Determination of Methylprednisolone (Medrol) Residues in Milk by High-Pressure Liquid Chromatography

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A high-pressure liquid chromatographic method (lc) for the determination of methylprednisolone is described which removes, through several partition steps, most of the interfering materials before injection of the sample onto the lc column for detection. The methylprednisolone is extracted from the sample by ethyl acetate, partitioned between hexane/acetonitrile, then hexane/water, and finally water/methylene chloride. The residue containing the methylprednisolone is dissolved in methanol and an aliquot injected onto a 1-m hydrocarbon polymer column using 3:1 water–methanol as the mobile phase. Detection is by a precision photometer (254 nm) and quantitation is by peak height measurement. Observed recovery ± estimated standard deviation for methylprednisolone in milk over a 5–50 ppb range was 94 ± 4%. Following three consecutive intramammary infusions of Lincocin Forte in Holstein cows, methylprednisolone was detected in the milk 12 hr posttreatment, but not at 24, 36, and 48 hr posttreatment.

Methylprednisolone (1-dehydro-6α-methylhydrocortisone, or Medrol) is a potent corticosteroid possessing greater anti-inflammatory action than prednisolone. It is one of the active ingredients of Lincocin Forte, which is used in the treatment of infectious bovine mastitis. The optimal dose of 5 mg of methylprednisolone per quarter (20 mg per udder) would, by necessity of safety, require that the milk be free of the steroid following the treatment. Consequently, a residue method was needed that had good specificity, low background, negligible interference from other steroids, and a sensitivity of 10 ppb.

While preliminary work in our laboratory had shown that methylprednisolone could be detected by glc–flame ionization, this procedure required a tlc step which caused considerable loss of sample. This paper describes the necessary cleanup steps for quantitation of methylprednisolone by high-pressure liquid chromatography which eliminates the tlc step.

EXPERIMENTAL

Animal Studies. Five lactating Holstein cows were selected for the study. Each cow was first milked and then treated with 10 cm^3 of Lincocin Forte in each quarter. Treatments were repeated for the three consecutive milkings at −24 hr, −12 hr, and 0 hr. Zero designated the start of the posttreatment sample collection. Each cow was milked with a commercial milker twice a day, the production noted, and 200-ml aliquots frozen in 1-pt polyethylene freezer cartons. All samples were stored in the deep freeze until assayed (less than 5 weeks).

Apparatus. Waring Blendor, Model 702B, was refitted with polyethylene bowl gaskets cut from polyethylene freezer containers. A Du Pont 820 Liquid Chromatograph, equipped with a precision photometer (sensitivity of 0.005 absorbance units full scale deflection at 254 nm), was used with a 1-m × 2.1-mm i.d. hydrocarbon polymer column (HCP, Du Pont #820950008).

Reagents and Solutions. Lincocin Forte Aqueous Solution (Lot Number 1JU75), The Upjohn Co. Milligrams of drug per 10-cm^3 dose: Lincomycin hydrochloride monohydrate equivalent to lincomycin base, 200 mg; neomycin sulfate (286 mg) equivalent to neomycin base, 200 mg; methylprednisolone, 5 mg. Methylprednisolone standard, 99.5% purity, The Upjohn Co.

Solvents. Burdick and Jackson Laboratories, distilled in glass grade, or equivalent source. Solvents should be lc pure, *i.e.*, have no peaks at the retention time of methylprednisolone.

Extraction. Place 200 ml of (@ 200 g) frozen milk (Figure 1) into a Waring Blendor with 200 ml of ethyl acetate, 1.0 ml of methanol, and blend at low speed until slushy. Then add 200 ml of additional ethyl acetate and blend for 5 min at low speed. Pour the homogenate into a 1-l. separatory funnel and drain off the ethyl acetate layer. Repeat the extraction two more times with 300 ml of ethyl acetate. To the combined extracts add 50–60 g of anhydrous sodium sulfate (washed with $CHCl_3$ and dried at 120°C) and shake vigorously for 1 min. Filter the mixture through a sintered glass funnel (medium porosity) and rotary evaporate the solvent.

Partition the residue between 100 ml of hexane and 100 ml of acetonitrile. *All partitions here and throughout the method are shaken vigorously for 1 min and allowed to stand 15–30 min.* Drain the lower layer and wash the hexane two more times with acetonitrile. Combine the acetonitrile extracts and rotary evaporate the solvent at 45°C. *Prolonged evaporation on the roto-dryer, after all solvent is gone, will not cause loss of the steroid.*

Transfer the residue to a 1-l. separatory funnel with deionized water and hexane, and save the flask for further rinsing. Partition the residue between 500 ml of water containing 1 ml of saturated sodium sulfate solution and 100 ml of hexane. Drain the water phase into another 1-l. separatory funnel. Repeat washing the hexane two more times with 100 ml of water containing 0.2 ml of saturated sodium sulfate solution (prepared with $CHCl_3$ washed Na_2SO_4). *Rinse the original flask with each solvent prior to its addition to the separatory funnel. Also, save the flask for the next partition step below.*

Rinse the flask saved from the previous step with 100 ml of methylene chloride and transfer this to the separatory funnel containing the combined water phases (700 ml). Add 1.0 ml of saturated sodium sulfate solution and shake for 1 min. Drain the methylene chloride phase and reextract the water phase four more times with 100 ml of methylene chloride. Rotary evaporate the combined extracts.

Liquid–Liquid Chromatography. Operating conditions. First stage air, 50 lb per $in.^2$; second stage air, 22–25 lb per

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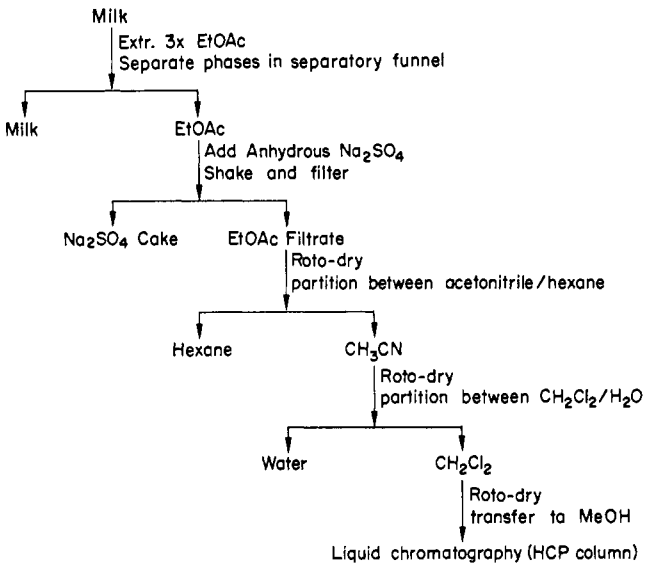

Figure 1. Flow diagram for cleanup steps

in.^{2}; flow rate, 0.5 ml per minute; uv-detector setting, 1×10^{-2}; *low ground* switch in *open* position; *filter-normal* switch in *normal* position; *column flow* control in *drain position*.

Mobile phase was 3:1 water–methanol. The degassing procedure steps were as follows. Operate with vacuum and recycle on for 3 min, close recycle valve and operate with vacuum only for 2 min, then resume vacuum and recycle operation for 1 min more.

The sample residue from the partition steps was dissolved in 1 ml of methanol and 3 μl were injected into the column with the pump pressure at zero. The pump was turned on and the eluted peak measured to the nearest 0.5 mm. Quantitation was based on the peak height of 3 μl of a 5-ppm standard solution.

RESULTS AND DISCUSSION

Recovery Studies. These were based on the amount of methylprednisolone found after fortification of 200-ml milk samples with a known amount of steroid. Nearly all recovery values were in the 90% range (Table I) for fortification levels of 5, 10, 15, 20, and 50 ppb. Calculations of percent recovery and standard deviation for all values over the 5 to 50 ppb range were 94 ± 4%. Since the number of samples was too small, calculation at each level of fortification was omitted. These samples were fortified directly in the Waring Blendor because previous data in our laboratory indicated that samples fortified, frozen, and even stored for 5 weeks showed no change from the direct fortification method.

Linear regression analysis of the ppb added (x) and found (y) showed that the regression equation was $y = 0.3160 \times 0.968x$, with a standard error of 0.125. Analysis of variance showed the regression factor to be highly significantly related to the results.

Sensitivity was approximately 1 ppb of methylprednisolone/mm of scale deflection. Consequently, with the noise and sample background observed in this study, chromatograms

Table I. Recovery of Methylprednisolone from Fortified Milk[a,b]

ppb		Average
Added	Found[c]	percent
0	0	0
5	4.73	94
10	9.3	93
15	13.9	92
20	19.1	96
50	48.1	96

Overall average for the 5 to 50 ppb range 94 ± 4

[a] Fortified by pipetting various amounts of the 5.0-ppm solution directly into the blender. [b] Actual assay order was determined from a randomized series table. [c] Amount found was based on the comparison of the peak height of the sample to the peak height of the standard.

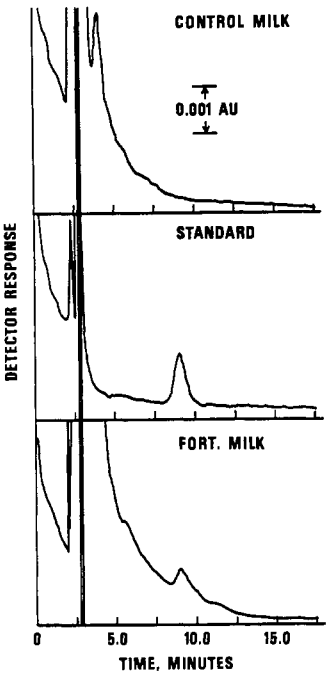

Figure 2. Typical chromatogram with the following conditions: 3-ft HCP column on Zipax; 3:1 water–methanol as the mobile phase; 0.01 absorbance units full scale; sample in 1.0 methanol; standard concentration was 5 ppm in methanol; injection was 3 μl standard or 15 ng on column or equivalent to 20 ppb in milk. Fortified milk was equivalent to 10 ppb

Table II. ppb Methylprednisolone in the Milk

	Treatment hr[a]			Hr posttreatment				Total μg[b] recovered	Percent[c] of total
Cow no.	−24 a.m.	−12 p.m.	0 a.m.	+12 p.m.	+24 a.m.	+36 p.m.	+48 a.m.	in the milk	dose
1	0	14	...	8	0	0	0	128	0.20
2	0	36	0	29	0	0	0	333	0.56
3	0	44	5	22	0	0	0	395	0.66
4	0	40	0	78	0	0	0	618	1.03
5	0	98	0	37	0	0	0	797	1.33

[a] Three treatments approximately 12 hr apart. Treated immediately after each milking. [b] Calculated from the ppb and milk production. [c] Total of 60 mg given (10 mg/quarter for three doses).

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

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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

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