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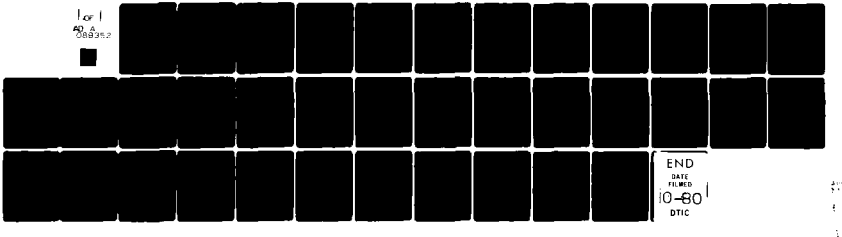

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Interim Report No. 4

A New Procedure for Preparing Uniform Fecal Samples for LSC

Carl C. Smith, Ph.D.
Steele F. Mattingly, D.V.M.

Geraldine F. Wolfe, M.S.
David H. Bauman, D.V.M.
Gary L. Keller, D.V.M.

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Department of Environmental Health and
Department of Laboratory Animal Medicine
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267

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feces	organic solvents	2,4-diamino-6-(2-naphthylsulfonyl)-quinazoline
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ball-mill	monkey	bile-duct cannulation
extraction	WR-158,122	bile-duct ligation
digestion	sulfonyl quinazoline	LSC and ^{14}C and metabolites

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We have developed a procedure for preparing uniform fecal samples for LSC counting and study of fecal metabolites. The procedure requires milling of a rat or monkey feces sample with anhydrous Na_2SO_4 in a stainless steel ball mill. The fine off-white flours th[illegible] result are passed through a fine wire sieve (18-8 stainless steel wire, 3[illegible]oles/inch). This sieving increases homogeneity by removing traces of unground material and dietary fiber. Digestion of samples with NaOH and counting total ^{14}C in dioxane-toluene-naphthalene with Cab-O-Sil (DTN-C) gave reproducible recoveries. This procedure was adequate for

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0.25 g and 0.5 g samples but inadequate for 1.0 g samples. In a study of 7 solvent/digestants 1) Soluene, 2) NaOH and 3) Protosol extracted the most dpm (^{14}C) from feces samples and showed the best reproducibility. Protosol and Soluene digests counted as efficiently in Biofluor as in DTN-C. Ten replicate Soluene digests in Biofluor and 10 replicate NaOH digests in DTN-C showed mean % recoveries which matched well (NaOH, 40.6 ± 1.0 SD; Soluene, 41.3 ± 1.35 SD). Combustion assays for total ^{14}C gave recoveries that agreed well with values obtained by digestion in NaOH, Soluene, and perchloric acid - H_2O_2. One gram aliquots of 4 milled feces samples were extracted sequentially 3 times with THF and extracted once with Soluene. The THF recoveries matched the digestion methods for monkey feces but recovery of ^{14}C in the rat feces samples was 10 to 20% lower than digestion assays. Six feces samples were extracted sequentially with benzene, ethyl acetate, acetonitrile and tetrahydrofuran to see whether this procedure would give complete recovery of fecal ^{14}C. 96% of total dpm's were recovered in the combined ethyl acetate and benzene extracts for all samples except for a non-operated rat sample (CT-4). Acetonitrile extracted 15% of the total dpm's in CT-4 feces suggesting that the metabolites in this rat's feces are different from those in monkey 75-5♀ (bile-duct cannulated) and some are soluble in acetonitrile. Further work is planned to find the best series of solvents for sequential extraction and separation of fecal ^{14}C containing metabolites. This will be followed by purification of the metabolic fractions on HPLC and eventual structural studies using GC-MS and other analytical techniques such as IR and NMR if sufficient material can be generated.

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A New Procedure for Preparing Uniform Fecal Samples for LSC

Introduction

Pursuing the identity of the fecal products following the oral (or parenteral) administration of WR-158,122-^{14}C posed technical problems. We were unaware of a method of homogenizing (and digesting) feces that did not require chemical and probably degradative action on the sample. In other words how can one get homogeneous fecal samples without changing the composition of the feces themselves? Standard methods including homogenization with acid or aklaki or solvent extraction (MeOH) after dessication did not appear satisfactory for our needs; therefore, we began the development of a new procedure.

Preliminary Experiment

Our first experiment consisted of digesting fecal samples (ground with mortar and pestle using various solvents.

Feces Sample

The first sample, spiked rat feces (SRF), was prepared by grinding 6.0 g of control rat feces with 60 g of anh. Na_2SO_4 and 0.5 ml of WR-158,122-^{14}C treatment suspension with a mortar and pestle and subsequently ball-milling this. The final milled sample was calculated to contain 8,518 dpm/g.

The second sample (BL-10-24) was prepared by grinding the 24 hr feces sample from bile-ligated rat #10 with anh. Na_2SO_4 (3.78 g feces + 20 g Na_2SO_4).

Solvent/Digestants

1) Dimethylsulfoxide (DMSO)
2) DMSO: tetrahydrofuran (THF), (1:1)
3) THF
4) Protosol®*

*Registered trademark for a tissue solubilizer, obtained from New England Nuclear, 549 Albany St., Boston, Massachusetts 02118.

5) Soluene 100 ®*

6) Tetramethylammonium hydroxide 24% in methanol (TMH)

Procedure

Samples (0.25 g) were weighed into LSC vials, 1 ml of each of the above solvent/digestants was added and the vials placed in a 70°C incubator. Because digestion appeared incomplete at 24 hr we added 0.5 ml of 0.5 N NaOH and 0.5 ml of tertiary butyl hydrogen peroxide (TBHP) bleach to each sample and incubated an additional 10 hr. Samples were counted after vigorous shaking with 15 ml of a counting medium consisting of dioxane, toluene, naphthalene plus 5% Cab-O-Sil ®** (DTN-C) and the counts corrected for quenching with an internal standard.

Results

As seen in Table 1, recovery of fecal ^{14}C in dpm/g varied from 8730 to 9790 for SRF and from 70,800 to 90,000 for the BL-10-24 sample; the lowest value for the first sample was obtained with TMH, the lowest for BL-10-24 with DMSO-THF. This unacceptable variability suggested we were encountering either 1) sample inhomogeneity, 2) incomplete digestion or 3) poor gel formation in the counting vials.

Table 1

Recovery of Fecal ^{14}C in Various Solvent/Digestants

Solvent/ Digestant	SRF dpm/g***	BL-10-24
DMSO	8740	89,200
DMSO-THF (1:1)	9790	70,800
THF	9170	78,500
Protosol	9650	86,400
Soluene	9460	83,900
TMH	8730	90,000

*Registered trademark for a sample solubilizer obtained from Packard Instrument Company, Inc., 2200 Warrenville Rd., Downers Grove, Illinois 80515

**Registered trademark for thixotropic agent (Silicon dioxide), available at Cabot Corp., 21010 Center Ridge Rd., Rocky River, Ohio 44116

***Gram of final milled powder

The next step was to develop a better fecal homogenization technique. In 1936 one of us (C.C.S.) had used a ball mill to prepare powdered dehydrated muscle samples. Using a stainless steel ball mill* loaned to us by Dr. Wm. E. Kuhn, Dept. of Material Sciences and Metallurgical Engineering, Univ. of Cincinnati, we found that the best sample preparations from rat and monkey feces were obtained by grinding the feces with 4 to 8 parts anh. Na_2SO_4 for 30 to 60 min. using a combination of ½ and 1 inch stainless steel or chrome plated steel balls. The final preparations were fine, off-white flours which appeared homogeneous. Later (Exper. 5 and after) all flours were passed through a fine wire sieve (18-8 stainless steel wire, 30 holes/in.) which removed traces of unground material and dietary fiber. With almost no changes this is the procedure we have adopted for preparing fecal samples.

*Model 611 unlined steel grinding jar, Jar size 0 (0.50 gal), Norton Chemical Process Products Division, Akron, Ohio 44309.

Experiment 1

Introduction

From the preliminary experiment we concluded that NaOH may be as good a digestant as any of the others we had tried and could be counted adequately in DTN-C. Therefore, we used NaOH in the next experiment.

Purpose

To study the linearity and reproducibility of alkali-digested milled feces.

Sample Preparation

The feces used were the 24 and 48 hr samples from 6 bile-duct ligated (BL) rats treated with 10 mg/kg oral doses of WR-158,122-^{14}C. They were weighed and milled with known amounts (4 to 8 parts) of anh. Na_2SO_4 in a stainless steel ball mill for 30 to 60 min.

Procedure

The milled feces aliquots (0.25, 0.5 or 1.0 g weighed on an analytical balance) were put in LSC vials and 1 ml of 1 N NaOH was added. After 24 hr 1 ml of H_2O was added to 0.5 g and 1.0 g samples to increase the liquid volume and assist digestion. After 24 hr the oven temperature was increased from 70°C to 80°C and incubation was continued to 72 hr. Glass beads were placed in some vials to assist in suspending sample adhering to vial walls. The recovery of fecal ^{14}C was expressed as % dose.

Results

The figures in Table 2 indicate that NaOH digestion followed by counting in DTN-C worked adequately for 0.25 and 0.5 g samples of these milled feces. The high results obtained with some of the samples suggested that the procedure is inadequate for counting 1 g samples. The difference among 6 duplicate 0.25 g samples varied from 1 to 5%. For 0.5 g samples the variation

was from 1 to 11%. The 1 g samples were occasionally higher (4/12) or lower (2/12) or similar (6/12) to the values we obtained for the 0.25 and 0.5 g aliquots of the same sample. The higher apparent recovery values for certain 1 g aliquots were due to abnormally low estimates of efficiency using the internal standard method. For this reason there appear to be advantages in restricting the sample size to 0.25 g (or possibly 0.5 g).

Table 2

Reproducibility and Linearity of Alkaline Digests of Milled Rat Feces in % Dose

Wt. of milled feces (g)	BL-9		BL-10		BL-11	
	24*	48*	24	48	24	48
0.25	78.8	5.2	82.3	10.8	56.1	4.7
	79.4	5.1			58.7	4.5
0.5	77.0	5.4	71.7	10.5	59.7	4.9
	76.7	5.4	79.8	10.9		
1.0	88.3	5.3	68.0	10.7	68.8	5.1

Wt. of milled feces (g)	BL-12		BL-13		BL-14	
	24	48	24	48	24	48
0.25	69.8	11.0	71.2	6.4	65.3	14.6
					65.6	14.4
0.5	67.0	10.5	72.9	6.4	70.9	15.0
			72.8	6.6		
1.0	58.7	10.6	86.9	6.0	98.1	14.0
	56.1	10.6				

*24 and 48 hr samples.

Experiment 2

Introduction

In Experiment 1 we proved that 1.0 N NaOH is a good digestant for fecal samples. We also established that it is best to limit sample size to 0.25 g samples. In this study we digested 0.25 g samples of 5 milled feces in a series of 7 solvent/digestants.

Purpose

To find out which of a series of solvent/digestants extracts the most dpm from milled feces samples and does so most consistently. To check reproducibility of 0.25 g samples.

Sample Preparation

Five feces samples prepared by milling with 4-8 parts anh. Na_2SO_4 in a stainless steel ball mill were used.

1) SRF as described on page 1

2) BL-10-24. 24-hr feces from bile-duct ligated rat #10 treated with WR-158,122-^{14}C (10 mg/kg oral dose).

3) CT-4-48. 48-hr feces from a non-operated rat treated with WR-158,122-^{14}C (10 mg/kg oral dose).

4) Spiked monkey feces (SMF). Control monkey feces (39.14 g) from rhesus 75-5 were ground with 123.13 g of anh. Na_2SO_4 and 4.0 ml of WR-158,122-^{14}C treatment suspension with a mortar and pestle and subsequently were ball-milled. The final milled sample was calculated to contain 27,250 dpm/g.

5) 75-5-48. 48-hr feces sample from bile-duct cannulated rhesus 75-5 ♀ treated with WR-158,122-^{14}C (5 mg/kg oral dose).

Solvent/Digestants

1) DMSO

2) DMSO-THF (1:1)

3) THF

4) Protosol

5) Soluene

6) Tetramethylammonium hydroxide (TMH)

7) NaOH, 0.5 N

Digestion Protocol

a. Weigh milled feces samples (approximately 250 mg) on an analytical balance and place in 20 ml LSC vials.

b. Add 1 ml of solvent/digestant.

c. Digest in oven set at 68-70^{o}C for 24-48 hr.

d. Bleach with 0.5 ml tertiary butyl hydrogen peroxide (TBHP) at 2-24 hr.

e. Add either 15 ml of 5% DTN-Cab-O-Sil (DTN-C) or 15 ml of Biofluor.

f. Cool and dark-adapt samples at least 2 hr at 4^{o}C before counting.

g. Internally standardize with toluene ^{14}C, if desired.

Procedure

In this experiment digestions were carried out as described in the Digestion Protocol except incubation was terminated at 24 hr and all samples were counted in 15 ml of DTN-C.

Results

Total ^{14}C recovery expressed as % dose in Table 3 showed total ranges shown below:

	% dose range	Coefficient of Variation, %
SRF*	95.1-103.2	8.1
BL-10-24	71.1-84.2	16.8
CT-4-48	5.1- 6.7	26.7
SMF*	97-102.3	5.3
75-5-48	35.4-38.9	9.4

*Calculated to a mean recovery of 100%.

In Table 4 we have listed the results we obtained with each solvent/digestant (solv/dig) with symbols (L, l, =, h, H) and in parentheses, the differences between each pair of duplicates expressed in percent variation to make them comparable. On the basis of these two criteria and giving major weight to the solv/dig which gave the best recoveries (most H or h, least l or L) we classified the solv/dig as "good" or "poor". Thus, we classified Protosol, Soluene and 0.5 N NaOH as "good" and the rest as "poor".

Discussion

An undissolved salt residue was present in all the solv/dig except 0.5 N NaOH which dissolved most of the salt. We are uncertain whether this salt residue interferes with LSC. Bleach was effective in all the solv/dig except TMH which bleached poorly. There were traces of brown particles in many of the digested samples. These may represent "hot spots" of undigested feces and/or drug. All the samples formed good gels except THF which required the addition of 0.5 ml 0.5 N NaOH.

Conclusions

From our evaluation of this series of solvent/digestants we concluded that the best were 1) Soluene, 2) NaOH and 3) Protosol.

Table 3

Extraction of Milled Feces with a Series of Solvent/Digestants

Solvent/Digestant	SRF*	BL-10-24	CT-4-48	SMF*	75-5-48
	Total ^{14}C in % of Dose				
DMSO	95.3	83.3	5.9	100.6	36.7
	101.5	78.4	6.1	100.9	37.7
DMSO-THF (1:1)	97.7	75.6	5.8	101.7	37.3
	98.5	78.1	5.5	100.4	37.8
THF	101.1	75.5	5.1	102.0	37.9
	102.2	76.3	6.1	98.5	36.9
Protosol	102.2	64.2	6.4	102.0	37.4
	103.2	75.0	5.8	99.1	37.2
Soluene	99.8	78.5	6.0	98.8	37.8
	102.4	79.3	6.4	97.8	38.2
TMH	98.6	74.6	6.7	97.2	35.7
	95.1	71.1	5.9	97.0	35.4
NaOH	99.8	78.7	6.1	102.3	38.9
	102.4	82.6	6.2	101.5	38.8

*Calculated to a mean recovery of 100% for all 7 samples.

Table 4

Classification of Solvent/Digestants Based on Recovery of Fecal ^{14}C From Five Milled Feces Samples

Solvent/ Digestant	SRF	BL-10-24	CT-4-48	SMF	75-5-48	Classification
DMSO	L (6.2)	H (4.9)	= (0.2)	h (0.3)	l (1.0)	Poor av. 2.52
DMSO-THF	L (0.8)	l (2.5)	l (0.3)	h (1.3)	h (0.5)	Poor av. 1.08
THF	= (1.1)	L (0.8)	L (1.0)	= (3.5)	= (1.0)	?Poor av. 1.48
Protosol	H (1.0)	H (9.2)	= (0.6)	= (2.9)	= (0.2)	Good av. 2.78
Soluene	h (2.6)	h (0.8)	h (0.4)	l (1.0)	H (0.4)	Good av. 1.04
TMH	L (3.5)	L (3.5)	H (0.8)	L (0.2)	L (0.3)	Poor av. 1.66
NaOH(0.5 N)	h (2.6)	H (3.9)	= (0.1)	H (0.8)	H (0.1)	Good av. 1.5

L = lower than mean recovery figure

l = slightly lower than mean recovery figure

= equal to mean recovery figure

h = slightly higher than mean recovery figure

H = higher than mean recovery figure

() = difference between duplicate samples

Experiment 3

Introduction

Previously we had counted feces digests primarily in DTN-C. This is a good medium but often results in very solid gels which are tedious to use and to internally standardize. Therefore we decided to try a different medium.

Purpose

To determine whether the counting efficiency of Protosol and Soluene digests of milled feces in Biofluor ®* equals that of NaOH digests.

Samples

The four milled feces samples used in Experiment 2 were employed: 1) SRF, 2) SMF, 3) CT-4-48 and 4) 75-5-48.

Solvent/Digestants

1) Protosol

2) Soluene

Procedure

See Digestion Protocol.

Results

The results in Table 5 indicate that for these four milled feces samples, the values obtained with Protosol and Soluene in Biofluor were essentially the same and had the same variability as those obtained using NaOH in DTN-C. Standard deviations varied from 0.3 to 2.2. If we expressed these figures as coefficient of variation (in %), the values ranged from 2 to 7%, which we considered acceptable.

Conclusions

We can employ Biofluor for future assay work with Protosol and Soluene digests. This circumvents the need to prepare the more time consuming DTN-C medium.

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*Registered trademark for a liquid scintillation counting fluid obtained from New England Nuclear (NEN), 549 Albany Street, Boston, Massachusetts 02118.

Table 5

Comparison of Two Media for Counting Feces Digests

Feces Sample	Solvent	LSC MEDIA Biofluor	5% DTN-Cab-O-Sil
SRF*	Protosol	100.1, 99.9	99.6, 100.5
	Soluene	97.3, 102.7	98.7, 101.3
		100.0 ± 2.2**	100.0 ± 1.12
CT-4-48	Protosol	5.7, 6.7	6.4, 5.8
	Soluene	6.0, 6.0	6.0, 6.4
		6.23 ± 0.46	6.15 ± 0.30
SMF*	Protosol	99.8, 100.2	101.4, 98.5
	Soluene	100.3, 99.7	100.5, 99.5
		100.0 ± 0.29	100.0 ± 1.25
75-5-48	Protosol	38.7, 38.4	37.4, 37.2
	Soluene	39.5, 38.8	37.8, 38.2
		38.8 ± 0.47	37.7 ± 0.44

*Calculated to a mean recovery of 100% for all four samples.

**Mean ± S.D.

Experiment 4

Introduction

Having established in Experiment 3 that Biofluor is a good medium for Soluene digests of milled feces samples we decided to compare % recovery of 10 Soluene digests in Biofluor medium with % recovery of 10 NaOH digests in DTN-C medium.

Purpose

To compare the reproducibility of NaOH and Soluene digests of a milled feces sample.

Sample

The 48 hr milled feces sample from bile-duct cannulated monkey 75-5 ♀ given a single 5 mg/kg oral dose of WR-158,122-^{14}C.

Solvent/Digestants

1) 0.5 N NaOH

2) Soluene

Digestion Procedure

The protocol was modified as follows: Samples were incubated 48 hr. NaOH digests were counted in 15 ml of DTN-C, Soluene digests in 15 ml of Biofluor.

Results

The 10 replicate NaOH digests showed a mean % recovery of 40.6 ± 1.0 SD. The 10 replicate Soluene digests showed a mean % recovery of 41.3 ± 1.35 SD (see Table 6). Thus the results with the two types of digests were quite comparable. The average counting efficiency of the 10 NaOH digests was 81.6%; for the 10 Soluene digests the efficiency was 82.6%.

Discussion

Although the 10 replicates for each solvent match well in this experiment, we noted that some earlier recovery figures for this same feces sample were a

bit discrepant.

Exp. #2	NaOH (DTN-C)	38.9 38.8	Exp. #3	Soluene (DTN-C)	37.8 38.2
			Exp. #3	Soluene (Biofluor)	39.5 38.8
Exp. #4	NaOH (DTN-C)	40.6 ± 1.0	Exp. #4	Soluene (Biofluor)	41.3 ± 1.35

Although the variability was only 1.7 to 1.8% for NaOH and 1.8 to 3.5% for Soluene, several questions remained to be answered. How do these values compare with the results that would be obtained by combustion assay of the same samples? Our second question was: Are the milled samples really homogeneous? To answer this latter question in part we sieved all of the previously milled samples using a stainless steel wire screen sieve (18-8, 30 holes/inch). This usually removed only unground fibers but in some samples there were residues which suggested incomplete milling. These latter residues were reground and mixed with the original powder. This slight inhomogeneity could possibly have contributed to our aliquot to aliquot variability but probably did not account for the differences among the the different experiments listed above. Nevertheless, passing the feces through a fine sieve is a valuable check on homogeneity of the samples and adequacy of the milling process. We have adopted it as the last step of our standard feces preparation procedure.

Conclusions

1. Sample replication of 10 NaOH digests counted in DTN-C and 10 Soluene digests counted in Biofluor is very good within the same experimental run.

2. There are variations in sample recovery values among different experimental runs which led us to question whether our digestion procedures are giving recoveries as good as those one would expect from combustion assays.

Table 6

Reproducibility of NaOH Digests and Soluene Digests of a Milled Monkey Feces Sample

	% Recovery (as ^{14}C)		
	NaOH Digests in DTN-C		Soluene Digests in Biofluor
1	41.6	11	43.7
2	39.2	12	40.7
3	40.7	13	41.2
4	40.1	14	40.3
5	40.3	15	41.2
6	40.7	16	39.4
7	39.1	17	40.2
8	40.5	18	40.6
9	42.1	19	42.4
10	41.7	20	43.0
	$\bar{x}$ 40.6 ± 1.0*		$\bar{x}$ 41.3 ± 1.35

*Mean ± S.D.

Experiment 5

Introduction

The variation we had observed among different experimental runs of milled feces samples led us to question whether our digestion procedures were giving recoveries comparable to those one would obtain from a combustion assay.

Purpose

To compare the results following digestion of fecal samples with NaOH, Soluene, and perchloric acid-hydrogen peroxide ("PH") of Mahin and Lofberg (1) with the results of combustion assays of the same samples.

Feces Samples (all milled)

1) 75-5-24 - 24 hr feces sample from rhesus 75-5 ♀ (bile-duct cannulated). (5 mg/kg oral dose of WR-158,122-^{14}C)
2) 75-5-48 - described on page 7
3) BL-13-24 - 24 hr feces sample from bile-duct ligated rat #13 (10 mg/kg dose of WR-158,122-^{14}C)
4) CT-4-24 - 24 hr feces sample from non-operated rat #4 (10 mg/kg oral dose of WR-158,122-^{14}C)

Solvent/Digestants

1) 0.5 N NaOH
2) Soluene
3) "PH": 60% perchloric acid* (0.3 ml) plus 30% hydrogen peroxide** (0.4 ml) (1,2).

Procedures

Combustion Assays.

The 4 milled feces samples were submitted for combustion assay to

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*60% perchloric acid - Reagent A.C.S. - Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, New Jersey 07410.

**30% H_2O_2 - "Baker Analyzed" Reagent - J.T. Baker Chemical Co., Phillipsburg, N.J. 08865.

the New England Nuclear LSC Applications and Assay Laboratories.* Sample weights employed for the combustion assays ranged from 95 to 160 mg and samples were run in triplicate. The samples were combusted in a Packard 306B oxidizer and counted in a Packard 3385 counter at 30% gain, 50-1000 window. Results were corrected for quench by the channels ratio procedure.

Digestion

The Digestion Protocol was modified as follows:

NaOH digests were counted in 15 ml of DTN-C and Soluene digests were counted in 15 ml of Biofluor. The "PH" digests were incubated for 4 hr at 70°C and counted in 15 ml of Scintiverse®.**

Results

The combustion assays gave results which were in general agreement with values obtained by other digestion procedures (see Table 7). Most of the NaOH values compare closely with combustion values. Most Soluene values were somewhat higher than combustion values. Some of the "PH" values were slightly lower than combustion values and this might be explained by $^{14}CO_2$ loss in this digestion method, if any of the fecal ^{14}C was labile (2).

Conclusions

This experiment established that the recoveries of fecal ^{14}C by combustion and digestion procedures were very similar. These results also indicated that the digestion procedures were probably reliable and that any of the three digestants, Soluene, NaOH, or Protosol, were applicable.

The next step was to find out whether an extraction procedure would give us equally good recoveries. This was a necessary step in preparing extracts for identification of fecal metabolites. An adequate extraction procedure should extract at least 80-90% of the ^{14}C content of the feces.

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*We wish to thank Dr. Yutaka Kobayashi for his assistance with these assays.

**Scintiverse is the registered trademark for a scintillation counting fluid obtained from Fisher Scientific Co., Chemical Manufacturing Div., Fair Lawn, N.J. 07410.

Table 7

Comparison of Recovery of Fecal ^{14}C by Four Methods

Feces Sample	Combustion Assay*	Total ^{14}C as dpm/mg NaOH Digestion DTN-C	Soluene Digestion Biofluor	"PH" ** Digestion Scintiverse
75-5-24	84.3 ± 0.5	84.3	88.8 91.8	81.3 84.3
75-5-48	102.0 ± 2.4	106.1	108.5 112.3	105.0 107.2
BL-13-24	97.8 ± 2.0	97.8	96.7 95.6	93.3 91.4
CT-4-24	65.5 ± 1.0	64.4	68.7 62.8	65.3 63.9

* New England Nuclear, Inc.

** Perchloric acid - H_2O_2

Experiment 6

Introduction

In the preceding experiment we established that our recoveries of fecal ^{14}C by combustion and digestion procedures were reasonably similar. The next question was to find out whether an extraction procedure would provide equally good recoveries.

Purpose

To determine whether tetrahydrofuran (THF) completely extracts the total ^{14}C in milled feces samples.

Samples

The four milled feces samples (75-5-24; 75-5-48; BL-13-24; CT-4-24) used in Experiment 5 were employed in this experiment.

Solvent/Digestants

1) Tetrahydrofuran - Fisher Scientific Co. (HPLC grade)

2) Soluene

Protocol

One gram quantities of the 4 milled feces samples were placed in 15 ml graduated centrifuge tubes and extracted three times with 5 ml THF as follows: tubes were shaken for 15 min on an automatic shaker, centrifuged for 10 min and each solvent layer poured into a LSC vial. The THF was evaporated with N_2 in a Meyer N-evaporator ®* in separate LSC vials labeled extract 1, 2, and 3. Each residue was dissolved with 1 ml of THF, bleached, if necessary, with 0.5 ml TBHP, and counted in 15 ml of Biofluor.

The residues of the 1 g feces aliquots (after THF extraction) were digested with 2 ml of Soluene for 26 hr at 70°C. TBHP (0.5 ml) was added and the tubes

*Meyer N-evaporator, Model III, obtained from Organomation Assoc., Inc., Northborough, Mass. 01532.

were centrifuged for 30 min. The supernates were poured into LSC vials and counted in 15 ml of Biofluor.

Results

THF extracted almost the same dpm/mg (see Table 7) as were obtained by combustion assay or digestion by NaOH, 0.5 N, Soluene, or "PH", for the two monkey feces samples. Soluene digests of the 1.0 g residues of monkey feces samples recovered only an additional 1.8% for 75-5-24 and 3.2% for 75-5-48. However, THF extracted only 82% of total ^{14}C in CT-4-24 and 92% in the case of BL-13-24. Soluene added an additional 18% in the case of CT-4-24 and 7.8% for BL-13-24.

When Soluene digest recovery was added to THF recovery, the total recovery figures for monkey feces samples were about 7% higher than combustion figures but agreed well with the Soluene values. For the rat feces samples, the total recovery figure was slightly lower than combustion figures but probably not significantly different from the digestion values.

Conclusions

Recovery of fecal ^{14}C with THF extraction matched recovery obtained by combustion, NaOH digestion, Soluene digestion, and "PH" digestion very well for the two monkey feces samples. Recovery of fecal ^{14}C for rat feces with THF was about 20% lower than combustion recovery for the CT-4-24 sample and about 10% lower than combustion recovery for the BL-13-24 sample. This suggests that rat feces samples contain metabolites of WR-158,122 not extracted by THF.

Table 8

THF Extraction Compared with Combustion and Three Digestion Methods

Feces Sample → / Method ↓	Total ^{14}C in dpm/mg			
	75-5-24*	75-5-48*	CT-4-24*	BL-13-24*
THF Extract 1	79.6	92.9	46.7	76.0
2	8.5	11.3	5.4	10.5
3	0.9	1.3	0.9	1.5
Soluene digestion	1.6	3.5	11.8	7.4
Total	90.6	109.0	64.8	95.4
Combustion**	84.3 ± 0.5	102.0 ± 2.4	65.5 ± 1.0	97.8 ± 2.0
NaOH Digestion**	84.3	106.1	64.4	97.8
Soluene Digestion**	88.8	108.5	68.7	96.7
	91.8	112.3	62.8	95.6
"PH" Digestion**	81.3	105.0	65.3	93.3
	84.3	107.2	63.9	91.4

* Milled feces sample

** Results from Exp. 5

Experiment 7

Introduction

In Experiment 6 we found that THF extraction recovered as much fecal ^{14}C from monkey feces samples as did the combustion and digestion procedures. On the other hand, THF extraction of rat feces gave recoveries that were somewhat lower. We decided to do sequential extraction of several feces samples with 4 organic solvents to see whether a different extraction procedure would give us complete recovery of fecal ^{14}C.

Purpose

To determine whether sequential extraction of feces samples with four organic solvents (benzene, ethyl acetate, acetonitrile, tetrahydrofuran) will give complete recovery of fecal ^{14}C.

Feces Samples

The milled (and sieved) feces samples used in this experiment were the same feces samples employed in Experiments 2 and 6. They were SRF, BL-13-24, CT-4-24, CT-4-48, SMF, and 75-5-48.

Solvents

Benzene - ACS Reagent (Matheson, Coleman & Bell)

Ethyl Acetate - ACS Certified (Fisher Scientific Co.)

Acetonitrile - Reagent Grade (Matheson, Coleman & Bell)

Tetrahydrofuron - HPLC Grade (Fisher Scientific Co.)

Protocol

Placed 1 g aliquots of milled feces in 15 ml screw cap centrifuge tubes and extracted with organic solvents sequentially as follows:

A. Extracted with 10 ml benzene followed by extraction with 5 ml benzene.

B. Extracted residual solids with 10 ml ethyl acetate followed by extraction with 5 ml ethyl acetate.

C. Extracted residual solids with 10 ml acetonitrile followed by extraction with 5 ml acetonitrile.

D. Extracted residual solids with 10 ml tetrahydrofuran followed by extraction with 5 ml tetrahydrofuran.

For each extraction the tubes were shaken for 30 min, centrifuged for 10 min, and the supernatant solvent phases poured into a LSC vial. The extracts for each solvent were combined and evaporated under N_2 in a Meyer N-evaporator. Each residue was dissolved in 1 ml THF, bleached, if required, and counted in Biofluor.

Results

1) Ethyl acetate extracted most of the fecal ^{14}C in all samples. (See Table 9).

2) 96% of total dpm's was recovered in the total benzene and ethyl acetate extracts for all samples except the feces samples of treated but non-operated rat CT-4.

3) Acetonitrile extracted 15% of total dpm's in both feces samples from rat CT-4.

4) Tetrahydrofuran recovered little more than 1% of total dpm's except in rat CT-4 (CT-4-24, 4.1%; CT-4-48, 6.6%).

5) Two previously assayed samples, CT-4-48 and 75-5-48 showed lower recoveries in this experiment when compared to recoveries in earlier studies (See Table 5).

Discussion

Sequential extraction with the 4 organic solvents studied did not give total recoveries of ^{14}C in two treatment feces samples as high as those we obtained in earlier studies. This indicates that we might have recovered additional ^{14}C by digesting the residues with NaOH or Soluene.

It is quite interesting that 15% of the total dpm's in both non-operated rat samples (CT-4-24 and CT-4-48) was recovered in acetonitrile. This suggests that acetonitrile will extract fecal metabolites present in rat treatment feces samples.

Conclusions

The 4 organic solvents extracted most of the fecal ^{14}C, but % dose recoveries were lower for 2 samples than those obtained in earlier studies. This difference is under investigation.

Further work is planned to find the best series of solvents for sequential extraction and separation of ^{14}C - containing metabolic fractions of feces.

Table 9

Sequential Extraction of Six Feces Samples with Four Organic Solvents

Solvent (Sequential)	dpm/g					
	SRF	BL-13-24	CT-4-24	CT-4-48	SMF	75-5-48
1. Benzene	1042 (12.3)*	18685 (20.9)	5660 (12.2)	353 (21.3)	7863 (29.5)	10963 (10.0)
2. Ethyl Acetate	7228 (85.3)	66980 (74.9)	32314 (69.5)	932 (56.3)	17889 (67.2)	94283 (86.3)
3. Acetonitrile	161 (1.9)	3105 (3.5)	6614 (14.2)	260 (15.7)	624 (2.3)	3381 (3.1)
4. Tetrahydrofuran	42 (0.5)	655 (0.7)	1921 (4.1)	109 (6.6)	260 (1.0)	578 (0.5)
Total	8473	89425	46509	1654	26636	109205
	(100.0)	(100.0)	(100.0)	(99.9)	(100.0)	(99.9)

* % of total dpm's in each extract

Summary

1. We have developed a procedure for preparing uniform fecal samples for LSC counting and study of fecal metabolites. The procedure requires milling of a rat or monkey feces sample with anhydrous Na_2SO_4 in a stainless steel ball mill. The fine off-white flours that result are passed through a fine wire sieve (18-8 stainless steel wire, 30 holes/inch). This sieving increases homogeneity by removing traces of unground material and dietary fiber.
2. Digestion of samples with NaOH and counting in dioxane-toluene-naphthalene with Cab-O-Sil (DTN-C) gives reproducible recoveries. This procedure was adequate for 0.25 g and 0.5 g samples but inadequate for 1.0 g samples.
3. In a study of 7 solvent/digestants we concluded that the best solv/dig were 1) Soluene, 2) NaOH and 3) Protosol. These three solv/dig extracted the most dpm from feces samples and showed the best reproducibility for 0.25 g aliquots.
4. Protosol and Soluene digests count just as efficiently in Biofluor as in DTN-C.
5. Ten replicate Soluene digests counted in Biofluor and 10 replicate NaOH digests counted in DTN-C showed mean % recoveries which matched well (NaOH, 40.6 ± 0.32 SE; Soluene, 41.3 ± 0.43 SE).
6. Recovery of ^{14}C by combustion assay was compared with results obtained by digestion in NaOH, Soluene, and perchloric acid-H_2O_2. The combustion assays gave recoveries that agreed well with values obtained by our digestion procedures.
7. When 1.0 g aliquots of 4 milled feces samples were extracted sequentially 3 times with THF and digested once with Soluene, the THF extraction recoveries matched the digestion methods for monkey feces. Recovery of ^{14}C in rat

feces samples was 10 to 20% lower than digestion assays.

8. Six feces samples were extracted sequentially with benzene, ethyl acetate, acetonitrile and tetrahydrofuran to see whether this procedure would give complete recovery of fecal ^{14}C. 96% of total dpm's were recovered in the total ethyl acetate and benzene extracts for all the samples except the non-operated rat CT-4. In this rat only 78 to 82% was extracted by these solvents, suggesting that the metabolites in this rat's feces are different from those in monkey 75-5♀ (bile-duct cannulated). Most of the undissolved fraction in rat CT-4 was soluble in acetonitrile.
9. Further work is planned to find the best series of solvents for sequential extraction and separation of fecal ^{14}C containing metabolites. This will be followed by purification of the metabolic fractions on HPLC and eventual structural studies using GC-MS and other analytical techniques such as IR and NMR if sufficient material can be generated.

References

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Appendix

Procedure for Preparing Uniform Fecal Samples
and Measuring Total Radioactivity by LSC

Milling Protocol

1. If feces are moist remove water by blotting. Then weigh the frozen feces sample.
2. Place the feces sample and a known amount (4 to 8 parts) of anh. Na_2SO_4 in a 0.5 gal. stainless steel ball mill. Add approximately 100 ½" and 8 - 12 1" stainless steel balls. (The number of balls is increased or decreased in accordance with sample size).
3. Mill the sample for 30-60 min. (Time may be increased for very large samples).
4. Inspect the milled feces to see that there are no grossly discernible large unmilled particles.
5. Pass the milled feces sample through a fine wire sieve (18-8 stainless steel wire, 30 holes/in.). Significant amounts of unground material are reground and combined with the rest of the powder.
6. Store feces powder at -20°C.

Digestion Protocol

1. Weigh milled feces samples (Approximately 250 mg) on an analytical balance and place in 20 ml LSC vials.
2. Add 1 ml of solvent/digestant (Soluene, NaOH (0.5 N) or Protosol).
3. Digest in oven at 68-70°C for 24-48 hr.
4. Bleach by adding 0.5 ml tertiary butyl hydrogen peroxide (TBHP) at 2-24 hr.
5. Add either 15 ml of 5% DTN-Cab-O-Sil (DTN-C) or 15 ml of Biofluor.
6. Cool and dark-adapt samples at least 2 hr at 4°C before counting.
7. Internally standardize with toluene ^{14}C, if desired.

SIGNATURE PAGE

Marvin Wilson Tabor

Marvin Wilson Tabor, Ph.D.
Acting Principal Investigator
1980-1981 Contract Period

Carl C. Smith

Carl C. Smith, Ph.D.

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