Disappearance of Betamethasone Esters from Calf Synovial Fluid and Its Correlation with *In Vitro* Behavior

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The disappearance of betamethasone phosphate and betamethasone acetate from calf synovial fluid was studied. Thin-layer chromatography was used for determining the presence of both steroids. It was found that betamethasone acetate remained in synovial fluid in relatively high concentrations for at least 24 hr. after intra-articular administration. With betamethasone phosphate, however, little or no steroid was present after 2 hr. The disappearance rate of both steroids was studied in an *in vitro* system, and the results supported the *in vivo* findings.

INTRA-ARTICULAR steroid treatment is widely used for local anti-inflammatory therapy. It would be desirable for preparations used in this manner to display both immediate and prolonged local effect, little or no systemic action, and to be devoid of local irritation. Several products containing insoluble steroid esters have been reported to cause "post injection flare" (1, 2). This may be a non-specific effect due to the particulate nature of the material injected. Such effects have been reported after the administration of microcrystalline sodium urate, calcium oxalate, and other substances (1). McCarty and Faires (1) indicate that corticoid crystals may induce an initial irritation while steroid solutions elicit only the expected anti-inflammatory response.

In this study, a mixture of steroids combining a water soluble steroid (betamethasone phosphate) and a water insoluble steroid (betamethasone acetate) was investigated. The concentration of steroids in calf synovial fluid at various times after injection was determined in order to estimate the relative persistence of the two steroids after intra-articular administration. The disappearance rate of betamethasone phosphate and betamethasone acetate was also obtained using an *in vitro* model and were correlated with the *in vivo* data.

EXPERIMENTAL

In Vitro Study.—Three formulations were studied:

	A	*B*[1]	*C*
Betamethasone disodium phosphate	...	2.1 mg.	4.2 mg.
Betamethasone acetate	1.5 mg.	1.5 mg.	...
Vehicle *q.s.*	1.0 ml.	1.0 ml.	1.0 ml.

The vehicle contained mono- and dibasic sodium phosphate, ethylenediaminetetraacetic acid, and benzalkonium chloride. The same vehicle was used throughout the study.

PROCEDURE

In Vitro Study.—Twenty milliliters of steroid formulation was placed in a Visking No. 4465-A2 dialysis casing (1-in. width) and immersed in 1000 ml. of distilled water maintained at 37 ± 0.1°. The casing was moved in a vertical plane beneath the surface of the test fluid by means of a Stoll-Gershberg apparatus set at 30 strokes/min. The surrounding fluid was periodically assayed for steroid content. With formulations *A* and *C*, an aliquot was diluted, if necessary, and the ultraviolet absorbance determined at 240 mμ, using a Beckman model DU spectrophotometer. When both the phosphate and acetate esters were present (as in formula *B*), an aliquot was first assayed for total steroid content by the above method. The same solution was then extracted four times with an equal volume of reagent grade chloroform, and the absorbance of the aqueous extract was once again determined spectrophotometrically at a wavelength of 240 mμ. The absorbance of the aqueous layer after extraction is proportional to the concentration of betamethasone phosphate. The absorbance before extraction, less the absorbance after extraction, is proportional to the concentration of betamethasone acetate.

In Vivo Study.—Young calves were used in which 1.5 ml. of synovial fluid was withdrawn from the knee joint at 0 time to serve as control. This volume was immediately replaced with an equal volume of steroid formulation containing either betamethasone acetate (30 mg.) or betamethasone phosphate (30 mg. alcohol equivalent). After 5 min., 0.5 ml. of synovial fluid was withdrawn from the joint in order to determine the early steroid levels. Two hours after the administration of betamethasone phosphate and 24 hr. after administration of betamethasone acetate, the remaining synovial fluid (about 5 ml.) was removed from each joint for analysis.

Analytical Procedure.—Initially, isonicotinic acid hydrazide was used as a reagent for the determination of Δ^4-3-ketosteroids (3), but frequent contamination of synovial fluid with blood made the analysis by this method unreliable. Thin-layer chromatography proved to be a useful tool for analysis of both steroids. Chromatographic plates were covered with silica gel (G. F. 254) and developed for 30 min. in benzene–ethyl acetate (1:1). The plates were subsequently heated at 100° for 10 min. and after cooling sprayed with a 1:1 mixture of concentrated sulfuric acid and ethyl alcohol. The steroids were visualized as black spots after heating the plates for 15 min. The color intensity of the spots decreased with time.

Samples of synovial fluid containing betamethasone acetate were lyophilized from the frozen state and the residue extracted with 0.3–0.5 ml. of ethyl alcohol and then applied on the chromatographic plate. Since betamethasone phosphate does not migrate in the selected solvent system, it was con-

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[1] This preparation, having twice the concentration of both steroids, is available commercially as Celestone Soluspan from Schering Corp., Bloomfield, N. J. The chemical name for betamethasone is 16β-methyl-9α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione.

verted to the free alcohol, prior to chromatography, by the following procedure.

Samples of synovial fluid containing the steroid phosphate were lyophilized from the frozen state and the residue mixed with 0.5 ml. of Tris buffer at pH 8.6. Alkaline phosphatase (0.1 ml.) from calf mucosa (Sigma Chemical Co., 20 mg./ml.) was added, and the samples were incubated for 30 min. at 37°. The samples were then immediately lyophilized and the residue extracted with 0.3–0.5 ml. of ethanol. Subsequent steps were the same as described for betamethasone acetate.

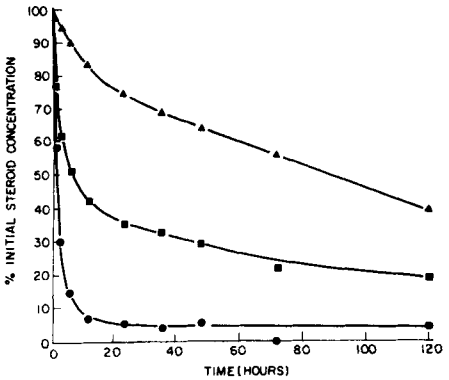

Fig. 1.—A plot of the disappearance rate of several betamethasone esters from a dialysis cell immersed in 1000 ml. of distilled water maintained at 37 ± 0.1°C. Key: ▲, formula *A;* ■, formula *B;* ●, formula *C.*

RESULTS AND DISCUSSION

The relative *in vitro* release behavior of the three steroid formulations is illustrated in Fig. 1. As anticipated, the water soluble betamethasone phosphase (formula *C*) rapidly passes through the membrane and is equilibrated with the surrounding fluid in 6 to 12 hr. The betamethasone acetate present in formula *A* escapes from the casing at a much slower rate, with approximately 40% of the initial steroid content remaining within the sack after 5 days of treatment. The transfer of steroid from within the membrane to the surrounding fluid, in this case, is probably limited by the dissolution rate of the solid steroid.

Formula *B* contains both the water soluble and water insoluble betamethasone esters. The release behavior of this formulation, as expected, represents a composite of the individual steroids.

The *in vivo* results obtained with betamethasone phosphate (after conversion to the alcohol) are illustrated in Fig. 2. The chromatographic mobility of betamethasone alcohol is shown in the last strip to the right of this figure. The steroid level in synovial fluid was found to be very high in all samples 5 min. following the intra-articular injection of betamethasone phosphate (each sample represents the assay of fluid from a single calf knee joint). At the 2-hr. period, however, there was very little steroid phosphate left in sample 1 and 2 and practically none in samples 5 and 6. The control samples of synovial fluid (0 hr.) are presented to the left.

The synovial fluid showed appreciable amounts of betamethasone acetate (Fig. 3) 5 min. and 24 hr.

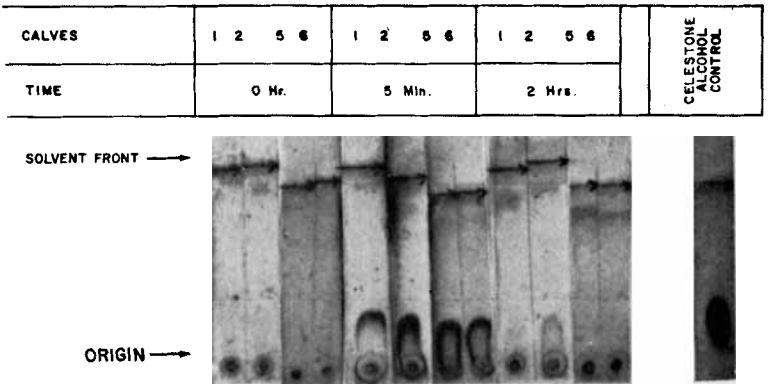

Fig. 2.—Thin-layer chromatography of synovial fluid at various time intervals after injection of betamethasone phosphate. The steroid ester was converted to the free alcohol prior to chromatography.

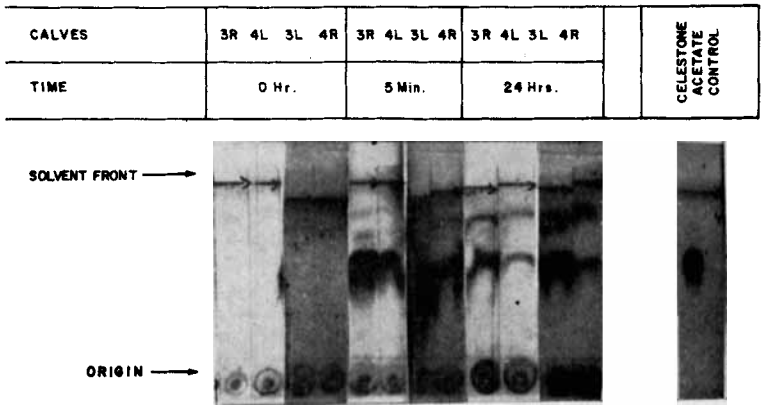

Fig 3.—Thin-layer chromatography of synovial fluid at various time intervals after injection of betamethasone acetate.

after the intra-articular administration of this steroid in all samples analyzed. The mobility of betamethasone acetate standard is shown in the strip to the extreme right. No betamethasone alcohol is visible in the chromatograms. This either suggests no appreciable conversion of betamethasone acetate to the free alcohol in synovial fluid or very rapid elimination of any free alcohol formed. The additional spots visible in both figures could not be identified, but are probably due to blood contamination of synovial fluid.

To evaluate the relative amounts of steroid present in the samples, experiments were conducted to determine the minimum sensitivity of the analytical method. The described procedure clearly distinguishes 20 mcg. of betamethasone acetate and 50 mcg. of betamethasone phosphate (converted to the free alcohol) from background. A direct relationship between the intensity of the corresponding chromatographic spot and the concentration of the steroid (20–300 mcg.) was also observed. In another experiment, the effect of the steroid vehicle on analysis of betamethasone phosphate was tested. The vehicle was injected intra-articularly, and the total content of synovial fluid was withdrawn after 2 hr. Betamethasone phosphate (500 mcg.) was added to this as well as to a control sample of synovial fluid. Both samples were then treated as described for the betamethasone phosphate determination. The intensity of the chromatographic spots so obtained appeared similar, indicating that this amount of the vehicle did not interfere with the analysis of betamethasone phosphate.

The *in vivo* results thus demonstrate that the concentration of both betamethasone acetate and phosphate in synovial fluid is very high 5 min. after intra-articular administration. Betamethasone phosphate, however, very rapidly disappears, evidenced by the small amount of this drug present after 2 hr. This is particularly clear if one considers that the 5-min. sample represents the drug content of 0.5 ml. of synovial fluid while the 2- and 24-hr. samples represent the drug content of the total synovial fluid available from the joint (approximately 5 ml.).

Betamethasone acetate persists in the knee joint much longer than the phosphate ester as demonstrated by the appreciable levels of the steroid acetate in the joint even after 24 hr. This observation is consistent with the *in vitro* data and can probably be similarly rationalized on the basis of the slow dissolution rate of the drug.

The results obtained in this investigation could conceivably explain the clinical data (4) obtained with a formulation containing betamethasone acetate and phosphate (formulation *B*). The onset of activity with this formulation was found to be rapid and to have a prolonged duration (5).

The rapid onset of anti-inflammatory action is most logically explained as an effect of the readily available betamethasone phosphate. The persistance of betamethasone acetate in the joint is probably responsible for the prolonged activity of the formulation. This is consistent with the suggestion of Will and Murdoch (6) that longer persistence of a steroid in a joint cavity may account, at least in part, for its prolonged duration.

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Relationship Between Hematocrit and Solubility of ^{133}Xe in Blood

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The relationship between the hematocrit and the solubility of ^{133}Xe in blood at 37° was measured in 64 samples, and an excellent linear correlation was found. The partition coefficients for erythrocytes/water and plasma/water were 2.14 and 1.02, respectively.

MEASUREMENTS of regional blood flow from the wash-out curves of the radioactive inert gas, ^{133}Xe, from a tissue has become a valuable method in clinical studies (1, 2). To convert the clearance values of the gas to quantitative values for blood flow, the relative xenon solubility in tissue and blood must be known. The present work concerns the relationship between hematocrit and solubility of ^{133}Xe in blood.

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EXPERIMENTAL

Materials.—^{133}Xe was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. According to the specification, the maximal impurities of the gas with other radioactive isotopes, mainly ^{133}Xe m, were below 2%; only traces of ^{131}Xe m and ^{85}Kr were present. These data were confirmed by spectral analysis, where activity above the mentioned levels from elements other than the 0.081 Mev. γ emission of ^{133}Xe was not found. Nor by decay studies was contaminating radioactivity recognized.

Heparinized or citrated blood samples were obtained from voluntary donors.

The water used was redistilled.

Manipulation.—Blood samples with hematocrit values over a wide range were prepared by removal of plasma or erythrocytes from whole blood after centrifuging. The hematocrits of the samples were