A method for measurement of nanogram quantities of 3-methylcholanthrene in stool samples

William C. Duane,[1] Jerome C. Behrens, Susan Green Kelly, and Allen S. Levine

Department of Medicine, University of Minnesota and Veterans Administration Hospitals, Minneapolis, MN 55417

Summary The carcinogen 3-methylcholanthrene can be produced from deoxycholic acid and is postulated by some investigators to play a role in the pathogenesis of colon carcinoma. The small quantities of this compound which could be carcinogenic have been difficult to measure in feces because of many potentially interfering compounds. Using 3-[6-^{14}C]methylcholanthrene as an internal standard, petroleum ether extraction, C-18 SepPak separation, preparative high performance liquid chromatography, and gas–liquid chromatography–mass spectrometry with selected ion monitoring, we developed an assay capable of detecting <35 ng of 3-methylcholanthrene per gram of stool. Application of this technique to stools of five patients with colon carcinoma and two normal controls revealed no detectable 3-methylcholanthrene in any stool sample. This negative result was confirmed by incubating radiolabeled cholic acid in fecal homogenates. Although >90% of this radiolabeled bile acid was converted to deoxycholic acid, none of the radioactivity was found in the thin-layer chromatography fraction corresponding to 3-methylcholanthrene. These observations provide evidence against a role for 3-methylcholanthrene in pathogenesis of human colon carcinoma. Similar assays could be used for analysis of other carcinogens in stool samples.—**Duane, W. C., J. C. Behrens, S. G. Kelly, and A. S. Levine.** A method for measurement of nanogram quantities of 3-methylcholanthrene in stool samples. *J. Lipid Res.* 1984. **25:** 523–526.

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3-Methylcholanthrene is an extremely potent carcinogen believed by some investigators to play a role in pathogenesis of human colon cancer (1, 2). In the test tube, 3-methylcholanthrene can be synthesized from deoxycholic acid (3), a major secondary bile acid formed in the human colon by dehydroxylation of cholic acid. Since 100–300 mg of deoxycholic acid passes through the human colon each day and since 3-methylcholanthrene is carcinogenic in microgram quantities (4), occurrence of this reaction, even to a small extent, in the human colon could contribute to development of carcinoma. Moreover, as suggested by Hill et al. (1, 5, 6), this reaction would provide a mechanistic link between the association of high fecal concentrations of deoxycholic acid and colon carcinoma. It would also explain the observation that some populations with colon cancer have a high incidence of fecal bacteria capable of initial dehydrogenation of the sterol ring nucleus (1, 5, 6).

A sine qua non for implicating 3-methylcholanthrene in pathogenesis of colon carcinoma would be the demonstration of this carcinogen in stool. This is an especially difficult analytical task because of the need to identify very small amounts of 3-methylcholanthrene amidst a virtual sea of potentially interfering compounds. The present report describes a method for determining nanogram quantities of 3-methylcholanthrene in stool using selected ion monitoring during gas–liquid chromatography–mass spectrometry (GLC–MS) following a preparative procedure including high performance liquid chromatography (HPLC). Negative results of this method in two normal controls and five patients with colon carcinoma were further confirmed by incubation of radioactive cholic acid in fecal homogenates and searching for labeled 3-methylcholanthrene.

METHODS

Materials

Freshly passed stool samples were obtained from two normal volunteers and five patients with colon carcinoma. Analysis and incubations were begun within 1 hr of obtaining stool.

Standard 3-methylcholanthrene (Eastman Kodak, Rochester, NY) and 3-[6-^{14}C]methylcholanthrene (57 mCi/mmol; New England Nuclear, Boston, MA) were purchased and further purified by thin-layer chromatography (TLC) on silica gel 60 with a mobile phase of hexane–tetrahydrofuran 95:5. [2,4-^{3}H]Cholic acid (16 Ci/mmol) was purchased (New England Nuclear) and further purified by TLC on silica gel G with a mobile phase of isooctane–ethyl acetate–acetic acid 5:5:1. [4-^{14}C]Cholesterol (53 mCi/mmol) was purchased (New England Nuclear) and found to be 98% isotopically pure by TLC on silica gel 60 using a mobile phase of ethyl ether–hexane 1:1. [4-^{14}C]Cholic acid was prepared by administration of [4-^{14}C]cholesterol to a chronic bile fistula rat. Bile rich in radioactive cholic acid was pooled and the bile acid was deconjugated as previously described (7). Cholic acid was extracted with two volumes of chloroform–methanol 2:1 and purified by TLC using the solvent system described above. The resulting labeled cholic acid

Abbreviations: GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

[1] To whom reprint requests should be addressed at Gastroenterology Section (111 D), VA Medical Center, 54th Street and 48th Avenue South, Minneapolis, MN 55417.

had a specific activity of 5.2 μCi/mmol. Solvents suitable for use on high performance liquid chromatography (HPLC) were purchased from MCB Manufacturing Chemist, Inc., Cincinnati, OH.

Analysis of 3-methylcholanthrene

A single stool specimen from each subject was weighed and homogenized with an equivalent volume of 150 mM $NaHCO_3^-$ saturated with CO_2 under an atmosphere of 100% CO_2. Part of this homogenate was used for incubation with radioactive bile salt (vide infra). Duplicate 2-ml aliquots of the homogenate were removed for analysis. One of these was spiked with 0.02–0.20 μCi of 3-[6-^{14}C]methylcholanthrene plus 100 ng of unlabeled 3-methylcholanthrene. The other was analyzed in parallel without spiking. These duplicate aliquots were immediately combined with 100 ml of 70% methanol in water and extracted with two 100-ml volumes of petroleum ether. The petroleum ether was then evaporated to dryness, and the residue was dissolved in acetonitrile–water 50:50. This solution was transferred to a C-18 SepPak (Waters Associates, Milford, MA) which was sequentially eluted with 4 ml, 10 ml, and 10 ml of 50%, 90%, and 100% acetonitrile–water, respectively. The 90% fraction, which contained all the radioactivity, was concentrated to dryness and redissolved in 100 μl of acetonitrile. One-half of this was injected onto a Hewlett Packard 1084B HPLC equipped with a column of LiChrosorb RP-8. The mobile phase was acetonitrile–water 80:20. Detection was accomplished by a variable wavelength detector set at 304 nm. Fractions of the HPLC eluate were quantitatively collected. Those fractions containing radioactive 3-methylcholanthrene were pooled, evaporated to dryness, and redissolved in acetonitrile. This entire sample was injected onto an LKB 9000 gas chromatograph/mass spectrometer equipped with a 3% OV-17 column at 290°C. Selected ion monitoring was done at m/e of 268 for unlabeled 3-methylcholanthrene and 270 for ^{14}C-enriched 3-methylcholanthrene.

Incubation studies

Half of each fecal homogenate, prepared as described above, was spiked with known amounts of either [2,4-^{3}H]cholic acid (10–15 μCi) or [4-^{14}C]cholic acid (0.05–0.10 μCi) and incubated under an atmosphere of 100% CO_2 at 37°C with constant gentle agitation for 24 hr. The other half of the homogenate was autoclaved for 45 min prior to spiking with labeled bile acid and incubated in identical fashion. At the end of the incubation each homogenate was made to 100 ml of 70% methanol and extracted twice with 100 ml of petroleum ether. The petroleum ether extracts were combined and concentrated to dryness under N_2. The residue was redissolved in a small volume of petroleum ether for application to a silica gel 60 TLC plate which was developed with ethyl ether–heptane 1:1. After development the plate was divided into strips which were scraped into counting vials containing 10 ml of Aquasol (New England Nuclear) and 1 ml of water for determination of radioactivity on a Packard Tricarb 4640 liquid scintillation counter.

RESULTS

Fig. 1 shows typical HPLC runs for 3-methylcholanthrene standard (Fig. 1A) and for a fecal extract (Fig. 1B). Fractions of the fecal extracts corresponding to the retention time of 3-methylcholanthrene were collected and further analyzed by GLC–MS.

Analysis of serial dilutions of standards by selected ion monitoring indicated a well-defined peak with injection of 10 ng of 3-methylcholanthrene (**Fig. 2**). Sequential analysis of radioactivity in fecal homogenate samples spiked with 3-[6-^{14}C]methylcholanthrene indicated that 62 ± 8% of the radioactivity was retained through the entire procedure used in preparation for GLC–MS. Thus, the assay would be expected to easily detect 10 ÷ 0.6 or about 17 ng of 3-methylcholanthrene in a given extract. Since our extract up to the point of injection onto the HPLC represented 1.0 g of stool and one-half this extract was injected onto the GLC–MS, the assay should have detected at least 34 ng of 3-methylcholanthrene per gram of stool.

Fig. 3 shows selected ion monitoring analysis of fecal sample spiked with 3-methylcholanthrene (Fig. 3A) and a sample not spiked. In none of the unspiked stool samples from the two normal volunteers or the five colon cancer

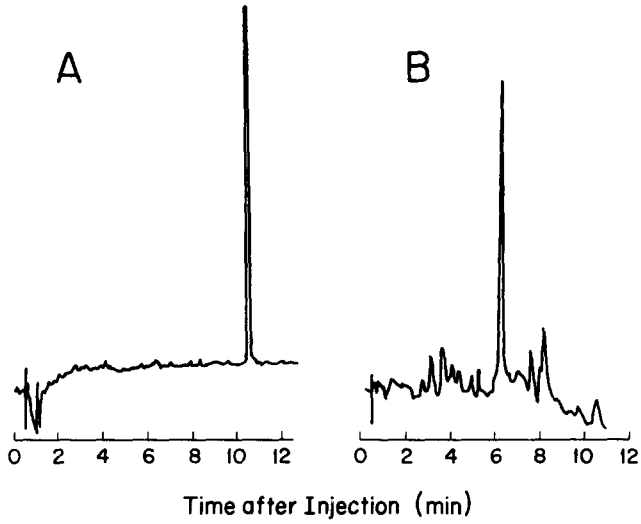

Fig. 1. High performance liquid chromatograms of standard 3-methylcholanthrene (A) and a fecal extract (B). Retention time of 3-methylcholanthrene was about 9.9 min (K′ = 14.3).

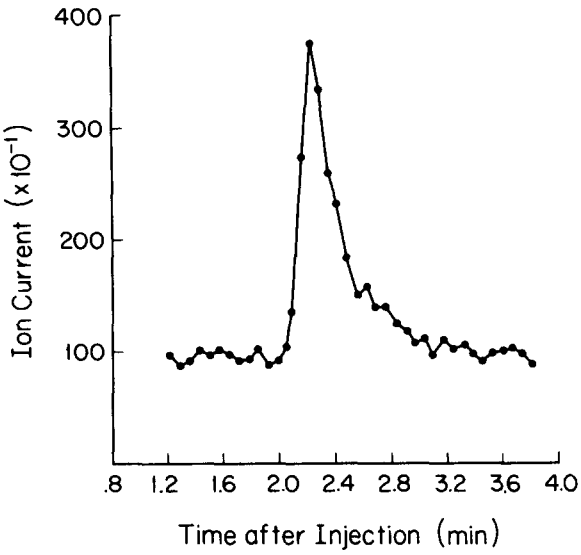

Fig. 2. Selected ion monitoring at m/e of 268 during gas–liquid chromatography–mass spectrometry of 10 ng of unlabeled 3-methylcholanthrene.

patients was any 3-methylcholanthrene detected by GLC–MS. In all cases the parallel aliquot spiked with 3-[6-^{14}C]methylcholanthrene and unlabeled 3-methylcholanthrene yielded the appropriate peaks on selected ion monitoring. After correction for loss of isotopic tracer, total mass of 3-methylcholanthrene assayed in these spiked aliquots averaged 103 ± 12% of that added prior to analysis.

In the incubation studies, fecal homogenates which had been autoclaved demonstrated no conversion of labeled cholic acid to deoxycholic acid and no counts extractable into petroleum ether. In contrast, fecal homogenates which had not been autoclaved converted >90% of the [2,4-^{3}H]cholic acid to deoxycholic acid as judged by TLC. Also, between 0.5 and 1.0% of the radioactivity after a 24-hr incubation was extractable into petroleum ether. A TLC analysis of this extractable radioactivity is shown in **Fig. 4.** No counts from any of the stools incubated were found in the area of the TLC plate corresponding to 3-methylcholanthrene standard. Two incubations using [4-^{14}C]cholic acid yielded results indistinguishable from incubations using [2,4-^{3}H]cholic acid.

Finally, to estimate the ability of fecal bacteria to metabolize 3-methylcholanthrene, 3-[6-^{14}C]methylcholanthrene was added to two stool homogenates and incubated for 24 hr as described above. In both instances, >70% of the labeled 3-methylcholanthrene was recovered unchanged from the homogenates.

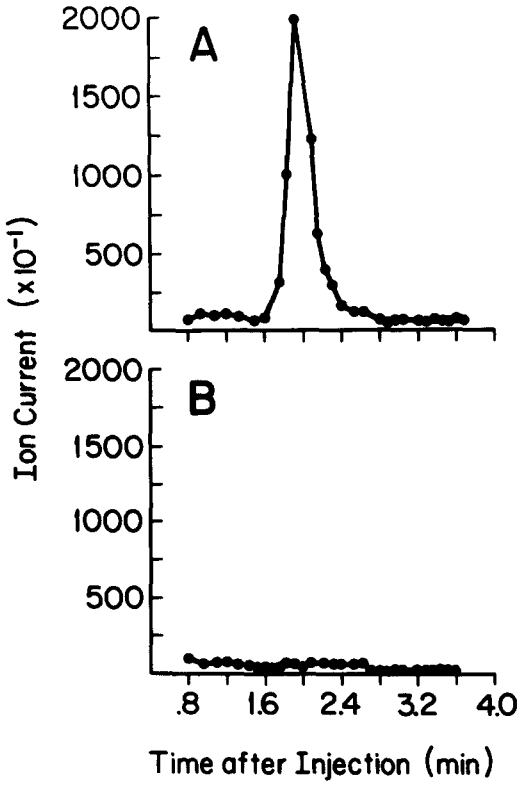

Fig. 3. Selected ion monitoring at m/e of 268 of a fecal extract spiked with 3-methylcholanthrene (A) and an extract not spiked (B).

DISCUSSION

GLC–MS with selected ion monitoring is a highly specific and sensitive technique for analysis of small quantities of a given compound, even in the presence of many similar compounds. It is possible to analyze small quantities of

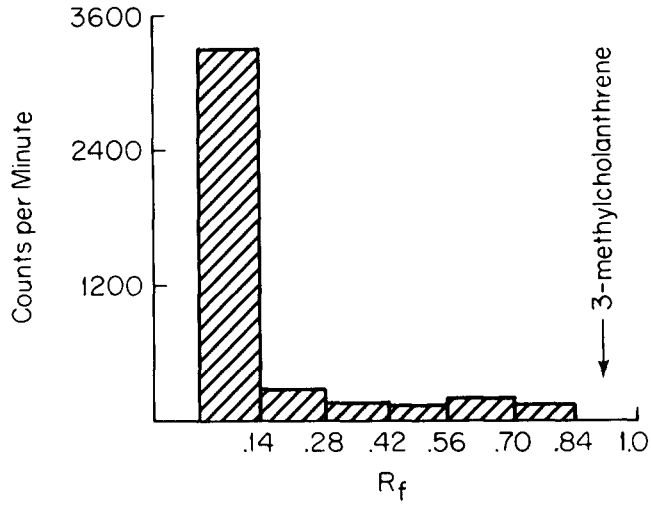

Fig. 4. Thin-layer chromatography of the petroleum ether-extractable counts after a 24-hr incubation of [2,4-^{3}H]cholic acid in a fecal homogenate. The stationary phase was silica gel 60 and the mobile phase was ethyl ether–heptane 1:1. No counts were found in the band corresponding to 3-methylcholanthrene.

3-methylcholanthrene (~10 ng) by HPLC alone; however, in many of our samples one or more peaks with retention times similar to 3-methylcholanthrene were seen on HPLC (Fig. 1). Only after collection and analysis of these peaks by GLC–MS could we be confident that they did not represent 3-methylcholanthrene. This same technique with little or no modification could be used for analysis of a variety of minor sterol metabolites or potential carcinogens in stool and other biological fluids.

Because 3-methylcholanthrene is readily synthesized from deoxycholic acid in the test tube (3), it has been suggested that conversion of deoxycholic acid to this potent carcinogen by gut bacteria might be important in the etiology of colon cancer (1, 2, 5, 6). Supporting this possibility is the association of high fecal concentrations of deoxycholic acid with colon carcinoma (2) and the high incidence in colon cancer patients of bacteria capable of initial desaturation of the sterol ring (1). In addition, bacterial aromatization of rings A and B of the bile acid molecule has been demonstrated in vitro, although saturation of ring C has not been demonstrated (8).

In the present study no 3-methylcholanthrene could be detected in the stools of five colon cancer patients and two normal controls despite sensitivity of our assay to below 35 ng/g of stool. This observation does not exclude the possibility that these subjects' bacteria produced 3-methylcholanthrene which was subsequently completely absorbed or metabolized to other compounds. Against this possibility, however, is our finding that incubation of labeled cholic acid in fecal homogenates revealed rapid and nearly quantitative conversion to deoxycholic acid but no conversion to 3-methylcholanthrene. Moreover, incubation of labeled 3-methylcholanthrene with fecal homogenates revealed only partial (<30%) conversion to other compounds (Results). While we cannot be sure that some people do not have fecal bacteria capable of producing 3-methylcholanthrene from deoxycholic acid, our observations suggest that this reaction is not a common etiological factor in carcinoma of the colon.

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REFERENCES

1. Hill, M. J., B. S. Drasar, V. Aries, J. S. Crowther, G. Hawksworth, and R. E. O. Williams. 1971. Bacteria and aetiology of cancer of large bowel. *Lancet.* **1:** 95–100.
2. Wynder, E. I., and B. S. Reddy. 1974. Metabolic epidemiology of colorectal cancer. *Cancer.* **34:** 801–806.
3. Cook, J. W., and G. A. D. Haslewood. 1933. The conversion of a bile acid into a hydrocarbon derived from 1:2-benzanthracene. *Chem. Indust.* **38:** 758–759.
4. Mirvish, S. S., P. Ghadirian, L. Wallcave, C. Raha, S. Bronczyk, and J. P. Sams. 1981. Effect of diet on fecal excretion and gastrointestinal tract distribution of unmetabolized benzo(a)pyrene and 3-methylcholanthrene when these compounds are administered orally to hamsters. *Cancer Res.* **41:** 2289–2293.
5. Hill, M. J. 1974. Bacteria and the etiology of colonic cancer. *Cancer.* **34:** 815–818.
6. Hill, M. J. 1980. Bacterial metabolism and human carcinogenesis. *Br. Med. Bull.* **36:** 89–94.
7. Duane, W. C., R. L. Ginsberg, and L. J. Bennion. 1976. Effects of fasting on bile acid metabolism and biliary lipid composition in man. *J. Lipid Res.* **17:** 211–219.
8. Hill, M. J. 1975. The role of colon anaerobes in the metabolism of bile acids and steroids, and its relation to colon cancer. *Cancer.* **36:** 2387–2400.