Bioassay of cholecystokinin

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SUMMARY An *in vitro* bioassay for cholecystokinin which is superior to the previously described *in vivo* assays has been properly validated mathematically. The specificity of the assay has been assessed by measuring the potency of other polypeptides which share the same C-terminal pentapeptide sequence with cholecystokinin (gastrin, caerulein, octapeptide of cholecystokinin, and pentagastrin). The assay is shown to be quite specific for cholecystokinin. Secretin does not interfere with the assay. Comparison of the relative potency of the two commercially available preparations of cholecystokinin indicates that the two major units of measurement are almost identical in cholecystokinetic potency. One Crick Harper Raper unit of cholecystokinin (Boots) is equivalent to 1.22 ± 0.12 Ivy dog units of cholecystokinin (Karolinska Institute). The lower limit of sensitivity for the assay was 2.5m IDU/ml.

Radioimmunoassay is now universally used for the assay of all biologically active gastrointestinal peptides. There are, however, no simple sensitive bioassays for any gastrointestinal peptide except cholecystokinin.

Cholecystokinin (CCK) acts predominantly on the gall bladder to produce contraction and on the pancreas to produce pancreatic secretion. The biological activity of cholecystokinin is consequently expressed in two quite different units. The first is the Crick Harper Raper unit (CHRU), which is a measure of pancreatic secretion rate in an intact anaesthetised cat. The second unit is the Ivy dog unit (IDU). This is a measure of the rise in gall bladder pressure produced in an intact anaesthetised dog. Both of these units are assayed *in vivo* and the assays are difficult, tedious, and expensive.

There are two commercially available preparations of CCK and the activity of each is expressed in different units. The Boots preparation is standardised in Crick Harper Raper units, the GIH Laboratories preparation is standardised in Ivy dog units.

An *in vitro* bioassay of CCK has recently been described¹⁻³ using isolated strips of rabbit gall bladder which has a clear advantage over the previously *in vivo* bioassays. However, the authors claimed that this simple bioassay was not influenced by variations in tissue sensitivity. Our

experience is quite contrary to this view. An identical dose of pure cholecystokinin administered to the preparation repeatedly over several hours has given widely different magnitudes of response by the tissue, sometimes changing by a factor of four or five. This change in sensitivity of the preparation would often occur suddenly after several hours of reasonably stable responsiveness. These biological variations in tissue sensitivity are well recognised and occur in all biological assay systems. Indeed, it would be surprising if rabbit gall bladder were spared this. Because of this biological variation in tissue response, a result for an unknown sample can not simply be read from a dose response curve constructed from the response to standard concentrations administered earlier in the experimental period, because the tissue sensitivity frequently changes between these two points in time. This problem is obviated by the standard technique of a four point assay—two concentrations of unknown assayed and two concentrations of standard assayed in a fixed time interval and then mathematically validated to ensure that no significant variation occurred during that time interval.

We have studied the validity, precision, and specificity of such an *in vitro* bioassay for cholecystokinin. The specificity of the assay has been estimated by comparing the relative potency of polypeptide hormones showing the same C-terminal pentapeptide sequence. Each assay has been validated by statistical criteria.

Method

The method is based on that described by Johnson and McDermot¹ in which oxygenated Krebs solution, to which the standard or test substance is added, is continuously superfused over isolated rabbit gall bladder strips at 37°C and pH 7.4. The gall bladder contractions were relayed via a custom built isotonic transducer, and the signal amplified and recorded on a Bryan's Flatbed XY recorder. The substances used in this assay were: (1) GIH Research Laboratories (Karolinska Institute) cholecystokinin (GIH CCK), (2) Boots pancreozymin (Boots CCK), (3) human synthetic gastrin (G1, 17), (4) caerulein (Farmitalia), (5) octa peptide of cholecystokinin kindly donated by Squibb Europe Ltd, (6) pentagastrin (Peptavlon, ICI), (7) secretin.

ANALYSIS OF RESULTS

In the absence of biological variation in tissue sensitivity, a standard dose response curve could be constructed from which the unknown values could be estimated. This is the method used in the published assays. However, we have found that the same standard dose produced a response in the tissue at different times which may vary by up to a

factor of five. Because of this, we have used a four point (2×2) assay. Each unknown is estimated on a minimum of three occasions at two different concentrations, one high and one low. The mean of these estimations is then calculated. These six points were assayed in random sequence and interspersed with the assay of two different concentrations of the standard preparations (GIH CCK). The concentration of the standard was chosen so that the height of the contractions produced was approximately the same as that produced by the unknowns. The dose interval between the high and low concentration of standard and the high and low concentration of unknown were identical.

The mean results could be expressed diagrammatically as shown (Figure), and the relative potency of the unknown with respect to the standard would be given by the horizontal distance between the two lines obtained, if the lines are parallel.

The validity of each assay was checked statistically by the methods recommended by Gaddum.⁴ The assay was considered invalid and the results discarded if the lines showed a statistically significant deviation from parallel or if

Figure S_L : low concentration of standard cholecystokinin solution. S_H : high concentration of standard cholecystokinin solution. U_L : low concentration of unknown solution. U_H : high concentration of unknown solution. Each concentration of standard and unknown is assayed on a minimum of three occasions. Assays are performed in random sequence and mean $\pm$ standard error of mean is calculated. The relative potency of the unknown with respect to the standard is then given by the horizontal distance D between the two lines which are constructed as indicated in this diagram. This diagram is provided to illustrate the concept only as these values are calculated mathematically. For a valid assay, the lines must be demonstrated mathematically to show no statistically significant deviation from parallel and the slopes to be statistically significantly greater than zero.

the slope of either line were statistically insignificant at the level $P < 0.05$. The accuracy of each assay was assessed by calculating (1) the index of significance of slope (Finney's g), (2) the index of precision for the assay (Gaddum's λ), and (3) the 95% fiducial limits.

If a valid assay showed relatively large errors as assessed in this way, then analysis of variance was performed and, where the error was caused by a systematic drift, it was eliminated and the result recalculated using the new error terms.

Results

SPECIFICITY

Significant dose-related contractions occurred with both preparations of cholecystokinin, octapeptide of cholecystokinin, caerulein, and pentagastrin.

No significant contractions were recorded with secretin, or with human synthetic gastrin (sulphated, G1, 17) in physiological doses. Dose dependent responses to gastrin were seen only at massive concentrations of 200 to 400 ng/ml.

RELATIVE POTENCY OF PEPTIDES

The results are expressed as the means ($\pm$ SEM) of the best estimates for each assay, as the range of fiducial limits is cumbersome.

One CHRU of Boots = 1.22 ± 0.12 IDU of GIH CCK. These data are derived from a total of eight valid assay runs. The arithmetical mean of Gaddum's λ for the eight assays was 0.119 (range 0.07-0.18). Mean Finney's g (for the eight assays) was 0.29.

One IDU of GIH was equivalent to 101 ng pentagastrin—that is, 6.15 pM—and 7.68 ng octapeptide—that is, 6.55 pM.

Thus the relative potency on a molar basis with respect to GIH Laboratory CCK is as follows:

Caerulein	:	Octapeptide	:	Cholecystokinin	:	Pentagastrin
		is				
14		13		1		0.006

This assumes a molecular weight of 3883 for cholecystokinin. One IDU of GIH CCK was contained in 333 ng or 86 pM.

SENSITIVITY

The assays were consistently invalid at CCK concentrations below 2.5 m IDU/ml.

Discussion

We have found that the sensitivity of isolated rabbit gall bladder strips to superfused cholecystokinin is not constant as previously suggested.² In contrast, a marked variation in tissue sensitivity has been

consistently observed during the process of bioassay, which was a major source of error. We, therefore, consider previous assays which have ignored this variability to be inadequate.

The assays reported here have been individually validated mathematically by internationally accepted criteria.

The Ivy dog unit of CCK activity is defined as that amount of dry material which, dissolved in normal saline and injected intravenously during 10-15 seconds into an anaesthetised dog weighing approximately 15 kg, causes a more or less immediate rise in intra gall bladder pressure of 1 cm of bile. The Crick Harper Raper unit is that amount of activity which was present in 0.1 mg of an arbitrarily chosen standard preparation of secretin prepared by the method of Harper and Raper (1943).⁵ In the original description one unit produced an average flow of 1.2 ml of pancreatic juice in 12 minutes in an anaesthetised cat.

In our validated assay system, we find that these two units of CCK activity are fortuitously very similar, 1 CHRU being equivalent to 1.22 IDU.

It is well known that peptides which show a similar C-terminal pentapeptide sequence with cholecystokinin possess some cholecystokinetic properties. To assess the specificity of the assay we have studied some of these peptides.

The amphibian decapeptide caerulein was shown in this assay system to have 14 times the potency of cholecystokinin. The octapeptide of CCK was only slightly less potent than caerulein on a molar basis. This finding is in good agreement with Vagne and Grossman⁶ whose *in vivo* assay was well validated.

Cross-reactivity with gastrin often interferes with radioimmunoassays for CCK. In this bioassay the synthetic pentapeptide in the form of peptavlon was a very weak stimulant of gall bladder activity (0.0006 of the potency of CCK). Similarly, human synthetic (sulphated) gastrin (G1, 17) did not produce dose dependent contractions except when grossly unphysiological concentrations (200-400 ng/ml) were used.

Secretin is believed to contaminate some commercial preparations of cholecystokinin. Pure synthetic secretin did not produce any contractions in this assay.

The assay, therefore, appears to be relatively specific for CCK when assayed as a superfusate in buffer. However, degradation of the CCK molecule liberating the octapeptide would clearly cause serious over-estimation of the CCK concentration by this method.

This assay used purified CCK in a buffer solution. Cholecystokinin in serum will, of course,

cause the rabbit gall bladder preparation to contract. However, when all cholecystokinin has been removed from serum before assay, the resulting serum will still cause a contraction. This has been called 'the blank effect'.³ There are, therefore, factors in serum other than cholecystokinin which cause gall bladder contraction and which have not yet been accurately identified. The parallel line assay assumes that there is a single substance—in this case, CCK—in the assay medium which produces a dose-related response. This is clearly not the case for serum where several different constituents—for example, histamine—can produce a cholecystokinin-like response—the 'blank effect'. Identification and elimination of these factors is essential before the concentration of CCK can be assayed biologically in serum.

The assay reported here is sensitive and relatively

specific for CCK assayed in buffer but more work is required before the assay can be applied to serum.

References

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Books

Practical Gastroenterology Edited by Ronald L Koretz. (Pp. 395. Illustrated. £22.00). John Wiley: Chichester. 1982.

This book is part of a programme of publications entitled 'Family Practice Today: A Comprehensive Postgraduate Library'. I am unfamiliar with the series but if the other volumes are as enjoyable as the one on practical gastroenterology then the series would repay closer attention.

Practical Gastroenterology has six contributors who have produced a most readable book. The style is relaxed, almost chatty, the points are made clearly, and the emphasis is on down-to-earth everyday gastroenterological practice. The book is divided into two sections, the first dealing with the nine most prominent symptoms of gastrointestinal disease, and the second with a baker's dozen of the more important abdominal diseases. The chapters have been very carefully structured, asking and answering specific points. The reader is taken through a clinical problem by a clinician who is familiar with his field. One would not necessarily agree with all the statements made: I have seldom found the shape of the stool of diagnostic value; and the flow-diagram of the diagnostic approach does not fit exactly with my clinical practice, but all the advice offered is sound and reasonable. This is not a conventional textbook of gastroenterology, rather it is a practical guide to the evaluation of the clinical situation and as such is in competition with at least three other recently published texts. *Practical Gastroenterology* compares most favourably and can be recommended to undergraduates and to graduates who do not have a specialist interest in gastroenterology.

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Large Bowel Cancer Edited by Jerome J. de Cosse (Pp. 225. Illustrated. £9.75). Churchill Livingstone: Edinburgh. 1981.

This first volume of a new long-awaited series, 'Clinical Surgery International', has not been an anticlimax. The series starts with the 'Cinderella' specialty of surgery and is well priced, easy to read and digest, and each chapter is sensibly referenced. It does not attempt to be comprehensive but, instead, highlights areas of contemporary interest. Edited by an American, there are contributions from Australia, from six United Kingdom centres, from six North American 'centres', including Hawaii, and also from Japan. The aetiology of large bowel cancer and familial polyposis are refreshingly discussed by workers of the Japan-Hawaii Cancer Study and the recently established Japanese Polyposis Centre, while the pathology of polyps and the evidence for the adenocarcinoma sequence are authoritatively presented by the pathologists of St Mark's. The methods of early diagnosis and the importance of preventive screening are thoughtfully and honestly evaluated. The current areas of debate in operative technique are focused in chapters on prevention of sepsis, extent of resection, staplers, and the management of obstructing large bowel cancers, which illogically separates a chapter on sphincter-preserving operations from a chapter on local treatment of rectal cancers. Throughout, the volume is appropriately illustrated with particular commendation for the illustrations with the discussion of stapling devices by Rothenberger and Goldberg. Subsequently Goligher sagely reviews the results of surgery and Hughes usefully reviews the management of recurrent large bowel cancer with suitable references for further consultation. In my opinion the added bonus of this volume is the truly comprehensive reviews of adjuvant radiation therapy, chemotherapy, and immunotherapy.

J G PAYNE

News

Steroid Reference Collection

The Steroid Reference Collection, which is maintained by the UK Medical Research Council and the US National Institute of Arthritis and Metabolic Diseases, NIH, provides milligram or microgram samples of reference steroids free of charge for use in clinical or biochemical studies, including the development and stan-

dardisation of microassays, chromatography, and mass spectrometry. The Collection also offers NMR services (^1H and ^{13}C) for the identification of new steroidal compounds, and for structural studies. Requests for information, lists of available steroids, or offers of samples for the Collection, should be addressed to: Professor D N Kirk, Curator of the Steroid Reference Collection, Chemistry Department, Westfield College, Hampstead, London, NW3 7ST.

Correction

The Crick, Harper, Raper Unit for pancreaticozym was incorrectly defined in the paper, 'Bioassay of cholecystokinin', by E L Giannoulis and R E Barry, in the February edition of *Gut*, 23, 146-9. The correct definition is: one Crick, Harper, Raper Unit is that amount of activity in 1mg of an arbitrarily chosen standard preparation. (Ref.: Crick J, Harper AA, Raper HS. On the preparation of secretin and pancreaticozym, *Journal of Physiology* (1949), 110, 367-76).