	88	85	76	68	79	76
G27	100	90	95	71	76	90	86	90	90	100
G28	100	84	79	95	100	100	100	100	100	100
G29	100	92	86	92	96	86	96	79	73	79
G30	100	86	73	86	73	82	86	77	82	82
G31	100	74	84	63	68	63	74	63	63	79
G32	100	86	81	90	100	96	81	94	96	100
G33	100	86	76	88	86	98	69	75	82	92
G34	100	74	63	63	59	80	83	100	100	100
G35	100	88	91	84	59	96	86	62	66	76
G36	100	98	89	94	72	100	100	94	100	98
G37	100	100	95	75	70	63	73	65	60	60
G38	100	91	91	83	86	80	71	71	80	83

G39	100	92	84	90	100	100	97	90	89	80
G40	100	88	81	78	78	81	100	59	78	97
G41	100	94	100	91	80	66	66	71	68	74
G42	100	90	76	72	69	72	65	76	65	83
G43	100	95	99	85	71	67	74	64	56	60
G44	100	87	90	93	80	68	74	68	80	-
G45	100	92	100	88	76	91	87	76	65	65
G46	100	100	100	100	94	97	75	81	78	83
G47	100	95	98	87	76	91	87	76	65	65
G.C.										
G48	100	100	89	65	43	35	24	32	37	26
G49	100	53	42	47	53	35	52	41	69	54
G50	100	90	66	48	38	31	33	38	48	49
G51	100	89	78	70	63	39	76	76	62	89
G52	100	80	88	76	68	63	41	54	45	38
G53	100	76	80	68	56	39	44	43	40	44
G54	100	59	66	50	49	53	59	59	59	78
G55	100	67	65	61	63	53	41	47	76	76
G56	100	92	48	80	71	71	57	52	69	78
G57	100	85	63	48	34	42	52	53	75	59
G58	100	100	100	76	38	30	28	26	30	34
G59	100	100	91	63	56	60	42	36	42	28
G60	100	84	67	44	44	35	52	39	34	33
G61	100	75	79	61	61	35	40	31	21	20
G62	100	98	100	90	71	45	49	41	53	68
G63	100	94	97	86	72	69	58	47	44	47
G64	100	86	74	55	43	51	70	58	55	62
G65	100	80	66	56	70	52	45	56	52	66

Appendix J

	0	5	10	15	20	25	30	40	50	60
H1	100	72	63	56	40	53	53	63	94	91
H2	100	100	76	48	45	26	16	10	22	13
H3	100	90	88	85	85	80	80	90	80	100
H4	100	82	43	31	15	11	6	11	13	26
H5	100	94	61	32	23	36	25	12	11	14
H6	100	94	100	100	100	89	100	83	100	89
H7	100	80	58	22	18	20	17	26	40	38
H8	100	90	78	82	69	45	57	40	29	29
H9	100	62	30	24	52	63	81	100	75	100
H10	100	99	88	66	52	58	66	55	41	47
H11	100	83	63	50	53	33	43	28	20	39
H12	100	57	44	31	18	13	16	26	23	40
H13	100	93	84	89	62	34	23	17	30	44
H14	100	76	73	65	46	25	14	12	15	19
H15	100	91	52	35	17	12	16	21	17	29

APPENDIX K

Bk (μM)	0	0.1	1	5	10	20	50	100
I1	0	0	21	40	58	76	84	100
I2	0	11	26	51	70	78	95	100
I3	0	13	34	62	73	-	97	100
I4	0	7	15	34	45	-	84	100
I5	0	6	15	65	97	-	97	100
I6	0	1	1	3	12	-	53	100

Bradykinin CRCs for gallbladder strips from good contracting patients.

Bk (μM)	0	0.1	1	5	10	20	50	100
J1	0	11	23	38	50	59	82	100
J2	0	1	4	22	32	47	76	100
J3	0	22	36	59	68	-	92	100
J4	0	27	36	59	68	-	92	100
J5	0	0	6	40	55	95	96	100
J6	0	0	16	47	56	63	94	100
J7	0	1	18	53	61	66	73	100
J8	0	0	3	11	17	38	81	100

Bradykinin CRCs for gallbladder strips from non contracting patients.

Bk (μM)	0	0.1	1	5	10	20	50	100
K1	0	1	1	7	16	29	64	100
K2	0	3	10	34	51	63	85	100
K3	0	13	15	26	69	71	79	100
K4	0	3	9	10	35	50	64	100
K5	0	26	43	53	67	89	97	100
K6	0	1	11	43	65	68	77	100
K7	0	24	74	93	96	98	100	100
K8	0	24	74	93	96	98	100	100
K9	0	8	20	41	52	60	70	100
K10	0	7	46	59	74	88	91	100
K11	0	48	80	85	88	92	96	100
K12	0	26	36	51	58	69	94	100
K13	0	0	15	34	54	63	81	100
K14	0	2	13	44	59	87	100	100
K15	0	1	30	44	56	82	90	100
K16	0	10	17	43	52	63	84	100
K17	0	1	11	16	33	54	88	100
K18	0	2	12	24	47	68	95	100
K19	0	4	4	18	48	74	82	100
K20	0	9	15	23	31	67	95	100
K21	0	13	16	35	38	50	60	100
K22	0	5	25	47	49	69	78	100
K23	0	6	12	40	62	85	96	100
K24	0	0	3	16	42	58	79	100
K25	0	4	12	23	28	-	37	100
K26	0	0	0	33	48	-	100	100

Bradykinin CRCs in gallbladder strips from poor contracting patients.

CCK (nM)	0	10	20	50	100	200	500	1000
I1	0	9	28	31	38	48	68	100
I3	0	0	5	10	10	12	24	100
I4	0	0	0	0	0	16	61	100
I5	0	0	0	0	1	28	51	100
I6	0	9	9	9	9	24	73	100

Cholecystokinin CRCs for gallbladder strips from good contracting patients.

CCK (nM)	0	10	20	50	100	200	500	1000
J1	0	0	1	3	16	36	80	100
J2	0	3	13	27	43	52	90	100
J5	0	0	0	0	0	47	98	100
J6	0	0	0	0	0	25	48	100
J7	0	34	57	70	74	87	98	100
J8	0	26	29	38	59	64	86	100

Cholecystokinin CRCs for gallbladder strips from non contracting patients.

CCK (nM)	0	10	20	50	100	200	500	1000
K1	0	7	10	24	50	79	100	
K2	0	20	46	53	67	77	83	100
K3	0	0	0	0	3	33	65	100
K4	0	6	14	27	39	65	80	100
K5	0	16	28	41	50	72	84	100
K11	0	11	13	18	69	89	96	100
K12	0	0	24	60	69	88	92	100
K13	0	0	34	34	34	38	62	100
K14	0	46	49	60	65	84	90	100
K15	0	25	44	49	49	58	76	100
K16	0	0	0	0	0	7	19	100
K18	0	0	1	2	18	29	62	100
K19	0	0	9	42	74	96	100	100
K20	0	27	50	61	83	96	100	100
K21	0	37	55	64	81	88	90	100
K22	0	19	23	52	67	72	87	100

Cholecystokinin CRCs for gallbladder strips from poor contracting patients.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adrian TE, Ferri GL, Bacrarese-Hamilton AJ, et al. Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 1985; 89: 1070-7.

Amer MS and McKinney GR. Studies with cholecystokinin *in vitro*. J. Pharmacol. Exp. Ther. 1972; 183: 535-8.

Bailey IS, Walsh T, Hill A et al. Effect of cholecystectomy on plasma CCK. Br. J. Surg. 1992; 79: 456.

Bainbridge FA and Dale HH. The contractile mechanisms of the gallbladder and its extrinsic nervous control. J. Physiol. 1995; 12: 138-55.

Behar J, Rhim B, Thompsom W and Biancani P. Inositol triphosphate restores impaired human gallbladder motility associated with cholesterol stones. Gastroenterology 1993; 104:563-8.

Behar J, Lee K, Thompson W and Biancani P. Gallbladder contraction in patients with pigment and cholesterol stones. Gastroenterology 1989; 97: 1479-84.

Boyden E.A. A study of the behaviour of the human gallbladder in response to the ingestion of food; together with some observations on the mechanism of the expulsion of bile in experimental animals. Anat. Rec. 1926; 333: 201-55.

Braverman DZ. Gallbladder contraction in patients with the irritable bowel syndrome. Israeli J. Med. Sci. 1987; 23: 181-4.

Burleigh D. N-Nitro-L-Arginine reduces nonadrenergic noncholinergic relaxations of human gut. Gastroenterology 1992; 102:679-83.

166

Cantor P and Rehfeld J. Radioimmunoassay of cholecystokinin: comparison of different tracers. J.Immunol Methods 1985; 82:47-55.

Cantor P, Olsen O, Gertz BJ et al. Inhibition of cholecystokinin stimulated pancreaticobiliary output in man by the cholecystokinin receptor antagonist MK-329. Scand. J. Gastroenterol. 1991; 26: 627-637.

Catnach S. Fairclough P, Trembath R et al. Effect of oral erythromycin on gallbladder motility in normal subjects and subjects with gallstones. Gastroenterology 1992; 102: 2071-6.

Chen Q, Amaral J, Oh S et al. Gallbladder relaxation in patients with pigment and cholesterol stones. Gastroenterology 1997; 113: 930-7.

Clave RA and Gaspar MA. Incidence of gallbladder disease after vagotomy. Am J. Surg. 1969; 118: 169-76.

Davies F and Harding H.E. Pouch of Hartmann, Lancet 1942; 1: 193-5.

Davis M, Ryan J. Influence of progesterone on guinea pig gallbladder motility in vitro. Dig. Dis. Sci. 1986; 31:513.

De Ketham J. Fasciculus Medicinae. Facsimilie of 1491 Venetian edition. Translation by Luke Demaitre. Published by Griffin editions 1988.

Dlugosz J, Folsch UR, Creutzfeld W. Inhibition of intraduodenal trypsin does not stimulate pancreatic exocrine secretion. Digestion 1983;26: 197-204.

Dockray GJ. The integrative functions of CCK in the upper gastrointestinal tract. In Hughes J, Dockray G, Woodruff G (eds.) The neuropeptide cholecystokinin (CCK) Horwood Chichester, 1989, pp: 232-239.

Dockray GJ. Vagally mediated actions of CCK. In: Adler G, Beglinger C (eds). Cholecystokinin antagonists in gastroenterology. Springer Verlag, Berlin, 1991, pp:56-62.

Dodds W, Groh W, Darweesh R et al. Sonographic measurement of gallbladder volume. Am J. Rad. 1985; 145: 1009-11.

Doty J, Pitt H, Kuchenbecker S and Den Besten L. Impaired gallbladder emptying before gallstone formation in the Prairie Dog. Gastroenterology 1983; 85: 168-74

Doyle JS, Farrar JT. A sphincteric mechanism in the cystic duct of dogs. Irish J. Med. Sci. 1969; 2: 109.

Doyon M. A L'etude de la contractilite de voies bilaires. Arch de physiol 1893; 5: 678-685.

Ellenbogen S, Grime SJ, Mackie CR, et al. The mechanisms initiating the preduodenal phase of gallbladder emptying in man. Br. J. Surg. 1988; 78: 940-5.

Everson G, Braverman D, Johnson M and Kern F. A critical evaluation of real time ultrasonography for the study of gallbladder volume and contraction. Gastroenterology 1980; 79: 40-46.

Everson G, McKinley C, Lawson M et al. Gallbladder function in the human female: effect of the ovulatory cycle, pregnancy and contraceptive steroids. Gastroenterology 1982; 82: 711-9.

Everson GT, Lawson M, McKinley C, et al. Gallbladder and small intestine regulation of biliary lipid secretion during intraduodenal infusion of standard stimuli. J. Clin. Invest. 1983; 71: 596-603.

Everson GT. Gallbladder function in gallstone disease. Gastroenterol. Clin. North Am. 1991; 20: 85.

Festi D, Frabboni R, Bazzoli F et al. Gallbladder motility in cholesterol gallstone disease. Effect of ursodeoxychilic acid administration and gallstone dissolution. Gastroenterology 1990; 99: 1779-1785.

Fiorucci S, Santucci L, Morelli A. 5-HT3 receptor antagonist modulates gallbladder emptying and motilin release induced by erythromycin. Dig. Dis. Sci. 1993; 38: 2236-2240.

Fiorucci S, Distrulli E, Quintieri A, Sarpi L et al. L-Arginine/Nitric oxide pathway modulates gastric motility and gallbladder emptying induced by erythromycin and liquid meal in humans. Dig. Dis. Sci. 1995; 40: 1365-71

Fisher RS, Rock E, Malmud LS. Gallbladder emptying response to sham feeding in humans. Gastroenterology 1986; 90: 1854-7.

Fisher R, Stelzer F, Rock E and Malmud L. Abnormal gallbladder emptying in patients with gallstones. Dig. Dis. Sci. 1982; 27: 1019-24.

Fisher RS, Rock E, Levin G, Malmud L. Effects of somatostatin on gallbladder emptying. Gastroenterology 1987; 92: 885-90.

Fridhandler T, Davison J, and Shaffer E. defective gallbladder contractility in the ground squirrel and prairie dog during the early stages of cholesterol gallstone formation. Gastroenterology 1983; 85: 830-6.

Funch-Jensen P, Sorensen S. Influence of graded distension of the gallbladder on sphincter of Oddi activity in dogs. Dig. Dis. Sci. 1991; 9: 408-13.

Furness JB, Kuramoto H, Baker RA, Toouli J. Direct neural projections from the duodenum to the gallbladder and the sphincter of Oddi. Gastroenterology 1990; 98: A379.

169

Glambek I, Arnesjo B, Soreide O. Correlations between gallstones and abdominal symptoms in a random population. Results from a screening study. Scand J. Gastroenterol. 1989; 24: 277-81.

Graham E and Cole WH. Roentgenologic examination of the gallbladder. J.A.M.A. 1924; 82: 613-4.

Gracie WA, Ransohoff DF. The natural history of silent gallstones: the innocent gallstone is not a myth. N Engl J Med. 1982; 307: 798-800.

Green GM, Lyman RL. Feedback regulation of pancreatic enzyme secretion as a mechanism for trypsin inhibitor induced hyper secretion in rats. Proc. Soc. Exp. Biol. Med. 1972; 140: 6-12.

GREPCO. The epidemiology of gallstone disease in Rome, Italy Parts 1 and 2. Hepatology 1988; 8: 904-913.

Gomez G, Townsend CM, Maani R et al. Down regulation of pancreatic growth and gallbladder contractility by bile salts. Am J. Surg.1989; 157:20-26.

Gullo L, Bolondi I, Priori P, et al. Inhibitory effect of atropine on CCK induced gallbladder contraction in man. Digestion 1984; 29: 209-213.

Harper AA, Raper HS. Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. J. Physiol. 1943; 102: 115-25.

Harvey R and Read A. Effect of cholecystokinin on colonic motility and symptoms in patients with the irritable bowel syndrome. Lancet 1973;7793:1-3.

Hess JF, Borkowski JA, Young GS et al. Cloning and pharmacological characterisation of a human bradykinin BK2 receptor gene. Biochem. Biophys. Res. Commun. 1992; 184: 260-268.

Howard P, Murphy G and Dowling R. Gallbladder emptying patterns in response to a normal meal in healthy subjects and patients with gallstones: ultrasound study. Gut 1991; 32: 1406-11.

Hotz J, Ho S, Go VW, Di Magno EP. Short term inhibition of duodenal tryptic activity does not affect human pancreatic, biliary or gastric function. J. Lab. Clin. Med. 1983; 101: 488-495.

Hutton S, Sieveret C, Vennes J, et al. Spontaneous passage of glass beads from the canine gallbladder: facilitation by sphincterotomy. Gastroenterology 1988; 94: 1031-5.

Ivy AC and Oldberg E. A hormone mechanism for gallbladder contraction and evacuation. Am. J. Physiol. 1928; 86: 599-613.

Jebbink M, Masclee A, Lamers CHBW. Effect of Loxiglumide and atropine on erythromycin induced reduction in gallbladder volume in human subjects. Hepatology 1992; 16: 937-942.

Jensen RT, Wank SA, Rowley WH, Sato S, Gardner JD. Interaction of CCK with pancreatic acinar cells. Trends Pharmacol Sci. 1989; 10:418-423.

Johnson C.D., Hillier K, Reid J.M., Rushton P, Borman R.A. The contractile action of bradykinin on human gallbladder *in vitro*. J. Physiol.1997; 499:107P

Jonderko K, Nowak A, Kasicka-Jonderko A et al. Effect of nifedipine on interdigestive gallbladder volume and postprandial gallbladder emptying in man. Dig. Dis. Sci. 1991; 36: 1434-40.

Jorgensen T. Abdominal symptoms and gallstone disease: an epidemiological investigation. Hepatology 1989; 9: 856-60.

Jorpes J, Mutt V. Cholecystokinin and pancreozymin one single hormone? Acta Physiol. Scand. 1966; 66: 196-202.

171

Jungst D, Lang T, Von Ritter C, et al. Cholesterol nucleation time in gallbladder bile of patients with solitary or multiple cholesterol gallstones. Hepatology 1992; 15: 804-8.

Kanayama S, Liddle RA. Somatostatin regulates Duodenal CCK and somatostatin messenger RNA. Am J. Physiol 1990; 258: G358.

Kato K, Hayashizaki Y et al. Molecular cloning of the human cholecystokinin cDNA. Poster presented at the first international conference on neuronal Cholecystokinin, Brussels 1984.

Kellow JE, Miller LJ, Phillips SF, et al. Altered sensitivity of the gallbladder to CCK octapeptide in irritable bowel syndrome. Am J. Physiol 1987; 253: G650-655.

Kim CK, Lee KY, Wang LT, Sun G, et al. Role of endogenous cholecystokinin on vagally stimulated pancreatic secretion in dogs. Am. J. Physiol. 1989; 257: G944-949.

Kishk S, Darweesh R, Dodds W et al. Sonographic evaluation of resting gallbladder volume and postprandial emptying in patients with gallstones. Am J. Rad. 1987; 148: 875-9.

Koop I, Koop H,Gerhardt C, Schafmayer A, Arnold R. Do bile acids exert a negative feedback control on cholecystokinin release? Scand. J. Gastroenterol 1989; 24: 315-320.

Kotwall CA, Clanachan AS, Baer HP, Scott GW. Effect of prostaglandins on motility of gallbladders removed from patients with gallstones. Arch Surg. 1984; 119: 709-712.

Kratzer W, Mason R, Haag U, et al. Effect of extracorporeal shock-wave lithotripsy on gallbladder emptying in patients with solitary and multiple gallstones. Dig. Dis. Sci. 1995; 40: 1179-84.

172

Lanzini A, Jazrawi R, Northfield TC. Simultaneous quantitative measurements of absolute gallbladder storage and emptying during fasting and eating in humans. Gastroenterology 1987; 92: 852-61.

Lee KY, Bianchi P, Behar J. Calcium sources utilised by cholecystokinin and acetylcholine in the cat gallbladder muscle. Am. J. Physiol. 1989; 256: G785-88.

Lembeck F, Griesbacher T and Legat F. Lack of significant unspecific effects of HOE-140 and other novel bradykinin antagonists *in vivo* and *in vitro*. Recent Progress on Kinins; 414-422

Lennon F, Feeley TM, Clanachan AS, Scott GW. Effects of histamine receptor stimulation on diseased gallbladder and cystic duct. Gastroenterology 1984; 87: 257-262.

Liebmann C, Schnittler M, Stewart J and Reissmann S. Antagonist binding reveals two heterogeneous B_2 bradykinin receptors in rat myometrial membranes. Eur J. Pharmacol 1991; 199: 363-5

Lilja P, Fagan F, Wiener I, Inoue K et al. Infusion of pure cholecystokinin in humans. Correlation between plasma concentrations of cholecystokinin and gallbladder size. Gastroenterology 1982; 83: 256-61.

Luman W, Williams A, Pryde A, et al. Influence of cholecystectomy on sphincter of Oddi function. Gut 1997; 41: 371-4.

Mack A J and Todd JK. A study of human gallbladder muscle *in vitro*. Gut 1968; 9: 546-9

Mann FC. A physiological consideration of the gallbladder. J.A.M.A. 1924; 83: 829-32.

Marzio L, Capone F, Neri M, et al. Effect of cholinergic agonists and antagonists on gallbladder volume in fasting man.Eur. J. Clin. Pharmacol. 1987b; 33: 151-3.

Mate L, Sakamoto T, Greeley GH, Thompson JC. Effect of substance P on contractions of the gallbladder. Surg. Gynecol. Obstet. 1986; 163: 163-6.

McCafferty D, Mudgett J, Swain M and Kubes P. Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. Gastroenterology 1997; 112: 1022-1027.

McKirdy M, McKirdy H and Johnson C.D. Non-adrenergic non-cholinergic inhibitory innervation shown by electrical field stimulation of isolated strips of human gallbladder muscle. Gut 1994; 35: 412-6.

McKirdy M, Johnson C D, McKirdy H. Inflammation impairs neurally mediated responses to electrical field stimulation in isolated strips of human gallbladder muscle. Dig.Dis.Sci. 1994; 39: 2229-34.

McLaughlin JT, Luca MG, Jones A et al. CCK release by fatty acids is a function of acyl chain length but not saturation. Gut 1995; 37 (suppl 2) :A19.

Meltzer S.J. The disturbance of the law of contrary innervation as a pathogenetic factor in the diseases of the bile ducts and gallbladder. Am. J. Med. Sci. 1917; 153: 469-77.

Menke JG, Borkowski JA, Bierlo KK et al. Expression cloning of a human B1 bradykinin receptor. J. Biol. Chem 1994; 269: 21583-21586.

Mincsev, M Bilocular gallbladder. Orvoskepzes 1967; 42: 286-98.

Moncada S, Radomski MW, Palmer RMJ. Endothelium derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function . Biochem pharmacol 1988; 37: 2495-501.

Mourad F, O'Donnell L, Andre E. et al. L-Arginine, nitric oxide and intestinal secretion: studies in rat jejunum. Gut 1996; 39: 539-44.

Mourelle M, Guarner F, Moncada M and Malagelada J. The arginine/nitric oxide pathway modulates sphincter of Oddi motor activity in guinea pigs and rabbits. Gastroenterology 1993; 105: 1299-1305.

Mourelle M, Guarner F, Molero X et al. Regulation of gallbladder motility by the arginine nitric oxide pathway in guinea pigs. Gut 1993; 34: 911-5.

Mutt V, Jorpes JE. Structure of porcine cholecystokinin-pancreozymin. Eur. J. Biochem. 1968; 6: 156-62.

Nilsson B, Delbro D, Hedin L et al. Role of nitric oxide in induction of inflammatory fluid secretion by the mucosa of the feline gallbladder. Gastroenterology 1996; 110: 598-606.

O'Donnell LJ, Wilson P, Guest P, Fairclough PD. Indomethacin and post prandial gallbladder emptying. Lancet 1992; 339: 269-271.

Oddi R. D'une dispositiona sphincter speciale de l'ouverture du canal choledoque. Arch. Ital. Biol. 1887; 8: 317-32

Owyang C, Archem-Karam SR, Vinik AI. Correlation between pancreatic enzyme secretion and plasma concentration of human PP in health and in chronic pancreatitis. Gastroenterology 1982; 83: 55-62.

Owyang C, Dexter SL, Tatum D. Feedback regulation of pancreatic enzyme secretion. Suppression of cholecystokinin release by trypsin. J. Clin. Invest. 1986; 77: 2042-2047.

Patankar R, Ozmen M, Sanderson A and Johnson C.D. Effect of cisapride on gallbladder emptying and plasma CCK in normal and vagotomised human subjects. Dig. Dis. Sci. 1996; 41: 543-8.

Patankar R, Bailey IS, Sanderson A, Johnson CD. Biliary motility and CCK levels after truncal vagotomy. Gut 1993; 34: F201.

Patankar R, Ozmen M and Johnson C.D. Relative contributions of vagotomy and pyloroplasty to raised postoperative CCK levels in humans. J. Physiol 1994; 47: 74P.

Pauletzki J, Althaus R, Holl J et al. Gallbladder emptying and gallstone formation: a prospective study on gallstone recurrence. Gastroenterology 1996; 111: 765-771.

Pauletzki J, Cicala M, Holl J et al. Correlation between gallbladder fasting volume and postprandial emptying in patients with gallstones and healthy controls. Gut 1993; 34: 1443-7.

Perkins MN, Campbell E and Dray A. Anti nociceptive activity of the bradykinin B1 and B2 antagonists des-Arg^9, (Leu^8)- BK and HOE –140, in two models of persistent hyperalgesia in the rat. Pain 1993; 53: 191-7.

Pomeranz IS, Davison JS, Shaffer EA. In vitro effects of pancreatic polypeptide and motilin on contractility of human gallbladder. Dig. Dis. Sci. 1983; 28: 539-544.

Pomeranz I and Shaffer E. Abnormal gallbladder emptying in a subgroup of patients with gallstones. Gastroenterology 1985; 88: 787-91.

Portincasa P, Di Ciaula A, Baldassarre G, Palmieri V, Gentile A, Cimmino A and Palasciano G. Gallbladder motor function in gallstone patients: sonographic and in vitro studies on the role of gallstones, smooth muscle function and gallbladder wall inflammation. Journal of Hepatology 1994; 21: 430-440.

176

Portincasa P, Van de Meeberg P, Van Erpicum K et al. The pathogenesis and treatment of gallstones. Scand. J. Gastroenterol. 1997; 32 Suppl 223: 60-9.

Rapoport RM, Murad F. Agonist induced endothelium dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circ. Res. 1983; 52: 352-7.

Ravdin I.S., and Morrison J.L. Gallbladder function. The contractile function of the gallbladder. Arch Surg., 1931; 22: 810-28.

Regoli D, Barabe J. Pharmacology of bradykinin and related kinins. Pharmacol. Rev. 1980; 32 1-46.

Rehfeld J, Kruse-Larsen C. Gastrin and cholecystokinin in human cerebrospinal fluid. Immunochemical determination of concentrations and molecular heterogeneity. Brain Res 1978;155: 19-26.

Ritchie J. Pain from distension of the pelvic colon by inflating a balloon in the irritable bowel syndrome. Gut 1973; 14: 125-32.

Ryan J. Motility of the gallbladder and biliary tree. In   Physiology of the gastrointestinal tract. New York, Raven Press. 1981; pp: 473.

Ryan J, Cohen S. Gallbladder pressure volume response to gastrointestinal hormones. Am. J. Physiol. 1976; 230: 1461-65.

Sackmann M, Niller H, Kluepellberg U et al. Gallstone recurrence after shock wave therapy. Gastroenterology 1994; 106: 225-230.

Sakamoto T, Fujimara M, Newman J et al. Comparison of hepatic elimination of different forms of CCK in dogs: bioassay and RIA comparison of CCK sulphate and – 33 sulphate. J. Clin Invest 1985; 75: 280.

Sand J, Arvola P, Jantti V, Oja S et al. The inhibitory role of nitric oxide in the control of porcine and human sphincter of Oddi activity. Gut 1997; 41: 375-380.

Sauerbruch T, Stellaard F and Paumgartner G. Effect of endoscopic sphincterotomy on bile acid pool size and bile lipid composition in man. Digestion 1983; 27: 87-92.

Schneider H, Benninger JR, Rabes U, et al. Recurrent gallstone formation after successful extracorporeal shockwave lithotripsy. Am J. Gastroenterology 1993; 88: 1399-1404.

Scott G and Otto W. Resistance and sphincter like properties of the cystic duct. Surg. Gynae. Obst. 1979; 149: 177-182.

Singer MV, Niebel W, Jansen JBMJ. Pancreatic secretory response to intravenous caerulein and intraduodenal tryptophan: studies before and after stepwise removal of the extrinsic nerves of the pancreas in dogs. Gastroenterology 1989; 96: 925-34.

Snape W, Matarazzo S and Cohen S. Effect of eating and gastrointestinal hormones on human colonic myoelectrical and motor activity. Gastroenterology 1978;75:373-8.

Solcia E, Pearse A, Grube O, et al. Revised Wiesbaden classification of gut cells. Rendic. Gastroenterol. 1972; 5: 13.

Stark M and Szurszewski J. Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology 1992; 103: 1928-1949.

Stewart J. The present and future of bradykinin antagonists. Braz. J. Med. Biol. Res. 1994; 27: 1699-1706.

Stolk M, van Erpicum K, van Berge Henegouwen G et al. Gallbladder volume and contraction measured by sum of cylinders method compared with ellipsoid and area length methods. Acta Radiologica 1990; 31: 591-6.

Sugiyami M and Atomi Y. Long-term effects of endoscopic sphincterotomy on gallbladder motility. Gut 1996; 39: 856-9.

Tang C, Biemond I and Lamers C. Cholecystokinin receptors in human pancreas and gallbladder muscle: a comparative study. Gastroenterology 1996; 111: 1621-1626.

Tankurt E, Yegen BC, Biren T, et al. Influence of pirenzepine on gallbladder contraction in man induced by sham feeding or an intraduodenal meal. Digestion 1992; 51: 103-9.

Thompson J, Fried G, Ogden D et al. Correlation between release of cholecystokinin and contraction of the gallbladder in patients with gallstones. Ann. Surg. 1982; 195: 670-6.

Thompson WG, Dotevall G, Drossman DA, Heaton KW, Kruis W. Irritable bowel syndrome: guidelines for the diagnosis. Gastroenterol Int 1989; 2: 92-5.

Thornell E, Jansson R, Svanvik J. Indomethacin reduces raised intraluminal gallbladder pressure in acute cholecystitis. Acta Chir Scand 1985; 151: 261-265.

Tokunaga Y, Cox K, Coleman R et al. Characterisation of CCK receptors on the human gallbladder. Surgery 1993; 113: 155-62.

Toouli J. Sphincter of Oddi motility. Br. J. Surg. 1984; 71: 251-6.

Toouli J, Bushell M, Stevenson G et al. Gallbladder emptying in man related to the fasting duodenal migrating motor contractions. Aust. N.Z. J. Surg. 1986; 56: 147-51.

Upp J, Nealon W, Singh P et al. Correlation of cholecystokinin receptors with gallbladder contractility in patients with gallstones. Ann. Surg. 1987; 205: 641-8.

179

Wank SA, Pisegna JR, De Weerth A.Brain and gastrointestinal cholecystokinin receptor family: Structure and functional expression. Proc. Natl. Acad. Sci. USA 1992; 89: 8691-8695.

Wedmann B, Scmidt G, Wegener M et al. Sonographic evaluation of gallbladder kinetics: *in vitro* and *in vivo* comparison of different methods to assess gallbladder emptying. J. Clin. Ultrasound 1991; 19: 341-9.

Wegge C, Kjaergaard J. Evaluation of symptoms and signs of gallstone disease in patients admitted with upper abdominal pain. Scand. J. Gastroenterol. 1985; 20: 933-6.

Wiener I, Inoue K, Fagan C et al. Release of cholecystokinin in man. Ann. Surg. 1981;194: 321-7.