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Full Research Paper

Detailed faecal fat analysis using Fourier transform infrared spectroscopy: Exploring the possibilities

Anne-Sophie De Koninck [a], Karen Nys [a], Brent Vandenheede [a], Stephanie Van Biervliet [b], Marijn M. Speeckaert [c], Joris R. Delanghe [a,*]

[a] Department of Clinical Chemistry, Ghent University Hospital, Gent, Belgium
[b] Department of Paediatrics, Ghent University Hospital, Gent, Belgium
[c] Department of Nephrology, Ghent University Hospital, Gent, Belgium

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ABSTRACT

Objectives: Fourier transform infrared (FTIR) spectroscopic determination of faecal fat is a simple and elegant alternative for the classical Van De Kamer approach. Besides quantification of the total amount of fat, analysis of the lipase hydrolysis efficiency (fatty acid/triglyceride ratio), fatty acid chain length and trans-unsaturated fatty acids could provide a better monitoring of dietary treatment.

Design and methods: Stool samples (26 routine samples and 36 cystic fibrosis patients) were analysed with the Perkin Elmer Spectrum Two® spectrometer (3500–450 cm^{-1}). Fatty acid/triglyceride ratio was calculated using the absorbance ratio at 2855:1746 cm^{-1}. To estimate lipase hydrolysis efficiency, sample ratios were compared with the ratio of butter and pure free fatty acids. Mean fatty acid chain length was calculated using the absorbance ratio at 2855:1709 cm^{-1}. The absorbance at 966 cm^{-1} was used to trace the presence of trans-type unsaturated fatty acids.

Results: Butter showed a low fatty acid/triglyceride ratio (1.21) and pure free fatty acids a high fatty acid/triglyceride ratio (6.76). Mean fatty acid/triglyceride ratio of routine stool samples was 4.16 ± 1.01. The applicability of fatty acid/triglyceride ratios was also tested in cystic fibrosis patients under treatment with a mean of 4.92 ± 0.98. Relative absorbance contribution per carbon atom was 0.06 (ratio 1.06 for C18 standard, 0.91 for C16 standard). The mean ratio of the stool samples was 1.12 (mean acyl chain length of C19), with values ranging from 0.73 (C12) to 1.68 (C28). The presence of traceable amounts of trans-unsaturated fatty acids was also demonstrated.

Conclusions: For the analysis of faecal material, FTIR provides unique information, difficult to obtain using other techniques. These findings offer perspectives for diet monitoring in patients with (non-)pancreatic malabsorption.

1. Introduction

The human diet contains fats with a broad spectrum of fatty acids of varying chain lengths and degree of saturation. The main compound of dietary fats (92–96%) are triacylglycerols, which are composed of long-chain fatty acids. Two processes are involved in the absorption of these fats. Hydrolysis of triacylglycerols by predominantly pancreatic lipolytic enzymes (=lipolysis) leads to the formation of fatty acids and 2-monoacylglycerols. In addition, mixed micelles composed of bile components and lipolytic products are formed for the intestinal uptake. The fatty acid chain length, degree of saturation and hydrophobicity are the major determinants of the efficiency with which dietary fatty acids are absorbed by the adult small intestine [1,2].

In patients with steatorrhea, faecal fat analysis plays a role in the differential diagnosis of intestinal fat malabsorption or maldigestion [3–6]. The use of faecal fat analysis is mainly based on the landmark paper by Van De Kamer et al. [4]. This laborious and time consuming method, which was introduced in 1949, is still considered to be the golden standard. However, important interlaboratory differences have been observed due to a lack of standardisation [5].

More recently, attention has been paid to infrared (IR) spectroscopy as a simple alternative for the classical Van De Kamer approach. Fourier transform infrared (FTIR) spectroscopy has the potential to determine the amount of faecal fat in faeces based on the IR absorbance of chloroform extracts (wavenumbers 2928, 2855 and 1709 cm^{-1}) [5,6]. Next to quantification of the total amount of fat, IR spectroscopy allows a detailed analysis of the chemical nature of fats by molecular fingerprinting.

* Corresponding author at: Department of Clinical Chemistry, Ghent University Hospital, De Pintelaan 185, 9000 Gent, Belgium.
E-mail address: joris.delanghe@ugent.be (J.R. Delanghe).

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The abnormal fatty acid/triglyceride ratios in faeces could be identified using the absorbance ratio observed at 2855 cm^{-1} (C—H bond symmetric stretch vibration): 1746 cm^{-1} (C═0 vibration of glycerol esters). These ratios could elucidate the efficiency of fat digestion by pancreatic lipase (hydrolysis efficiency). Theoretically, it should also be possible to determine the fatty acid chain length of free fatty acids and fatty acid glycerol esters using the absorbance ratio observed at 2855 cm^{-1}: 1709 cm^{-1} [5]. Trans double bounds generate a highly characteristic absorbance peak in the vicinity of 966 cm^{-1}, due to the out-of-plane C—H deformation. The area under the peak/peak height at 966 cm^{-1} can possibly be used to quantify the trans-unsaturated fatty acid content [7].

Assessment of the lipase hydrolysis efficiency, fatty acid chain length and analysis of trans-unsaturated fatty acids could provide a better monitoring of dietary treatment. Hitherto, these analytical possibilities have not yet been fully explored in a clinical context. In the present study, an in-depth exploration of the possibilities of FTIR spectroscopy for detailed analysis of faecal fats was performed.

2. Materials and methods

2.1. Stool sample preparation and analysis

Palmitic acid (≥99%, $CH_3(CH_2)_{14}COOH$) and stearic acid (≥98.5%, $CH_3(CH_2)_{16}COOH$) (Sigma Live Science, St. Louis, USA) were used as standards. Reagents used for the faecal extraction were HCl (37%) (Tailor Made Chemicals, Rekkem, Belgium), ethanol (Merck, Darmstadt, Germany), petroleum ether (≥95%, between 40 and 60 °C) (Sigma-Aldrich, St Louis, USA) and chloroform (≥99.5%) (Sigma-Aldrich, St Louis, USA). To determine the trans-unsaturated fatty acid content, triglycerides with a single cis-type double bound (triolein) and trans-type double bound (trielaidine) were analysed (Nu-Check Prep, St Elysian, USA). The flow cell was cleaned using chloroform and aceton (≥99.9%, Sigma-Aldrich, St Louis, USA). Calibration curves were created using simple linear regression analysis. Standard mixtures (65:35) of stearic (C18; $CH_3(CH_2)_{16}COOH$) and palmitic acids (C16; $CH_3(CH_2)_{14}COOH$) were used for calibration. The standards were handled in the same way as the faecal samples (dissolved in 1 mL chloroform). The calibration curve was calculated using the Perkin Elmer Spectrum Quant software (Waltham, MA, USA). The calibration curve showed an excellent linearity up to 15 g fat/100 g faeces.

Human stool samples ($n = 41$), on which routine fat analysis was requested, were collected over 24 h and homogenized. Samples were analysed within 2 days or stored frozen until analysis at −20 °C. We also used archived residual human samples refrigerated at −20 °C from patients with cystic fibrosis ($n = 44$). Samples with a fat amount below 1 g/100 g faeces were excluded ($n = 15$ in the group of routine stool samples and $n = 8$ in the group of cystic fibrosis patients), due to limited clinical value and loss of interpretability of the ratios. That was also the reason why no collection of stool samples from healthy control subjects was performed. The authors complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. Samples were analysed using a method based on the one described by Jakobs et al. [5]. An aliquot of the stool sample (1 g) was transferred into a test tube and suspended with 1 mL of water, 100 μL of HCl (37%) and 3 mL of ethanol (96%). After homogenisation, 5 mL of petroleum ether was added and the mixture was vigorously shaken for 10 min, followed by centrifugation for 5 min at 3000g. A 4 mL portion of the organic layer (supernatant) was transferred to a new test tube and evaporated for at least 30 min at room temperature under a stream of nitrogen. The dried lipid extracts were dissolved in 1 mL of chloroform and transferred to an infrared transmission flow cell (path length 0.1 mm) (Perkin Elmer, Waltham, MA, USA). FTIR analysis was carried out on a Spectrum Two® spectrometer (Perkin Elmer, Waltham, MA, USA). IR spectra of the faecal extracts were recorded in the range 3500–450 cm^{-1}. Chloroform was used for background subtraction. Quantification was based on the absorbance band of the CH_2 group (2855 cm^{-1}, C—H bond symmetric stretch vibration) of free fatty acids and fatty acid glycerol esters (area under the peak, measured between 2841 cm^{-1} and 2881 cm^{-1}). Absorbance values were baseline corrected. After analysis of the sample, the flow cell was cleaned using chloroform and aceton, and was dried under a stream of nitrogen.

2.2. Determination of the lipase hydrolysis efficiency

To elucidate the efficiency of fat digestion by pancreatic lipase by hydrolyzing triglycerides, fatty acid/triglyceride ratios in faeces were calculated using the absorbance ratio observed at 2855 cm^{-1} (C—H bond symmetric stretch vibration): 1746 cm^{-1} (C═0 vibration of glycerol esters). Areas under the peak were automatically determined on each sample using manually preprogrammed software equations. The ratios were also calculated for pure butter (containing 82% total fat) and for pure free FA (i.e. stearic acid, palmitic acid and mixture of stearic: palmitic acid 65:35).

2.3. Determination of the fatty acid chain length

The FA chain length ($CH_3(CH_2)_xCOOH$) was determined using the absorbance ratio based on the peak height observed at 2855 cm^{-1} (C—H bond symmetric stretch vibration): 1709 cm^{-1} (C═O bond stretch vibration). The absorbance due to C═O bond stretch vibration (1709 cm^{-1}) is very similar for each type of fatty acid because each fatty acid contains 1 aldehyde group (—COOH), whereas the absorbance at 2855 cm^{-1} resembles the fatty acid chain length ($(CH_2)_x$). Peak heights were automatically determined on each sample using manually preprogrammed software equations. The ratios were also calculated for pure butter and for pure free FA.

2.4. Determination of trans-unsaturated fatty acids

Trans double bonds generate a highly characteristic absorbance peak in the vicinity of 966 cm^{-1}, due to the out-of-plane C—H deformation. The area of the peak/height at 966 cm^{-1} can possibly be used to quantify the trans-type unsaturated fatty acid content (trans content) in the stool samples [7]. To determine the specific absorbance peaks of trans double bounds, triglycerides with a single cis-type double bond (triolein) or trans-type double bond (trielaidine) were analysed.

3. Results

3.1. Fourier transform infrared spectrum of a stool sample

In Fig. 1, an IR spectrum of an extracted stool sample is depicted. In agreement with the findings of Jakobs et al. [5], main absorbance peaks were observed at 2928 cm^{-1}, 2855 cm^{-1} and 1709 cm^{-1}. C—H stretch vibrations of the fatty acid methylene residues were detected at 2928 cm^{-1} (antisymmetric) and 2855 cm^{-1} (symmetric), whereas the band at 1709 cm^{-1} corresponded with the C═O stretch vibration of the carboxylic acid functionality. No interference of chloroform was observed in the spectral region of interest (2950 ± 1650 cm^{-1}). Evaluation of the measured IR absorbance bands of faeces samples showed that the quantity of fatty acids could be predicted at 2855 cm^{-1}, directly followed by the band at 1709 cm^{-1}. The single band at 2855 cm^{-1} proved to be sufficient for calibration (highest F-value).

3.2. Determination of the lipase hydrolysis efficiency

The fatty acid/triglyceride ratios, determined on human stool samples on which routine fat analysis was requested, were compared with the ratio of pure butter (low fatty acid/triglyceride ratio) and with the ratio of free fatty acids (high fatty acid/triglyceride ratio) to estimate

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