On premises and principles for measurement of gastrointestinal peptide hormones

Nicolai J. Wewer Albrechtsen, Jens F. Rehfeld *

Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Denmark

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ABSTRACT

Gastrointestinal hormones are peptides, and the gastrointestinal tract is the largest endocrine organ in the body for production of peptide hormones. As a premise for accurate measurement of gastrointestinal hormones, the present review provides first an overview over the complex biology of the hormones: The structures and structural homologies; biogenetic aspects; phenotype variabilities; and cellular expression in- and outside the digestive tract. Second, the different methodological principles for measurement are discussed: Bioassay, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), mass-spectrometry (LC–MS/MS) and processing-independent analysis (PIA). Third, the variability of secretion patterns for some of the gut hormones is illustrated. Finally, the diagnostic value of gut hormone measurement is discussed. The review concludes that measurement of gastrointestinal peptide hormones is relevant not only for examination of digestive functions and diseases, but also for extra-intestinal functions. Moreover, it concludes that, so far, immunoassay technologies (RIA and ELISA) in modernized forms are still the most feasible for accurate measurements of gastrointestinal hormones in biological fluids. Mass-spectrometry technologies are promising, but still too insensitive and expensive.

1. Introduction

Gastrointestinal hormones are peptides released from endocrine cells, paracrine cells, and neurons spread out in an ordered manner in the mucosa of the digestive tract all the way from and including the stomach to the distal colon. The endocrine cells are intermingled with non-endocrine mucosa-cells, and not collected in gland-like structures. More than 30 hormone genes are known to be expressed in the gastrointestinal mucosa, which makes the gut the largest endocrine organ in the body [1–4].

In order to provide an overview about measurement of gastrointestinal hormones, it is essential to understand the biology of the hormones. The biology is categorized under five headings: The structural homology collects a majority of the hormones into nine families, each of which is assumed to originate from one ancestral gene. The individual gene often has multiple phenotypes due to alternative splicing, tandem organization, or – not least – a cell-specific differentiated posttranslational processing of the prohormone. A combination of these mechanisms results in release of more than 100 different hormonally active peptides from the gut. The hormone genes, however, are also widely expressed outside the digestive tract in other endocrine cells, in cerebral and peripheral neurons, in myocytes, immune cells and other cells. These extraintestinal cells may release different bioactive fragments of the same prohormone due to cell-specific maturation pathways. Moreover, the cells – including tumor cells – may secrete the peptides differently so that the same peptide may act not only as a classic blood-borne hormone, but also as a neurotransmitter, a local growth factor both for normal cells and cancer cells or as a fertility factor. Thus, today we see gastrointestinal hormones as potent regulatory, intercellular peptide systems that operate in the whole organism, and in fact also between organisms [4,5].

The described biological complexity has to be taken into account when gastrointestinal hormones are to be measured in a specific, sensitive, and accurate way. This review also deals with the options and limitations of methods available for such measurements.

* Corresponding author at: Dept. of Clinical Biochemistry (KB3014), Rigshospitalet, 9 Blegdamsvej, DK-2100, Copenhagen, Denmark. E-mail address: jens.f.rehfeld@regionh.dk (J.F. Rehfeld).

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2. Biological premises for measurements of gastrointestinal hormones

2.1. The structural homology

The gastrointestinal tract expresses, as mentioned, a large number of hormones, neuropeptides, and growth factors (Fig. 1). Not only have new hormones been found in gut extracts, but also neuropeptides from the central nervous system and hormones first identified in other endocrine organs have been found to be expressed in endocrine cells and/or neurons in the gut. Moreover, peptides originally believed to be classical hormones but later shown to be neurotransmitters in the central and peripheral nervous system have been isolated from gut extracts. The complexity is high, also because several genes for gut peptides encode different peptides released in a cell-specific manner.

In addition, there are still physiological, hormonal activities in the gut that are not yet structurally identified. Some of the activities can be explained by already identified peptides. Hence, the stimulation of insulin secretion from the gut, originally called the “incretin” effect [6], is today often explained by two hormones – glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) – but they also act in combination with other gut hormones [6,7]. Likewise, intestinal inhibition of gastric secretion, the “gastrone” effect, may be explained by combinations of CCK, GIP, GLP-1 and somatostatin. However, activities named villikinin, duocorin, enterocorin, and gastrcalcin still await structural identification. Fortunately, however, structural identifications have shown striking homologies between groups of peptides. Consequently, many of the biologically active gut peptides can now be classified into nine families (Table 1). The dual expression of several hormone genes both in the gut and pancreas reflects the intestinal origin of the pancreas. The nature of the homology varies. It may be an overall similarity in the primary structure as, for example, the PP-fold family [8]. Another type of homology is that of the PP-fold family [8]. Alternative splicing was discovered when it was shown that the calcitonin gene generates mRNAs encoding either calcitonin peptides or calcitonin-gene related peptides (CGRPs) [13]. CGRPs are now known to be abundantly expressed in intestinal neurons. Moreover, a tachykinin gene transcript [14] and the transcript encoded by the secretin gene [15] are also spliced alternatively in the gut.

2.2. The multiple phenotypes

Four decades ago, one gene was believed to encode one hormonal peptide in accordance with what we know about the master hormone, insulin. However, more intricate dimensions were added when it became obvious that a hormone gene often expresses different bioactive peptides. Today, we know three ways in which a gastrointestinal hormone gene can vary its expression into peptides.

2.2.1. Alternative splicing of transcripts

Alternative splicing was discovered when it was shown that the calcitonin gene generates mRNAs encoding either calcitonin peptides or calcitonin-gene related peptides (CGRPs) [13]. CGRPs are now known to be abundantly expressed in intestinal neurons. Moreover, a tachykinin gene transcript [14] and the transcript encoded by the secretin gene [15] are also spliced alternatively in the gut.

2.2.2. Multiple products of prohormones with one active sequence

The somatostatin and gastrin families represent peptide systems in which the gene encodes only one prohormone that contains one active site, but where the prohormone is processed in a way to release peptides of different lengths with the same epitope for receptor-binding, the bioactive C-terminus. Although the different products of the same precursor are bound to the same receptor, their varying clearances from
Table 1
Peptide hormones, neuropeptides, and growth factor families in the gastrointestinal tract and the pancreas.

| Families and members | Major regulatory activities |
|----------------------|---------------------------|
| **Secretin family**  |                           |
| Secretin             | Stimulates pancreatic bicarbonate secretion |
| Glucagon             | Increases glucose production and amino acid metabolism |
| Glucagon-like peptide 1 (GLP-1) | Stimulates insulin and inhibits glucagon secretion and gastric emptying |
| Glucagon-like peptide 2 (GLP-2) | Stimulates mucosal cell growth in intestinal crypts |
| Gastric inhibitory/glucose-dependent insulinotropic polypeptide (GIP) | Enhances glucose-stimulated insulin secretion and inhibits gastric secretion |
| Vasoactive intestinal polypeptide (VIP) | Inhibits gastrointestinal motility and stimulates fluid secretion |
| Peptide histidine isoleucine (PHI) | VIP-like actions |
| Growth hormone releasing hormone (GH) | Stimulates growth hormone secretion |
| Pituitary adenyl cyclase-activating peptide (PACAP) | Contributes to the regulation of gastric acid secretion and gastrointestinal motor function |
| **Gastrin Family**   |                           |
| Gastrin              | Stimulates gastric acid secretion and gastric mucosal cell growth |
| Cholecystokinin (CCK) | Stimulates pancreatic enzyme secretion, cell growth, and gallbladder emptying, but inhibits gastric acid secretion. |
| Caerulein and cionin, not expressed in mammals | Cholecystokinin-like activities |
| **Tachykinin Family** | Stimulates motility |
| Substance P           |                           |
| Neurokinin A          |                           |
| Neurokinin B          |                           |
| **Ghrelin Family**   |                           |
| Ghrelin              | Stimulates appetite and growth hormone secretion |
| Obestatin            | Suppresses food intake(?) |
| Motilin              | Contracts gastrointestinal smooth muscles to stimulate motility |
| **PP-fold Family**   |                           |
| Pancreatic polypeptide (PP) | Involved in feeding behavior (?) |
| Peptide YY (PYY)     | Reduces gastric emptying, pancreatic exocrine secretion, delays intestinal transit, and induces satiety |
| Neuropeptide Y (NPY) | Modulates the contractility in smooth muscle cells |
| **Somatostatin Family** | Inhibits gastric acid, gastrin secretion and other gut functions through endocrine, paracrine and neurocrine release |
| Somatostatin         | Somatostatin-like activities |
| Cortistatin          |                           |
| **Insulin Family**   | Establishes energy resources in fat, liver, and muscle cells |
| Insulin              |                           |
| Insulin-like growth factor I (IGF-I) | Stimulates growth and differentiation in interaction with other growth factors |
| Insulin-like growth factor II (IGF-II) | Stimulates growth and differentiation in interaction with other growth factors |
| Relaxin              | Function in the gastrointestinal tract uncertain |
| **EGF Family**       |                           |
| Epidermal growth factor (EGF) | Stimulates growth of epithelial cells and inhibition of gastric acid secretion |
| Transforming growth factor α (TGF-α) | EGF-like activities |
| Amphiregulin         | Growth regulation of epithelial cells |

Table 2
Singular peptide hormones, neuropeptides, and growth hormones in the gastrointestinal tract.

| Hormones and growth factors | Major regulatory activity |
|-----------------------------|--------------------------|
| **Apelin**                  | Stimulates gastric mucosal growth and cholecystokinin secretion |
| Bradykinin                  | Contributes to control alkaline secretion in the duodenal mucosa |
| Calcitonin gene-related peptide (GGRP) | Modulates blood flow, secretion and motility |
| Cocaine and amphetamine regulated transcript (CART) | Increases satiety |
| Galanin                     | Stimulates motility and luminal secretion |
| Gastrin-releasing peptide (GRP) | Stimulates atrial gastrin secretion |
| Neurotensin                 | Increases the ileal brake |
| Orexin                      | Stimulates gut motility (?) |
| Transforming growth factor β (TGF-β) | Growth, differentiation and inflammation |
| Thyrotropin-releasing hormone (TRH) | Releases TSH from epithelial cells in the gut |

For further information about these peptides, see refs. [1–3].

**Fig. 2.** Multiple phenotypes of three gut hormone genes. The cholecystokinin (CCK) gene encodes a prepropeptide which is processed to six CCK peptides varying in length from 83 to 8 amino acid residues through differentiated endoproteolytic cleavage. The six peptides have the same C-terminal bioactive octapeptide sequence. The secretin gene encodes a prepropeptide that through endoproteolytic cleavages and variable C-terminal trimming is processed to three bioactive secretin peptides of almost similar size (secretin-27, -28, and -30). In addition, bioactive secretin-71 is produced by splicing out RNA, encoding the midsequence of preprosecretin (i.e., broken line of secretin-71). The glucagon gene encodes a prepropeptide that through cell-specific endoproteolytic cleavages is processed to either genuine pancreatic glucagon (in pancreatic α-cells) or to glucagon-like peptides I and II (GLP-1, GLP-II) (see also ref. [4]).
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particularly thoroughly. It is, therefore, a useful illustration of the sec-
far, the biosynthesis of gastrin in antral G-cells has been examined.
prosomatostatin is processed to somatostatin-28 or somatostatin-14. So
mones and neuropeptides comprise many examples of such genes of
but often homologous peptide hormones or neuropeptides. Gut hor-
tides occurs when the gene encodes a propeptide containing different
sequences (Fig. 3, see also ref. [16]).

Proglucagon is an example of a poly-protein precursor that contains
three similar peptide sequences in mammals (Fig. 2, [17]). In pancreatic
α-cells, proglucagon is processed to release the well-known pancreatic
glucagon, whereas the C-terminal part of proglucagon remains silent. The
L cells of the gut also express proglucagon but process it in a different
way to release glicentin, oxyntomodulin, GLP-1 and GLP-2 [18,
19]. Although glucagon and, for instance, GLP-1 are highly homologous
and both glucoregulatory, they have separate activities and receptors
(Table 3). Proglucagon also tells another story, namely that bioactive
peptide structures cannot be predicted from cDNA and protein precursor
sequences. Identification requires exact examination of amino acid
sequence and derivatizations of the released peptides and studies of their
activities. The specificity of the methods used to measure prohormone-derived peptides are therefore of great importance.

2.3. The widespread gene expression

The expression cascade for gastrointestinal hormones involves many
processing steps. Each step contributes to control whether the initial
gene transcription results in a bioactive peptide. It is important, how-
ever, to realize that no processing step is complete, neither in normal nor
in diseased cells. Transcription can also occur without translation of the
transcript, and lack of parallelism between mRNA, propeptide, and the
mature bioactive peptide has also been encountered.

All gut hormones are expressed also in tissues outside the gastroin-
testinal tract, some mainly in neurons and extraintestinal endocrine
cells. However, several gut hormones are also expressed in other cell
types and tissues. The literature on extraintestinal expression of
gastrointestinal hormones has become overwhelming. Therefore, the
phenomenon will be described for a single hormonal system only.

The gastrin gene is expressed in several other cells than the G cells in
the antral antro-duodenal mucosa of the stomach and the proximal small
intestine. Quantitatively, these other cells release only little gastrin to
the blood in the normal adult organisms because the extra-antral
secretion may serve local purposes. Besides that, biosynthetic process-
ing is often so different that bioactive gastrins may not even be syn-
thesized. So far, extra-gastro-duodenal expression of the gastrin gene has
been encountered in the distal small intestinal and colorectal mucosa,
endocrine cells in the fetal and neonatal pancreas, pituitary cortico-
trophs and melanotrophs, hypothalamo-pituitary and vagal neurons and
spermatogenesis (for recent review, see ref. [16]).

The rationale for extraintestinal synthesis of gut hormones is often
unknown, but local growth stimulation is a possibility. It is also possible
that the low concentration is without significant function in the adult,
but is a relic of a more comprehensive fetal synthesis. A third possibility
is that low cellular concentration reflects constitutive secretion where
the peptides are not stored in secretory granules.

2.4. The cell-specific prohormone processing

The processing of gastrointestinal prohormones is so elaborate that
the result of gene expression is unpredictable. Thus, the cellular equip-
ment with processing enzymes and their cofactors that determine the
structure of the particular peptide hormone, varies [20]. This cell-specific maturation of prohormones applies to all gut hormones.
However, once again gastrin is also one of the most extensively studied
hormones in this respect.

Almost every tissue in which progastrin is expressed has its own
processing pattern. Four different patterns are shown in Fig. 3. For
members of the gastrin family, the processing varies with respect to
dendroproteinic processing and amino acid derivatizations such as
tyrosyl sulfations, seryl phosphorylations and phenylalanyl amidations.
In this context, it is worth realizing that the different types of processing
may influence each other. Thus, tyrosyl sulfation, the earliest post-
translational modification for the gastrin family of prohormones,

![Fig. 3. Schematic illustration of cell-specific processing of preprogastrin in antral G-cells, G-cells in fetal and neonatal pancreas, in pituitary corticotrophic cells and in unidentified cells in the colorectal mucosa (see also ref. [4]).](image-url)
increases endoproteolytic cleavage efficiency [21].

2.5. The cell-specific peptide release

To understand the specific effects of gastrointestinal hormones, it is necessary to realize that the different types of cells that express the genes also release the peptides in different ways. Secretion of gut hormones was supposed to be endocrine only, until 40 years ago. Today, however, three alternative routes of secretion to neighboring cells and one to the secretory cell itself have been discovered (Fig. 4). Firstly, the peptides synthesized in neurons are released from synaptosomal vesicles in the nerve terminals to the receptors on adjacent target cells as neurotransmitters. It is also possible that some peptidergic neurons expressing gut hormonal peptides, such as hypothalamo-pituitary neurons, release the peptides directly to blood vessels as neurocrine secretion. Secondly, it has been shown that there are specific paracrine cells that release, for instance, somatostatin in the gastrointestinal mucosa [22]. These cells carry peptidergic granules through cytoplasmic extensions to specific target cells in the neighborhood.

Cells stimulate their own growth through autocrine secretion. Trophic peptides bind to specific receptors in the membranes of cells in which they are also synthesized (Fig. 4). Autocrine secretion is supposed to play a decisive role in tumor and cancer development [23].

Cellular release of gastrointestinal peptides also occurs in a fifth way: Spermatozoa in mammals express gastrin, CCK, and pituitary adenylate cyclase activating peptide (PACAP) genes [24–26]. The peptides are concentrated in the acrosome. In accordance with the acrosomal reaction, the peptides are released from the spermatozoa by contact with the jelly-coat of the egg and subsequently bind to receptors in the egg membrane. Defects of the reproductive functions have now been found in PACAP-deficient mice [27]. The release of bioactive peptides from acrosomal granules could be termed spermiocrine release (Fig. 4).

3. Methodological principles for measurement of gastrointestinal hormones

Measurement of gastrointestinal peptide hormones began 120 years ago in tissue extracts using bioassays because hormones then were defined exclusively by their bioactivity. Their chemical structures were still unknown. However, the bioassays were rather insensitive and unspecific. Since the 1960s, however, massive advances in antibody production, detection-technologies and mass-spectrometry based approaches have changed the methodology spectrum. However, basic analytical quality criteria required for accurate measurement are frequently omitted with the potential of misinterpretation of scientific questions, as well as a wrong clinical diagnosis [28].

3.1. The Bioassay

The first discovered gastrointestinal hormones have been detected via their biological activities as reflected by the names (secretin, gastrin, cholecystokinin, incretin, enterogastrone, pancreozymin, gastric inhibitory polypeptide, motilin etc. (see Fig. 1)). For their original discovery, functional identification and purification from gastrointestinal extracts, as well as their subsequent structural identification as specific peptide hormones, bioassays have been indispensable tools. Some of the
bioassays have also, in terms of biology, been quite sophisticated. However, since hormones – gastrointestinal as well as extra-intestinal hormones – are defined by their occurrence in blood, where the plasma concentrations for most hormones are in the picomolar or even lower range, bioassays have failed. In comparison with immunoassays (RIA and ELISA) bioassays are far too insensitive and structurally less specific. In addition, they are also technically demanding, labor intensive and consequently expensive. Therefore, they are rarely used today. There are singular reports about bioassays for gut hormones that are sufficiently sensitive and specific for plasma measurements. Hence, in the 1980s, Liddle et al. managed to develop a cholecystokinin bioassay based on amylase secretion from exocrine pancreatic cells that could measure the low picomolar concentrations of CCK in plasma without cross-reactivity from any gastrin [29,30]. However, the complex and demanding labor intensity and costs of this assay prevented establishment in other laboratories. Thus, even Liddle et al. have today switched to a sensitive and specific CCK-RIA [31].

3.2. The radioimmunoassay (RIA)

The story of the breakthrough of peptide hormone measurements in plasma began in Bronx, New York, in the late 1950s. In these years, Solomon Berson and Rosalyn Yalow, based on studies of protein metabolism [32], developed what became known as the radioimmunoassay (RIA) – first developed for insulin [33]. The RIA technology changed biology and medicine in a fundamental way. Thus, RIA made it possible to measure substances in plasma in nanomolar, picomolar and, interestingly, even femtomolar concentrations. And at these concentration levels, a multitude of molecules essential for life and health circulate in blood: For instance hormones (proteins, peptides, steroids and thyronins), drugs and many other substances. Subsequently, already in the early 1960s, several laboratories began to develop RIAs for peptide hormones from the pancreas, the pituitary, the thyroid, the parathyroid glands, and the gastrointestinal tract. With the introduction of the RIA technology, fundamental discoveries in biology and many specialties of medicine have been achieved, including both diagnostic and therapeutic implications.

RIA is based on the competition between the peptide of interest in the sample and the tracer (the same peptide radioactively iodinated) for binding to an antibody present in deficient concentration. The epitope of the antibody used is crucial for specific measurements. In many cases whole length sequence of the peptide of interest is used as for the immunization, but this approach may be problematic due to the different molecular forms in which peptide hormones often occur. Therefore, synthetic peptide haptons of 6–10 amino acid residue sequences covalently coupled to carrier proteins have been used to raise antibodies in order to help ensure the exact specificity of the antibody. Furthermore, there is also the technical term of a ‘C- or N-terminal wrapping’ antibody which refers to the fact that the antibody used is highly sensitive for these termini. This is essential when differentiating between the bioactive peptides belonging to a given gut hormone system, because gut hormones, as mentioned above, circulate in different molecular forms. An example is the C-terminal or N-terminal modifications, of which there are many, are important to consider in order to fully reach specificity. Epitope mapping is another way to assess the specificity of the antibody used but is not frequently used due to cost. Then there is the question about the tracer (the radiolabeled peptide). There are various ways to iodinate a peptide. A frequent method is the stepwise chloramine T oxidative method, where iodine 125 is incorporated in the tyrosine amino acids. Another, but entirely non-oxidative way is the Bolton-Hunter moniodination, where the risk of decay catastrophe is reduced. Finally, iodogen – a mild oxidative reagent – can be used without reducing agents. Preferably, moniodination of peptides containing one (or more) tyrosyl residue(s) should be attempted in order to ensure stability of the tracer and hence reliability of the assay [34,35]. Finally, one should consider the synthetic peptide used both as substrate for iodination but also as calibrator/standard. The measured concentration may vary considerably across batches (inter-assay variation) and across laboratories using different synthetic peptides. A few companies are using WHO traceable calibrators that may decrease variability in absolute quantification across time/batches.

3.3. The enzyme-linked immunosorbent assay (ELISA)

The first reports on the ELISA technology were published in the early 1970s [36]. Development of the enzyme-linked immunosorbent assays (ELISAs) has been instrumental in clinical biochemistry due to the scalability of the ELISA. The ELISA appeals to some laboratories, as compared to the RIA. The ELISA is typically performed in a 96-well format plate and often requires less sample material (5–50 μL) compared to RIA. Several versions of the ELISA exist and include a direct, an indirect, a sandwich and a competitive ELISA. The detection system is flexible and ranges from chromogenic (absorbents) to laser (the LumineX technology) and is therefore without radioactive material, which some laboratories wish to avoid. Another aspect is the pleiotropic ways in which ELISAs can be designed. From a direct competitive ELISA to a Sandwich ELISA that each has different strengths and limitations. For example, the advances of the sandwich ELISA are the use of two antibodies (hence sandwich) that may increase the specificity of the peptide to be measured. Classic examples of peptides measured with a sandwich ELISA approach are biological intact parathyroid hormone (PTH) and glucagon. In other words, when the molecular heterogeneity of a peptide hormone is essential for understanding or diagnosing a disease, one must carefully assess analytical features such as specificity. Several immunoassays used in the literature have been flawed due to the lack of specific antibodies. Therefore, when considering the methodologies for measuring peptide hormones, one must carefully consider their analytical reliability and shortcomings. Unfortunately, many commercial immunoassay kits for gastrointestinal hormones are insufficiently evaluated and consequently often inaccurate [35,37–39]. This shortcoming may have dire clinical consequences and may lead to much confusion and problematic interpretation of the published literature.

3.4. Mass-spectrometry approaches

Antibody independent methodologies have also been described and used. A particular perspective has mass-spectrometry based detection of peptide hormones [40]. Mass-spectrometry is in principle superior to antibody-based assay methods because cross-reactivity and plasma interference are eliminated and as the method enables the differentiation of molecules down to 1 Da [40]. The current mass-spectrometry based approaches may in general be divided into ‘unbiased MS-based peptidomics’ and targeted MS-based detection [41]. For the latter, several approaches have been published, including both top-down (undigested peptides) and bottom-up (undigested and/or digested peptides) with and without the use of prior enrichment or fractionation steps. Enrichment-based strategies include the use of antibodies coupled to magnetic beads, whereas the fractionation steps typically include size exclusion chromatography but limit the sensitivity. Therefore, a large volume of material is necessary. Antibody-based enrichment is powerful but comes with the price of analytical variability and the same issues as described for the RIA and the ELISA. Of note is the challenge with variation in recovery (enrichment) that may vary up to 50 % and thereby introduces a considerable inaccuracy [38].

For the unbiased MS-based peptidomic approach one major concern is the ‘search space’. The search space refers to the number of combinations that amino acid sequences may occur in and given the large heterogeneity in not only the cell-specific processing of peptide hormones, but also includes posttranslational modifications and protease-induced cleavage sites, the ‘search space’ increases drastically and so does the analytical time. Furthermore, given that many peptide hormones are cleaved in vitro in plasma, protease inhibitors for sample
collection have become important, but also complex as the peptidome will vary accordingly [42]. In summary, although MS-based approaches in theory should be superior to RIA and ELISA, similar considerations regarding analytical reliability, costs and practicability are necessary. Moreover, for gut hormones systems with basal plasma concentrations in the femtomolar range, however, MS-technologies are not yet sensitive enough.

3.5. Processing-Independent analysis (PIA)

A major problem for quantitation of gastrointestinal hormones is their biogenetically determined molecular heterogeneity. The heterogeneity comprises - as mentioned above - different hormonally active products of the prohormone as well as biologically inactive precursor fragments and the often multiple processing-intermediates [43]. Consequently, a measure of the total hormone-gene expression at peptide level is complicated, laborious and costly to ascertain just by conventional RIA, ELISA, or MS-technology – even though it is a valuable parameter both in basic and clinical endocrinology. Fortunately, there is a simple method that solves the problem, i.e. processing-independent analysis (for recent review, see ref. [44]).

The antibody-based version of PIA is developed in the following way: A sequence of 8–10 amino acid residues of the propeptide that is neither modified nor cleaved during cellular processing, but on the other hand neighbors in vitro cleavage sites (typically a trypsin-sensitive basic residue) is synthetized. The peptide is coupled through either its N- or C-terminal residue to a suitable carrier protein (for instance bovine serum albumin). In order to facilitate coupling, a cysteine residue is added during synthesis at the terminus of the peptide through which coupling is going to take place. The peptide carrier complex is mixed in a suitable vehicle (Freund adjuvant) and then injected conventionally for production of antibodies specific for the peptide. The function of hormones in the digestive tract is primarily to allow release of one appropriate hormone when released into the bloodstream in response to food intake. The differentiated response to food is, however, not only a question in theory should be superior to RIA and ELISA, similar considerations regarding analytical reliability, costs and practicability are necessary. Moreover, for gut hormones systems with basal plasma concentrations in the femtomolar range, however, MS-technologies are not yet sensitive enough.

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### Table 4

| Name    | Origin of Secretion | Plasma concentration at fasted condition | Plasma concentration at fed condition (mixed meal) | Reference |
|---------|---------------------|-----------------------------------------|-----------------------------------------------|-----------|
| Ghrelin | Gastric             | 100                                     | 70                                             | [45]      |
| Gastrin | Antrum              | 10                                      | 30                                             | [46]      |
| CCK     | Duodenum and jejunum| 2                                       | 8                                              | [47]      |
| Secretin| Duodenum            | 10                                      | 125                                            | [48]      |
| GLP-1   | Ileum               | 5                                       | 40                                             | [49]      |
| GLP-2   | Ileum               | 10                                      | 50                                             | [50]      |
| Neurotensin | Ileum       | 20                                      | 60                                             | [51]      |

The concentrations shown are peak-concentrations 15–30 min after the onset of a meal. For ghrelin, the concentration is at nadir.

PIA-designed radioimmunoassay or ELISA where by definition proprotein, processing intermediates and end-products are included in the quantitation with equimolar potency. Hence, each translated molecule of protein is measured irrespective of the degree of processing (see also Fig. 5).

4. The concentration levels of gastrointestinal hormones in plasma

The function of hormones in the digestive tract is primarily to respond to food in order to initiate digestion, increase motility of the gut, which again further enzymatic digestion, absorption and metabolism of the digested food elements. Subsequently, some gut hormones – mainly CCK, GLP-1, and PYY – contribute to terminate food intake by acting as satiety signals in due course. The overall response pattern of the individual hormone varies considerably with the size and composition of the meals. Also, the secretory pattern is to be tightly coordinated and balanced in relation to the remaining gut hormones. Metaphorically, the entire secretory process of all the gut hormones in association with a meal may therefore remind of a symphony played with finely tuned coordination of each individual instrument’s participation and volume in different parts of the symphony.

The differentiated response to food is, however, not only a question of interacting timing and magnitude. The concentration levels of the different hormone systems vary significantly. This is illustrated by Table 4, which shows the basal and meal-stimulated concentrations for...
Fig. 6. Some gastrointestinal hormone responses to oral glucose and fructose challenge in healthy humans. Mean values + 1 SE are shown in response to oral (75 g in 300 mL water) glucose (black) and fructose (gray) intake. Total AUC values are shown for comparison between treatments. A: plasma total glucagon-like peptide-1 (GLP-1_total, pM); B: plasma GLP-1_total AUC values (min × pM); C: plasma total glucose-dependent insulinotropic polypeptide (GIP_total, pM); D: total plasma GIP_total AUC values (min × pM); E: plasma peptide YY (PYY)_{3-36} values (pM); F: plasma (PYY)_{3-36} AUC values (min × pM); G: plasma cholecystokinin (CCK) values (pM); H: plasma CCK AUC values (min × pM); I: plasma neuropeptide (NTS)_{1-13} values (pM); J: plasma NTS_{1-13} AUC values (min × pM). *Within-group significance compared with base level (mean value for 15 and 0 min control samples). #Between-group significance. *P < 0.05; **P < 0.01; ***P < 0.001; P < 0.0001 (See also ref. [58]).
endocrinology. Particularly remarkable is the development of de
type 2 diabetes [18,19,48,52].
become potent drugs due to the incretin effect of GLP-1 in therapy of
rivatives of the gut hormone GLP-1 and DPP-4 inhibitors, which have
instance ref [51]). Besides, major metabolic diseases such as type 2
gastrinomas often express one or more gastrointestinal hormones (see for
intestinal hormones assumed to contribute to the carcinogenesis in an
Gastroenteropancreatic neuroendocrine tumors*.

| Tumor type       | Main sites of primary tumor | Major Symptoms | Hormones                        |
|------------------|----------------------------|----------------|--------------------------------|
| Carcinoid        | Gut                        | Flushing       | Serotonin                      |
|                  | Pancreas                   | Diarrhea       | Substance P                    |
|                  | Lungs                      | Weight loss    | Neurokinin A                   |
| Cholecystokininoma (CCKoma) | Pancreas                  | Duodenal ulcer | Cholecystokinin (CK)           |
|                  | Duodenum                   | Gallstones     |                               |
|                  | Pancreas                   | Weight loss    |                               |
| Gastrinoma       | Duodenum                   | Duodenal and jejunal ulcers | Gastrin |
|                  | Pancreas                   | Diarrhea       |                               |
| Ghrelinoma       | Pancreas                   | Increased growth hormone secretion | Ghrelin |
| Glucagonoma      | Pancreas                   | Skin rash      | Glucagon                       |
| Insulinomas      | Pancreas                   | Hypoglycemia   | Insulin                        |
| Somatostatinomas | Pancreas                   | Diabetes       | Somatostatin                   |
|                  | Small intestine            | Diarrhea       |                               |
|                  |                            | Weight loss    |                               |
| VIPomas          | Pancreas                   | Watery diarrhea | Vasactive intestinal polypeptide (VIP) |
|                  |                            | Weight loss    |                               |

* For further information, see ref. [56].

which there is reasonable consensus for eight of the major gastrointestinal hormone systems [31,45-50]. The timing and size of the secretory response pattern to a carbohydrate meal (glucose or fructose) in healthy humans is shown in Fig. 6. These data emphasize the necessity of timing of for instance blood sampling or tissue biopsy. In other words, you have to know whether the experimental animal, control subject, or the patient to be examined is fasting. You also have to know the time-relationship to the latest meal, as well as the composition of the meal. Otherwise, measurement of gastrointestinal hormones becomes meaningless.

5. The clinical diagnostic application of measurement of gastrointestinal hormones

For years, measurement of gastrointestinal hormones has been essential in examination of the pathophysiology and diagnosis of severe and common neoplastic and metabolic diseases. Hence, the major gastrointestinal cancers (esophageal, gastric, pancreatic, small intestinal and colorectal) express to various degrees growth-regulating gastrointestinal hormones assumed to contribute to the carcinogenesis in an autocrine manner [51]. In addition, common extraintestinal cancers (brain tumors, lung cancer, breast cancer, and ovarian cancers) as well as sarcomas often express one or more gastrointestinal hormones (see for instance ref [51]). Besides, major metabolic diseases such as type 2 diabetes mellitus and obesity have also been in focus of gastrointestinal endocrinology. Particularly remarkable is the development of derivatives of the gut hormone GLP-1 and DPP-4 inhibitors, which have become potent drugs due to the incretin effect of GLP-1 in therapy of type 2 diabetes [18,19,48,52].

Gastrointestinal hormones are, however, also measured in the daily diagnostic routine of neuroendocrine tumors. Some of the specific tumor syndromes have in fact been uncovered by gut hormone measurements. The clinical syndromes caused by hypersecretion of gastrointestinal hormones from neuroendocrine tumors are listed in Table 5. Although patients with tumor syndromes are relatively rare with annual incidences of 1–2 per million inhabitants, they are common in the national centers for neuroendocrine tumors. That is because most of the tumors –although slow-growing – are malignant and require regular control visits with measurements of the plasma concentrations of the actual, specific gastrointestinal hormone, and – occasionally – also its precursors. For instance, the Danish Center for Neuroendocrine Tumors (which serves a population of 5.8 million inhabitants) requires around 1000 measurements per year of the concentrations of plasma gastrin and its precursors [53].

The complex and variable pathobiochemistry for gut hormone producing tumors may be illustrated by the glucagonoma syndrome: clinically the symptoms of a glucagonoma may be hyperglycemia and necrolytic migratory erythema consistent with biological actions of glucagon on glycogenolysis, glyconeogenesis and ureagenesis [54]. However, patients diagnosed immunohistochemically as having a glucagonoma may also present with hypoglycemia and hyperinsulinemia (without the tumor being insulin-producing). Accurate measurement of proglucagon-derived peptides such as glucagon and GLP-1 has revealed that this is indeed due to the differential splicing of proglucagon in such patients. The patient having a pancreatic phenotype with hyperglucagonemia resulting in hyperglycemia and enhanced amino acid catabolism whereas the patient with hypoglycemia having an intestinal phenotype that results in increased concentrations of GLP-1 and insulinotropic effects resulting in hypoglycemia [55].

6. Conclusion

If in the 1960s you wished to measure peptide hormones such as insulin and for instance gastrin in plasma (as one of us did), it looked simple. You just had to establish a RIA for each hormone by immunizing guinea pigs or rabbits with purified insulin and a synthetic gastrin (gastrin-17) and then label a tyrosyl residue in the peptides with [125I] using the chloramin T technique). With antiseras, isotope-labelled tracer and pure peptide as standard, you had your RIAs. However, in the 1970s and 80 s, the premises for measurement changed radically as described in the first part of this review: A hormone was not only a singular bioactive peptide. The hormone gene expressed itself in plasma and tissue in different molecular forms and the identified gastrointestinal hormone genes were widely expressed not only in endocrine gut cells, but also in extraintestinal endocrine cells, neurons, myocytes, immune cells, and fat cells etc., where the expression cascade moreover might be cell-specific and dependent on the specific cellular expression of relevant processing-enzymes. In other words, you had to realize that most peptide hormones, originally conceived as gastrointestinal or pancreatic, were complex, more or less ubiquitous multiple systems that might act as intercellular messengers all over the body, - and sometimes also between bodies (in fertility and defense mechanisms).

Alongside with the recognition of the molecular and cell-biological complexity of peptide hormones, also the methodology for measurement has been developed as described in the second part of the review. While bioassays were necessary in the early purification and identification of the first gastrointestinal hormones, they are generally too insensitive, unspecific and labor intensive for today’s measurements in plasma and tissue extracts. However, the immunoassay technology has been considerably refined since the 1960s, using purified monolabelled tracers and nonspecific antibodies with precisely defined sequence-specificity, often used together for N- and C-terminal epitopes, or even as complementary sequence-specific panels. Thus, today sophisticated, highly sensitive immunoassays with precisely defined epitope-specificity (RIA and ELISA) prevail for plasma measurement of the gastrointestinal hormone systems – also when it comes to quantitation of the entire posttranslational phase (PIA). Finally, the MS-technology is widely used in the identification of new and species-specific gastrointestinal peptides. Furthermore, in the coming decade it is likely that the
MS-technology with high specificity may reach a sensitivity-level sufficient to measure also gut hormones that circulate in femtomolar and low picomolar concentrations in plasma.

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**References**

[1] V. Mutt (Ed.), Gastrointestinal Hormones. Adv. Metab. Disorders 11 (1988) 1-545.
[2] J.F. Rehfeld, The new biology of gastrointestinal hormones, Physiol. Rev. 78 (1998) 1087–1108.
[3] J.F. Rehfeld, The endocrine gut, in: A. Belfiore, D. Le Roith (Eds.), Principles of Endocrinology and Hormone Action, Springer Publishers, New York, 2018, pp. 517–531.
[4] J.F. Rehfeld, The origin and understanding of the incretin concept, Front. Endocrinol. 9 (2018) 1–17.
[5] J.F. Rehfeld, Incretin physiology beyond glucagon-like peptide 1 and glucose-dependent insulinoctropin polypeptide, Actions Physiol. 201 (2011) 405–411.
[6] L.D. Glover, D.J. Barlow, J.E. Pitt, S.P. Wood, J.L. Tickle, T.L. Blundell, Conformational studies on the pancreatic polypeptide hormone family, Eur. J. Biochem. 142 (1984) 379–385.
[7] A.H. Johnsen, J.F. Rehfeld, Cionion: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the protochondrine, Ciona intestinalis, J. Biol. Chem. 265 (1990) 3064–3068.
[8] A. Anastasi, V. Ersparer, R. Enzle, Isolation and amino acid sequence of caerulein, the active decapeptide of the skin of hydra caerulea, Arch. Biochem. Biophys. 125 (1968) 57–68.
[9] R.F. Doolittle, D.F. Feng, S. Tsang, G. Cho, E. Little, Determining divergence times of the major kingdoms of living organisms with a protein clock, Science 271 (1996) 174–177.
[10] J.R. Bundgaard, J. Vuust, J.F. Rehfeld, Tyrosine O-sulfation promotes proteolytic conversion of proglucagon to glucagon, EMBO J. 23 (2004) 4561–4569.
[11] R.A. Liddle, I.D. Goldfine, M.S. Rosen, R.A. Taplitz, J.A. Williams, Cholecystokinin bioactivity in human plasma: molecular forms, responses to feeding, and relationship to gallbladder contraction, J. Clin. Invest. 75 (1985) 1144–1152.
[12] J.F. Rehfeld, Accurate measurement of cholecystokinin in plasma, Clin. Chim. Acta 6 (1966) 233–239.
[13] J.F. Rehfeld, On measurement of cholecystokinin in plasma with reference to radioimmunoassay, J. Clin. Invest. 35 (1956) 170–174.
[14] R.A. Liddle, I.D. Goldfine, M.S. Rosen, R.A. Taplitz, J.A. Williams, Cholecystokinin bioactivity in human plasma: molecular forms, responses to feeding, and relationship to gallbladder contraction, J. Clin. Invest. 75 (1985) 1144–1152.
[15] J.F. Rehfeld, Preparation of 125I-labelled synthetic human gastrin I for radioimmunoassay, Scand. J. Clin. Lab. Invest. 30 (1972) 361–368.
[16] M.J. Bak, N.W. Albrechtsen, J. Pedersen, B. Hartmann, M. Christensen, T. Vibbøll, F.K. Knop, C.F. Deacon, L.O. Dragsted, J.J. Holst, Specificity and sensitivity of commercially available assays for glucagon and oxyntomodulin measurement in humans, Eur. J. Endocrinol. 170 (2014) 529–538.
[17] E. Engvall, P. Perlmann, Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G, Immunchemistry 8 (1971) 871–874.
[18] J.F. Rehfeld, M.H. Gingras, L. Bardram, L. Hilsted, J.J. Holst, The Zollinger-Ellison syndrome and mismeasurement of gastrin, Gastroenterology 140 (2011) 1444–1453.
[19] J.F. Rehfeld, J. Broedboek, J.J. Holst, L. Keijger, L.M. Hilsted, High Chromogranin A concentrations in plasma from patients with small intestinal neuroendocrine tumours, Scand. J. Gastroenterol. 55 (2020) 565–573.
[20] J.F. Rehfeld, On measurement of cholecystokinin in plasma with reference to other inositols, Nutr. Res. 10 (1990) 1–4.
[21] N.J. Wewer Albrechtsen, D. Hornburg, R. Albrechtsen, B. Svendsen, S. Torang, S. L. Jepsen, R.E. Kuhre, M. Hansen, C. Janus, A. Floyd, A. Lund, T. Vibbøll, F. Knop, H. Vestergaard, C.F. Deacon, F. Reimann, J.A. Williams, N. Wewer Albrechtsen, J.J. Holst, M. Hartmann, Oxyntomodulin identified as a marker of type 2 diabetes and gastric bypass surgery by mass-spectrometry based profiling of human plasma, ElBioMedicine 7 (2016) 112–120.
[22] A. Sina, M. Mann, A beginner’s guide to mass-spectrometry-based proteomics, Biochemist 42 (2020) 64–69.
[23] R.G. Kay, B.G. Challis, T.R. Casey, G.P. Roberts, C.L. Meek, F. Reimann, M. N. Gribble, Peptidic analysis of endogenous plasma peptides from patients with pancreatic neuroendocrine tumours, Rapid Commun. Mass Spectrom. 32 (2018) 1414–1424.
[24] J.F. Rehfeld, J.R. Bundgaard, J.F. Goetze, L. Friis-Hansen, A.H. Johnsen, Naming progastrin-derived peptides, Regul. Pept. 9 (1998) 2405–2406.
[25] A.S. Kopin, M.B. Wheeler, J. Nishi, E.W. Weir, R.M. Chay, The secretin gene: evolutionary history, alternative splicing and development, Proc. Natl. Acad. Sci. USA 89 (1991) 5335–5339.
[26] M.L. Schubert, J.F. Rehfeld, Gastric peptides – gastrin and somatostatin, Compr. Physiol. 10 (2019) 217–228.
[27] G.I. Bell, R.P. Saraste, G.T. Mullerbach, Hamster preproglucagon contains the sequence of glucagon and two related peptides, Nature 302 (1983) 716–718.
[28] J.J. Holst, C. Orskov, O.V. Nielsen, T.W. Schwartz, Truncated glucagon-like peptide 1, an insulin-releasing hormone from the gut distal ileum, FEBS Lett. 211 (1987) 169–174.
[29] S. Mejova, G.C. Weir, J. Habener, Insulinoctropin: glucagon-like peptide 1 (7-37) co-encoded in the glucagon gene is a potent stimulator of insulin release in the perfused rat pancreas, J. Clin. Invest. 97 (1991) 616–619.
[30] J.F. Rehfeld, J.R. Bundgaard, J. Hambsal, K. Zheng, C. Nordrum, D.F. Steiner, L. Friis-Hansen, The cell-specific pattern of cholecystokinin peptides in endocrine cells versus neurons is governed by the expression of prohormone convertase 1/3, 2 and 5/6, Endocrinology 149 (2008) 1600–1608.
[31] J.R. Bundgaard, J. Vuust, J.F. Rehfeld, Tyrosine O-sulfation promotes proteolytic processing of proglucagon, EMBO J. 14 (1995) 3073–3079.
[32] L.L. Larsson, N. Goltermann, L. de Magistris, J.F. Rehfeld, T.W. Schwartz, Somatostatin cell processes as pathways for paracrine secretion, Science 205 (1979) 1393–1395.
[33] M.B. Sporn, A.B. Roberts, Autocrine growth factors and cancer, Nature 313 (1985) 745–747.
[34] M. Li, M. Mihayk, K. Nakayama, A. Mitya, A. Aimura, Prohormone PC4 processes the precursor of PACAP in the testes, Ann. N. Y. Acad. Sci. 921 (2000) 333–339.
[35] H. Persson, J.F. Rehfeld, A. Ericsson, M. Schalling, M. Pelto-Huikko, T. Høfkt, Transient expression of the cholecystokinin gene in male germ cells and accumulation of the peptide in the acrosomal granule: possible role of cholecystokinin in fertilization, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 6166–6170.
[36] M. Schalling, H. Persson, M. Pelto-Huikko, L. Ohdm, P. Ekman, C. Gottlieb, T. Høfkt, J.F. Rehfeld, Expression and localization of gastrin messenger RNA and peptide in human spermatogenic cells, J. Clin. Invest. 86 (1990) 660–669.
[56] G. Mamikunian, A.I. Vinik, T.M. O’Dorisio, E.A. Woltering, V.L.W. Go, Neuroendocrine Tumors: A Comprehensive Guide to Diagnosis and Management, Inter Science Institute, 2009, pp. 1–217, 4th ed.

[57] J.F. Rehfeld, Beginnings: a reflection on the history of gastrointestinal endocrinology, Regul. Pept. 177 (2012) S1–S5.

[58] R.E. Kuhre, F.M. Gribble, B. Hartmann, F. Reimann, J.A. Windelev, J.F. Rehfeld, J. J. Holst, Fructose stimulates GLP-1 but not GIP in mice, rats and humans, Am. J. Physiol. (Gastrointest. Liver Physiol.). 306 (2014) G622–G630.