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Development and Validated Bio-analytical Method for Quantification of Fats in Fecal and Food Samples and its Incurred Sample Reanalysis: Application in Clinical Study

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Abstract

A selective, accurate, and reproducible novel fourier transform infrared spectroscopy method was developed and validated for quantification of fats in fecal and food sample. Fatty acids were extracted from fecal samples with acidified petroleum ether-ethanol and the extracts were dried and redissolved in chloroform. Quantification was based on the absorbance band of the CH₂ group (2855 cm⁻¹) of free fatty acids and fatty acid glycerol esters. The calibration curve of fats in food and fecal matrix showed excellent linearity. The method was proved to be specific, accurate, precise and linear over the concentration ranges of 1.00 g% to 14.98 g% for food and 1.00 g% to 14.96 g% for fecal matrix. The method was validated according to the Guidance for Industry, Bioanalytical Method Validation, US-FDA. The method also showed good performances in terms of accuracy (94.05-101.36% for food matrix and 89.84-103.40% for fecal matrix) and precision (4.9-10.9% for food matrix and 3.1-11.1% for fecal matrix) for fats. The percent recovery of fats ranged from 55.7-60.4% for food matrix and 54.4-70.4% for fecal matrix. Results of all stability studies were within acceptable limits. Incurred sample reanalysis (ISR) was performed by random selection of fecal and food samples. Incurred sample reanalysis data indicated good acceptance and reproducibility of the method and met ISR acceptance criteria, 94% of food and 88% fecal matrix.

The validated bioanalytical method was successfully applied to measure fats concentration in fecal and food matrix samples obtained from clinical study of orlistat capsule, an anti-obesity medication. A total of approximately 1200 fecal and 300 food samples were successfully analyzed. The new FT-IR method is simple, reproducible and enables rapid analysis of fats in clinical samples.

Keywords: Fecal Matrix, Food Matrix, Fats, Fourier Transform Infra Red Spectrometry, Incurred Sample Reanalysis.

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Introduction:

The detection of steatorrhoea is useful for the diagnosis of pancreatic insufficiency and small bowel disorders causing malabsorption. Steatorrhoea due to chronic pancreatitis and intestinal malabsorption is quite prevalent in India [1].

Steatorrhoea is defined as an increase of fat in stool. Intestinal malabsorption of fat, which is the most frequently reported cause of steatorrhoea, is a problem caused due to dysfunctions in bowel absorption [2,3]. In some clinical studies, fecal fat excretion is the pharmacodynamic end point to evaluate treatment therapeutic efficacy. Accordingly, fecal fat quantitation is important in diagnosing steatorrhea, as well as monitoring the efficacy of treatments. To date, the classical gold standard laboratory method used to quantify fecal fat, involved collection of stool samples for a duration of 72 h and measurement of fecal fat by traditional techniques such as titrimetric, gravimetric or acid steatocrit methods [4-6]. These methods are based on a common three-stage principle: saponification (alkaline hydrolysis) to transform to soaps, acid hydrolysis and extraction of fatty acid, and finally gravimetric or titrimetric assay of fatty acids [7]. However, these methods required prolonged manipulation of stools and are rather complex and time consuming [8].

A Nuclear magnetic resonance method has also been reported however it is difficult to adapt to daily laboratory practice[9]. Taken together a reliable, quick, precise and more efficient method to quantitate fecal fat is needed.

The main aim of this study was to develop and validate a bio-analytical method for quick, precise and accurate quantification of fat in fecal and food matrices using fourier transform infrared (FT-IR) spectroscopy to support clinical studies. The method has been developed and validated for both inter-batch and intra-batch precision and incurred sample reanalysis as per current bio-analytical method, US-FDA guidance and successfully used in clinical study of orlistat capsule for quantification of food and fecal fats.

Materials and Methods**Chemicals and reagents:**

Palmitic acid and Stearic acid were obtained from Fluka. Human fecal material was collected from healthy volunteers. Chloroform, petroleum ether and water were HPLC grade. Other chemicals were analytical grade.

FT-IR conditions:

FT-IR system was used for quantification of fats in food and fecal matrices. This instrument was manufactured

by Thermo Scientific (model no. Nicolet 6700). A summary of the FT-IR spectrometric conditions used during validation and bio-analysis are summarized below:

Target compounds:	Free Fatty acids and Fats Glycerol esters (Lipids)
Quantification:	Based on absorbance band of CH ₂ group
Absorbance Wave Number:	2855 cm ⁻¹ ± 2 cm ⁻¹
Scanning Range:	4000 to 650 cm ⁻¹
No. of Scans Co-added:	16
Optical Resolution:	4 cm ⁻¹
Transmission Flow Cell:	Zinc Selenoid Crystals (0.5mm)
Background Subtraction:	Chloroform

Preparation of fatty acids standard solution:

Stearic acid and palmitic acid (in 65:35 ratios respectively weight by weight) were weighed and dissolved in chloroform to prepare standard stock solution.

Preparation of calibration curve (CC) and quality control (QC) standard samples:

All the CC and QC samples standards were prepared by freshly spiking the required amount of blank matrices (food and fecal matrix) with known volume of standard fatty acid stock solution (solution of stearic acid and palmitic acid 65:35). The calibration curve standards at concentrations of 1.00, 2.00, 4.00, 6.00, 8.00, 10.00, 12.00, 15.00 g% and quality control samples i.e. 1.00 (LLOQ), 2.00 (LQC), 5.00 (MQC), 11.00 (HQC) were prepared on each day of analysis using their respective stock solution.

Preparation of human fecal blank matrix:

10 ± 0.5g of fecal matrix was collected from healthy volunteers and treated with hydrochloric acid (35%) and petroleum ether to make them fat free. This procedure was repeated two times to make fecal samples fat free.

Preparation of food blank matrix:

Homogenized pulp of cucumber was used as food blank matrix. As per reported literatures cucumber possess the lowest amount of fat content (0.06g fat /52g matrix) in it [11].

Method description:

Approximately 1 ± 0.05g of food and fecal material were taken in 50-ml centrifuge tube resuspended in 600 µL of chloroform. Hydrochloric acid (0.2mL, 35%) was added and vortexed to thoroughly mix the content. 1ml of water and 3ml of ethanol was added to it and mixed well. 5ml of petroleum ether was added to the tube and it was capped. The tubes were spun at 25 ± 5 °C in a refrigerated centrifuge at 5000 rpm for 5 min. 3ml of clear supernatant organic layer was transferred

into a glass tube. The samples were dried and evaporated under a current of nitrogen at 20psi and 50 ± 5 °C, till complete dryness. The dried lipid extract was reconstituted with 0.4ml of chloroform and vortexed to dissolve it completely. 0.1ml volume of the solution was transferred to clean transparent crystal plate placed on the FTIR instrument for analysis.

Quantification and assay validation:

The spectra were acquired and processed using computer based Omnic software version 8.1 to obtain the response of analyte as maximum height of absorbance band between the wave number 2855 ± 2 cm^{-1} . The absorbance height of the CH_2 plotted on Y-axis and known concentration of fats on X-axis. For each analytical run, a calibration curve was derived from maximum height of absorbance against the respective known concentration values of fats using a weighed ($1/x^2$) linear least-square regression. The regression equation was then used to calculate the concentration of fats in food and fecal samples. The present method was validated following the Guidance for Industry, Bioanalytical Method Validation, US-FDA document for inter- batch and intra-batch precision and accuracy, linearity, selectivity, sensitivity, short-term long term sample stability and incurred sample reanalysis [12].

RESULTS

Linearity, limit of quantification of food and fecal fats:

The linearity of the calibration curve was evaluated from three individual batches for fats in food and fecal matrix. Linearity was established from 1.00 g% to 14.98 g% in food and 1.00 g% to 14.96 g% in fecal matrices. The coefficient of determination (r^2) was consistently greater than or equal to 0.9919 for food matrix and 0.9892 for fecal matrix respectively. Eighteen replicates of the lower limit of quantification (LLOQ) samples were used to evaluate the inter-batch precision and accuracy at LOQQC. The between inter-batch precision and accuracy at LOQQC concentration for fats was 10.9 % and 94.05%, respectively in food matrix and 11.1% and 89.84%, respectively in fecal matrix. Representative spectra of quality control sample (lower quality control) for fatty acid in food and fecal matrices are shown in Fig 1 to Fig 2.

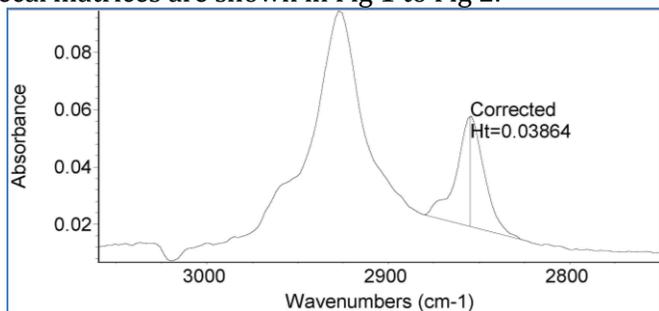


Fig 1: Spectrum of LQC food matrix

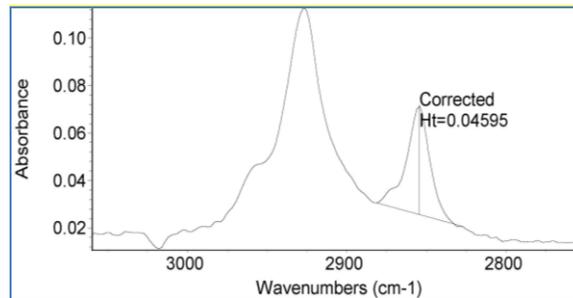


Fig 2: Spectrum of LQC fecal matrix

Accuracy, precision of food and fecal fats:

Six replicate of QC samples for each of runs were used to evaluate the precision and accuracy at low, mid, and high QC (LQC, MQC, HQC) concentrations levels. The intra- batch accuracy ranged from 87.00 to 105.40% in food matrix and 84.20 to 107.25% in fecal matrix for fats where as inter-batch accuracy ranged from 94.05 to 101.36% in food matrix and 89.84 to 103.40% in fecal matrix for fats, respectively. The intra-batch precision ranged from 1.4 to 13.0% in food matrix and 1.6 to 14.4% in fecal matrix where as inter-batch precision was ranged from 4.9 to 10.9% in food matrix and 3.1to 11. % in fecal matrix, respectively. The results are summarized in Table 1.

Recovery of fats:

The percentage recovery of fats was determined by measuring the peak area response of spiked (extracted) quality control samples (LQC, MQC and HQC) against the peak area response of aqueous (unextracted) quality control samples (LQC, MQC and HQC) of equivalent concentrations. Mean percent recovery of fats at LQC, MQC and HQC quality control samples were 60.4%, 58.0%, and 55.7% respectively in food matrix and 70.4%, 56.8% and 54.4% , respectively in fecal matrix. The results are summarized in Table 1.

Selectivity:

Twenty lots of fecal and food matrices were prepared and one sample of each cleaned matrix lot was screened for lowest response at absorbance band of CH_2 . The clear lot was selected for the preparation of selectivity samples. Six lower quality control (LOQ) samples were prepared and evaluated by comparing the response of each blank sample against mean response of LOQ samples and none showed significant interfering peaks at the wave number of 2855 ± 2 cm^{-1} .

Sample stability:

Stability test were conducted to evaluate the analyte stability in stock solution, food and fecal matrix samples under different conditions. The bench top, freeze- thaw and long term frozen storage stability were tested and the results are presented in Table 1.

As shown in table 1, percent stability observed after three freeze thaw cycle ranged between 95.6 to 104.4% in food matrices and 96.5to 98.4% in fecal matrices for

fats which confirmed that fats in food and fecal matrix samples were found to be stable for three freeze and thaw cycles. For bench top stability low and high QC levels were kept at room temperature for a period of 24.98 hours and 31.42 hours respectively (stability samples). These stability samples were processed along with the freshly spiked calibration curve and quality control samples (comparison samples). The comparative stability ranged from 96.3 to 104.3% in food matrices and 96.8 to 97.3% in fecal matrices. Stock solution stability was established for duration of 2.37 hrs at room temperature and the percentage stability of the stock solution was found to be 113.2%. Long term stability for food matrix and fecal matrix was established for 109 days and 110 days respectively for which four replicates of QC samples at low and high quality control samples (stability samples) were stored (frozen) at temperature below -20°C and -50°C. These samples were analyzed against freshly spiked calibration curve. The percent stability ranged from 100.4 to 102.1% (109 days) in food matrices and 102.7 to 102.9% in fecal matrices (110 days) for samples stored below -20°C where as the stability ranged from 98.6 to 99.0% (109 days) in food matrices and 99.2 to 106.7% in fecal matrices (110 days) for samples stored at below -50°C.

Experimental Parameters	Results	
	Food- Matrix	Feces- Matrix
Analytical range (g%)	1.00 - 14.98	1.00 - 14.96
Minimum quantifiable (g%)	1.00	1.00
Intra-batch accuracy (%)	87.00 - 105.40	84.20 - 107.25
Inter-batch accuracy (%)	94.05 - 101.36	89.84 - 103.40
Intra-batch precision (% CV)	1.4 - 13.0	1.6 - 14.4
Inter-batch precision (% CV)	4.9 - 10.9	3.1 - 11.1
% Recovery		
Lower quality control sample (LQC)	60.4	70.4
Medium quality control sample (MQC)	58.0	56.8
High quality control sample (HQC)	55.7	54.4
% Recovery (Mean)	58.04	60.54
% Stability (Mean)		
% Stock solution Stability (2.37 hrs)	113.2	
% Freeze-thaw stability (after 3 cycles)		
% Bench-top stability (in hrs.)	96.3 - 104.3 (24.98)	96.8 - 97.3 (31.42)
% Long term stability (below -20°C) (in days)	100.4 - 102.1 (109 days)	102.7 - 102.9 (110 days)
% Long term stability (below -50°C) (in days)	98.6 - 99.0 (109 days)	99.2 - 106.7 (110 days)

Table 1: Result of validation parameters

Incurred sample reanalysis (ISR)

Incurred sample reanalysis (ISR) was performed by random selection of fecal and food samples. 60 study

samples in fecal matrix and 50 study samples in food matrix were re-analyzed for incurred sample reanalysis. ISR data are acceptable if the % difference between initial and repeated concentration is $\leq 20\%$ for at least 67 % of repeated samples. The 94% of food and 88% of fecal matrix repeated samples met the acceptance criteria of ISR.

DISCUSSION

This new method allowed rapid determination of fats in fecal and food samples by FT-IR where as traditional techniques such as titrimetric, gravimetric or acid steatocrit methods relatively required long processing time of sample to obtain results [4-6].

The method was validated over the concentration range of 1.00 g% to 14.98 g% for fats in food and 1.00 g% to 14.96 g% for fat in fecal matrices according to the Guidance for Industry, Bioanalytical Method Validation, US-FDA. None of methods previously reported has been validated as per Bioanalytical Method Validation Guidance, US-FDA and our method has met all the current regulatory standards.

Water is the major component of stools (amounting to $60 \pm 90\%$). Our approach of using a simple extraction of fatty acids from stool prevented the interfering effect of water. Another advantage of this separation of fatty acids from stool is the exclusion of water soluble interfering substances, and the opportunity to use a primary standard (stearic \pm palmitic acids) for calibration. We used primary standards consisting of a mixture of the most prominent fatty acids in human stools (stearic and palmitic acids, 65:35) [13].

We used the absorbance band of the CH_2 group (2855 cm^{-1}) of free fatty acids and fatty acid glycerol esters of the primary fatty acid standard for quantification. However other reported methods based on FT-IR without sample purification used complex procedures to obtain a reliable model to derived the results [14-16].

Regulatory agencies such as US-FDA, EMA and TGA recommended to evaluate accuracy and reproducibility of method through incurred samples Reanalysis (ISR), by reanalysis of study samples in separate runs at different days [17].

Incurred Sample Reanalysis (ISR) was performed for our new method and incurred sample reanalysis met regulatory ISR acceptance criteria. Results of incurred sample reanalysis further confirmed that our method has showed reproducible results where as reproducibility was one of the major concerned in previously reported methods. None of the previously reported methods performed incurred analysis.

To demonstrate the applicability of the FT-IR method, the procedure was successfully used for quantification of fats in food and fecal matrix in clinical study of orlistat.

A total of approximately 1200 fecal and 300 food study samples were collected during the clinical phase of the study and these samples were analyzed for fats content. The fats content of fecal and food matrix samples were used to derive fecal fat excretion. We have published the pharmacodynamic and statistical results of clinical study. The results from clinical study proved that the test formulation of Orlistat capsule is bioequivalent to the reference marketed Alli™ when administered to healthy volunteers as a single dose under fed conditions [18].

CONCLUSION

A new FT-IR method for quantification of fats in food and fecal matrices was developed. The method was completely validated showing satisfactory data for all the validation parameters tested as per current regulatory standards. Incurred sample reanalysis met the regulatory standard and confirmed the reliability of the new method. The new FT-IR method is simple and enables rapid analysis of clinical samples for fats. The developed FT-IR method was successfully used for determination of fats in food and fecal matrices in clinical study of orlistat capsules.

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