CHAPTER 24

BIOLOGICAL SAMPLE COLLECTION AND BIOLOGICAL MARKERS OF DIETARY COMPLIANCE

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Biological samples are collected in virtually every controlled feeding protocol; their analysis provides the *primary outcome data* for evaluating the effects of the defined dietary manipulation. Many feeding study hypotheses are tested by evaluating changes in physiologic pools of nutrients or metabolites in response to the research diet. The concentrations or half-lives of various compounds in the pools are determined by laboratory assay, and estimation of pool volume is achieved for internal compartments (such as blood) using algorithms, whereas for excretory compartments (such as urine and feces) pool volumes can be measured directly. (Also see Chapter 16, ''Compartmental Modeling, Stable Isotopes, and Balance Studies.'')

Biological samples are also collected in search for an objective measure of *compliance* with diet. Every controlled feeding study raises the question ''Did the participant eat all of the study food, and no other foods?'' Investigators can make observations of the behavior of the participants, and they can ask questions of the participants, but this information is subject to bias. Thus, it is valuable to have an independent laboratory-based indicator of compliance as well.

Valid interpretation of data for any of these measurements, whether as study outcomes or as measures of compliance, requires assurance that the collection of the biological sample was complete. When members of the research staff collect the sample (such as during phlebotomy) and otherwise have control of the collection process, it is relatively easy to provide the assurance that no sample was lost. Collection of urine and feces presents a different set of challenges, however; the study participant ''collects'' the samples at intervals that may extend for hours or days, and sometimes in a variety of locations. Verification that collection is complete likely will require use of markers of the completeness of collection.

A variety of markers and measurements can be used to provide qualitative and quantitative estimates of dietary compliance and sample collection. A biological marker has been defined by Bingham as ''any biochemical index in an easily accessible biological sample that in health gives a predictive response to a given dietary component'' (1). Markers used for measuring dietary compliance and collecting biological specimens can be either naturally occurring nutrients ingested within the diet or physical or chemical markers taken orally or added to the food. A good biomarker is nontoxic, accurately and easily measurable, excreted rapidly without being metabolized, minimally invasive, and inexpensive. When the marker is added to food, it should be colorless, odorless, and tasteless, and it should not change the appearance of the food.

When planning the study protocol and its associated laboratory analyses, investigators must consider which analytes in which samples will serve as primary endpoints, as markers of compliance, and, if urine and fecal samples are to be collected, as markers of completeness of collection. To avoid confounded interpretations of data, it is crucial that each

purpose be achieved through analysis of a different component; that is, the various measures must be independent of each other. The components can be analyzed in the same biological sample, but they cannot serve two purposes at once. For example, a study evaluating fecal calcium levels as a primary outcome variable should not also use fecal calcium levels to determine compliance with research diets containing various levels of calcium; yet another marker would be needed to assess whether all of the fecal samples had been collected.

This chapter will review several currently used measures of compliance with diet and will discuss, for several of the most frequently analyzed tissues and body pools, the logistics of sample collection in the context of feeding protocols and the assurance of completeness of this sampling. These additional procedures not only add time and cost to the study, but also may affect adherence to the overall protocol. All methods have their limitations for application and effectiveness, and their usefulness must always be evaluated within the context of individual research projects.

Biological Markers of Dietary Compliance

At present there is no ideal biomarker of compliance with a research diet regimen. Such a marker would be easily measured in an easily obtained specimen, and the resulting data could be readily compared with expected values based on available information about the diet. This ideal marker would indicate, with perfect sensitivity and specificity, whether any study foods were not consumed (''omissions''), whether any unauthorized foods were consumed (''additions''), and whether any study foods had been replaced with unauthorized foods (''substitutions''). The available methods for evaluating compliance generally permit a judgment to be made about whether omissions have occurred, but it is difficult to detect additions or substitutions with any certainty.

It would be convenient to have compliance markers that could be measured in blood samples, but the metabolic transformations associated with nutrient metabolism, and the homeostatic regulation of metabolite pools among the blood and other compartments, mean that there are few dietary components whose intakes are reflected in blood levels with sufficient precision to meet the purposes of feeding studies. Thus, as a method of monitoring compliance, blood samples at present are of little value in ''catching'' those who have not complied with the dietary protocol. Similarly, many of the classic nutritional status indicators are seldom useful as markers of dietary compliance. These indicators may be more useful in epidemiological studies that require the assessment of nutrient status over long periods of time to classify subjects into broad categories of intake (2–4). For feeding studies whose duration is relatively long (for example, several months), however, some nutritional status indicators might be useful for indicating compliance with a specific treatment; the sensitivity of the measure will be increased if the dietary treatments are markedly different.

Even though the composition of plasma or blood generally does not reflect dietary intake with sufficient specificity to serve as a *quantitative* measure of dietary compliance, blood samples occasionally can be used as *qualitative* markers of dietary compliance for certain nutrients. For example, participants taking fish oil are expected to have a plasma fatty acid profile that reflects this intake. The presence of the marker fatty acids indicates dietary compliance; unexpectedly low levels or the absence of a given marker is more difficult to interpret, however, because it is impossible to distinguish noncompliers from nonresponders under the circumstances of most dietary interventions.

Role of Compliance Markers in Feeding Studies

One of the greatest challenges in conducting well-controlled feeding studies is ensuring and then documenting dietary compliance. This is particularly true for metabolic studies in which food is consumed outside the research facility. Subjective methods such as oral reporting, written records, and visual observation can enhance compliance, but objective methods are necessary to verify dietary adherence. Biological collections can provide the medium for such quantitative determinations and documentation.

The most commonly used methods for evaluating compliance entail the collection and analysis of 24-hour urine samples (see below). Fecal samples are seldom used in monitoring compliance with diet, although this can be considered if feces are being collected for other required analyses. (In the case of either urine or feces, additional markers indicating the completeness of sample collection would be needed, as described later.)

When evaluating the results of a compliance assessment and before concluding the study participant is ''guilty'' of noncompliance, investigators must rule out other reasons for the findings. These include the potential for interference from other physiologic processes, as well as various types of protocol errors, such as errors in laboratory processing and analysis, omission of the marker from the food, errors in sample collection, and wrong dosage given to the participant. Nevertheless, quantitative markers add another level of confidence to controlled feeding studies, which otherwise have to rely upon subject compliance and subjective measures only.

Urinary Measures of Dietary Compliance

Most qualitative and quantitative measures of dietary compliance require measurements made from 24-hour urine collections. Commonly used methods involve measuring the

urinary excretion of para-aminobenzoic acid, urinary osmolality, or individual nutrients such as sodium.

Para-aminobenzoic Acid

The potassium salt of para-aminobenzoic acid (PABA) is a physiologically inert indicator that is readily excreted in the urine.

Advantages of using PABA as a biological indicator of dietary compliance are (1) it is excreted in the urine within 24 hours; (2) it may be taken orally in tablet or liquid form; (3) it can be added to individual foods; and (4) it has no detectable taste. In addition, PABA is inexpensive and can be analyzed by colorimetric assay with a minimum of technical expertise. It is also used as a means of assessing the completeness of 24-hour urine collections.

Limitations include the time-consuming procedures needed to incorporate PABA into the different foodstuffs and the fact that the method only assesses the intake of foods that have been marked with the PABA. The consumption and omission of unmarked foods will go undetected.

The PABA solution is incorporated into foods in exact amounts delivered by pipette. It can be added to many different foods such as beverages, bread products and other baked goods, meats, and salads. The appearance of the food does not change. It is difficult, however, to add PABA to high-fat foods such as peanut butter, cream cheese, margarine, and oils, because the water-based PABA solution tends to separate. There does not appear to be any difference in recovery if the PABA is incorporated into the food item such as a cookie before baking or afterward.

Because PABA and its metabolites are subject to degradation by ultraviolet light, the PABA solution should be kept in dark containers, and all foods containing the added PABA need to be covered immediately with foil or placed in a dark area. Collected urine should be kept in brown jugs or opaque containers and away from light. Certain drugs (such as acetaminophen, furosemide, paracetamol, phenacetin, and sulphonamides) and vitamins (such as folic acid) can interfere with the assay for determining PABA recovery; study participants should be instructed to avoid these compounds.

The usefulness of PABA to verify food intake in controlled dietary studies was first described by Bingham and Cummings (5) and further developed by Roberts et al (6). Roberts et al (6) concluded that PABA was a sensitive index of dietary compliance when multiple-day collections are pooled. In a 3-day validation study the recovery of the PABA, administered daily in 4 test foods, was 98.7%. The between-participant variability was much greater in individual collections (coefficient of variation [CV] 9%) than in combined 3-day collections (CV 3.7%). Therefore, multiple-day collections are recommended to monitor actual compliance.

Osmolality

The measurement of urine osmolality to monitor dietary compliance in metabolic studies has been described by Roberts et al (7). This method is based on the hypothesis that the urine osmol excretion rate (OER), which reflects the urinary solute load (made up primarily by nitrogen-containing compounds, sodium, and potassium), can be compared to the OER predicted from a known nitrogen, sodium, and potassium intake. A complex series of polynomial-regression equations is used for the calculations. The OER predicted from dietary intake is reported (7) to be most precise for dietary periods of more than 6 days (CV 6.9%).

Using OER as a compliance measure of dietary intake requires that all known sources of sodium, potassium, and nitrogen be controlled and measured. Advantages of using urinary osmolality to monitor dietary compliance are (1) both over- and underconsumption of food intake can be determined with high accuracy; (2) chemical analysis is simple and relatively inexpensive; and (3) there is no need to add an extrinsic marker to the food. Disadvantages include the need for multiple 24-hour urine collections and the inability to detect food substitutions that either have a similar nitrogen, sodium, and potassium content or unauthorized foods that are void of these nutrients. Also, the calculations for determining the predicted dietary OER can be time consuming.

Sodium

Twenty-four hour urinary sodium levels can be used as an indicator of dietary compliance when the dietary sodium intake is known and kept constant. This measure is based on the body's homeostatic tendency to maintain ''sodium balance'' in which urinary sodium output is equivalent to that of intake after several days on a constant sodium intake. For example, a participant living under controlled conditions, consuming 10 mEq of sodium per day, and in a state of sodium balance will excrete in the urine approximately 10 mEq or less of sodium per day. Typical urinary sodium recovery is approximately 95%; 2% is accounted for by fecal loss, and the remainder by sweat. Depending on environmental temperature and level of physical activity, however, the amount of sodium lost in sweat can vary from 2% to 5%, making it difficult to determine sodium balance in free-living participants. This loss should be considered and controlled when investigators use urinary sodium as a compliance marker.

Advantages of urinary sodium monitoring are its simplicity, speed of laboratory measurement, and its minimal cost. For certain types of investigations, such as endocrinology studies requiring controlled sodium intake, this simple measure is all that is needed for checking dietary compliance.

The primary limitation of urinary sodium as an indicator of dietary compliance is that it reflects only total sodium intake. Because sodium is a natural component of almost all foods and water, both in the natural state and as an additive, intake of actual food items consumed may not be discernable unless weighed salt is incorporated into selected foods. Likewise, overconsumption or failure to eat foods not containing sodium goes undetected.

Sodium concentrations in drinking water, whether from a municipal water supply or a well, can vary considerably because of various environmental factors. Therefore, the sodium content of the water supply should be known and accounted for in evaluating sodium balance. Providing distilled or deionized water for drinking can eliminate this variable; however, bottled water can be cumbersome to supply for extended periods of time.

Other Measures: Riboflavin, Potassium, Nitrogen

Riboflavin (vitamin B-2) excretion in urine can serve as a marker of dietary compliance. Riboflavin is a yellow to orange-yellow crystalline powder having a slight odor. When a high dose is added to foods, the excess vitamin appears in the urine and can be detected by fluorometric methods. Wolraich et al (8) reported the addition of 16.7 mg riboflavin to 100 g sugar that was then incorporated into a variety of foods. This was estimated to provide about 10 times the RDA for riboflavin when sweetened foods are consumed in typical quantities. Although this technique is not quantitative, it does verify that specific test foods are consumed.

Riboflavin has the advantages of being water-soluble and being easy to incorporate into foods. Because of its color, riboflavin is difficult to incorporate into a blinded study; its deep yellow color can easily be observed in lightly colored foods and in the urine. Participants do not usually know, however, what causes the yellow color. In the study by Wolraich et al (8), most thought a dye was added to the diet.

Riboflavin should be stored in airtight containers and protected from light to prevent degradation. The recovery of the load dose of riboflavin must be evaluated against the expected recovery from the 1 mg to 2 mg riboflavin present in an ordinary, riboflavin-adequate diet. The use of multivitamins, especially therapeutic doses of riboflavin, should be avoided. Also, certain drugs or substances found in food such as quinine or tonic water should be avoided because they can result in the excretion of fluorescent compounds, which may give false positive assay results.

Urinary *potassium* excretion can be compared with intake to qualitatively or semiquantitatively assess dietary compliance. Approximately 30% of potassium intake is excreted in the stool, however, reducing its accuracy as a urinary compliance measure.

Measurement of urinary nitrogen often is performed for studies of protein metabolism. Protein is not the sole source of urinary nitrogen; nonprotein nitrogen sources must also be considered. However, if total dietary nitrogen can be measured, urinary nitrogen can provide a means of assessing compliance.

URINE SAMPLES

Urine is analyzed in feeding studies to obtain primary outcome data and evaluate compliance with a specific dietary protocol. An advantage of using urine for measuring biological parameters is that the collection procedure is noninvasive and can be done in free-living participants. Interpreting the resulting data, however, also requires documenting the completeness of the urine collection.

Collection Procedures

In some research studies, random daytime or morning urine samples are used. More often, however, urine samples collected in feeding studies are obtained over 24-hour periods. Usual urine output ranges between 1,200 ml and 2,500 ml per day and depends on individual fluid intake, temperature, exercise, and kidney function. Some investigators using 24-hour urine measures encourage a fluid intake of 2,000 ml to 2,500 ml per day to ensure adequate hydration, renal perfusion, and a good urine output.

Over- or undercollection of urine samples results in data that are difficult to interpret. Overcollection can occur when a participant collects urine beyond the time frame specified in the protocol, whereas undercollection can result from missed collections or spillage. The protocol should incorporate methods to estimate the completeness of the collection, such as urinary creatinine or para-aminobenzoic acid. The accuracy of a complete 24-hour urine collection requires that the participant understand thoroughly the collection technique and the timing of the sample. The collection procedure should be explained orally to the participant, who should then have an opportunity to ask questions. Written instructions must also be provided (Exhibit 24–1).

The process of collecting 24-hour urine samples can be tedious and awkward. Participant cooperation may be easier to obtain if there is some flexibility as to the day(s) the urine is to be collected. For example, a weekend day may not be better than a week day because schedules may be less predictable. Participants' motivation can also can be enhanced if they are told they will be informed of the results of the analyses and what those results mean to them or in the context of the study. Some potential participants may find urine collections difficult because of problems with motor coordination or remembering and following procedures accurately.

A research participant involved in 24-hour urine collections usually is encumbered with a gallon jug, and, for females, some type of collection device. If this equipment presents a particular problem, it may be helpful to provide several smaller collection containers from which urine can be pooled. A brown paper shopping bag, gym bag, or opaque plastic bag with handles can be used to carry containers.

As with all biological sample collections, storage and processing techniques for urine must be appropriate to the analytes of interest. Samples collected for analysis of ascorbic acid, for example, require cold storage and stabilization with acid to prevent oxidation. Some organic analytes require protection from ultraviolet light; some are destroyed by bacterial growth, a process that can be prevented or decreased by refrigerating urine samples. Interfering sub-

EXHIBIT 24-1

Procedure for 24-hour Urine Collection

Materials: Collection container—1 gallon brown jug or several smaller collection containers (labeled and preservatives added, if necessary); funnel, urinal, or collection ''hat''; large safety pin to attach to undergarment as a reminder; brown paper or opaque plastic bag with handles; written instructions to give to participant; cooler; and ice.

Instructions

1. The collection time begins immediately after the first void of the morning. Do *not* save that first specimen as part of the beginning day's collection. For multiple-day collections, this void should complete the collection from the previous day, otherwise, it should be discarded. Write the time of the first void (that you threw away or that you saved as the end of the previous day's collection) on the collection container as your START TIME. Remember also to put the date that you began the collection.
2. Collect each sample in the urinal or ''hat,'' then pour it into the brown jug. Females can also use a funnel to urinate directly into a container when it is inconvenient to carry around a gallon jug. Males can void directly into the collection container.
3. Keep the brown jug on ice in the cooler provided and replenish ice as needed.
4. The final specimen collected will be the first void upon awakening on the second morning (24 hours after you began). Be *sure* to write this date and the time on the jug.
5. Be especially careful not to spill any of your collection. Keep the jug upright to prevent leaking. All urine, except the first void, is to be collected.
6. If a collection is missed or spilled, estimate the amount, if possible, and be sure to report *any* uncollected urine to the investigator.
7. Upon completion of your 24-hour collection, bring the container to the _____. Be sure that the label is filled out completely indicating your name, the date and time of starting the collection (the time you threw the specimen away), and the date and time of finishing the collection.
8. If you have any questions, please call us at _____.

Remember: the accuracy of your collection determines the accuracy of your results.

SAMPLE LABEL FOR 24-HOUR URINE COLLECTION

Participant Name: _____ ID: _____
Investigator: _____
Start date: ___/___/___ End date: ___/___/___
Start time: _____AM End time: _____AM

stances can leach from urine collection containers. Glass containers are required when certain analytical techniques are used because many plastics contribute fluorescent compounds or minerals to samples. Specific instructions for collection, storage, and processing of urine and other biological specimens are available from reference texts (eg, *Clinical Guide to Laboratory Tests* by Toetz [9]).

Urine Collection Markers

The two most commonly used markers for assessing the completeness of urine collection are creatinine (an intrinsic physiologic marker) and para-aminobenzoic acid (an extrinsic dietary marker).

Creatinine

Creatinine is the most frequently used quantitative measure of complete 24-hour urine collections in participants consuming a controlled diet. Urinary creatinine is a by-product of muscle metabolism and reflects lean body mass. Its excretion by a given healthy individual is expected to be consistent from day to day when dietary intake of protein is held constant. Age, sex, body mass, and kidney function affect creatinine excretion, making it invalid for interparticipant comparisons. Creatinine excretion may be less suitable for long-term studies with participants who are in positive nitrogen balance, such as children or pregnant women.

Multiple consecutive 24-hour urine collections are necessary to use urinary creatinine as a measure of sample completeness. The coefficient of variation for creatinine excretion in consecutive daily urine collections was reported as 4% and 3.2% in studies by Bingham and Cummings (10) and McCullough et al (11). Other investigators, however, have found a much greater variability, ranging from 21% to 24% (12).

Advantages of using urinary creatinine to assess completeness of 24-hour urine collections are:

- Creatinine is excreted in the urine as a naturally occurring, measurable metabolite.
- No inert substances need to be taken by the research participant.
- No additional work or measures are necessary on the part of the research team.
- The measurement of urinary creatinine is inexpensive and can be done easily on standard laboratory autoanalyzers.

The usefulness of urinary creatinine as a collection marker is limited in studies that allow a varying dietary intake because of the sizable effect of protein intake on creatinine excretion.

Para-aminobenzoic Acid

In addition to its use as a marker of dietary intake compliance, PABA has also been used to validate the completeness of 24-hour urine collections. The use of PABA for this purpose was first described by Bingham and Cummings (5) based on the hypothesis that the amount of PABA excreted in the urine is directly related to the dose. An advantage of using PABA to determine completeness of 24-hour urine collections is that it is physiologically inert, unaffected by food intake or physiological characteristics of the participant, easily analyzed, and relatively inexpensive. Because PABA is light sensitive, urine collections must be stored in brown jugs or opaque containers and in a dark area.

Bingham and Cummings (5) showed that in a group of four individuals, 93% of a single 80-mg tablet of PABA given with a single meal was recovered after 5 hours. In order for a marker to represent a complete 24-hour collection, the marker must be present in significant quantities in each individual specimen. These same researchers determined that 240 mg PABA given by mouth with the day's three meals was an effective dose to monitor 24-hour urine collections. A recovery of less than 205 mg PABA (85%) was considered to represent an incomplete collection.

Because of the large measurement variance for PABA, a single 24-hour urine collection may not be as useful as a sample pooled from 3 to 4 days of collection. As mentioned earlier, Roberts et al (6) reported a CV of 9% for 1-day collections but only 3.7% for pooled 3-day measurements. Therefore, PABA may be more useful in evaluating the completeness of multiple-day, pooled collections.

Stool Samples

Controlled diet studies frequently collect and analyze stool samples (13, 14). Such analyses provide information about the digestion, absorption, bioavailability, and balance of nutrients, as well as about the actions of colonic flora and other aspects of gastrointestinal physiology. Fecal markers are an important component of stool collection protocols because, as Davignon, Simmonds, and Ahrens explained, "In metabolic balance studies stools collected during a given period of time do not necessarily reflect the biological events that take place in the intestine during the collection period; even 4-day collections rarely represent exactly 96 hr in the transit of any particular portion of the intestinal contents. Hence there is a need for an inert marker which can be incorporated into the food intake, the excretion of which can indicate the completeness of stool collections and permit corrections for variations in fecal flow" (15).

Collection Procedures

Fecal collections are physically and psychologically demanding for research study participants. For many prospective participants, stool collection goes against cultural norms and years of social conditioning. Carrying collection containers and samples can be embarrassing, awkward, and tedious; and cooperation of participants can be difficult to achieve. For these reasons, extra financial incentives may be necessary for participants in studies requiring fecal collection.

It is particularly important to realize that stool collections are difficult if a participant is prone to diarrhea or constipation or if either is a complication of the study. As with urine collections, some individuals may have difficulty in understanding the protocol and physically collecting the specimen.

Participant cooperation is imperative in obtaining a complete stool collection. Careful and detailed instructions, both verbal and written, as well as all the collection materials and storage containers should be provided. As with urine collections, compliance is enhanced when there is some flexibility about the timing of the collections.

Stool collections usually need to be frozen. The temperature (0°C, −20°C, −70°C) will vary depending on which analytes will be assayed. Some study investigators provide a small home freezer to their free-living participants, particularly if daily collections are made over a significant time period. Other investigators provide participants with an insulated food storage style or Styrofoam container (48 qt to 54 qt) containing dry ice (frozen CO_2). Fifty pounds of dry ice (pellets or block) will last approximately 5 to 7 days in a tightly sealed insulated container; however, in warm weather, dry ice will need to be replenished more frequently. Care should be taken not to handle dry ice without gloves because direct skin contact can cause frostbite. Dry ice should be held in a tightly sealed box in a well-ventilated room; the CO_2 vapors can reduce oxygen concentrations to dangerous levels.

When specific freezing temperatures are required, participants must understand the distinction between the freezing temperatures of dry ice (−78.5°C) and water ice (−0°C). Otherwise a participant might mistakenly "thaw" the sample in his or her home freezer when the dry ice runs out.

Finally, containers for storing stool collections need to be designated for that use only and should never be used for storing or transporting food. Containers must also be tested in advance to make sure that there is no leaching of inter-

fering compounds into the sample. Stool collection procedures are outlined in Exhibit 24–2.

Fecal Collection Markers

Criteria for a good fecal marker were described by Whitby and Lang (16). The substance should be inert, nontoxic, and easily measured. It should have very little bulk, mix well with the intestinal contents, be completely unabsorbed, and not undergo any metabolism in the body or the intestinal lumen. Many years later, these criteria still hold.

Two types of markers have proved themselves useful in controlled feeding studies. *Intermittent markers* such as inert colored dyes and radio-opaque pellets are given in single bolus doses to define the *timespan* (ie, the beginning and end) of the fecal collection period. Occasionally dyes of differing colors or pellets of differing shapes are used to distinguish between time points or collection periods. Some dyes may contain minerals and thus may not be suitable for trace element studies (13); laboratory analysis in advance is recommended to avoid generation of confounded data.

Continuous markers such as polyethelene glycol, chromic oxide, and barium sulphate are given in multiple doses at regular intervals throughout the feeding period to determine the *completeness* of fecal collection. By defining the quantity of feces in relation to a given quantity of food, they allow the investigator to account for intra- and interindividual differentials in fecal flow due to variability in transit time and intestinal pooling (intraluminal sequestration of intestinal contents).

The type of fecal marker should be selected judiciously for a given study so that the desired time course, collection, and endpoint can be measured according to the study design. For example, not all fecal markers traverse the gastrointestinal tract at the same rate, and some are more lipid- or water-soluble than others. Likewise, different luminal contents,

EXHIBIT 24–2

Procedure for Fecal Collections

Materials: Underseat collection frame (''hat''), clear plastic collection bags with twist ties (numbered and labeled) or ''deli-style'' pint containers with lids, resealable bags (eg, Ziploc®), disposable gloves, brown paper or opaque plastic bag with handles, insulated or Styrofoam container containing dry ice (if necessary).

Procedure

1. Obtain a numbered, large plastic collection bag or container, being careful that it is in numerical order. Complete label with date and time.
2. Urinate (if necessary) and collect as usual prior to bowel movement.
3. Put on disposable gloves.
4. Open the large plastic bag/container (in numerical order) and place inside collection ''hat.'' If a collection container is not available, attach the bag to the outside of the toilet bowl or hold the bag, whichever is easier.
5. During the bowel movement, make sure that the entire stool falls into the bag/container *without any urine.*
6. *After the bowel movement, remove the plastic bag/container, remove air from collection bag (air insulates sample and slows the freezing process), gently twist the top and place the bag inside the self-sealing freezer bag.*
7. *Seal the freezer bag and immediately place the bag inside the dry ice chest or the freezer provided by the study.*
8. Remove and discard gloves.
9. Replace ice chest lid tightly.
10. Use a new large plastic bag in numeric sequence for each stool sample.

Important:

- Immediately freeze stool sample.
- No urine in stool sample.

Caution: Do not let dry ice come in contact with your skin. It can cause burns! Keep dry ice tightly sealed and in a well-ventilated room.

SAMPLE LABEL FOR FECAL COLLECTION

Stool Sample Number _____
Participant Name: _____ ID: _____
Investigator: _____
Date: ___/___/___
Time: _____ AM/PM (Please circle AM or PM)

such as fats, move through the intestinal tract at different rates than those transported in an aqueous medium.

There may be occasions when an investigator does not want the subject to take biochemical marker capsules or wants the subject to be unaware of a specific collection period. Real foods, such as corn kernels, which are usually only partially digested and readily visible in the stools; beets, which usually color the stool red; or seeds can be used to mark a diet intake period without the subject's being aware of the intent.

Extrinsic markers given in capsule or added to food must have food- and pharmaceutical-grade sanitary clearances and handling procedures so that they cannot be a source of illness (as described later).

Intermittent Markers

Coloring Agents (Inert Dyes)

The inert dyes *carmine red (CI Natural Red 4)* and *brilliant blue (FD&C Blue 1)* are two of the most commonly used fecal markers for defining dietary periods. They are usually administered in powdered form (available from Spectrum Chemical, New Brunswick, NJ). They are not absorbed in transit and do not undergo secondary metabolism by gastrointestinal bacteria, and thus are recovered fully in the stool. Another dye, *methylene blue*, is also used as an intermittent fecal marker, although it is partially absorbed in the gut, with some urinary excretion and less than complete recovery in the stool sample. Methylene blue can be administered in two forms, a dark green crystalline powder and a deep blue liquid (available from Fisher Scientific, Pittsburgh, Pa).

Capsules containing dye markers can be purchased commercially. They can also be prepared to study specifications by the investigators. The dyes are obtained from laboratory suppliers, mixed with methylcellulose microcrystals (Avicel), and encapsulated in size 00 or 000 capsules. The methylcellulose helps to keep the dye stationary and prevents the blending of colors. Carmine red is frequently autoclaved before being encapsulated, as contamination with *Salmonella* has been reported. (Formulations for carmine red and methylene blue capsules are shown in Exhibit 24–3.)

Radio-opaque Pellets

The use of radio-opaque pellets (ROP) for measuring stool transit was first described by Hinton, Leonard-Jones, and Young (18) and further described by Cummings and Wiggins (19). Radio-opaque pellets have since been used successfully by a variety of investigators (20, 21). The method provides a valid quantitative measure of a complete fecal collection because 100% recovery of the pellets is expected. As with all human feeding studies, however, the unexpected can happen. For example, in a study where all participants were to receive 40 pellets, one participant excreted only 20 while another excreted 60 pellets, suggesting a dosage error.

Barium-impregnated radio-opaque polyethylene pellets may be obtained from Konsyl Pharmaceutical Co, Fort Worth, Texas (Sitzmark brand pellets). Using pellets of differing geometric shapes allows more specific demarcation of time periods (13). The tiny ROP are incorporated into gelatin capsules and swallowed at the beginning and end of each collection period or with certain meals. All stools for a designated time period are then collected and examined under fluoroscopy for retrieval of the pellets. An advantage of using ROP is that this method of measurement, which uses X-rays, does not alter the stool, and fecal handling is kept to a minimum. Disadvantages of using ROP include the additional cost of fluoroscopy and participant reluctance to swallow the pellets.

Continuous Markers

Polyethylene Glycol

Polyethylene glycol (PEG) (Spectrum Chemical, New Brunswick, NJ) is a white, crystalline, water-soluble, high molecular weight polymer. It is neither absorbed nor decomposed by the intestinal tract during transit. PEG has been used as a fecal marker to follow time and completeness of collections, to determine when experimental diets have been eliminated, and to correct for differences in the day-to-day variation of fecal transit time (22, 23).

PEG is easily dissolved in water, juice, or other fluids and has little taste. PEG is not the marker of choice when study participants are taking vitamin and mineral supplements because it is used as an inert filler in some prepara-

EXHIBIT 24-3

Formulations for Fecal Dye Markers

Courtesy of General Clinical Research Center, Massachusetts Institute of Technology, Cambridge, Mass

Carmine Red Markers
10 g sterilized carmine red dye
10 g Avicel (methylcellulose)
Size 00 gelatin capsules
(approximately 500 mg dye each)
Yield: 20 capsules
Dose: 1–2 capsules

Methylene Blue Markers
5 g methylene blue
20 g Avicel (methylcellulose)
Size 00 gelatin capsules
(approximately 250 mg dye each)
Yield: 20 capsules
Dose: 1 capsule

tions. A typical dosage schedule for PEG is 3.0 g per day provided as 1.0 g dissolved in 120 cc water and given at breakfast, lunch, and dinner.

Barium Sulphate

Barium sulphate ($BaSO_4$) is a nonabsorbable, nontoxic, insoluble compound (Spectrum Chemical, New Brunswick, NJ; Fisher Scientific, Pittsburgh, Pa) that can easily be measured and given in capsule form. Its usefulness for correcting the variability of fecal flow in metabolic balance studies was described by Figueroa, Jordan, and Bassett (24). A 0.5-g dose of flocculating $BaSO_4$ was divided equally among 3 capsules and administered at 3 daily meals. Recovery of the barium sulphate from 5 participants throughout the 5-day balance was 97.7% to 103%, indicating its usefulness in validating complete collections. Barium sulphate should not be used in the presence of antacids containing aluminum hydroxide and magnesium hydroxide as they are partially recoverable in the stool and may interfere with subsequent chemical assays.

Chromium Oxide

Chromium oxide (Cr_2O_3, chromium sesquioxide, Fisher Scientific Company, Pittsburgh, PA) is a bright green continuous fecal marker delivered in capsular form. Its usefulness in metabolic balance studies for calculating fecal pool size, turnover rates of unexcreted intestinal contents, and attainment of a steady state was well documented by Davignon, Simmonds, and Ahrens (15). Although varying doses in the range of 250-mg to 500-mg capsules or tablets have also been used, these authors determined that 300 mg per day given as one 60-mg tablet 5 times per day was an adequate dose for recovery determinations.

Radioisotopic chromium (as $^{51}CrCl_3$) has also been used as a fecal marker in balance studies, although it may undergo partial absorption in the gut (13).

Blood Samples

Blood samples in feeding studies often provide the main vehicle for assessing the effects of the controlled diet intervention. They can also provide information about nutrient utilization, nutritional status, and dietary patterns. An advantage of using blood samples is that they are less demanding of the research participant's time than are urine and stool collections. The sample can be collected quickly and in almost any off-site location. Another advantage of blood samples is that they can sometimes be obtained with no additional discomfort to the participant by increasing the volume drawn during an already scheduled venipuncture.

Blood drawing should only be done by a well-trained technician, nurse, or physician. Although there are no regulations governing who can draw blood, a good training program covering technique, infection control, and processing is critical, not only for the safety of the participants and staff but also for accurate data collection. Most medical facilities can provide training for researchers who will be drawing blood specimens. Technicians responsible for blood sampling should have this task written into their job descriptions. Such documentation will ensure that liability for any accident or resultant injury, although rare, would be covered by the insurance of the affiliated institution and/or researcher.

The informed consent document should clearly state that blood sampling will be a part of the study and should detail the associated risk. A simple venipuncture under sanitary conditions carries minimal risk; this risk is typically limited to minor discomfort during the blood draw and to the possibility of a slight hematoma. The frequency of the blood sampling and the amount to be drawn should also be clearly stated in the informed consent document, and blood sampling to be conducted outside the research facility (eg, at home, school, work) should be identified.

It is important to keep blood drawings to the necessary minimum. Overly frequent blood draws may become an aversive experience and make it hard for the participant to complete the protocol. In addition, study costs may be increased by the need for extra financial incentives for the participants. Finally, total volume of blood must be limited to prevent anemia, especially in children and reproductive-age women.

No person enjoys undergoing a venipuncture; therefore, rapport with the subject is important for compliance and participation. Some participants become light-headed or faint during or shortly after blood collections. A reclining chair, bed, or couch should be available so that a dizzy or faint individual may lie down. In addition, smelling salts and a supply of food and juice should be kept close to the blood drawing area. A source of rapidly absorbed carbohydrate, typically fruit juice, can quickly counter the light headedness that some people experience. Similarly, participants should be provided with a meal or snack if blood has been drawn under fasting conditions so that they are not in danger of fainting after they leave the facility. If a controlled food intake is part of the study, part of the participant's daily food should be reserved for this purpose.

Meticulous collection and processing of blood samples is critical for generating accurate data. The laboratory responsible for analyzing the sample should be consulted prior to the study for detailed collection, processing, and storage guidelines. These guidelines may entail the addition of various chemicals to stabilize the specimens; the temperature at which centrifugation should take place; and sample storage details such as temperature, light, and stability.

Both the day of the week and the timing of blood samples should be considered in the context of the study protocol. If fasting is required, participants should understand the exact time after which they can consume no food in the evening. They must also understand that they can eat no food the following morning until after the sample has been drawn. Some investigators elect not to collect blood samples on Monday mornings because participants who have take-out meals for weekends may eat their Sunday evening meals late

and therefore not comply with the typically required 12-hour fast. Others avoid collecting blood on Fridays or the day before holidays because this may delay the laboratory analysis of time-sensitive samples.

Blood specimen labels should be as detailed as possible with the participant's identification number, date, time of sample collection, and any other pertinent information. Other variables to consider are whether the participant should be supine or sitting, fasting, the time of day, and any medications the participant may be taking.

Other Biological Samples

Other types of samples, such as saliva, sweat, breast milk, and expired CO_2, are sometimes used for endpoint measurements, as indicators of nutrient intake, or for assessing nutrient status. Their range of applications is relatively narrow, but may include studies of mineral balance, energy balance, and endocrine physiology.

Subcutaneous adipose tissue samples obtained by either a needle or skin biopsy punch have been used to provide information on long-term fatty acid storage. A noninvasive technique using cheek cell membranes was developed by McMurchie, Potter, and Hetzel (25) to investigate tissue fatty acid composition reflective of a shorter duration. Cheek cell turnover is approximately 5 days; cells can be collected by having a participant lightly scrape the cheeks with the side of a plastic spoon, rinse the mouth with distilled water, and collect the sample in a container.

Hair and nail samples have the advantage of being easily collected. However, their use in nutrition research is limited primarily to assessing the status of long-term dietary intake or toxic exposure of trace elements and heavy metals. Contamination of hair and nails with cleaning agents, shampoos, and environmental pollutants can provide misleading data unless identified and controlled in advance.

Conclusion

Biologic samples, particularly of blood, urine, and feces, are commonly analyzed in feeding studies to assess primary endpoints of interest and to monitor dietary compliance. Valid interpretation of the analytical results depends on proper collection and storage of these biological samples. The data must also be interpreted with the knowledge that human motivation and compliance with collection procedures can be difficult to achieve. When selecting measures to use for assessing dietary compliance, one must carefully assess the application of the techniques as well as their advantages and logistics.

Most studies documenting the use of markers for dietary compliance and biological collections have been conducted using small, limited population groups with results about their usefulness extrapolated to the general population. More extensive studies are needed that include men and women, children, the elderly, and minorities, as well as clinical subpopulations. For example, individuals with impaired renal function and renal abnormalities could have altered excretion of urinary markers. Given the importance of verifying dietary adherence in human feeding studies, further research is greatly needed to expand the roster of specific and sensitive biochemical/biological indicators.

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