Morphine Radioimmunoassay Specificity Before and After Extraction of Plasma and Cerebrospinal Fluid

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Abstract □ Currently available morphine radioimmunoassays, using antiserum to 3-*O*-carboxymethylmorphine, lack sufficient specificity for clinical pharmacokinetic studies following repeated oral doses due to the relatively high plasma concentrations of cross-reacting metabolites. A procedure is described for the recovery of the drug and removal of its polar metabolites. The single-step solvent extraction recovered 97% (*CV* 6.9%) morphine and none of the major inactive metabolite, morphine-3-glucuronide. Extracted and nonextracted morphine radioimmunoassay standard curves had comparable slopes, precision, and I_{50} values. Cross reactivity between morphine-3-glucuronide and the antiserum was eliminated when the radioimmunoassay was preceded by extraction. Without prior extraction, the apparent plasma morphine concentration following repeated oral doses was dilution dependent. In contrast, concentration was dilution independent when the radioimmunoassay was preceded by extraction. The plasma morphine concentration in 23 cancer patients at 4 hr following their previous dose (calculated to 10 mg of base) was 26 ng/ml (95% confidence interval (CI), 20–33 ng/ml) with prior extraction, as compared with the apparent concentration of 80 ng/ml (95% CI, 64–96 ng/ml) without extraction. These data indicate that by combining prior extraction with radioimmunoassay, specific, steady-state plasma morphine levels can be obtained following repeated oral doses. However, no significant differences were observed between extracted and nonextracted morphine concentrations in ventricular cerebrospinal fluid from four patients who had received a single intravenous dose. The extraction procedure, prior to radioimmunoassay, provides the specificity required for the measurement of morphine in biofluids that contain relatively high concentrations of cross-reacting metabolites.

Keyphrases □ Morphine—radioimmunoassay specificity before and after extraction of plasma and cerebrospinal fluid □ Radioimmunoassay—morphine, specificity before and after extraction of plasma and cerebrospinal fluid

While the development of a sensitive morphine radioimmunoassay (1) has allowed measurement of clinical pharmacokinetic parameters following single parenteral morphine dosing (2–9), there is limited pharmacokinetic information available following repeated and oral dosing (10). Morphine-3-glucuronide, the major biotransformation product, cross reacts with morphine antisera (1, 11–13). Cross reactivity does not appear to be a significant factor following single parenteral dosing due to the limited amount of metabolite present in plasma and to its relatively low affinity for morphine antisera (3). However, with repeated and oral dosing, the ratio of plasma morphine-3-glucuronide to morphine is likely to be much higher due to the first-pass effect (14) and consequent accumulation of the inactive metabolite. Under these circumstances, most currently available morphine radioimmunoassays are lacking in specificity. While there are other methods available for the measurement of morphine, such as those employing derivatization followed by GLC (15, 16) and that employing extraction followed by scintillation counting of radiolabeled drug (14), an appropriately specific radioimmunoassay would be desirable to many investigators for further studies of the clinical pharmacokinetics of morphine.

The objective of the studies reported here was to demonstrate that an extraction procedure, preceding an otherwise nonspecific radioimmunoassay, provides the specificity required for the measurement of unconjugated morphine in plasma following repeated oral dosing. The utility of the method is demonstrated by the measurement of extracted and nonextracted steady-state plasma morphine concentrations in hospice patients who had been receiving oral morphine doses for the relief of pain due to advanced cancer. These results are contrasted with those obtained by the morphine radioimmunoassay of extracted and nonextracted serial samples of cerebrospinal fluid from cancer patients with chronic pain who had received single intravenous injections of morphine.

EXPERIMENTAL

The extraction procedure is a modification of the first step in a previously reported multistep procedure (17).

Solutions—Glassware for morphine extraction and for storage of morphine standard solutions was siliconized[1]. Morphine standards[2] were added to control plasma in concentrations of 0.31, 0.62, 1.2, 2.5, 5.0, 10, and 20 ng of free base/ml. The extraction buffer, pH 8.6–8.7, contained 40% (w/v) K_2HPO_4 and was sodium chloride saturated. The extraction organic solvent was chloroform–2-propanol (3:1). The extract was reconstituted with 0.1% (w/v) gelatin[3] in 0.01 *M* phosphate-buffered saline, pH 7.3 (assay buffer).

Procedure—An aliquot (0.5 ml) of each plasma standard and of the patient's plasma (up to 0.5 ml) was added to distilled water (1.0 ml) and extraction buffer (1.0 ml) in 15-ml screw-top centrifuge tubes. The varied volumes of the patients' plasma were adjusted to a common volume (0.5 ml) by the addition of control plasma. Each tube was vortexed. The organic solvent (6 ml) was added, and the tubes were capped and shaken for 10 min. The tubes were then centrifuged for 5 min at 300×*g*. The upper aqueous phase and the lipid interphase were aspirated off, and an aliquot (3.0 ml) of the lower organic phase was transferred to a 12-ml conical centrifuge tube. The organic phase was evaporated to dryness in a water bath at 83°. The dry extract was reconstituted with assay buffer (0.5 ml). The tubes were vortexed, and the reconstituted extract was frozen until radioimmunoassay.

Morphine and Morphine-3-glucuronide Recovery Studies—[6-^{3}H]morphine[4] (1.0, 2.0, 4.0, 5.0, 8.0, and 25 ng) was added to control plasma and extracted. Results from scintillation[5] counting of these samples at full tritium window were compared with those of identical amounts of tritiated morphine which had been added directly to the organic phase (3 ml, which had been previously shaken with buffered control plasma and water), evaporated to dryness, and reconstituted. Extracted and nonextracted [^{14}C]morphine-3-glucuronide[6] (40 μg) was also treated as described above.

The radioimmunoassay is a modification of a previously reported procedure (1, 3).

Solutions—A suspension of 0.1% (w/v) dextran[7] and 1.0% (w/v)

[1] Prosil-28, PCR Research Chemicals, Inc., Gainsville, Fla.
[2] Applied Science Labs, Inc., State College, Pa.
[3] J. T. Baker Chemical Co., Phillipsbur;, N.J.
[4] NET-445, New England Nuclear Corp., Boston, Mass.
[5] Riaflour, New England Nuclear Corp., Boston, Mass.; model LS-3133P, Beckman Instruments, Fullerton, Calif.
[6] Supplied by Dr. Amand Misra, Narcotic and Drugs Research, Inc., Brooklyn, N.Y.
[7] Dextran T70, Pharmacia Chemicals, Uppsala, Sweden.

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Table I—Influence of Morphine-3-glucuronide Cross Reactivity on the Measurement of Unconjugated Morphine

Parts Morphine-3-glucuronide: Morphine Added to Control Plasma		Ratio of Morphine Equivalents Measured by Radioimmunossay to Morphine Added	
Parts	N	Extracted	Nonextracted
0:1	9	1.00 ± 0.03	1.01 ± 0.05
10:1	9	1.08 ± 0.07	1.79 ± 0.11
40:1	9	1.02 ± 0.06	2.51 ± 0.21

charcoal[8] in assay buffer was used for the adsorption of unbound morphine. The radioligand, [7,8-^{3}H]dihydromorphine[9] with a specific activity of 49 Ci/mmole at a concentration of 1.0 mCi/ml of ethanol, was diluted (1:2000) in ethanol to obtain ~20,000 cpm/50 μl. Morphine antiserum[10] was prepared from the blood of New Zealand white rabbits immunized by the administration of 3-carboxymethyl-morphine–bovine serum albumin every 2–3 months for 1 year (18). Antibody dilution studies resulted in 50% binding of the radioligand at an antibody dilution of 1:6000 in 0.01 *M* phosphate-buffered saline (pH 7.3) when 100 μl of diluted antibody was incubated in a final assay volume of 450 μl.

Procedure—Diluted antiserum (100 μl) was added to assay tubes (10 × 75-mm disposable culture tubes) containing the reconstituted plasma extract (200 μl of the original 0.50 ml for standards and 50–200 μl of the original 0.50 ml for patients' samples), radioligand (50 μl), and control plasma (100 μl) to yield a final volume of 450 μl. If necessary for patients' samples, assay buffer was added to yield the 450-μl final volume. Two sets of duplicate background tubes containing no antibody or unlabeled morphine and two sets of triplicate maximum-binding tubes containing no unlabeled morphine were included. Standards and unknowns were assayed in duplicate. The tubes were incubated at room temperature for 1 hr. After incubation, the dextran–charcoal suspension (0.50 ml), which was kept stirring in an ice bath, was added to each tube. The tubes were vortexed and placed in an ice bath for 10 min. The tubes were then centrifuged at 4° and 1500×*g* for 15 min. An aliquot (0.50 ml) of the supernatant was removed from each tube for scintillation counting. A logit-log transformation[11] was used for the linearization of the standard curve and the calculation of the amount of morphine in patients' samples.

Nonextracted radioimmunoassay conditions were identical to those described above for the extracted radioimmunoassay except that 100-μl aliquots of the plasma morphine standards were added to the assay buffer (200 μl) along with the usual amounts of radioligand and antibody solutions. Patients' plasma samples were added in 10–100-μl volumes with any volume differences adjusted by the addition of control plasma to yield a final plasma volume of 100 μl and a final incubation volume of 450 μl as in the extracted radioimmunoassay. Morphine concentrations in cerebrospinal fluid were determined by radioimmunoassays before and following extraction as described for plasma.

Specificity Studies—*Morphine-3-glucuronide Affinity Study*—A morphine-3-glucuronide[12] standard curve was generated by the substitution of the metabolite for morphine in plasma standards which were prepared in 10 times the concentrations (weight basis) as the morphine plasma standards. Conditions of the radioimmunoassay were identical to those described above for the nonextracted morphine standard curve.

Morphine-3-glucuronide Cross Reactivity Study—Three different sets of standards in control plasma were prepared as follows: set I, morphine standards in concentrations of 0.62, 5, and 20 ng/ml; set II, morphine standards as in set I with 10-fold amounts of morphine-3-glucuronide added; set III, morphine standards as in set I with 40-fold amounts of morphine-3-glucuronide added. Triplicates of each standard were assayed before and after extraction as described above for patients' plasma.

Variable Dilutions Study—Plasma samples from two hospice patients (described in the following section) were assayed prior to extraction in graded dilutions of 1:10, 1:50, and 1:250 and following extraction in graded dilutions of none, 1:5, and 1:25.

[8] Norit-A Decolarizing Carbon, Fisher Scientific Co., Fair Lawn, N.J.
[9] New England Nuclear Corp., Boston, Mass.
[10] Supplied by Drs. S. Spector and B. Berkowitz, Roche Institute of Molecular Biology, Nutley, N.J.
[11] Radioimmunoassay Program of Clinical Lab and Nuclear Medicine Pac for HP-97 calculator, Hewlett-Packard Co., Cupertino, Calif.
[12] Supplied by Dr. R. Willette, National Institute on Drug Abuse, Rockville, Md.

Table II—Morphine Equivalents Determined by Radioimmunoassay in Varied Dilutions of Patients' Plasma Before and Following Extraction

	Extracted Plasma		Nonextracted Plasma	
Patient	Dilution	Morphine, ng/ml	Dilution	Morphine, ng/ml
1	None	19.0	1:10	65
	1:5	17.8	1:50	122
	1:25	17.4	1:250	226
2	None	17.0	1:10	74
	1:5	19.6	1:50	130
	1:25	18.6	1:250	226

Applications—Extracted and nonextracted radioimmunoassay procedures were applied to the measurement of morphine equivalents in two sets of biofluids: a set of plasma samples in which the ratio of morphine-3-glucuronide to morphine might be expected to be relatively high and a set of cerebrospinal fluid samples in which the ratio would be expected to be considerably lower.

Plasma Morphine after Repeated Oral Dosing—The patients[13] (Group I) were being treated with morphine for relief of pain due to advanced cancer. There were 23 patients (10 men and 13 women) ranging in age from 42 to 84 years. They had been receiving oral doses of either 5, 10, 20, 30, 45, or 60 mg of morphine sulfate every 4 hr for at least 48 hr and had been receiving oral morphine for at least 1 week before being stabilized at their given doses. Blood samples were obtained at 4 hr following their previous medication. The plasma was recovered and assayed for morphine equivalents prior to and following extraction.

Cerebrospinal Morphine after a Single Intravenous Dose—The patients[14] (Group II) were being treated for the relief of chronic pain. There were four men (34, 45, 56, and 67 years old) who had received a single intravenous injection of 10 mg of morphine sulfate. An indwelling intraventricular catheter and Ommaya reservoir had been implanted in these patients for the introduction of chemotherapeutic agents as part of their treatment for cancer. This allowed the collection of ventricular cerebrospinal fluid samples (5, 10, 15, and 30 min and 1, 2, 4, 8, and 24 hr postdrug). Samples were assayed for morphine prior to and following extraction. The results of an initial nonextracted assay of these samples were previously reported (19).

RESULTS AND DISCUSSION

The extraction of added radiolabeled morphine (1.0–25 ng) to plasma resulted in a mean recovery of 97% (range, 88–104%; *CV*, 6.9%), with no indication of any trend between the amount of morphine added to plasma and the amount recovered. The extraction of 40 μg of radiolabeled morphine-3-glucuronide from plasma resulted in a consistent recovery of 0%. These data indicate that the extraction procedure effectively eliminates the metabolite while recovering the parent drug with acceptable loss and variation. The consistency of morphine recovery is more important than the absolute percentage recovery, since the assay is designed to treat the standards and unknowns in the same manner. Any losses of morphine in the extraction procedure would be reflected in the standards and unknowns to the same extent. High and consistent recovery is achieved, however, with thorough siliconization of the extraction tubes.

Assuming 100% recovery of extracted morphine and taking aliquot losses into consideration, the assays had been designed to yield the same amounts of morphine in each of the standard curves (0.031, 0.062, 0.12, 0.25, 0.50, 1.0, and 2.0 ng). Extracted and nonextracted standard curves were comparable with respective mean slopes of 2.4 ± 3.3% (*CV* from day to day) and 2.6 ± 6.7%, regression correlation coefficients of 0.996 ± 0.3% and 0.997 ± 0.1%, and amounts of morphine which inhibit 50% of radioligand binding equivalent to 0.16 ng ± 3.0% and 0.18 ng ± 14%. The intra-assay variation (*CV*) for duplicate standards was 4.5 and 2.8% for extracted and nonextracted assays, respectively. These data demonstrate that the assays are quite reproducible with acceptable variations in the various parameters for both standard curves. In addition, the data for extracted assays support the consistency of extraction recovery results obtained with radiolabeled morphine standards.

When morphine-3-glucuronide was substituted for morphine in the nonextracted standard curve, a slope of 1.2 was obtained as compared with the slope of 2.6 for morphine. At 25, 50, and 75% displacement of

[13] Inpatients at St. Christopher's Hospice in Sydenham, England.
[14] Inpatients at Memorial Sloan-Kettering Cancer Center, New York, N.Y.

Table III—Morphine Equivalents in Plasma after Repeated Oral Dosing (Group I) and in Cerebrospinal Fluid after a Single Intravenous Dose (Group II) Before and Following Extraction

Patient Group	N	Extracted Samples Morphine, ng/ml	Nonextracted Samples Morphine, ng/ml
Group I	23	26 ± 3.2	80 ± 7.6
Group II	4		
5 min	3	3.2 ± 1.4	3.3 ± 2.1
10 min	4	3.9 ± 1.0	3.8 ± 1.5
15 min	3	5.9 ± 1.6	7.0 ± 2.3
30 min	4	7.1 ± 0.8	7.4 ± 2.0
1 hr	4	7.8 ± 0.8	7.6 ± 1.8
2 hr	4	5.1 ± 1.1	5.2 ± 1.1
4 hr	4	4.8 ± 0.9	5.2 ± 1.4
8 hr	4	3.6 ± 1.2	4.1 ± 1.2
24 hr	2	0.4 ± 0.3	1.1 ± 0.4

radioligand binding, 9.4, 28, and 84 times as much glucuronide as morphine (weight basis) was required. Thus, the ratio of metabolite to parent drug which displaces equivalent amounts of radioligand from the antibody is dependent on the total extent of radioligand displacement. It has previously been reported that standard curves for morphine and its major metabolite (when substituted for morphine in a morphine radioimmunoassay) are not parallel (13), and that the commonly used index of cross reactivity, the ratio of the amounts of two compounds at I_{50}, can be misleading (13).

A more direct approach to examining the cross reactivity of two compounds is to assay varying combinations of them. When morphine-3-glucuronide was added to selected plasma morphine standards in 10- and 40-fold amounts, significantly higher morphine equivalents were obtained as compared with when no metabolite was added in the assays not preceded by extraction (Table I). Ten- and 40-fold amounts of the metabolite resulted in overestimates of 1.8 and 2.5 times as much morphine, respectively. The degrees of overestimation of morphine, when graded amounts of metabolite are added and assayed without prior extraction, are reasonably consistent with what would be predicted on the basis of the ratio of amounts of compounds at I_{50} derived from separate standard curves. However, when the assay was preceded by extraction of duplicate samples of the above, no significant differences were observed among morphine equivalents. These data indicate that the extraction effectively removes morphine-3-glucuronide and are consistent with the results of the recovery study.

One of the methods employed for the validation of the specificity of a radioimmunoassay is the demonstration that the final concentration of the component being measured in a particular sample is independent of the dilution at which it is assayed (20). Table II illustrates that this criterion is fulfilled when the assay is preceded by extraction, but that this is not the case when the assay is performed without prior extraction. These data indicate that the extraction removes morphine metabolites from plasma which cross react with the morphine antibody. The higher dilutions resulted in higher morphine equivalents in the nonextracted assay. This is consistent with the greater degree of cross reactivity between morphine and its metabolite at lower degrees of label displacement observed in the comparison of their standard curves.

The mean morphine concentration (calculated as a common dose of 10 mg of base) in the 23 hospice patients' plasma was 26 ng/ml (95% confidence internal (CI), 20–33 ng/ml) when assayed following extraction as compared to 80 ng/ml (95% CI, 64–96 ng/ml) when assayed without extraction (Table III). Nonextracted morphine equivalents ranged from ~1 to 10 times the extracted levels, but since these ratios are highly dependent upon the dilution of nonextracted plasma, which varied, they cannot be taken as an estimation of the actual ratios of metabolite to parent drug. Nevertheless, the average threefold difference ($p < 0.001$) indicates considerable cross-reacting metabolite(s) in plasma as compared with unconjugated morphine. These data are consistent with the significant first-pass effect for oral morphine, which results in 20–33% bioavailability (14). A molar ratio of glucuronide to unconjugated morphine of 1.8 has been reported at 4 hr following a single intravenous dose of morphine (3).

Steady-state 4-hr trough plasma levels of morphine in a comparable patient group receiving morphine according to a comparable dosing regimen have been reported to be 34 ng/ml (95% CI, 21–48 ng/ml) when adjusted to a common dose of 10 mg of morphine base (10). A nonextracted radioimmunoassay was employed in these latter studies. The mean morphine concentration was significantly ($p < 0.05$) higher (31%) than the concentration reported here. The earlier report used morphine antiserum which had been raised against 6-succinyl morphine–bovine serum albumin, rather than against a 3-position antigen. Thus, the antiserum would have a lower affinity for morphine-3-glucuronide as compared with the antiserum used in this study. While they reported a cross-reactivity of <10% in their radioimmunoassay, even this degree of specificity would not be acceptable for situations in which there is a high ratio of glucuronide to free morphine.

Table III details the results of the radioimmunoassay of extracted and nonextracted cerebrospinal fluid samples which had been obtained from four cancer patients with chronic pain following the intravenous injection of 10 mg of morphine sulfate. In contrast to the extracted and nonextracted plasma levels of patients receiving repeated oral morphine doses, there is no significant ($p > 0.3$; n, 32; paired t test) difference (5.9%) between extracted and nonextracted morphine equivalents in the cerebrospinal fluid following a single intravenous dose. These data are consistent with reports of the limited access of morphine metabolites to the cerebrospinal fluid (21).

A most specific morphine antiserum, raised against *N*-carboxymethylmorphine, has a morphine-3-glucuronide–morphine ratio of ~350–I_{50} (22). This antiserum, however, is not readily available. But even the same antigen in the same individual animal can result in batches of morphine antisera of widely differing specificity (13). Thus, short of the generation of a readily available monoclonal antibody specific for morphine, it is not likely that the numerous investigators of the clinical pharmacokinetics of morphine will be able to employ a common antiserum. While radioimmunoassays may be sufficient for accurate determination of plasma morphine following single parenteral doses, studies of the clinical pharmacokinetics of morphine following repeated and/or oral dosing should incorporate more specific methodology. Because the sensitivity and relative ease of carrying out the radioimmunoassay has made this method more acceptable to many investigators than the use of other methods, the single-step extraction procedure, prior to radioimmunoassay, provides an alternative to the adaptation of other methods for morphine measurement in human biofluids.

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Radiolytic Degradation Scheme for ^{60}Co-Irradiated Corticosteroids

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Abstract □ The cobalt 60 radiolytic degradation products have been identified in the following corticosteroids: cortisone, cortisone acetate, hydrocortisone, hydrocortisone acetate, hydrocortisone sodium succinate, isoflupredone acetate, methylprednisolone, methylprednisolone acetate, prednisolone, prednisolone acetate, and prednisone. Two major types of degradation processes have been identified: loss of the corticoid side chain on the D-ring to produce the C-17 ketone and conversion of the C-11 alcohol, if present, to the C-11 ketone. Minor degradation products derived from other changes affecting the side chain are also identified in several corticosteroids. These compounds are frequently associated in corticosteroids as process impurities or degradation compounds. No new radiolytic compounds unique to ^{60}Co-irradiation have been found. The majority of corticosteroids have been shown to be stable to ^{60}Co-irradiation. The rates of radiolytic degradation ranged from 0.2 to 1.4%/Mrad.

Keyphrases □ Corticosteroids—^{60}Co-irradiated, radiolytic degradation scheme □ Degradation—scheme for ^{60}Co-irradiated corticosteroids □ Irradiation—radiolytic degradation of corticosteroids

The U.S. Food and Drug Administration (FDA) has proposed strict limits on allowable residual quantities of ethylene oxide and its major reaction products in drugs because of possible mutagenic and carcinogenic properties of ethylene oxide (1). Faced with the possibility of increased regulatory pressure, the replacement of ethylene oxide with steam or ^{60}Co-irradiation is a major goal of a sterilization alternative program. One phase of this program is to conduct the experimental work required to demonstrate the feasibility of sterilizing bulk drugs and formulated products with ^{60}Co-irradiation.

Recently, the FDA published a proposal regulating irradiated foods for human consumption (2). The proposal permits irradiation of any food at a dose not >100 Krads without the additional safety data. The proposal also permits irradiation of foods at a dose of ≤5 Mrads if the foods comprise only a minor portion (NMT 0.01%) of the daily diet. The proposal includes guidelines for toxicological testing of other foods irradiated at a dose >100 Krads. The proposal is based on projected daily human consumption of radiolytic degradation compounds (3).

The daily dose of pharmaceutical products is substantially less than the amount of foods consumed. Information determining the rate of degradation and identifying degradation compounds unique to irradiation, therefore, should be sufficient to examine the feasibility of ^{60}Co-irradiation as an alternate method for sterilizing pharmaceutical materials and products. The present report provides such information for corticosteroids.

EXPERIMENTAL

^{60}Co-Irradiation—The irradiation source[1], consisting of several rods containing ^{60}Co-pellets mounted on a plaque, was raised from a pool of water into a stationary position for irradiation. Samples were placed in totes on two tiers surrounding the cobalt 60 source. A shuffle–dwell system averaged the radiation gradient around the source by periodically shifting the tote horizontally and vertically. Other samples were stationed at calibrated points where the radiation intensity was more precisely known.

Absorbed irradiation doses were measured potentiometrically using a ceric-cerous dosimeter[2] (4, 5) and spectrophotometrically using a red perspex dosimeter[3].

High-Performance Liquid Chromatography (HPLC) Appara-

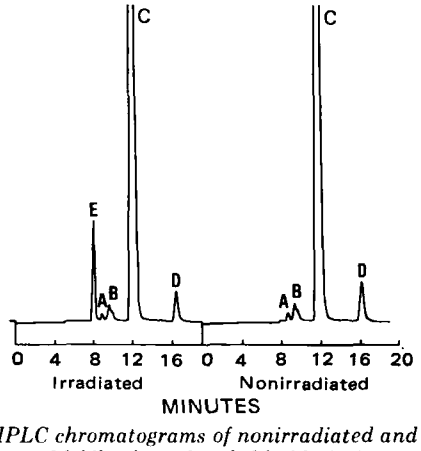

Figure 1—*HPLC chromatograms of nonirradiated and irradiated (6 Mrads) cortisone. Mobile phase: butyl chloride (50% water saturated)–tetrahydrofuran–methanol–glacial acetic acid (950:70:35:30). Column: Brownlee SI-100. Peak identification: (A) cortisone acetate; (B) hydrocortisone acetate; (C) cortisone; (D) hydrocortisone; (E) 4-androstene-3,11,17-trione.*

[1] All irradiations were conducted at a facility of Isomedix Inc., Morton Grove, Il.

[2] Atomic Energy of Canada Limited, Ottawa, Canada.

[3] United Kingdom Atomic Energy Authority, Harwell, Oxon, U.K.

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