## IN VITRO NUCLEAR MEDICINE

# Carcinoembryonic Antigen: Assay Following Heat Compared with Perchloric Acid Extraction in Patients with Colon Cancer, Non-neoplastic Gastrointestinal Diseases, or Chronic Renal Failure

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**Heat inactivation has been proposed as an alternative to perchloric acid (PCA) precipitation for the extraction of carcinoembryonic antigen (CEA) from human plasma. We examined a commercial RIA kit using heat inactivation, and compared results with those obtained with PCA precipitation. Adequate sensitivity (1.5 μg CEA/l plasma), satisfactory analytical recovery of CEA added to plasma, and dilutional linearity of samples found to have elevated CEA concentrations, were demonstrated for the heat-inactivation assay. Between-assay precision was better with the heat inactivation than with the PCA assay. Although the absolute concentration of CEA estimated after heat inactivation was consistently lower than that estimated after PCA extraction of plasma specimens, there was excellent correlation between results obtained with the two methods in colon cancer patients free of disease, colon cancer patients with residual or recurrent disease, patients with benign gastrointestinal disease, and in patients with chronic renal failure. We conclude that the heat-inactivation assay is an excellent alternative to the PCA assay.**

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Since first described by Gold and Freeman (*1*), the measurement of carcinoembryonic antigen (CEA) by radioimmunoassay (RIA) has been widely applied clinically as a marker for colon and other cancers (*2,3*). This measurement requires separation of CEA from nonspecific plasma proteins that prevent the direct RIA of the antigen. Most frequently the separation has been accomplished by perchloric acid (PCA) precipitation followed by dialysis (*4*) or by column chromatography (*5,6*) to remove the acid. Heating has been demonstrated to be a satisfactory alternative to PCA precipitation and has the advantage of requiring no further sample preparation (*7*). Recently, an assay based on heat inactivation has become available commercially (*8–11*). We have evaluated the characteristics of this assay and compared its results with those obtained with a perchloric acid extraction-dialysis assay in patients with colon cancer, benign gastrointestinal diseases, and chronic renal failure.

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## METHODS

**Measurement of CEA.** Reagent kits for extraction and quantitation of CEA by RIA were obtained commercially for the perchloric acid extraction and dialysis process* and for the heat-inactivation method.†

The former assay* involved the zirconyl phosphate procedure initially described by Hansen (*12*). Duplicate plasma samples were extracted with perchloric acid. Samples were then introduced into dialysis bags and dialyzed against 87 l of distilled deionized water in an automated tank. Six successive 2½ hr dialysis periods

were followed by a final dialysis against 0.01 *M* ammonium acetate buffer. CEA in the entire dialysate was then quantitated by RIA following the instructions provided by the manufacturer. If the CEA concentration exceeded the concentration of the highest calibrator (25 μg/l), the sample was re-extracted and re-dialyzed, and the dialysate diluted with EDTA (kit assay) buffer before re-assay.

The heat-inactivation assay† requires a 1:3 dilution of patient samples in 0.2 *M* sodium acetate followed by heating at 70°C for 15 min in a temperature-controlled water bath. This assay involves a solid-phase (glass bead) primary antibody (guinea pig) and a two-site immunoradiometric assay (IRMA). After incubation of the heat-treated plasma with the anti-CEA-coated bead, CEA bound to the bead is identified by the addition of goat I-125 tagged anti-CEA antibody. Activity associated with the bead is proportional to the CEA content of the plasma sample and is quantitated by comparison with similarly prepared calibrators. The only modification we made was the addition of a 0.5 μg/l standard to the manufacturer's calibration curve, permitting the detection of 1.5 μg/l CEA in undiluted patient plasma.

We heated aliquots of a plasma pool with elevated CEA concentration for 15, 30, 60, and 120 min at 70°C after 1:3 dilution with extraction buffer. We also heated the assay calibrators undiluted and diluted 1:3 with assay buffer for the same times.

Dilutions of heat-inactivated patients' sera with elevated CEA were prepared with assay buffer (zero calibrator) and assayed along with undiluted serum samples.

Concentrated calibrator (~5 mg CEA/l), provided by the manufacturer† in assay buffer, was diluted to permit concentration estimation by IRMA [10 μl CEA concentrate + 990 μl assay buffer (zero standard)]. Ten milliliters of CEA concentrate were then added to 1.0, 2.0, and 3.0 ml of serum obtained from three patients with low initial CEA concentrations. These nine samples were then subjected to heat inactivation and assayed for apparent CEA content. Recovery of added CEA was expressed as the percentage of exogenous CEA measured, 100 × (CEA added-endogenous CEA)/CEA added.

The standard dose/response curve expressed as logit percent (bound counts/total added activity) plotted against log calibrator concentration (μg/l) was found to be linear. Control or patient unknown dose concentrations were estimated by comparison with the standard curve using a computer program.

**Assay controls.** A total of six EDTA plasma pools were used for assay control. These were prepared by pooling patient samples in which CEA had been measured by the perchloric acid extraction method. The standard deviations for within-assay and between-assay replicates were calculated and expressed as the percentages of the means (coefficients of variation, CV). Samples from three plasma pools were re-assayed only in the heat-inactivation assay. Samples from the other three pools were assayed several times in both assays. All pooled plasma controls were found to be free of $HB_sAg$ by IRMA before use.

**Patient samples.** Eight plasma samples freshly collected from patients were assayed by both assays. These samples were then frozen, thawed, and reassayed by both assays twice more to determine whether freezing and thawing would alter the apparent CEA concentration.

Patients' blood specimens were collected into EDTA-coated tubes and the plasma was separated from the cells within several hours. Specimens were stored at −20°C before assay. Measurements were made by both assays in 109 specimens frozen for about 1 yr at −20°C, and in 81 specimens obtained prospectively during the course of this study. An additional 166 specimens obtained over the next 18 mo from patients with colon cancer were assayed for CEA using the heat-inactivation assay only. These specimens were stored at −20°C no longer than 1 wk before assay.

**Patients studied.** We studied 117 patients with adenocarcinoma of the colon or rectum. We excluded patients with squamous cell lesions or patients in whom there was history of a second primary carcinoma. Included were 29 patients with disease that did not penetrate the bowel wall (Dukes A), 42 whose carcinoma penetrated the bowel wall but who had no evidence of distant disease (Dukes B), 38 patients with tumor in regional lymph nodes (Dukes C), and eight other patients the extent of whose original disease was not recorded. Seven of these were referred with extensive metastatic disease and one was disease-free 4 yr after surgery. There were 60 males and 57 females who were 39 to 89 yr of age when we initiated our study. In 17 patients, a smoking history was not recorded; 59 patients never smoked, 22 were former smokers, and 27 smoked (22, cigarettes; three, cigars; two, pipe).

Plasma samples (138) collected from these 117 patients were assayed for CEA using perchloric acid extraction and the heat-inactivation procedures. One hundred four specimens were obtained from 93 patients who had no evidence of recurrent neoplasms. Ten samples were obtained preoperatively before resection of the primary tumor. Twenty-four samples were obtained from patients with documented recurrence of colon cancer.

We then followed these 117 patients for 18 mo, during which time an additional 166 CEA estimates were made using the heat-inactivation assay.

Twenty-three plasma samples were obtained from 22 patients with benign gastrointestinal disorders (polyps, seven; regional enteritis, five; diverticulitis, three; irritable bowel, three; bleeding ulcer, two; chronic ulcerative

colitis, one; nontropical sprue, one; appendicitis, one).

Plasma samples were obtained from 27 patients with chronic renal failure. All were chronically on hemodialysis. Samples were obtained immediately preceding dialysis, before administration of heparin. Two of the 27 have had malignancies (lymphoma, adenocarcinoma of the lung), but neither has evidence of residual disease. None of the other 25 has current evidence of malignancy.

All patients' medical records were reviewed to ascertain the diagnosis, clinical status, and smoking history. The medical records of the 117 patients with colon and rectal carcinoma were reviewed 18 mo later to document our initial impression of their clinical status and to obtain follow-up information.

## RESULTS

A representative calibration curve is shown in Fig. 1. We were reliably able to detect 0.5 μg CEA/l (1.5 μg CEA/l undiluted patient plasma). The 0.5 μg/l calibrator generally resulted in twice the zero calibrator counting rate. We observed similar CEA concentrations in samples of a plasma pool and assay calibrators heated for 15 min and 30 min. Minimal decreases in measurable CEA were seen after 60 min of heating and 25–50% lower concentrations were measured after 120 min.

Dilutions of 12 patients' sera parallel the standard dose/response curve. Results obtained in four of the 12 patients are shown in Fig. 2. Recoveries of CEA added to serum pools were 93–105%.

Replication of control pooled plasma samples for both assays is shown in Table 1.

Repeated freezing and thawing did not alter the apparent CEA concentrations as established by either assay. We observed no differences in the correlation between the two assays for samples collected prospectively and for samples stored frozen for about 1 yr.

CEA results obtained with both assays in 93 patients with no known residual or recurrent colon cancer are shown in Fig. 3. On the basis of these results, we chose 3.5 μg/l as the upper limit for "normal" for the heat-inactivation assay. The upper limit for the perchloric acid assay in our laboratory is 5 μg/l. Detection limits (1.6 μg/l for perchloric acid assay, 1.5 μg/l for heat-inactivation assay) are shown. These represent the lowest concentration calibrators used in each assay and are both greater than the zero calibrator + 2 s.d.

Of 117 patients with colon or rectal carcinoma, ten had CEA measured preoperatively, 11 had distant disease when we began our study, and ten developed recurrent or metastatic disease during the study period (21 mo). All 11 patients with distant disease had elevated CEAs by the heat-inactivation method (8.6 μg/l to 395 μg/l) when initially studied. In seven of the ten patients who developed recurrent or metastatic disease, CEA by heat inactivation was elevated (>3.5 μg/l) one to 13 mo before disease was otherwise clinically evident. CEA by heat inactivation remained normal in two patients 1 yr after recurrent disease was clinically obvious, and CEA was not measured again in the one other patient. There were two deaths due to colon carcinoma and one perioperative death, leaving 95 patients who were disease free 2 to 16 yr after surgery. There were three deaths not related to cancer in this group.

The distribution of CEA results obtained by heat inactivation in patients with no residual or recurrent carcinoma related to smoking history is shown in Fig. 4. We found no difference in CEA measurements made in males and females in this group.

CEA results obtained in 34 patients with known colon cancer (ten preoperative, 24 recurrent) are shown for both assays in Fig. 5.

CEA results in 22 patients with benign gastrointestinal diseases are shown in Fig. 6.

Results of CEA estimation in 27 patients on hemodialysis for chronic renal failure are shown in Fig. 7.

## DISCUSSION

Carcinoembryonic antigen has been associated with colon neoplasms and has been useful for the detection of recurrent malignancy (*2,3*). CEA is a glycoprotein whose measurement depends upon precipitation of interfering plasma proteins followed by quantitation by RIA. This has been accomplished by perchloric acid

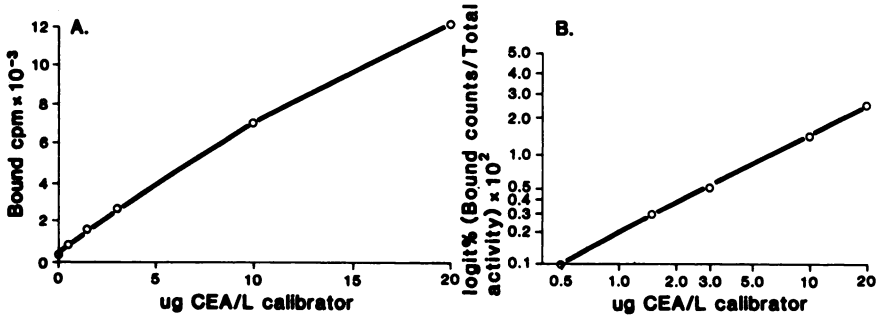


**FIG. 1.** CEA standard (calibration) dose/response curve obtained with heat-inactivation assay. (A) Linear-linear coordinates. (B) Logit-log coordinates.

**TABLE 1. PRECISION OF REPLICATE MEASUREMENTS OF PLASMA POOLS (HEAT INACTIVATION)**

| Plasma pool | Within-assay | | | Between-assay | | |
|---|---|---|---|---|---|---|
| | Replicates | $\bar{x}$(μg/l) | CV*(%) | Replicates | $\bar{x}$(μg/l) | CV(%) |
| PL | 12 | 2.1 | 11 | 18 | 2.3 | 10 |
| PM | 12 | 3.0 | 5 | 22 | 3.0 | 8 |
| PH | 12 | 13.5 | 10 | 22 | 13.8 | 9 |
| CL5 | | | | 27 | 1.8(3.0)† | 10(17) |
| CM6 | | | | 24 | 4.8(4.9) | 11(21) |
| CH6A | | | | 18 | 13.8(22) | 11(12) |

* CV = coefficient of variation.

† Values obtained by perchloric acid/dialysis are in ( ).

extraction followed by dialysis or by chromatography to remove the acid (*4–6*). The ionic strength and pH of the dialysate water critically affect the subsequent RIA results (*13*). The recovery of CEA following extraction and either dialysis or chromatography is not routinely measured but may be variable, contributing to imprecision (*14*). More recently, a procedure for CEA extraction by heating has been developed and forms the basis for commercial immunoassays using either radioactive or enzyme labels† (*7*).

The solid-phase anti-CEA heat-inactivation assay is able to detect about 0.5 μg/l CEA in the assay tube or 1.5 μg/l in undiluted patient plasma (Fig. 1). As marketed, the lowest concentration calibrator permits detection of 4.5 μg CEA/l in undiluted patient plasma. Our observations in patients with colon cancer but without residual or recurrent disease (Fig. 3) suggest that greater sensitivity is desirable. A 0.5 μg/l calibrator may be prepared by dilution of any of the calibrators of higher concentration. The counting rate in this 0.5 μg/l calibrator is about twice that of the zero calibrator.

In agreement with Kim et al. (*7*), extraction of CEA from a plasma pool was similar in samples heated 15 and 30 min. Further heating resulted in a decline in apparent CEA concentrations.

Precision of the heat-inactivation assay was better than that which we routinely obtained with the perchloric acid extraction assay (Table 1). Recovery of CEA (calibrator) added to plasma samples was satisfactory and patient samples diluted proportionately (Fig. 2).

We found no differences in apparent CEA concentrations after plasma samples were frozen and thawed twice. We have now used pooled plasma controls frozen for over 6 mo without observing changes in apparent concentration.

Concurrent measurements in pooled plasma controls, as well as in patient samples, suggest that results obtained following heat inactivation are quantitatively lower than those obtained after perchloric acid extraction. The correlation between results obtained with the two assays was nonetheless excellent (r = 0.88, 0.90 for colon cancer patients with disease and renal dialysis patients) in the patient populations we studied.

We have chosen an upper limit for "normal" for the heat-inactivation assay of 3.5 μg/l based on multiple measurements made in patients after colon cancer resection with long (2–16 yr) disease-free intervals. The only patients with CEA concentrations following heat inactivation >3.5 μg/l in this group of patients during a 21-mo period were smokers (Fig. 4). While smoking

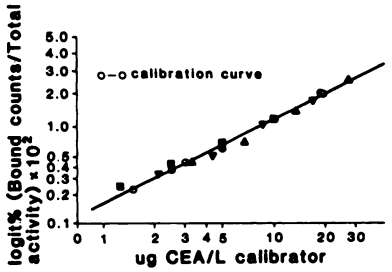


**FIG. 2.** Dilutions of heat-inactivated patients' sera compared with standard dose/response curve. (O—O-standards; other symbols represent dilutions of patients' sera).

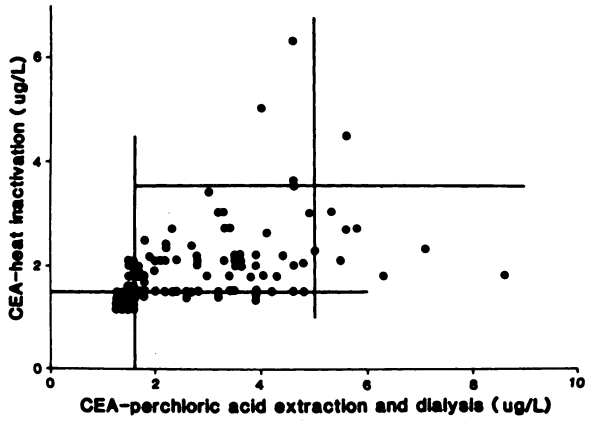


**FIG. 3.** CEA results obtained after PCA extraction and dialysis or by heat inactivation in patients with resected colon cancer and no known residual or recurrent disease.

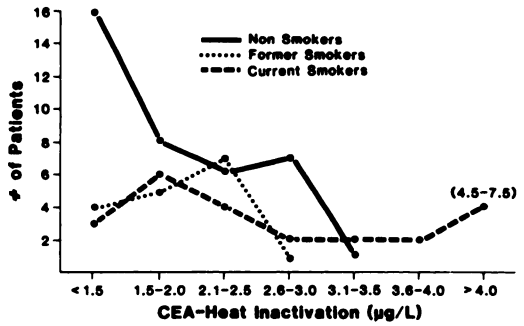


FIG. 4. CEA results (heat inactivation) observed in smokers, former smokers, and nonsmokers. Result for each patient is mean of several measurements. All patients are disease free after removal of colon cancer.

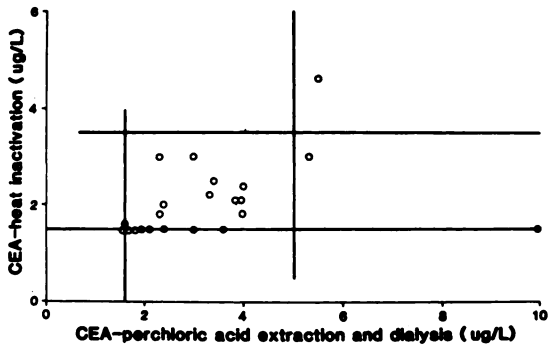


FIG. 6. CEA results obtained after PCA extraction and dialysis or by heat inactivation in patients with benign gastrointestinal diseases.

may account for the CEA concentrations >3.5 µg/l following heat inactivation shown in Fig. 3, only two of the five patients accounting for the eleven elevated CEA measurements made following perchloric acid extraction were smokers. While CEA measurements in aged patients without significant disease may be of interest, we believe a valid reference range can be derived from apparently disease-free patients who have had colon cancer. Patients whose results are shown in Fig. 3 were followed an additional 18 mo, which makes it unlikely that clinically significant disease was present at the time those measurements were made. At our institution, CEA measurements are used clinically to detect recurrent or metastatic colon or rectal adenocarcinoma. If CEA measurements are made in a clinically dissimilar population, then our reference range might not be applicable.

The ability to detect the presence of colon cancer was similar for both assays (Fig. 5). There was no significant difference between the results obtained by the two assays in patients with benign gastrointestinal diseases (Fig. 6). As has been reported previously, CEA may be elevated in plasma from some patients with non-neoplastic bowel diseases (*15*). As reported for the perchloric acid assay (*16*) that CEA concentrations may be elevated by the heat-inactivation assay in patients on hemodialysis for chronic renal failure (Fig. 7). As observed in other studies (*17*) using perchloric acid extraction, smoking may elevate the CEA estimate made using heat inactivation (Fig. 4).

In this study we demonstrated CEA elevation (heat inactivation) that preceded clinically apparent disease in seven of ten patients with recurrence during a 21-mo study period. Because we made no measurements using the perchloric acid extraction assay during the follow-up period, we cannot state whether either assay may detect recurrent colon cancer before the other. We have no experience with the recently modified Roche RIA (second antibody rather than zirconyl phosphate separation of bound and free fractions). This assay is still preceded by perchloric acid extraction followed by dialysis or column chromatography.

In conclusion, although the heat-inactivation assay systematically quantitates less "CEA" than does the perchloric acid assay, we find the clinical results of both assays are essentially equivalent in patients with colon cancer, benign gastrointestinal diseases, and chronic renal failure on hemodialysis. Because the heat-inactivation assay is technically easier, less time-consuming, and more precise than the perchloric acid extraction

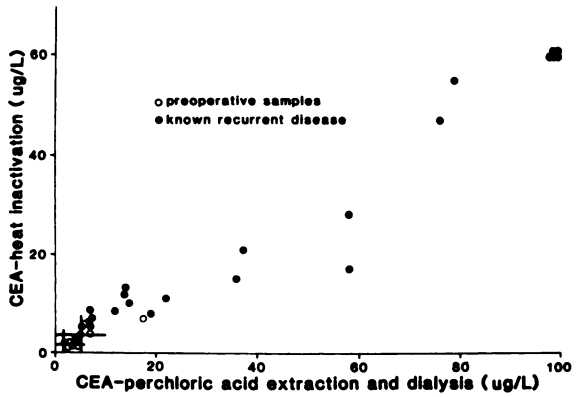


FIG. 5. CEA results obtained after PCA extraction and dialysis or by heat inactivation in patients with known colon cancer. (y = 1.7 + 0.55x; correlation coefficient—excluding samples above or below detection limits of either assay—was 0.88).

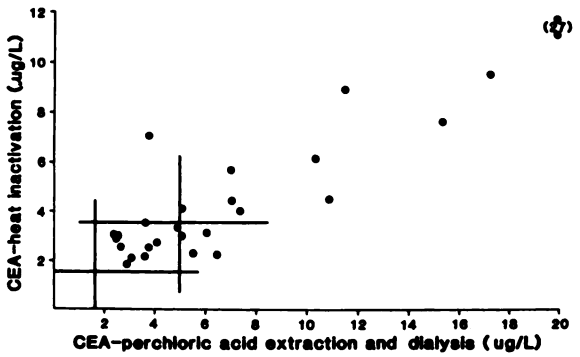


FIG. 7. CEA results obtained after PCA extraction and dialysis or by heat inactivation in patients on dialysis for chronic renal failure. (y = 1.4 + 0.42x; correlation coefficient was 0.90).

assay, we believe it offers an attractive alternative to the perchloric acid dialysis assay.

FOOTNOTES

* Hoffman La Roche, Nutley, NJ.
† Abbott Laboratories, North Chicago, IL.

REFERENCES

1. GOLD P, FREEDMAN SO: Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J Exp Med* 121:439-462, 1965
2. SHANI A, O'CONNELL MJ, MOERTEL CG, et al: Serial plasma carcinoembryonic antigen measurements in the management of metastatic colorectal carcinoma. *Ann Intern Med* 88:627-630, 1978
3. LADENSON JH, MCDONALD JM: Colorectal carcinoma and carcinoembryonic antigen (CEA). *Clin Chem* 26: 1213-1220, 1980
4. THOMSON DMP, KRYPEY J, FREEDMAN SO, et al: The radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system. *Proc Natl Acad Sci USA* 64:161-167, 1969
5. HAAGENSEN DE, JR, EASTERDAY RL, STOLLE CA, et al: Buffer-exchange column for rapid separation of carcinoembryonic antigen from perchloric acid. *Clin Chem* 24: 135-137, 1978
6. WEISSMAN EB: Carcinoembryonic antigen assayed by column chromatography on polyacrylamide gel. *Clin Chem* 26:770-771, 1980
7. KIM YD, TOMITA JT, SCHENCK JR, et al: Extraction of human plasma or sera by heat treatment for a solid-phase radioimmunoassay of carcinoembryonic antigen. *Clin Chem* 25:773-776, 1979
8. HIRAI H: A collaborative clinical study of carcinoembryonic antigen in Japan. *Cancer Res* 37:2267-2274, 1977
9. MAIOLINI R, BAGREL A, CHAVANCE C, et al: Study of an enzyme immunoassay kit for carcinoembryonic antigen. *Clin Chem* 26:1718-1722, 1980
10. FABRICATORIAN D, GALLAGHER ND: Evaluation of a commercial enzyme radioimmunoassay kit for serum carcinoembryonic antigen. *Ann Clin Biochem* 18:248-251, 1981
11. CHRISTENSON RH, MILES B, HAMMOND JE: Roche and Abbott Carcinoembryonic antigen assays compared. (lett) *Clin Chem* 28:561, 1982
12. GERFO PL, KRUPEY J, HANSEN HJ: Demonstration of an antigen common to several varieties of neoplasia. *N Engl J Med* 285:138-141, 1971
13. LADENSON JH, BELL CE, JR: pH measurement problems affecting assay of carcinoembryonic antigen. *Clin Chem* 21:255-257, 1975
14. WITHERSPOON LR, SHULER SE, ZOLLINGER LA, et al: An evaluation of prepackaged columns for the separation of carcinoembryonic antigen from perchloric acid: Concise communication. *J Nucl Med* 21:248-250, 1980
15. MOORE TL, KANTROWITZ PA, ZAMCHECK N: Carcinoembryonic antigen (CEA) in inflammatory bowel disease. *JAMA* 222:944-947, 1972
16. BRANDSTETTER RD, GRAZIANO VA, WADE MJ, SAAL SD: Carcinoembryonic antigen elevation in renal failure. *Ann Intern Med* 91:867-868, 1979
17. ALEXANDER JC, SILVERMAN NA, CHRETIEN PB: Effect of age and cigarette smoking on carcinoembryonic antigen levels. *JAMA* 235:1975-1979, 1976