Clinica Chimica Acta 400 (2009) 33–36

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Comparative analysis of fecal fat quantitation via nuclear magnetic resonance spectroscopy (^{1}H NMR) and gravimetry

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ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form 1 October 2008
Accepted 2 October 2008
Available online 17 October 2008

Keywords:
Fecal fat
Lipid
Feces
Nuclear magnetic resonance
Gravimetry

ABSTRACT

Background: Fecal-fat is typically measured by extracting lipid from homogenized feces with subsequent gravimetric/titrimetric analyses that are time-consuming and involve toxic solvents. Accordingly, an efficient and more safe method to quantitate fecal-fat is needed. The present objective was to adapt CEM SmartTrac® technology (i.e. ^{1}H NMR) to rapidly (<5 min) quantitate fecal-fat and compare ^{1}H NMR and gravimetric performance characteristics.

Methods: ^{1}H NMR and gravimetric measurements of stool-fat were conducted using excess stool samples (72 h collection; $n=107$) homogenized to semi-liquid consistency prior to analyses.

Results: The ^{1}H NMR method demonstrated acceptable linearity ($R^2=0.9999$) and recovery (mean=105%) with imprecision (intra-assay CV=1.2–6.5%; inter-assay CV=1.8–5.8%) comparable to or better than gravimetry (intra-assay CV=1.0–17.2%; inter-assay CV=3.8–6.5%). Excellent correlation between fecal-fat quantitation by ^{1}H NMR and gravimetry ($n=107$; $R^2=0.983$; $y=1.0173x-0.6859$) was exhibited; moreover, ^{1}H NMR demonstrated good sensitivity (92.3%), specificity (94.5%), negative-predictive value (92.9%) and positive-predictive value (94.1%) for malabsorption using the reference cut-off of ≤7 g fat/24 h.

Conclusions: These data demonstrate that ^{1}H NMR permits rapid and safe quantitation of fecal-fat while maintaining acceptable performance characteristics, thereby supporting the utility of ^{1}H NMR as an alternative method to gravimetry for fecal-fat quantitation.

1. Introduction

Malabsorption of fats/lipids may be a result of atrophy from malnutrition, or secondary to various pancreatic and intestinal diseases. Accordingly, fecal fat quantitation is important in diagnosing steatorrhea as well as monitoring the efficacy of treatments. To date, the classic *van de Kamer* extraction of lipids coupled with gravimetric or titrimetric methods is typically used to quantitatively measure total fecal fat in stool samples; however, these methods are laborious, time-consuming (>4 h), and potentially dangerous with the handling of toxic solvents during lipid extraction [1,2]. Several authors have proposed the use of infrared spectroscopy as an alternative method to measure fecal fat; however, the accuracy of fecal fat quantitation by this method still requires stool homogenization and/or lipid extraction prior to analysis and is unsuitable for quantitating fecal fat in stool samples containing >75% water content (e.g. diarrhea stool samples) [3–6]. Taken together, a reliable, more efficient and safer method to quantitate fecal fat is needed.

Hydrogen nuclear magnetic resonance spectroscopy (^{1}H NMR) is a standard technique in food industry for quantitating fat/lipid content in raw products [7–9]. Similarly, studies have used analogous ^{1}H NMR or ^{13}C NMR procedures to quantitate fat content in stool samples but required freeze-drying of the samples prior to analysis [10–13]. More recently, the CEM SmartTrac® System (Matthews, NC) was developed which combines microwave drying technology with ^{1}H NMR for fast (<5 min) and accurate measurement of fat and moisture in food products. In the present study, we proposed to adapt the CEM SmartTrac® system to quantitate fat content in stool thereby eliminating the preparation step of freeze-drying stool samples that was previously required in ^{1}H NMR measurement of fecal fat [10–13]. Accordingly, the aim of this study was to determine whether ^{1}H NMR using the CEM SmartTrac® System produces quantitative fecal fat results comparable to the reference method using gravimetric measurements, which would support the utility of ^{1}H NMR analysis of stool fat in diagnostic laboratories and future clinical studies.

2. Materials and methods

2.1. Stool sample collection and preparation

Comparison of the ^{1}H NMR and gravimetry analyses of stool fat was conducted using excess stool samples ($n=107$) sent for fecal fat analysis by gravimetry, wherein the

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doi:10.1016/j.cca.2008.10.001

patients' clinical information was not available. The stool samples were collected over a 72 h duration from 107 different individuals. Following collection the stool samples were homogenized to a semi-liquid consistency prior to fecal fat analysis. If necessary, a recorded volume of water was added to the stool sample to allow for complete homogenization.

2.2. ^{1}H NMR fecal fat measurement

The CEM SmartTrac® System (Matthews, NC) combines microwave drying with nuclear magnetic resonance (^{1}H NMR) to determine the fat content of samples. The method has been previously described using sample types other than stool [14,15]. Approximately 2.5 g of the well-homogenized stool sample was applied uniformly to an absorbent sample pad and weighed on the instrument balance pan. A second sample pad was placed over the sample at which point the sample was microwave-dried to remove the protons present due to moisture. The microwave contains infrared sensors that allow the maintenance of a steady temperature (<110 °C) without burning the sample and minimizing loss of the more volatile fats (free fatty acids) in the sample. The microwave time was determined by drying of a sample until a constant weight was achieved which prompted automatic cessation of drying (typically 2–4 min/sample). The sample was then wrapped in CEM Trac film, inserted into the CEM Trac tube, and analyzed via the ^{1}H NMR. The ^{1}H NMR pulses the sample with radio-frequency energy causing any remaining hydrogen protons to generate a signal (free-induction decay) that is detected and analyzed by the CEM SmartTrac® System software [14,15]. Free-induction decay of lipid protons occurs at a slower rate than that of protons from other substances in the stool, such as proteins and carbohydrates which permits determination of the percent fat in the weighed sample. The total analysis time after initial homogenization of stool was ~5.0 min per sample. The final results were expressed as % moisture, % solid and % fat of the aliquot analyzed. Finally, based upon the total weight of the homogenized stool specimen collected over 72 h, % fat was then converted to grams fat/24 h.

2.3. ^{1}H NMR calibration and quality control

The CEM SmartTrac® System balance was calibrated daily using certified 10.0 and 2.0 gram weights. Additionally, the CEM SmartTrac® system ^{1}H NMR magnet frequency and pulse width were adjusted daily and weekly, respectively, using the manufacturer's supplied oil standard. Two control samples with known values were analyzed daily to verify assay performance. Bi-yearly calibration of the ^{1}H NMR was performed using pooled fecal samples with known fat concentration as determined by the gravimetric method (see below).

2.4. Gravimetric fecal fat measurement

Gravimetric measurement of fecal fat was performed. Fat from the fecal samples was extracted as previously described [1,2] and the extract was evaporated to dryness and weighed. Ten grams of weighed, well-emulsified stool sample was saponified in 10 ml KOH (6.2 M) and 40 ml alcohol mixture (0.4% isoamyl alcohol and 95% ethanol) for 30 min with boiling and reflux to convert the esterified lipids to a potassium salt of a fatty acid. The sample was then acidified with 17 ml of 8.2 mol/l HCl to decrease the ionization of fatty acids. The total fecal lipids were extracted with 50 ml petroleum ether wherein the petroleum ether and the acid ethanol layers separated quickly (5–10 min). Following separation, 25 ml of the petroleum ether layer was transferred to a pre-weighed beaker and allowed to evaporate on a steam bath. The beaker was then dried at 110±5 °C in an oven for 30 min and the residue was weighed gravimetrically.

2.5. Gravimetric fecal fat quality control

The gravimetric balance was calibrated daily using certified 1.0, 10.0 and 50.0 g weights. Two control samples with known values were analyzed daily to verify acceptable assay performance.

2.6. Statistical analysis

The robustness of both assays was analyzed by calculating intra- and inter-assay precision, and agreement of the ^{1}H NMR method with the gravimetric method was assessed by performing linear regression analysis using Microsoft Excel software (Microsoft, Redmond, WA). The quantified fecal fat results determined by both methods were analyzed using Bland–Altman analysis.

3. Results

3.1. Linearity and recovery

To assess linearity of fecal fat quantitation by the ^{1}H NMR method, % fat was quantitated in serial-dilutions of 6 stool samples containing varying percentages of fat. Linear regression analysis indicates that fecal fat values (% fat) quantitated by ^{1}H NMR correlate well with the expected values, within the range of 0.1–21.0% fat producing a mean linear regression equation of $y=1.0009x+0.0004$, ($R^2=0.9999$) as shown in Fig. 1. This range of % fat comprises >99% of samples received in the laboratory for clinical testing. The percent recovery of fecal fat quantitated by the ^{1}H NMR was conducted using fresh-homogenized stool samples containing low (1.2–4.3% fat) or high (10.5–14.2% fat) lipid content as determined by ^{1}H NMR. Combinations of low and high lipid containing homogenized stool samples (25% low:75% high, 50% low:50% high and 75% low:25% high) were quantitated for lipid content by the ^{1}H NMR method and results were compared to the expected % lipid content to determine % recovery ($n=3$ combination sets or $n=9$). Expected lipid content ranged from 3.5–14.4% and data indicate a recovery range of 93–112% with a mean recovery of 105% ($n=3$ combination sets or $n=9$).

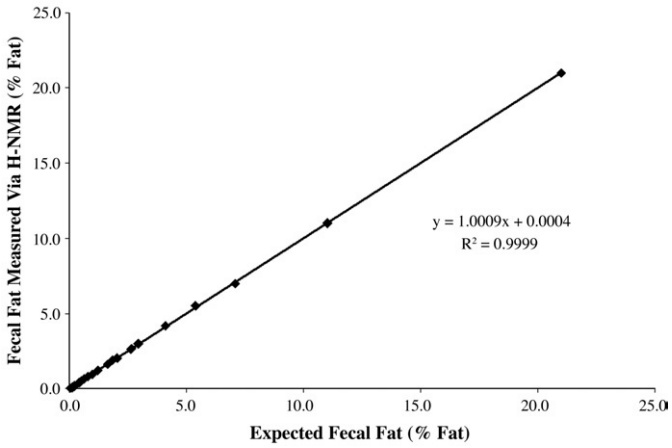

Fig. 1. Linearity of % fat in 6 serially diluted samples with varying fat concentrations analyzed by ^{1}H NMR. Mean linear-regression analysis of measured versus expected values, within the range of 0.1–21.0% fat is $y=1.0009x+0.0004$; ($R^2=0.9999$).

3.2. Imprecision

Imprecision results for fecal fat quantitation by the ^{1}H NMR and gravimetric methods are summarized in Table 1. Analysis of ^{1}H NMR and gravimetric intra-assay and inter-assay imprecision was conducted on the results obtained from different stool samples with varying levels of fecal fat (1.17–18.3 g fat/24 h). The CVs for ^{1}H NMR intra-assay (1.2–6.5%) and inter-assay (1.8–5.8%) imprecision are similar to, or better than imprecision results using the gravimetric method (intra-assay CV=1.0–17.2%; inter-assay CV=3.8–6.5%).

3.3. Method comparison

The method comparison results are presented in Fig. 2, that illustrate a strong positive correlation between fecal fat quantitation by the ^{1}H NMR method and the well-established gravimetric method ($R^2=0.98$; $y=1.0173x-0.6859$; $n=107$; Fig. 2A). The quantified fecal fat values ranged from 1.0–150.4 g 1fat/24 h by ^{1}H NMR and 0.3–130.6 g fat/24 h by gravimetry with a mean value of 18.5 g fat/24 h and 18.9 g fat/24 h, respectively. Moreover, ^{1}H NMR and gravimetric analysis of the stool samples produced median values of 7.0 and 6.6 g fat/24 h, respectively. A comparison of the percent of fat in the aliquot of homogenate measured by each method in these 107 samples was also performed ($R^2=0.95$; $y=0.9887x-0.0661$). Percent fat values ranged from 0.14 to 21.0%. As shown in Fig. 2B, comparison of fecal fat measurements by ^{1}H NMR and gravimetric analysis near the reference cut-off value of ≤7 g fat/24 h ($n=68$ of 107 samples measured fecal fat content <12 g fat/24 h and were included in Fig. 2B) indicates 3 false-positive and 4 false-negative results for malabsorption detected by ^{1}H NMR compared to the reference gravimetric method. Therefore, when compared to the gravimetric method, ^{1}H NMR measurement of fecal

Table 1
Impression of fecal fat assays by gravimetric and ^{1}H NMR methods

	Gravimetric								^{1}H NMR							
	Intra-assay					Inter-assay			Intra-assay					Inter-assay		
Sample:	A	B	C	D	E	F	G	H	1	2	3	4	5	6	7	8
Mean[a] (g/24 h)	1.17	2.55	4.21	13.9	17.9	1.2	2.7	4.3	1.2	2.6	4.3	14.2	18.3	1.2	2.6	4.4
SD[b]	0.20	0.06	0.08	1.73	0.18	0.05	0.17	0.16	0.08	0.04	0.05	0.25	0.30	0.04	0.05	0.25
CV[c] (%)	17.2	2.5	1.8	12.4	1.0	4.1	6.5	3.8	6.5	1.5	1.2	1.7	1.7	3.2	1.8	5.8

[a] Mean value of 5 determinations for intra-assay imprecision and 3 determinations over 1.5 years for inter-assay imprecision.
[b] SD is the standard deviation.
[c] CV is the coefficient of variance.

fat has a sensitivity of 92.3%, specificity of 94.5%, negative-predictive value of 94.1% and positive-predictive value of 92.9% for malabsorption using the reference cut-off value (≤7 g fat/24 h) previously established by the gravimetric method [16]. A Bland–Altman analysis of the method comparison data for fecal fat measurement by ^{1}H NMR and gravimetry is shown in Fig. 3. Fecal fat measured by the ^{1}H NMR method compared to the gravimetric approach demonstrated a mean absolute difference of 0.36 g fat/24 h.

4. Discussion

This study demonstrates a novel and simple method to quantitate fecal fat using CEM SmartTrac® technology (i.e. ^{1}H NMR). The CEM SmartTrac® System combines microwave drying technology with ^{1}H NMR, which permits rapid (<5 min) quantitation of lipid content in stool samples and eliminates the laborious and dangerous handling of toxic solvents that are required using the well-established gravimetric method. Moreover, the performance characteristics and fecal fat quantitation by ^{1}H NMR correlate well with the gravimetric reference method. Accordingly, this study supports the utility of the ^{1}H NMR to quantitate fecal fat in diagnostic laboratories and future clinical studies.

To date, the "gold standard" for fecal fat quantitation is the classic *van de Kamer* extraction of lipids coupled with titrimetric or gravimetric quantitation of total fecal fat in individual stool samples. However, this method is laborious, time-consuming (>4 h), and potentially dangerous as it involves handling of toxic, corrosive and flammable solvents during lipid extraction [1,2]. Conversely, our newly developed method for quantitating fecal fat uses ^{1}H NMR as part of the CEM SmartTrac® System, which eliminates the lipid extraction process including the handling and disposal of toxic solvents, thereby eliminating potential for errors and enhancing safety. Moreover, the quantitation of fecal fat via ^{1}H NMR can be accomplished in less than 5 min with minimal stool sample processing, as well as the fecal fat results can be automatically transferred to the laboratory information system (LIS) via interfacing the CEM SmartTrac® system with the LIS, which minimizes post-analytical errors. Consequently, ^{1}H NMR quantitation of fecal fat using the CEM SmartTrac® system offers numerous advantages compared to the reference gravimetric method.

In order to utilize ^{1}H NMR fecal fat results clinically, it was essential to appropriately validate the assay and determine if the results were acceptable and comparable to the fecal fat results obtained by the standard gravimetric method. Therefore, we first determined that the

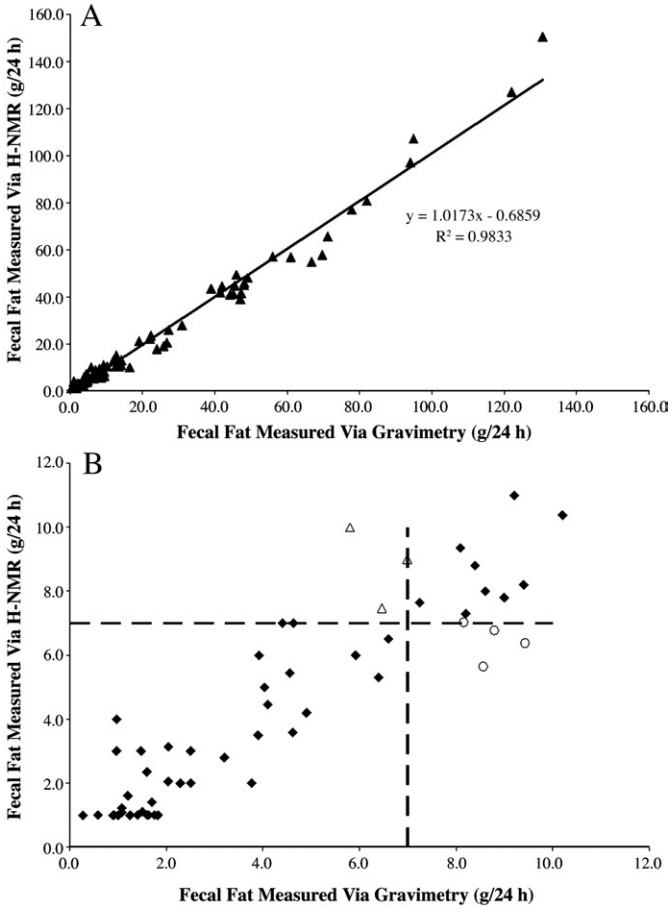

Fig. 2. Comparison between fecal fat content measured via gravimetry after *van de Kamer* extraction and by ^{1}H NMR. A) Linear-regression analysis of fecal fat results measured by gravimetry and ^{1}H NMR (R^2=0.98; n=107). B) Comparison of fecal fat measurements by ^{1}H NMR and gravimetric analysis near the reference cut-off value of ≤7 g fat/24 h (includes 68 of 107 samples measuring fecal fat content <12 g fat/24 h). The dashed-lines indicate the reference range cut-off value (≤7 g fat/24 h). Open triangles indicate false-positive results and open circles indicate false-negative results detected by ^{1}H NMR compared to the reference gravimetric method.

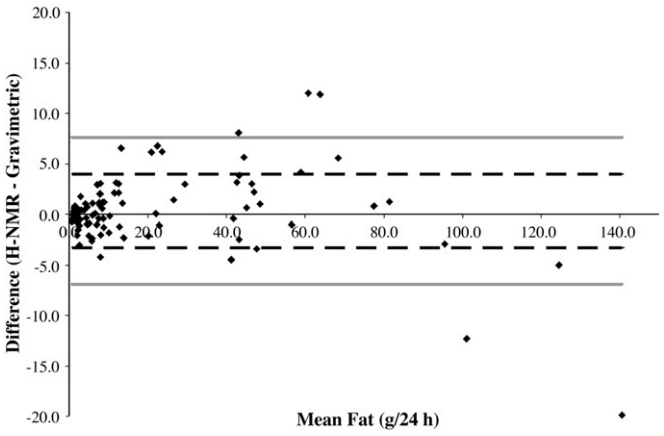

Fig. 3. Bland–Altman plot illustrating the limit of agreement between the gravimetric and ^{1}H NMR methods for fecal fat measurement. Fecal fat measured by the ^{1}H NMR method compared to the gravimetric approach indicates a mean difference of 0.36 g fat/24 h (upper limit of agreement: 12.0 g fat/24 h; lower limit of agreement: −19.8 g). The horizontal dotted and solid lines indicate the mean difference of fecal fat measured ±1SD and ±2SD, respectively.

linearity, recovery and imprecision of fecal fat quantitation by ^{1}H NMR were acceptable. The fecal fat linearity values quantified by ^{1}H NMR within the range of 0.1–21.0% fat correlated well with the expected values (Fig. 1). The coefficients of variance (CVs) for ^{1}H NMR intra-assay (1.2–6.5%) and inter-assay (1.8–5.8%) imprecision were comparable to or better than the gravimetric method imprecision results (intra-assay CV = 1.0–17.2%; inter-assay CV = 3.8–6.5%; Table 1). Additionally, the recovery of fecal fat quantitated by the ^{1}H NMR indicated a recovery range of 93–112% with a mean recovery of 105%. Taken together, these data indicate that ^{1}H NMR and gravimetric methods have comparable performance characteristics, thereby supporting ^{1}H NMR as a reasonable alternative to gravimetric quantitation of fecal fat.

Stool samples from 107 different patients with varying stool fat excretion were used for comparative analysis of fecal fat quantitation by ^{1}H NMR and gravimetric methods. A positive correlation between stool fat quantitation by ^{1}H NMR and gravimetry was exhibited ($R^2 = 0.98$; $y = 1.0173x - 0.6859$; Fig. 2A), demonstrating that the ^{1}H NMR method produces results similar to that generated by gravimetry. Bland–Altman analysis demonstrates a mean absolute difference of 0.36 g fat/24 h between ^{1}H NMR and gravimetric measurement of fecal fat (Fig. 3). Accordingly, with similar analytical performance characteristics between the two methods, it is not surprising that fecal fat measurement using the ^{1}H NMR also demonstrates good sensitivity (92.3%), specificity (94.5%), negative-predictive value (92.9%), and positive-predictive value (94.1%) for malabsorption when compared to the gravimetric method using the established clinical cut-point of ≤7 g fat/24 h established by gravimetry. Comparison of fecal fat measurements by ^{1}H NMR and gravimetric analysis near the reference cut-off value of ≤7 g fat/24 h ($n = 68$ of 107 samples measured fecal fat content <12 g fat/24 h) indicated 3 false-positive and 4 false-negative results for malabsorption detected by ^{1}H NMR compared to the reference gravimetric method (Fig. 2B). In view of the above results it may be stated that the utility of ^{1}H NMR-derived fecal fat results for identifying patients with malabsorption is similar to that of gravimetry. Regardless of the methodology, fecal fat results near the cut-off for malabsorption (7 ± 1 g) should be interpreted carefully with consideration of the patient's symptoms and repeated as necessary.

In summary, this study demonstrates a novel and simple method to quantitate fecal fat using ^{1}H NMR as part of the CEM SmartTrac® technology. This method permits rapid (<5 min) quantitation of lipid content in stool samples and eliminates the laborious and dangerous handling of toxic, corrosive and flammable solvents that are required when using the well-established gravimetric method. Fecal fat quantitation by ^{1}H NMR correlates well with the gravimetric reference method and demonstrates comparable performance characteristics, thereby supporting the utility of the ^{1}H NMR to quantitate fecal fat in diagnostic laboratories and future clinical studies.

Acknowledgements

We thank Dr. Lawrence K. Oliver and Shannon D. Hodel-Hanson for their suggestions and review of method validation studies and Sara J. Minnich, Aaron J. Stelpflug, John Christian, Jennifer E. Brown, and Karen Kloke for their technical support.

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