Pitfalls in Diagnostic Gastrin Measurements

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BACKGROUND: Gastrin measurements are performed primarily for the diagnosis of gastrin-producing tumors, gastrinomas, which cause the Zollinger–Ellison syndrome (ZES). Gastrin circulates as several bioactive peptides, however, and the peptide pattern in gastrinoma patients often deviates from normal. Therefore, it is necessary to measure all forms of gastrin.

CONTENT: Only immunoassays are useful for measurement of gastrin in plasma. The original assays were RIAs developed in research laboratories that used antibodies directed against the C terminus of gastrin peptides. Because the C-terminal tetrapeptide amide sequence constitutes the active site of gastrin peptides, these assays were well suited for gastrinoma diagnosis. More recently, however, most clinical chemistry laboratories have switched to commercial kits. Because of recent cases of kit-measured normogastrinemia in patients with ZES symptoms, the diagnostic sensitivity and analytical specificity of the available kits have been examined. The results show that gastrin kits frequently measure falsely low concentrations because they measure only a single gastrin form. Falsely high concentrations were also encountered, owing to overreactivity with O-sulfated gastrins or plasma proteins. Thus, more than half of the gastrin kits on the market are unsuited for diagnostics.

SUMMARY: Gastrinomas are neuroendocrine tumors, some of which become malignant. A delay in diagnosis leads to fulminant ZES, with major, even lethal, complications. Consequently, it is necessary that the diagnostic sensitivity of gastrin kits be adequate. This diagnostic sensitivity requires antibodies that bind the C-terminal epitope of bioactive gastrins without the influence of O-sulfation.

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Gastrin was the first gastrointestinal hormone to be measured in plasma (1–4). Great expectations surrounded gastrin research around 1970, because peptic ulcer was a widespread disease in which gastrin was assumed to play a central role (5, 6). Moreover, gastrin was expected to be part of the flawed incretin mechanism in type 2 diabetes (7). The subsequent discovery of gastrin gene expression in major cancers suggested that gastrin might be a carcinogentic growth factor (8). Finally, the Zollinger–Ellison syndrome (ZES)4 turned out to be caused by gastrinomas (9) that required gastrin measurements for proper diagnosis.

Today, the picture is changed: Peptic ulcer is caused by Helicobacter pylori infection and does not need gastrin measurement for diagnosis. The incretins glucagon-like peptide 1 and gastric inhibitory polypeptide have nearly eliminated the diabetes-related interest in gastrin (10). In addition, gastrin has not advanced to the major league of growth factors in cancer (11). On the other hand, small gastrinomas that produce mild ZES symptoms are now found with increased frequency (12), thus requiring gastrin measurement of at least 200 plasma samples per million individuals per year (13).

Analytical Issues

Alongside the clarification of the diagnostic indication for gastrin measurement, several analytical issues exist. First, there is the basic question of the molecular nature of circulating gastrin. Originally, gastrin was identified as a single peptide, gastrin-17 (14) (Fig. 1). We now know that normal antral G cells synthesize 6 bioactive gastrins, of which 5 (gastrin-71, −52, −34, −17, and −14) circulate as pairs of O-sulfated and nonsulfated peptides (15–17). Of these peptides, gastrin-34 and gastrin-17 are the major forms in normal plasma (15–17). Although each of the gastrins are equally bioactive in vitro (16, 17), their in vivo metabolic clearance from the circulation varies widely. For instance, the half-life of gastrin-34 is 40 min in humans, but that of gastrin-17 is only 4 min (18). Consequently, in states of gastrin hypersecretion, such as achlorhydria and gastrinomas.
nomas, the longer gastrin peptides predominate in plasma \(^{(19, 20)}\). Some plasmas even contain only long gastrins and no gastrin-17 \(^{(19)}\).

The second issue is the molecular heterogeneity of gastrin, which challenges the immunochemical specificity of gastrin assays. Most diagnostic assays use antibodies that have been produced against some form of gastrin-17 (Fig. 1). Gastrin-17 and its analogs are robust peptides that are easily directionally coupled to immunogenic carrier proteins for antibody production. Generally, conventional gastrin immunization in rabbits produces high-affinity antibodies with titers so high that the antisera can be used for decades. Our laboratory still uses a \(^{1}H1_{1022}\) 40-year-old antiserum from a single rabbit for routine diagnostic measurements \(^{(21)}\). The decisive question, however, is to what extent antibodies raised against gastrin-17 bind the other gastrins. The reactivity varies considerably with respect to both peptide length and amino acid derivatizations \(^{(22, 23)}\). Consequently, antibodies require careful evaluation to define both the epitope and reactivity against longer and shorter forms of gastrin, sulfated as well as nonsulfated. At an absolute minimum, the binding of sulfated and nonsulfated gastrin-17—as well as gastrin-34 and cholecystokinin-8 (CCK-8) (Fig. 1)—must be tested. Only antibodies that bind gastrin-17 and gastrin-34 equally, without the influence of O-sulfation of the tyrosyl residue, are useful in diagnostic immunoassays \(^{(24)}\).

The third issue is the biology of gastrinomas. Gastrinomas are generally slowly growing neuroendocrine tumors. In gastrinoma cells, progastrin is processed less efficiently than in normal gastrin cells \(^{(8, 25)}\). Although gastrinoma cells generally release more progastrin, processing intermediates, and long forms of gastrin (gastrin-71, \(-52\), and \(-34\)), each gastrinoma displays a highly individual biosynthetic pattern \(^{(19, 24)}\) with respect to both endoproteolytic cleavages and amino acid derivatizations \(^{(26–28)}\). Taken together with the differences in clearance from the circulation, the variable efficiency of the tumors to synthesize gastrin introduces large individual variation in the gastrin concentration and molecular gastrin pattern in the plasma of gastrinoma patients \(^{(19, 24, 27)}\).

The fourth issue is the shift from assays developed in academic research laboratories in the 1970s and early 1980s to kits produced by diagnostics companies. Thus, most diagnostic gastrin measurements carried out today in hospitals and private clinical chemistry laboratories are based on commercial assays (RIAs or ELISAs). An examination of the manufacturers’ instructions indicate that the new insights into gastrin biochemistry and gastrinoma biology that have occurred in recent decades have not been followed up. Consequently, the antibodies in some kits have not been adequately examined and selected for the diagnosis of ZES patients.

**The Clinical Situation**

In the 1960s and 1970s, most diagnosed ZES cases were clinically fulminant. Patients had massive hypersecretion of hydrochloric acid from an enlarged and folded gastric mucosa, severe and often multiple duodenal and jejunal ulcers, diarrhea, nanomolar gastrin concentrations in the plasma, frequent occurrence of metastases, and a high mortality rate \(^{(29, 30)}\). But in synchrony with the dissemination of the first sensitive RIAs, the clinical picture changed to more frequent incidences (5 new ZES patients per year in Denmark, which has 5 million inhabitants) and patients with milder symptoms and only moderate increases in gas-
trin concentrations in plasma [104–1040 pg/mL (50–500 pmol/L) gastrin; reference interval, <104 pg/mL (<50 pmol/L)]. The new gastrin RIAs were simply allowing ZES patients to be diagnosed at an earlier stage of the disease. Moreover, when proton pump inhibitors were introduced in the late 1980s, the symptoms of gastrinoma patients were treated efficiently (31), which allowed proper time for localization and removal of the gastrinomas and for management of the long-term complications of tumor growth. Proton pump inhibitor treatment itself increases the secretion of gastrin from normal antral G cells. Therefore, there is a risk of overdiagnosing proton pump inhibitor–treated patients with ZES symptoms and with moderately increased gastrin concentrations in the plasma. There are procedures, however, including the secretin provocation test, to rule out this risk (32).

The new picture, however, also included a group of patients with ZES symptoms but apparently normal gastrin concentrations in plasma. These patients have been estimated to constitute 0.3%–3% of all gastrinoma patients (33). Some of these patients developed multiple duodenal/jejunal ulcers, intestinal perforations, and/or bleeding, diarrhea, and severe acid vomiting, despite gastrin concentrations in the plasma that remained normal or near normal (24). Considering, on the one hand, the complex biosynthesis, the immunochemical specificity problems, and the gastrinoma biology, and, on the other hand, the increased use of commercial gastrin kits in the years with a growing incidence of patients with ZES symptoms but apparent normogastrinemia, an examination of the analytical specificity and diagnostic sensitivity of the kits seemed due.

**Kit Evaluation**

From our catalog and Internet searches, we discovered that the market offered 12 gastrin kits—7 RIAs and 5 ELISAs. We used these kits in accordance with the manufacturers’ instructions (24). For reference, we used a thoroughly evaluated in-house RIA. This RIA had been shown to measure all circulating bioactive forms of gastrin with equimolar potency, irrespective of the N-terminal sequence length and the degree of tyrosyl sulfation. In addition, the reference assay did not cross-react with CCK peptides (Fig. 1) (15, 21, 24, 34). We analyzed plasma samples from 40 fasting patients with a ZES diagnosis and who either had proven gastrinomas or were strongly suspected of having gastrinomas but that had not yet been localized. The reference assay showed that the plasma concentrations varied from <208 pg/mL (<100 pmol/L) to 129 605 pg/mL (62 340 pmol/L). The kits and the reference assay were also evaluated with known concentrations of synthetic gastrin peptides (gastrin-14, −17, −34, and −52, in both O-sulfated and unsulfated forms) spiked into plasma samples from which endogenous gastrins had been removed by immunosorption. Finally, plasma samples with “false” gastrin concentrations were subjected to gel chromatography monitored with the reference assay and the gastrin kits that had produced “false” concentrations.

The results showed that 4 kits frequently produced measured concentrations that were too low; 3 other kits often measured concentrations that were too high. Thus, only 5 kits exhibited acceptable diagnostic true-ness. The specificity test with synthetic peptides and the chromatographic analysis also provided an explanation: the “false” low concentrations were due to antibodies that recognized only gastrin-17 and not longer or shorter gastrins (Fig. 2). Three of the gastrin-17–specific kits also overreacted with sulfated gastrin-17 to different degrees, adding to the complexity and incom-
mensurability of the measurements. The 3 kits that produced falsely high results also overreacted with sulfated gastrins and/or were subject to nonspecific interference from plasma proteins (24).

The diagnostic sensitivities of the kits have been compared with respect to the probability of measuring truly increased gastrin concentrations that are diagnostic for ZES patients (Table 1). Note that the probabilities in the table are based on ZES samples with gastrin concentrations of only 104–1247 pg/mL (<600 pmol/L). It is these patients with slightly to moderately increased gastrin concentrations and moderate clinical symptoms who constitute today’s diagnostic challenge.

The problems for patients with a delayed ZES diagnosis are that their symptoms become fulminant and that they have a high mortality rate because of disseminated, metastatic gastrinomas. The diagnosis of patients with such fulminant ZES symptoms and nanomolar gastrin concentrations in plasma is straightforward and is not missed by any gastrin kit. But then it is often too late.

**Gastrin-17–Specific Antibodies**

Gastrin-17 constitutes only a minor fraction of the gastrins in ZES plasma and is, as previously mentioned, even absent in some samples. Assays that use gastrin-17–specific antibodies will therefore often produce concentration measurements that are too low. Why then were the antibodies used in the original gastrin RIAs (1–4, 21) all directed against the C-terminal epitope and hence measured all bioactive gastrins? The explanation may be simple. As shown in Figs. 1 and 3, the N terminus of gastrin-17 is a pyroglutaminyl residue. Consequently, the intact gastrin-17 peptide is without free NH₂ groups for carrier coupling, and gastrin-17 is without lysyl residues, with their ε-NH₂ group. The first useful gastrin RIA antibodies were raised by McGuigan against truncated gastrin 2–17 with a free N-terminal NH₂ group (35). Subsequent attempts to raise antibodies have often used McGuigan’s protocol. Therefore, the antibodies raised in this way were specific for the C-terminal epitope. If the intact gastrin 1–17 peptide is used for immunization instead, only the glutamic acid residues in the middle of the sequence are available for conventional coupling reactions (Figs. 1 and 3). The carrier-coupled gastrin 1–17 will then expose only the short N-terminal and C-terminal sequences, and, accordingly, some antibodies will require the combined N- and C-terminal epitope from gastrin-17 for binding (Fig. 3) and therefore become entirely specific for untruncated gastrin-17. Consequently, the lesson is to use either N-terminally truncated gastrin for immunization or N-terminally truncated gastrin-17 as a tracer peptide to pick up only C-terminally directed gastrin antibodies.

**CCK Interference**

CCK is the only naturally occurring peptide system in plasma that is closely related to gastrin. CCK and gastrin peptides have the same C-terminal sequence (Fig. 1); therefore, both are ligands for the gastrin/CCK-B receptor. Consequently, some C-terminally directed gastrin antibodies will also bind CCK, but the extent to which this occurs varies (21). Thus far, CCK interference in gastrin assays has been considered unimportant because CCK normally circulates at concentrations 10- to 20-fold lower than those of gastrin (36). A few CCK-producing tumors have been described (8, 37), but none have yet been reported in patients with enhanced plasma concentrations and hypercholesterolemia-related symptoms. Such tumors may occur, however. The bottom line, therefore, is that the reactivity with CCK peptides in diagnostic gastrin assays should also be examined.

**Second-Line Assays**

When immunoassays that measure the C-terminal epitope of bioactive gastrins (first-line assays) are used, as compared with measurements made with a validated reference RIA.a

<table>
<thead>
<tr>
<th>Gastrin kit</th>
<th>Probability, %</th>
</tr>
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<tbody>
<tr>
<td>Biohit</td>
<td>68</td>
</tr>
<tr>
<td>Assay Designs (Correlate-EIA)</td>
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<td>DiaSorin</td>
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<tr>
<td>DRG Diagnostics</td>
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<tr>
<td>US Biological</td>
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*a The probability calculations are based on individual measurements presented in Rehfeld et al. (24).
almost all patients with ZES are correctly diagnosed. A few gastrinomas, however, may process progastrin so poorly that the concentrations of bioactive amidated gastrins in plasma periodically fluctuate into the reference interval [104 pg/mL (<50 pmol/L)] while the concentrations of precursors are increased (27, 38). For these patients, we have designed a processing-independent analysis (39, 40). Processing-independent analyses use high-avidity antibodies that are monospecific for a non-processed precursor sequence. The sequence must be located immediately C-terminal to a trypsin-sensitive cleavage site. Monospecificity of the antibodies is ensured by immunization with short peptides (decapeptides to pentadecapeptides) directionally coupled at their C-terminus. A monoiodinated tracer is prepared by labeling a naturally occurring or a synthetically coupled tyrosyl residue in a position C-terminal to the antibody-bound sequence. By cleavage with trypsin (or another suitable endoprotease) before measurement, the selected epitope on the precursor is exposed for antibody binding. Moreover, the tryptic cleavage ensures that the peptide fragment to be measured always has the same size, i.e., the minimal tryptic fragment. With such a fragment used as a calibrator, the preanalytical trypsin treatment ensures optimal trueness, because the substance to be measured always corresponds to the calibrator. The described approach ensures that the translational product is quantified accurately, irrespective of the degree of posttranslational processing. In other words, 1 peptide fragment is measured stoichiometrically per translated propeptide molecule.

Processing-independent assays have been useful as second-line assays for a few gastrinomas with truly normal or only slightly increased plasma concentrations of amidated gastrins (27, 38). The ability to diagnose selected gastrinomas at an early stage of the disease via processing-independent analysis is therefore valuable.

Conclusion

It is challenging that peptide hormones display a biology and pathology considerably more complex than originally assumed. Gastrin illustrates the situation. Today, proper laboratory diagnosis requires assays that take the full biogenetic, immunochemical, and tumor-biological complexity into account. Gastrinomas are slowly growing neuroendocrine tumors that have to be diagnosed early, because the symptoms and the mortality rate for disseminated gastrinomas are severe. It is not acceptable that insufficient examination of assay specificity and diagnostic sensitivity may cause lifelong invalidity or early death. We hope the present review inspires a proper evaluation of immunoassays.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design,
References


