with peaks 3 mm or greater were considered to be significant. Peaks less than 3 mm, while discernible, were not reproducible and their usage could cause occasional interference from the background. Chromatograms at the 5-ppb level were free from extraneous peaks in the methylprednisolone area (Figure 2).

When five lactating Holstein cows were treated with three Lincocin Forte infusions in each quarter 12 hr apart, methylprednisolone was detected 12 hr after the last treatment (Table II). The residues were higher in those samples from the p.m. milking than from the a.m. milking. Although the study was set up for 12 hr, the actual time elapsed between the treatment and subsequent milking was less for the p.m. samples (10 hr) than it was for the a.m. samples (14 hr). This and the fact that smaller quantities of milk were obtained in the p.m. milking would account for the higher values. No detectable residues at the sensitivity of the method (5 ppb) were found in the milk from the five lactating cows collected at 24, 36, and 48 hr posttreatment. Since the samples did not exceed 5 weeks of storage (known period of stability), loss of methylprednisolone by degradation was not a factor.

A total of 60 mg of methylprednisolone was introduced into the udder over the treatment period. Based on the residue found and the quantity of milk excreted, the amount of the total dose recovered in the milk during the first 12 hr after treatment was 1.33% (Table II). Therefore, Lincocin Forte treatment does not result in excessive amounts of the dose in the milk.

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Gas Chromatographic Determination of Cyclophosphamide Residues in Sheep Tissues

Charlie Jackson, Jr.,* and Paul J. Reynolds

Tissue samples were extracted with methanol, cleaned up by isooctane and hexane extractions and Florisil chromatography, and gas chromatographed on 1% Versamid on 100/120 mesh Gas Chrom Q using a phosphorus-sensitive detector. Muscle tissue samples fortified to contain 0.100 to 0.010 ppm of cyclophosphamide {2-[bis(2-chloroethyl)-amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide} gave recoveries of 97 to 110%. Liver samples gave recoveries of about 60%.

The anti-tumor agent cyclophosphamide (CPA) has considerable promise as a practical chemical defleecing agent for sheep (Dolnick *et al.*, 1969; Lindahl *et al.*, 1970). A single oral dose of drug results in a gradual loosening of the fleece, which can then be removed within 7 to 21 days. Colorimetric methods for determination of CPA based on its acid hydrolysis followed by its reaction with 4-(*p*-nitrobenzyl)pyridine in alkaline solution (Hurata *et al.*, 1967; Friedman and Boger, 1961) are not sufficiently sensitive nor specific for measuring traces in tissues. The gas–liquid chromatography of CPA has apparently not been reported before.

REAGENTS AND APPARATUS

Methanol, hexane, 2,2,4-trimethyl pentane (isooctane), petroleum ether (30–60°C), and ethyl acetate were pesticide quality. Gas Chrom Q, Versamid 900, and Florisil (80–100 mesh) were obtained from Applied Science Laboratories, State College, Pa. Kuderna-Danish concentrators (500 ml) were obtained from Kontes Glass Co., Vineland, N.J. Celite 545 was used as received from Johns–Manville, Baltimore, Md. CPA standards were prepared in ethyl acetate from crystalline material.

The Versamid-Gas Chrom Q packing was prepared by a filtration technique. Thirty milliliters of a 1% solution of Versamid 900 in chloroform–methanol (85:15, v/v) was slurried with 10 g of Gas Chrom Q. The slurry was degassed under a bell jar using the partial vacuum of a water pump until the original evolution of gas had subsided, and was then filtered under vacuum on a medium porosity glass frit. Filtration was continued until no further visible filtrate was obtained. Drying was completed by passing a stream of N_2 at room temperature upward through the frit at a rate sufficient to fluidize the packing.

Florisil was slurried with distilled water and the slurry was filtered on a coarse porosity frit. Drying was completed on the filter using vacuum until the Florisil was free flowing. Storage was in air-tight containers.

GAS CHROMATOGRAPHY

A Varian Aerograph model 204-1B gas chromatograph fitted with an Aerograph phosphorus detector was used. The phosphorus detector, a modified flame ionization detector fitted with a CsBr tip, protrudes from the detector oven and is susceptible to ambient drafts and temperature changes; the protruding part was satisfactorily themostated in a metal box packed with glass wool. Chromatograms were recorded on a Texas Instrument 1 mV potentiometric recorder fitted with a 6000 count/minute Disc integrator which was used for quantitation. The column was 1.8 mm (i.d.) × 107 cm borosilicate glass packed with 1% Versamid 900 on 100/125 mesh Gas Chrom Q, operated isothermally at 240°C. The injector was fitted with a 1.8 mm (i.d.) borosilicate glass liner. Injector and detector were operated at 225°C. Ultrahigh-purity N_2

U. S. Department of Agriculture, Agricultural Research Service, Animal Science Research Division, Beltsville, Maryland 20705.

Figure 1. Chromatogram of 6.0 ng of cyclophosphamide; conditions were as described in text. Time scale: 1 min/division; Sensitivity: 1.3×10^{-9} AFS

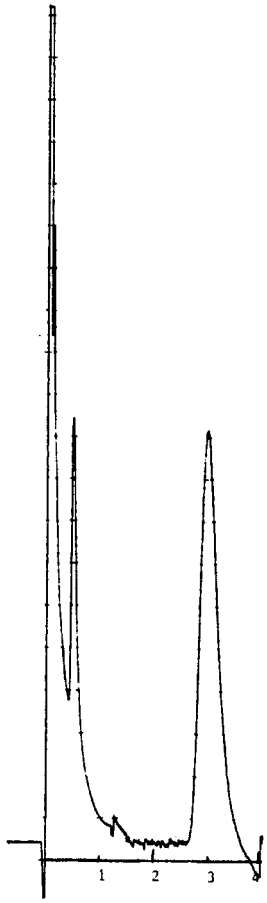

Figure 2. Chromatogram of 6.5 ng of cyclophosphamide recovered from muscle tissue fortified at 0.050 ppm. Conditions were as described in text. Time scale: 1 min/division; Sensitivity: 1.3×10^{-9} AFS

was the carrier gas, supplied at 18 ml/min. Breathing quality air (170 ml/min) and ultrahigh-purity H_2 were supplied to the detector through an Aerograph Model 403 dual back referenced flow controller. H_2 flow was adjusted to provide a background current of 1.3 to 2.6×10^{-9} AFS.

PROCEDURE

Ten-gram samples of frozen or fresh tissue, either previously ground or not, were blended with 60 ml of methanol in an Omni-mixer at full power. The blender cup was half immersed in water at room temperature as a heat sink. The homogenate was filtered with suction through a 2–3 mm bed of Celite 545 covering a coarse porosity glass frit. The blender cup and filter cake were washed 3× with small volumes of methanol. The methanol extract was evaporated to 2 to 5 ml on a rotary evaporator with the filtrate flask kept at 55 to 65°C in a water bath. A long-necked, half-liter flask was used to prevent losses from foaming. The evaporated extract was transferred to a 50-ml glass stoppered centrifuge tube with 25 ml of 4:1 (v/v) methanol:water. The evaporation flask was washed with 10 ml of isooctane and the washings were transferred to the centrifuge tube. After 2 min of manual shaking, the emulsion was broken by centrifugation for 5 min at 1000 rpm, and the isooctane layer was aspirated off and discarded. Three additional 10-ml isooctane extractions were performed, each aliquot of isooctane being used first to wash the evaporation flask.

The methanol layer was transferred to a 250-ml separatory funnel with 80 ml of water, or for liver samples, with 80 ml of 0.1 *N* NaOH; 5 ml of saturated NaCl solution and 80 ml of hexane were added. After manual shaking for 2 min, the separated hexane layer was aspirated off and discarded. CPA was removed from the methanol–water mixture by two successive extractions with 75 ml of ethyl acetate. The ethyl acetate extracts were combined in a separatory funnel and 60 ml of petroleum ether was added. The water layer (a few milliliters) was drained off. Remaining water was removed by the addition of 20–25 g of granular anhydrous Na_2SO_4. The dried extract was filtered through glass wool into a Kuderna-Danish concentrator and was reduced to 6–8 ml volume.

FLORISIL CHROMATOGRAPHY

A Florisil column (5 × 100 mm) was prewetted with ethyl acetate. The extract was transferred to the column and washed through with two void volumes of ethyl acetate. CPA was eluted with five void volumes of 1:10 (v/v) methanol:ethyl acetate. The eluate was taken nearly to dryness using a stream of N_2 and made to suitable volume for gas chromatography with ethyl acetate.

FORTIFICATION OF SAMPLES

Aliquots of a CPA standard in methanol were added to 10-g tissue samples prior to homogenization to supply 0.100, 0.050, 0.025, or 0.010 ppm of tissue.

RESULTS

Six nanograms of CPA standard injected in 3 μl of ethyl

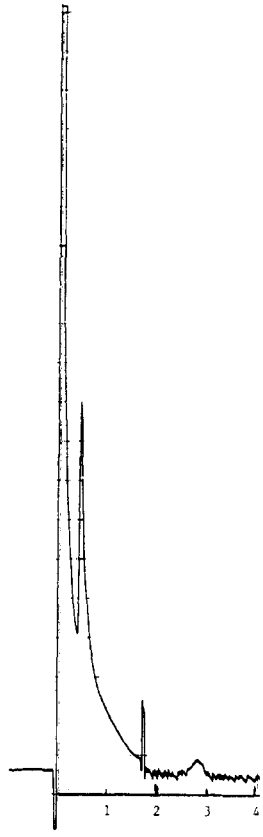

Figure 3. Chromatogram of 10 mg equivalent of unfortified muscle tissue extract. Conditions as described in text. Time scale: 1 min/division; Sensitivity: 6.6×10^{-10} AFS

acetate gave a response of about 45% of full scale at a sensitivity of 1.3×10^{-9} A full scale (AFS) (Figure 1); 6.5 ng of CPA in tissue extract at the same sensitivity gave a response of about 49% of full scale (Figure 2). An unspiked tissue extract equivalent to 10 mg of tissue at twice the sensitivity (6.6×10^{-10} AFS) of the standard yielded a small peak with the same retention time as CPA (Figure 3). Detector response was linear to 7 ng of CPA; greater quantities were not investigated.

The recoveries of 0.100, 0.050, 0.025, and 0.010 ppm of CPA added to sheep skeletal muscle were 97 to 110% (Table I), uncorrected for the small interfering peak of the unfortified sample. The recovery of CPA added to liver at 0.025 ppm was 60% (Table I).

DISCUSSION

The isooctane and hexane extractions each removed some interfering material which was not removed by the other nor by Florisil chromatography. Sodium hydroxide solution improved the cleanup of liver samples apparently by retaining in the aqueous layer some of the bile salts, which were visibly extracted with ethyl acetate when water was used in lieu of NaOH solution. Water and sodium hydroxide solution were equally effective with skeletal muscle samples, however.

The hexane extraction removes the least extraneous material and probably can be omitted for levels of CPA greater than 0.1 ppm.

Table I. Recovery of CPA Added to Tissue

Sample	CPA added, ppm	No. of determinations	% Recovery	s.d. %
Muscle	0.100	4	97	± 6.5
Muscle	0.050	5	100	± 5.1
Muscle	0.025	5	102	± 3.2
Muscle	0.010	5	110	±14.3
Liver	0.025	7	60	±10.4

Light petroleum ether is added to the combined ethyl acetate extracts to facilitate their drying over anhydrous Na_2SO_4. Because CPA is fairly unstable in the presence of water (Arnold and Klose, 1961), extracts were left overnight or longer in dry organic solvents.

The washing of Florisil with water was a deactivation step. Florisil thus deactivated required a lesser proportion of methanol in ethyl acetate to elute the CPA and resulted in an improvement of cleanup. Each lot of Florisil will require standardization. Retention of CPA can be increased by heating the Florisil and decreased by treating it with water as desired.

The Versamid columns were satisfactorily conditioned within 48 hr at operating temperatures. Versamid 900 is readily oxidized at elevated temperatures and the column must be purged with carrier gas before it is heated.

Using pure solvents, the proportions of CPA recovered in the upper phase of each of the extraction systems used in the cleanup were: 90% methanol/isooctane, none detectable; 10% methanol/hexane, <1%; 10% methanol/ethyl acetate (one extraction), 90%.

The cleanup was highly efficient in conserving CPA, as shown by the excellent recoveries from muscular tissue.

The increasing percentage recovery from muscle tissue with decreasing level of fortification is the result of the contribution to the chromatogram baseline of extraneous material not completely removed by the cleanup. This discrepancy is not serious, and the method may be considered to give essentially quantitative results in sheep skeletal tissue. Liver, however, yields more material not removed in cleanup, and results for this tissue are semiquantitative.

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