Pancreatology 16 (2016) 231–237

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Original article

Automated spectrophotometric bicarbonate analysis in duodenal juice compared to the back titration method☆

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ARTICLE INFO

Article history:
Available online 3 February 2016

Keywords:
Pancreatic disorders
Pancreatic function test
Pancreatic juice
Secretin
Short endoscopic secretin test
Bicarbonate

ABSTRACT

Objectives: We have recently evaluated a short endoscopic secretin test for exocrine pancreatic function. Bicarbonate concentration in duodenal juice is an important parameter in this test. Measurement of bicarbonate by back titration as the gold standard method is time consuming, expensive and technically difficult, thus a simplified method is warranted. We aimed to evaluate an automated spectrophotometric method in samples spanning the effective range of bicarbonate concentrations in duodenal juice. We also evaluated if freezing of samples before analyses would affect its results.

Methods: Patients routinely examined with short endoscopic secretin test suspected to have decreased pancreatic function of various reasons were included. Bicarbonate in duodenal juice was quantified by back titration and automatic spectrophotometry. Both fresh and thawed samples were analysed spectrophotometrically.

Results: 177 samples from 71 patients were analysed. Correlation coefficient of all measurements was $r = 0.98$ ($p < 0.001$). Correlation coefficient of fresh versus frozen samples conducted with automatic spectrophotometry ($n = 25$): $r = 0.96$ ($p < 0.001$)

Conclusions: The measurement of bicarbonate in fresh and thawed samples by automatic spectrophotometrical analysis correlates excellent with the back titration gold standard. This is a major simplification of direct pancreas function testing, and allows a wider distribution of bicarbonate testing in duodenal juice. Extreme values for Bicarbonate concentration achieved by the autoanalyser method have to be interpreted with caution.

Introduction

Direct pancreas function testing (DPFT) is invasive and thereby challenging. Used on correct indication it adds useful information in exocrine pancreatic function testing. The test probably serves best as a second line test in situations where primary tests, as faecal elastase 1, are insufficient. DPFT can discriminate primary from secondary pancreatic dysfunction [1,2]. Furthermore, direct tests may prove useful in detecting early exocrine dysfunction, before development of clinical obvious pancreatic exocrine insufficiency. In our short endoscopic secretin test (short EST), duodenal juice aspiration is performed in the period from 30 to 45 min after secretin stimulation, in the plateau phase of duodenal bicarbonate concentration. The whole endoscopic procedure, including a diagnostic gastroscopy, lasts normally not longer than 20 min [1–3], hence overcoming some of the disadvantages of the time-

Abbreviations: DPFT, direct pancreas function testing; MDH, malatdehydrogenase; PEPC, phosphoenolpyruvate carboxylase.

☆ The work was conducted at Haukeland University Hospital in Bergen, Norway.

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http://dx.doi.org/10.1016/j.pan.2016.01.010

consuming and cumbersome tube based tests [3–7]. To distinguish pancreatic exocrine failure from sufficient pancreas function a cut off of 80 mmol/L bicarbonate concentration in duodenal juice is generally accepted. Beneficial information about pancreatic exocrine insufficiency (PEI) can be obtained in a variety of patients with chronic pancreatitis, diabetes, cystic fibrosis, celiac disease. We have used our short EST to evaluate exocrine pancreatic function in patients with chronic pancreatitis, cystic fibrosis and diabetes [3,8–13].

Back titration has long been considered the gold standard for duodenal bicarbonate measurements [14,15]. This analysis is time consuming (minimum 2 h) and technically difficult, hence being expensive and vulnerable in routine diagnostics. As a consequence, only a few specialised or research centres perform this test today. Furthermore, back titration requires minimum 0.5 mL of duodenal juice. Such volumes are sometimes difficult to obtain from patients with severe ductal failure. In contrast, the autoanalyser used in this study requires only a few microlitres of duodenal juice and a short analysing time of 7 min. At present, autoanalysers are certified to quantify ingredients in blood or urine but not in duodenal juice. However, some earlier small studies have demonstrated a good correlation between autoanalysers and the back titration method [16,17] Automation of bicarbonate analyses is required to simplify short EST, but the method still needs further validation to replace back titration. Daily routine in a busy medical institution makes immediate analyses of duodenal juice to a challenge, and instant freezing of samples could be an option for institutions sending samples for analyses elsewhere.

In this study, we aimed to demonstrate the accuracy of an automated spectrophotometric method compared to back titration when analysing bicarbonate in duodenal juice. Additionally, we studied if freezing of samples affected bicarbonate concentrations.

Materials and methods

Patients

The use of samples from short EST in the following projects was approved by the local ethical committee: Chronic pancreatitis or other causes of abdominal pain (approval no. 3.2008.2516), cystic fibrosis (approval no.: 2010/2857-7) and celiac disease (approval no. 2011/1592). Short EST of these patients was performed between September 2012 and October 2014. Samples were chosen at random for comparison of back titration and automated spectrometry. Three consecutive aliquots of duodenal juice with different bicarbonate concentrations are collected in 15 min during the short EST, hence 1 to 3 samples per patient could be analysed.

Short endoscopic secretin test

Secretin was administered intravenous at a dose of 1 CU per kg bodyweight, maximum 70 CU. Gastroscopy started 25 min after secretin administration. A diagnostic gastroscopy was initially performed to identify or exclude other pathological findings. All gastric juice was aspirated and discharged. After 30 min the tip of the endoscope was placed distal to the papilla Vateri. Duodenal juice was aspirated in three 5 min sequences. The procedure is illustrated in Fig. 1 and described in detail elsewhere [3].

Handling of duodenal juice before analysis

The pH and volume of each sample was measured. Duodenal juice with pH < 6 was discarded due to probable pollution from gastric juice. One aliquot of duodenal juice from each sampling period was immediately placed on ice and bicarbonate concentration was immediately analysed using back titration and automated analysis. Otherwise samples were frozen to −196 °C. In the experiment

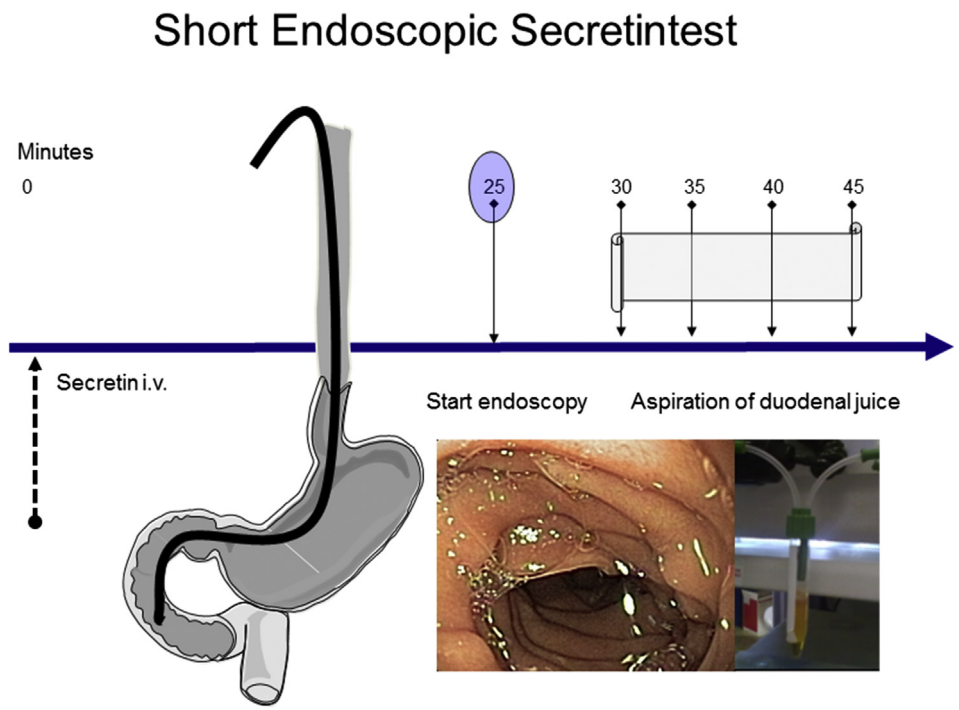

Fig. 1. Short endoscopic pancreas function testing (EST): 25 min after injection of secretin (1 CU/kg bodyweight, max 70 CU) an upper endoscopy was started. During the first 5 min a diagnostic gastroscopy was carried out. All juice from stomach and duodenum was discharged. Thereafter the tip of the endoscope was placed below the papilla for 15 min; duodenal juice was collected in three aliquots of 5 min. The intervention requires only 20 min.

comparing fresh and frozen samples, storage temperature was −80 °C before analyses with automated spectrophotometry.

Analysis of bicarbonate in duodenal juice by back titration

The principles of back titration are described elsewhere [14]. The back titration method for bicarbonate analyses in detail:

Before analyses, pH was measured. Ideally 1 mL of duodenal juice was needed to perform analyses but down to 0.5 mL was accepted. If the volume of duodenal juice was <1 mL, the sample was diluted to 1 mL using 0.9% NaCl. First, the pH of the sample was analysed. To acidify the sample, 1.5 mL of 0.1 N HCl was added, and the reaction between bicarbonate and HCl produced NaCl, H_2O and CO_2. The CO_2 (g) was evacuated by stirring the sample until pH was stable. Next, the solution was titrated ("back titrated") with 0.1 N NaOH until the original pH value was achieved. Based on the volume of NaOH used, the bicarbonate concentration in the duodenal juice was calculated using the formula.

(1000 × (0.1 mol/L HCl × 1.5 mL 0.1 mol/L HCl − 0.1 mol/L NaOH × Y mL 0.1 mol/L NaOH))/1 mL sample volume. Samples were analysed in parallels.

Automated measurement (spectrophotometry)

For automated analyses, the spectrophotometer COBAS® c111 (Roche Diagnostics GmbH; D-68298 Mannheim; Germany; www.roche.com) and the appropriate kit for measuring bicarbonate (CO2-L Bicarbonate liquid, Roche Diagnostics AG, CH-6343 Rotkreuz, Switzerland; www.roche-diagnostics.ch) were used. Analyses were performed using 15 μL of duodenal juice diluted with 30 μL 0.9% NaCl in water before it is pipetted by the instrument and added the start reagent containing phosphoenolpyruvate (≥40 mmol/L), NADH analogue (≥2.0 mmol/L), porcine malatdehydrogenase (≥314.3 ukat/L) and phosphoenolpyruvate carboxylase (≥30.8 ukat/L).

The principle of the test is as follows: Bicarbonate in the samples reacts with phosphoenolpyruvate in the presence of phosphoenolpyruvate carboxylase (PEPC), to produce oxaloacetate and phosphate. One hydrogen from a NADH analogue is transferred to oxaloacetate using porcine malatdehydrogenase (MDH) to produce malate and an NAD^+ analogue. The consumption of NADH results in a decreased absorbance at 409 nm, proportional to the bicarbonate concentration in the sample.

Statistical methods

Difference of groups was tested by t-test. Normality was tested by the Shapiro–Wilk test, and when it failed a Mann–Whitney rank sum test was run. Correlation was calculated with Pearson test, agreement with Bland Altman plot [18,19]. Adjustment was done by linear regression. ROC curves were drawn to evaluate sensitivity and specificity of spectrophotometry compared to gold standard back titration. A 5% level of statistical significance was used. Softwares used were ACCESS, EXCEL (Microsoft Office, Redmond WA, USA), and SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

Results

177 randomly chosen samples analysed by back titration and automated spectrophotometry

In the time period of the study short EST was performed in 94 patients and 257 samples of duodenal juice were obtained. 177 samples from 71 patients were chosen arbitrary and analysed by both back titration and automated spectrophotometry. Pearson test showed an excellent correlation of all measurements with a coefficient of $r = 0.98$, $p < 0.001$ (Fig. 2a). We performed supplementary a Bland Altman plot to further explore the agreement over the whole range: Mean difference (bias) was −4.4 with a 95% CI of −5.5 to −3.2. Agreement was excellent around the cut-off of 80 mmol/L but weak in extreme values: Compared to back titration as gold standard, the results from the autoanalyser showed higher levels for very low concentrations of bicarbonate, and lower levels for very high levels of bicarbonate (Fig. 2b). In a clinical setting, bicarbonate concentration of 80 mmol/L is used to differentiate between normal and insufficient ductal pancreatic function. Using back titration as gold standard, sensitivity in spectrophotometry was 97%, specificity 92% (AUC 0.99, $p < 0.001$). This implies that automated spectrophotometry can discriminate between PEI and non-PEI compared to back titration (Fig. 2c). Due to lack of agreement in the range of high and low values of bicarbonate concentration we calculated the following correction factor from linear regression: [HCO^3] back titration = −7172 + (1137*[HCO^3] autoanalyser) (mmol/L). When we recalculated the results using this correlation factor we got the following results: Correlation coefficient of all measurements was $r = 0.98$, $p < 0.001$. Linear regression did not change the excellent correlation (Fig. 2d). Bland Altman plot: Mean difference was −0.03 with a 95% CI −1 to 1. Adjustment by linear regression produced better agreement in extreme values (Fig. 2e). Sensitivity and specificity with a cut-off of 80 mmol/L using back titration as gold standard: sensitivity in spectrophotometry was 94%, specificity 94% (AUC 0.99, $p < 0.001$). ROC analyses show no difference in AUC after mathematical correction in the identification of PEI.

25 pairs of fresh and frozen samples analysed by automated spectrophotometry

Twenty-five samples from 9 patients were randomly chosen. In all measurements correlation after Pearson was excellent with a coefficient of $r = 0.96$ with $p < 0.001$ (Fig. 3). The agreement evaluated by the Bland Altman Plot was very good: Mean difference was −1.3 with a 95% CI of − 5.1 to 2.6. There was no bias in extreme values. Using the analysis of fresh samples as gold standard a ROC curve for the measurement of frozen samples for a cut-off of 80 mmol/L bicarbonate was drawn. Sensitivity and specificity was 100% (AUC 1; $p < 0.001$). Storing samples at −80 °C does not influence differentiation between PEI and non-PEI. Correlation and agreement between the pairs of fresh and frozen samples are excellent.

Standard solutions measured by back titration and spectrophotometric autoanalyser

A series of 7 standard solutions with bicarbonate concentrations of 20, 40, 60, 80, 100, 120, 140 mmol/L were analysed by back titration and spectrophotometric autoanalyser. Pearson test in back titration showed ideal correlation with a coefficient of 1 $p < 0.001$ (Fig. 4a). Bland Altman Plot pointed out nearly ideal agreement: Mean difference was −0.4 with a 95% CI of −0.6 to −0.3. Also with the spectrophotometric autoanalyser method an ideal correlation factor of 1 with $p < 0.001$ could be reached (Fig. 4b). Evaluation using the Bland Altman method revealed a bias analogue to experiment I: In higher bicarbonate concentrations the difference between standard solution and results by spectrophotometric autoanalyser enlarges. Mean difference was −3.2 with a 95% CI of −5.5 to −0.9 (Fig. 4c).

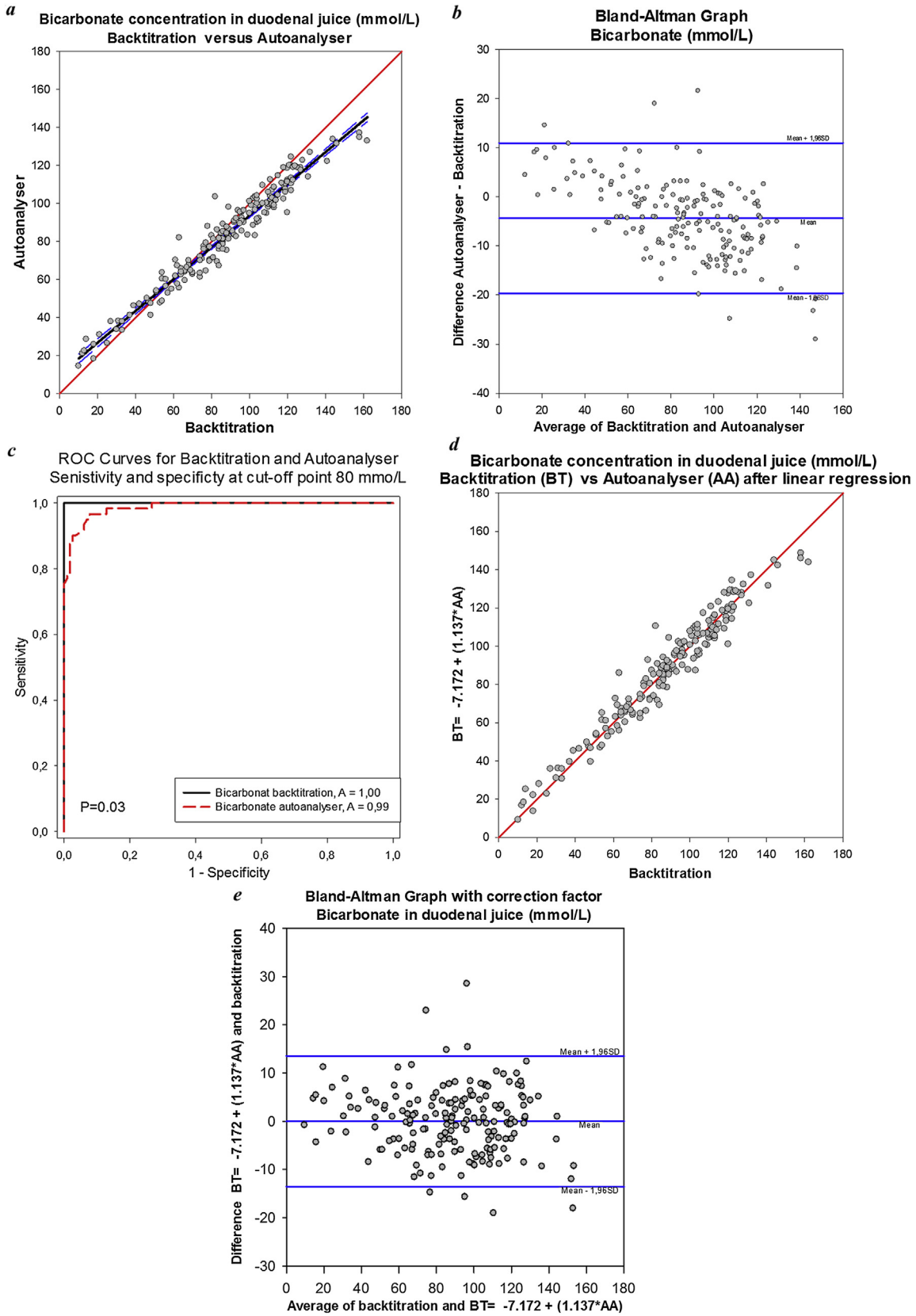

Fig. 2. Correlation and agreement evaluated by measuring Bicarbonate in duodenal juice with back titration (BT) or autoanalyser (AA). Sensitivity and specificity of spectrophotometry at a cut-off point of 80 mmol/L. Back titration is the gold standard. A cut-off point of 80 mmol/L is generally accepted to discriminate insufficient from sufficient ductal pancreatic function. N = 177. a: Excellent correlation between the two methods ($r = 0.98$; $p < 0.001$). b: Agreement between the two methods, excellent around the cut-off point of 80 mmol/L but weak in extreme values: In relation to back titration, autoanalyser measures in very low bicarbonate concentrations for high and in very high bicarbonate concentrations for low. We used therefore linear regression for correction: BT = −7.172 + (1.137*AA). c: Evaluation of the cut-off point of 80 mmol/L: Using ROC analyses with back

Discussion

In this study, we demonstrate that automated spectrophotometric measurement of bicarbonate in duodenal juice correlates excellent to analysis by the gold standard back titration method. Although the back titration method on standard solutions performed better than the spectrophotometric method, automated measurement discriminated sufficient from insufficient pancreatic function with good accuracy. Secondly, we also demonstrated that freezing and thawing did not influence the spectrophotometric result.

Changing from the gold standard back titration method to automated spectrophotometry presents an important simplification of the duodenal juice measurement. The excellent correlation and agreement around the generally accepted cut-off value for pancreatic exocrine failure makes it possible to replace the expensive and cumbersome back titration method by an automated test which can be run in common, basic laboratory services.

Our results are comparable to earlier small studies using an autoanalyser [16,17,20]. The present study comprises a larger number of samples over the whole physiological and pathophysiological range of bicarbonate concentration, thus adding information on reduced agreement in the lower and the higher concentration ranges. One possible explanation is that the high and low range measurements are a result of false measurements. However the same effect demonstrated in the standard solutions argue against this possibility. Over the range to higher bicarbonate concentrations the difference between standard solution and results by spectrophotometric autoanalyser enlarges. Back titration did not show this phenomenon. A possible explanation is the enzyme dependence in the autoanalyser method. Optimum pH of Phosphoenolpyruvate carboxylase (PEPC) is 8–9 [21] and optimum pH for malatdehydrogenase (MDH) is 7.4 (NADH —> NAD) [22]. We assume that pH in extreme bicarbonate concentration is outside the range of optimum pH of malatdehydrogenase. This would influence the consumption of NADH analogue and consecutively the absorbance of light at 409 nm leading to false measures in extreme bicarbonate concentrations.

Additionally, in both back titration and autoanalyser methods, dilution with NaCl 0.9% is substantial. Producers of NaCl 0.9% describe a range of pH between 4 and 7 at 20 °C (106404 | Sodium chloride, Merck Millipore Corporation. Merck KGaA, Darmstadt, Germany). Our measures on different batch numbers revealed a pH between 5.35 and 5.93. For this reason we analysed a small series of duodenal juice with autoanalyser and diluted with either buffer or NaCl; no difference was found. This indicates that results are not influenced by NaCl 0.9%, or buffer. Also other studies have demonstrated inferior agreement in bicarbonate values over 100 mmol/L. In these studies deionised water was used for dilution [17]. A solution to minimise bias in extreme low or high levels of bicarbonate concentration could be different dilution procedures. Explanations by chemistry are not sufficient and lead to new questions without answers. The size of our study using 177 samples with values distributed over the effective range, from very low to high bicarbonate concentrations made it possible to calculate a correction factor with the linear regression. In practice very low or very high concentrations of bicarbonate are not as interesting as the range between 60 and 100 mmol/L. Regardless of the demonstrated weaknesses in the agreement in the lower and the higher range, we demonstrate that this does not has substantial influence on the overall accuracy of the test. The use of a correlation factor is probably not necessary in clinical practice, but it implies that clinicians are aware of this fact.

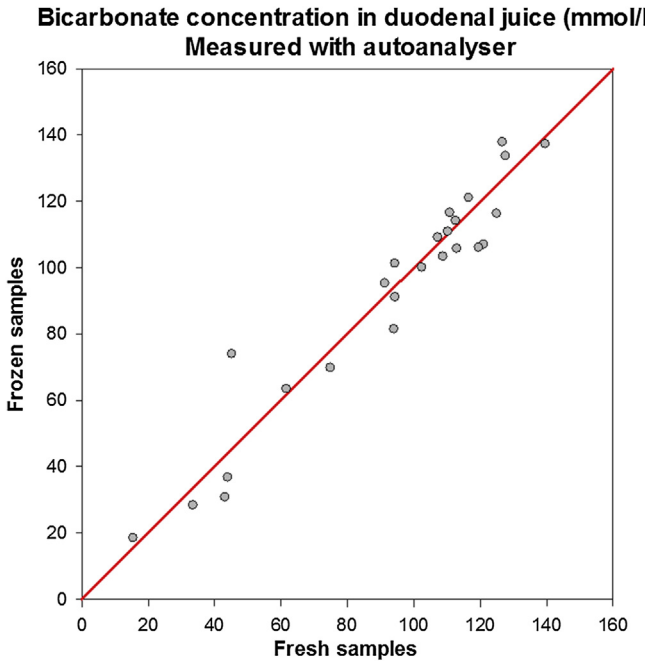

Fig. 3. Correlation and agreement of 25 samples analysed by autoanalyser under two conditions: 1) as fresh sample immediately after DPFT, 2) as frozen and thawed samples: excellent correlation ($R = 0.96$; $p < 0.001$).

The size of the samples over the whole effective range may explain that we can show the bias in the higher and lower values. This may explain that the bias in the lower values is not shown by other authors [16,17].

The amount of duodenal juice in a 5 min aliquot varies between 0 mL (dry tap) and 10 mL. In average we suctioned 2–5 mL per aliquot. In severe pancreatic exocrine insufficiency (PEI) the amount of duodenal juice is very poor. Bicarbonate analysis with back titration needs minimum 0.5 mL of duodenal juice. Consequently smaller amounts cannot be analysed with this method. In contrast, we performed the spectrophotometric method with only 15 μl. If necessary this method allows measuring a lower quantum of juice. The smaller volume requirement in the spectrophotometric analysis increases the possibility to analyse duodenal bicarbonate in all patients.

Limitations of the study

Most limitations of the study can be found in the handling and contents of the specimen. Samples were not centrifuged; consecutively pollution from debris or blood might affect the results of the analyses. On the other hand, we excluded obviously polluted samples and both methods will equally be affected.

For practical reasons in the sampling of juice from a large number of patients, frozen samples were stored up to 197 days at −196 °C before analysis. There are no data about long-term storage of samples at −196 °C and its possible influence on bicarbonate results. We evaluated bicarbonate concentrations in cohorts

titration as gold standard. Automated spectrophotometric method is nearly as good as back titration. d: After the linear regression there was no change of correlation after correction ($r = 0.98$; $p < 0.001$), but the regression line is very close to the equality line. e: Bias is corrected; no deviation of high and low values is shown. Good agreement over the whole range was reached.

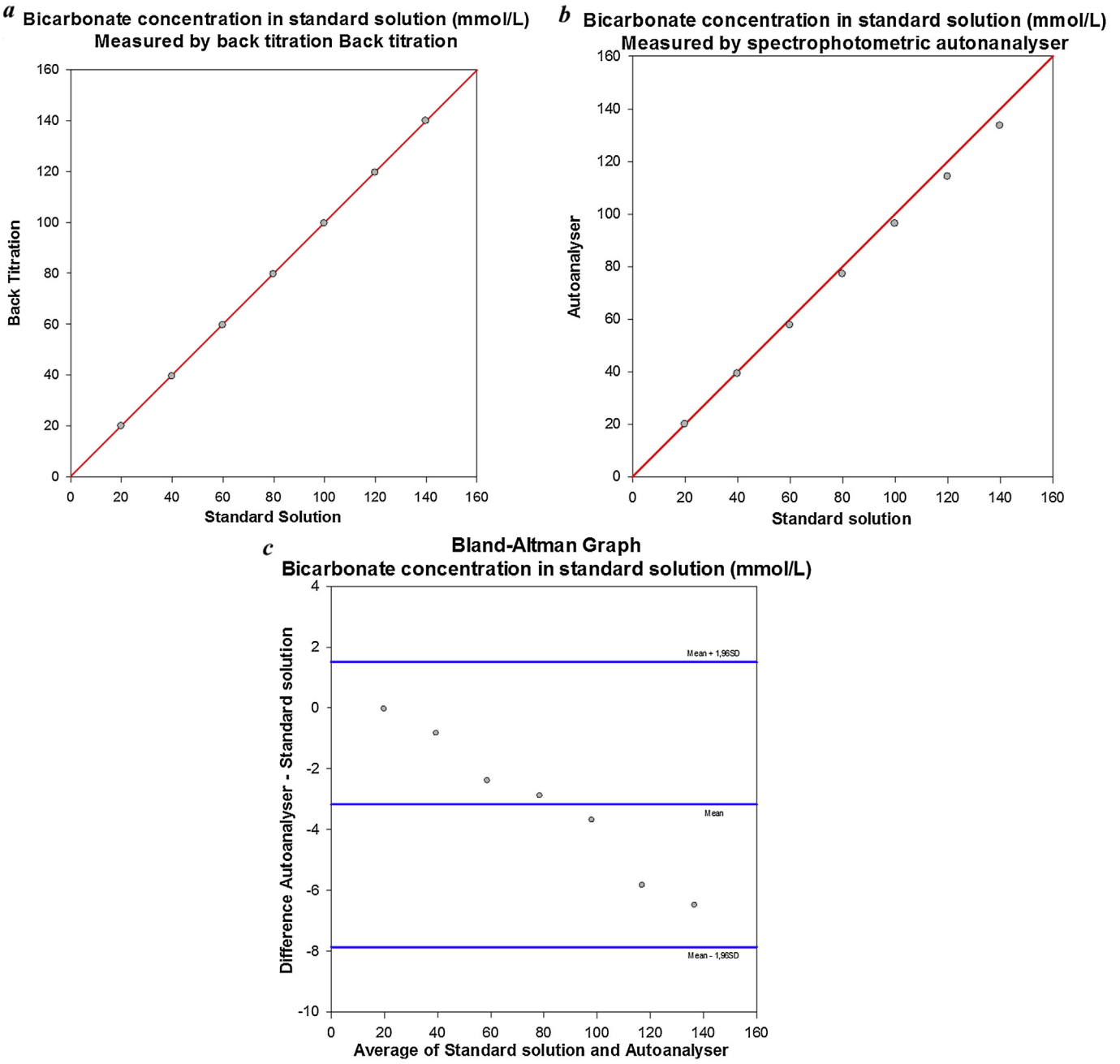

Fig. 4. 7 samples of standard solutions with bicarbonate concentrations (mmol/L) of 20, 40, 60, 80, 100, 120, 140 measured by back titration and spectrophotometric autoanalyser. a) Equality of standard solution and back titration ($r = 1$, $p < 0.001$). b) Equality of standard solution and autoanalyser ($r = 1$, $p < 0.001$). c) Bland Altman Plot unmasks a proportional bias: over the range to higher bicarbonate concentrations the difference between standard solution and results by spectrophotometric autoanalyser enlarges.

differentiated by storage time. No significant differences in correlation were shown. There was acceptable agreement up to 120 days. In experiment II, samples were frozen at minus 80 °C. As shown there was excellent correlation and agreement with fresh samples. This opens for storing duodenal juice in institutions without possibility of snap freezing to −196 °C.

Differentiations in physiological contents of the aspirated specimens might influence the results. The dye of aspirates of duodenal juice varied from colourless to all grades of yellow suggesting different contamination with biliary juice. Biliary acids may influence activity of enzymes used in the spectrophotometric method. However we think that this is negligible because back titration is a chemical method without enzymes and has excellent correlation and agreement to the spectrophotometric method in the range of interest.

Conclusion

Endoscopic secretin-stimulated pancreas function testing has not reached widespread distribution due to the invasive nature and the complexity of the analysing procedures. Easy accessible, simple and cost-effective indirect tests like faecal elastase 1 will probably still be the first line or screening tests. Nevertheless, invasive direct tests have a place in the second line testing due to the well-known pitfalls in the simpler tests. Back titration as old gold standard is time consuming and cumbersome. The validation of an automated

spectrophotometric analysis of bicarbonate in duodenal juice represents a significant simplification of the DPFT. We argue that simplifications of testing and analysis make standardisation of DPFT within reach in the years to come. It will be possible to perform DPFT globally in any health care institution with an endoscopy unit and basic laboratory services.

Acknowledgements

We thank the supporting lab technicians Aud Sissel Hjartholm and Liv Aasmul for preserving and running analyses on duodenal juice. The authors have received travelling grants and scholarships from the Norwegian Gastroenterological Association, Bergen Medical Research Foundation and MedViz.

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