A Simple Colorimetric Method to Quantify Total Fecal Oil Using Oil-soluble Dyes in Laboratory Animals and Its Correlation with Liquid Chromatography–Mass Spectrometry Analysis

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We developed a colorimetric method for measuring the amount of oil in mouse stool after co-administering an oil-soluble dye. When the amount of oil in the feces calculated from the amounts of Sudan III and Oil Red O was plotted against the amount of oil detected by liquid chromatography–mass spectrometry, the graph was linear, showing a one-to-one correlation between two analyses. This method may be utilized to determine the efficacy of lipase inhibitors, or to assess fat malabsorption in vivo.

Keywords Fecal fat analysis, oil-soluble dye, colorimetry, laboratory animals, liquid chromatography–mass spectrometry

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Introduction

Quantification of unabsorbed fats and oils in fecal matter is essential to develop lipase inhibitors and malabsorption drugs.1 The amount of excreted fat has been measured in various ways in the clinic to diagnose pancreatic diseases and intestinal disorders, as well as in animal studies.1,2 One method involves titration of the extracted fatty acid;3 however, this chemical analysis is complicated and time-consuming, and may require the use of harmful chemical materials.2 Another approach is a microscopic examination of fat droplets stained by oil-soluble dyes;4 however, this method does not provide reliable quantification estimates of the fecal fat.5 Other oil separation and detection techniques include chromatographic analysis methods, such as gas and gas–liquid chromatography using a flame ionization detection or mass spectrometry.5,7 These methods are accurate, but they require expensive equipment and highly trained technicians. To measure fecal fat easily, quickly, and cost-effectively, several other methods have been developed.28 These methods share a common approach in that lipid is directly quantified by various extraction methods. Since the results of lipid determination depend on the lipid extraction process, this extraction process assumes significance.

We have established a new method in which a mixture of oil and dye was administered orally to mice, and the total amount of oil in the feces was quantified using the colorimetric detection of the dye rather than of the oil. The determination of oil-soluble dyes was not affected by the presence of other extracted materials, because only the color of the dyes was quantified. The accuracy of our method depended on the oil-soluble dyes remaining in the digested oil in the gastrointestinal (GI) tracts of the mice. We performed experiments using eight oil-soluble dyes that are routinely used to stain fat in feces in order to examine the correlation between the amount of excreted oil and dye.9–12 This paper introduces our new simple method, a colorimetric assay,13,14 which enables the accurate quantification of oil in the excrement of laboratory animals.

Experimental

Reagents and chemicals

Triolein, Nile red, pyrene, Sudan I, II, III, and IV, Sudan Black B, Oil Red O, diolein, oleic acid, olive oil, corn oil, chloroform, and isopropyl alcohol were purchased from Sigma-Aldrich (St Louis, MO, USA). Monoolein (RYLO™ MG 19, >90% pure) was obtained from Danisco Ingredients (Copenhagen, Denmark). Orlistat was from Bicon (Bangalore, India). 1,1,1-13C3-Triolein was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Methanol, acetone, and isopropyl alcohol (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI, USA).

Dye solutions in triolein and lard

Oil-soluble dyes were completely dissolved in triolein at 0.21 mg g–1 (Nile red), 1.05 mg g–1 (pyrene), 3.16 mg g–1 (Sudan I), 2.11 mg g–1 (Sudan II), 1.05 mg g–1 (Sudan III), 2.11 mg g–1 (Sudan IV), 0.08 mg g–1 (Sudan Black B), and 0.84 mg g–1 (Oil Red O). Lard was extracted from pork-belly meat by baking, and purified using an equivolume mixture of
methanol and chloroform. Sudan III (2.25 mg g⁻¹) and Oil Red O (1.12 mg g⁻¹) were dissolved completely in lard at 30°C.

**Animal experiments**

Seven-week-old female BALB/c mice (18 - 20 g) were purchased from Nara Biotech (Seoul, Korea). Animal care and handling in this study followed the institutional guidelines of the Animal Care and Use Committee at the Korea Institute of Science and Technology. These dye/triolein or dye/lard solutions were administered orally to mice at various doses (10 – 100 μL/mouse) immediately after the administration of 20 mg kg⁻¹ orlistat, a gastrointesinal lipase inhibitor that reduces dietary fat absorption (n = 3 mice/group). Since dietary fat is an important nutrient and can be absorbed efficiently, we administered orlistat to ensure excretion of the oil, mimicking the fat malabsorption symptom in the animals. Since the mice had the habit of licking their bodies for grooming, they were fitted with protective collars to prevent licking. Since the mice had the habit of licking their bodies for grooming, they were fitted with protective collars to prevent licking.16

Feces and fur contaminated by oil were collected for 15 h, starting from the administration of dye/triolein or dye/lard.17 Dried feces and fur were weighed, mixed with an extraction solvent (chloroform/isopropyl alcohol = 1:19, v/v), homogenized and vortexed.9 These samples were centrifuged at 10000 × g for 5 min at 4°C and filtered to collect the supernatants. All samples containing the extracted dyes were stored at −70°C until analysis.

**Quantification of dyes**

The emission intensity of Nile red (ex: 560 nm, em: 631 nm) and pyrene (ex: 336 nm, em: 393 nm) in 1 mL of each extracted sample was measured using a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan). The absorbance of Sudan I (476 nm), Sudan II (493 nm), Sudan III (512 nm), Sudan IV (520 nm), Sudan Black B (605 nm), and Oil Red O (518 nm) in 100 μL of each extracted sample was determined by using a UV-visible spectrophotometric microplate reader (Spectra max 340, Molecular Devices, Sunnyvale, CA).

**Quantification of oils by LC-MS**

To measure the amount of the total excreted fecal oils, filtered samples (200 μL) were mixed with 25 μL of internal standard (1,1,1-¹³C-triolein, 10 μg mL⁻¹ in methanol) by vortexing, and 3 μL samples were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis, which was performed as described previously.18 LC-MS analysis was performed using a Shiseido Nanospace SI-2 high-performance liquid chromatograph (Tokyo, Japan) coupled to an ion-trap Thermo Finnigan LCQ Advantage mass spectrometer (San Jose, CA, USA). The mobile phase consisted of methanol and acetone-isopropyl alcohol (1:1, v/v), and the analytical column was Sunfire C8 (3.5 μm, 150 × 2.1 mm, Waters, Milford, MA, USA). The lipids (i.e., triolein, diolein, and monolein) in the extracted oil were simultaneously detected in the positive ionization mode ([M+Na]⁺). The total weight of oil was determined by adding the weights of triolein, diolein, and monolein.

**Gravimetric quantification**

To measure the amount of the total fecal fats excreted by mice that consumed the dye/lard solution, gravimetric analysis was used.28,11 The amount of lard in the excrement was weighed after the organic solvent in the supernatant of the excrement mixture was evaporated.

**Solubility measurement**

An excess of each dye was dissolved in triolein, diolein, monolein, oleic acid, olive oil, corn oil, and lard by vortexing at room temperature to measure the solubility of dyes in various oils. Oil solutions were centrifuged and filtered to remove undissolved dyes. These solutions were diluted in the extracted organic solvent mixtures to determine the solubilities using a fluorescence spectrophotometer or UV-visible spectrophotometric microplate reader, as detailed above.

**Results and Discussion**

We chose triolein and lard as representatives of vegetable and animal oils, respectively, to be quantified. Triolein is the main ingredient of most vegetable-based oils.19 Unlike triolein, lard is a mixture of tripalmitin, tristearin, triolein, and other triacylglycerides with melting points of 30 - 50°C.20 Nile red, pyrene, Sudan I, II, III, and IV, Sudan Black B or Oil Red O was dissolved completely in triolein to prepare oral solutions to be fed to mice. Depending on the dye, the oil solution showed deep red, blue, black, or no color. The oil solutions were orally fed to mice at various doses, and the feces and contaminated fur were collected after administration (Fig. 1). The dye and oil in the excrement were extracted with the extraction solvent and subsequently quantified by using fluorescence spectrophotometry or UV-VIS absorbance for the dyes, and LC-MS for the digested mixture of triolein. A quantification method was established for oleins in mouse feces in our previous study.18 Total weight of excreted oil was determined by adding the weights of triolein, diolein, and monolein by LC-MS. The total weights of excreted Sudan I and oil (oleins) were plotted as a function of oil dose in Figs. 1A and 1B, respectively, to generate the correlation between the total weights of excreted dye and oil (Fig. 1C).10 The correlations between the total weights of excreted dyes and oils are shown in Fig. 2A. In most cases, the data could be fitted by linear regression when the y-intercept was set to zero. The slope (m, dashed lines) represented the concentration of dye in the digested oils. The dye concentration in the excreted oil could be compared with that in triolein in the original solution.
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For Nile red, pyrene, Sudan I, and Sudan Black B, the correlation coefficients ($R^2$) were low, and the slopes deviated from the concentrations of dyes in triolein for oral administration. Nile red, Sudan I, and Sudan Black B were more selectively absorbed than oils, indicated by lower concentrations of dyes in the excrement than those in the oral solutions. In contrast, pyrene was less efficiently absorbed than oil. When Sudan II, III, and IV, and Oil Red O were used as probes, the concentrations of dyes in the excreted oils were similar to those in the oral solution, with $R^2$ values higher than 0.9. The correlations were best for Sudan III and Oil Red O.

Next, we examined whether Sudan III and Oil Red O could also be used as probes in fecal lard determination. Lard was mixed with these two dyes and fed to mice to determine the total excreted dyes in lard digestion products, which was quantified by using gravimetry (Fig. 2B). These two dyes could also be used as colorimetric probes in lard since good correlations were observed ($R^2 >0.95$). However, in the higher total excreted lard region (about 40 mg or higher), the correlation became worse than that observed in the lower region consistent to the results from the triolein data above (Fig. 2A).

Above a dose of 40 mg or higher, the mice showed symptoms of diarrhea. The dyes dissolved in the oils would experience environment changes in the GI tract, as the triglycerides were digested into diglycerides, monoglycerides, and fatty acids. As shown in Table 1, the solubility of pyrene, Sudan I, III, and IV, and Oil Red O in various oils did not change significantly, and the range of solubility difference was below ±15%. However, the solubility of Nile red was higher in monoolein and oleic acid than in triolein. Since monoolein and oleic acid could be absorbed into the body while triolein could not, the selective partition could explain the higher absorption of Nile red than the oils. Hydrophobic dyes were better probes than the dyes with lower log $P$ values, except for Sudan Black B (Table 1). The color of Sudan Black B in a triolein solution was faint due to its low solubility in triolein; it could therefore hinder color detection.

In conclusion, our experiments showed comparisons between colorimetric and LC-MS analyses using eight oil-soluble dyes as potential probes in order to find a quick and easy method to quantify fecal fat and oil. Colorimetric analysis, especially using Sudan III and Oil Red O, is an easy and accurate method for quantifying excreted oil, and is a good alternative to using LC-MS. This method allows for the rapid and simple quantification of the total excreted oil in stool after co-administration of oil-soluble dyes. As most of these have strong colors, one important advantage of this method is that their complete elimination from the body can be confirmed by the appearance of charcoal-colored feces. This method may also be utilized to determine the efficacy of lipase inhibitors such as orlistat and cetilistat or be used to assess fat malabsorption in experimental animals. In further experiments, we will investigate the utility of this colorimetric method with other fats and oils.

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