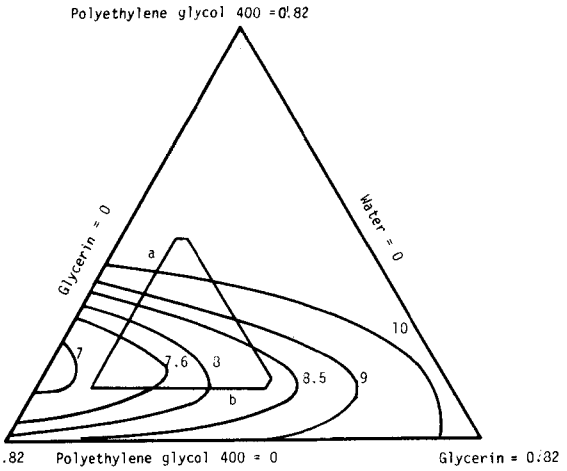

Figure 3—*Contour plot at polysorbate 60 = 0.08. Solubility = 10.1 + 38.0 (I) − 2.3 (water) − 59.9 (I)(II) − 74.2 (I)(water).*

The contour plot allows the formulator to choose any desirable solubility value and to determine the corresponding mixture. More importantly, it gives an overall picture of the response surface and the variation of the solubility as a function of the components. This, in turn, can be used to design a more robust formulation, that is, a region where the solubility does not vary greatly with small variations in component concentrations. Minor variations in production, therefore, would not result in drastic effects on the product. In addition, the following observations may be made from Fig. 3:

1. If one moves along the line glycerin = 0.1 (line a in Fig. 3) toward increasing polyethylene glycol 400, the solubility increases more slowly at lower polyethylene glycol 400 concentrations but increases more rapidly at higher levels.

2. If one moves along the line polyethylene glycol 400 = 0.1 (line b in Fig. 3) and replaces glycerin by water, the solubility changes only marginally. Hence, in changing the mixture from 40% glycerin to 10% glycerin by replacing with water, there is a change in solubility of only ~1 mg/ml. This information could be very useful if one were also concerned, for example, with the viscosity of the solution. The viscosity could be decreased considerably without compromising the solubility significantly.

Similar contour plots can be drawn holding different variables constant. These plots are not included here, but the interpretations are analogous to those made earlier.

From this analysis, it is evident that the extreme vertexes method in formulation development is a powerful approach to characterizing a formulation with a minimum number of experiments. (Factorial designs can be used for mixture problems only if one neglects the nth component, generally the least responsive, in generating the design. However, this approach may not give an optimum design.) This approach assumes even greater significance when more than one response must be studied since it allows optimization of the formulation with respect to the several responses simultaneously. This is easily done by overlapping contour plots of the different responses and choosing the region that satisfies all response criteria. An additional feature of the method is that one could first perform experiments only at the vertexes; if these experiments gave acceptable values for for the response, the design could be completed. Otherwise, the experimental region could be shifted to another area where the response might be more favorable, and the process could be repeated.

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Radioimmunoassay of Flunisolide in Human Plasma

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Received March 4, 1980, from the *Department of Analytical and Metabolic Chemistry, Syntex Research, Palo Alto, CA 94304.* Accepted for publication January 15, 1981.

Abstract □ A simple radioimmunoassay was developed for the measurement of flunisolide in human plasma or serum. Plasma extraction was not required. Antiserums were produced in rabbits by immunization against the flunisolide 21-hemisuccinate–bovine serum albumin conjugate. Cross-reactivities were determined for cortisol and a major metabolite of flunisolide and were 0.02 and 0.06%, respectively. Assay sensitivity is in the 100–200-pg/ml range. Accuracy studies gave regression lines of $y = 1.06x$, $r = 1.00$, for a 0.1-ml plasma aliquot and $y = 0.99x$, $r = 0.99$, for a 0.2-ml plasma aliquot. The accuracy of the method was estimated to be at least ±15%. The method was used to determine plasma concentration–time profiles in human subjects after the administration of a 1.0-mg iv dose.

Keyphrases □ Flunisolide—radioimmunoassay, human plasma or serum, plasma concentration–time profiles □ Corticoids—flunisolide, radioimmunoassay, human plasma or serum, plasma concentration–time profiles □ Radioimmunoassay—flunisolide, human plasma or serum

Flunisolide (6α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone, I) is a fast acting corticoid designed for use in the treatment of allergic rhinitis, asthma, and other respiratory disorders in humans (1–4).

Since the intended use of this drug was for the treatment of respiratory and upper respiratory disorders, its administration in an inhalation dosage form was a requirement. Since the amount of drug delivered by inhalation is generally small, the plasma levels achieved can be expected to be low.

This paper describes a selective and sensitive radioimmunoassay for the measurement of flunisolide in plasma or serum. This method was developed to provide data for the determination of certain pharmacokinetic parameters in humans, *e.g.*, plasma clearance, elimination half-life, and

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Table I—Radioimmunoassay Protocol

Tube Number	Tube Description	Number of Replicates	Buffer, ml	Labeled Flunisolide, ml	Cold Flunisolide, ml	Plasma Unknowns, ml	Antiserums, ml	Total Volume, ml
0	Zero std.	6	0.3	0.1	—	0.1[a]	0.1	0.6
1	10 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
2	20 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
3	50 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
4	100 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
5	200 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
6	300 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
7	500 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
8	600 pg std.	3	0.2	0.1	0.1	0.1	0.1	0.6
N1, N2, *etc.*	Plasma–nonspecific traps	3	0.4	0.1	—	0.1	—	0.6
NB	Buffer–nonspecific trap	3	0.5	0.1	—	—	—	0.6
TC	Total counts	3	0.5	0.1	—	—	—	0.6
U1, U2, *etc.*	Unknowns	3 or 6	0.3	0.1	—	0.1	0.1	0.6

[a] Standards and nonspecific traps received an aliquot of control plasma.

extent and rate of absorption. Other analytical methods such as GLC or GLC–mass spectrometry cannot be used for plasma level determinations because flunisolide is a thermally unstable compound.

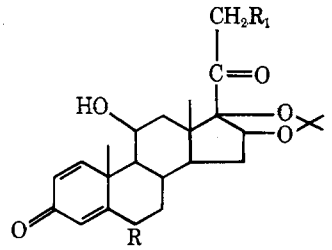
EXPERIMENTAL

Materials—Freund's complete and incomplete adjuvants were purchased in sealed glass ampuls[1]. Activated powdered charcoal[2] was used. The buffer was tris(hydroxymethyl)aminomethane–hydrochloric acid, 0.05 *M*, pH 7.2–7.4, with or without 0.1% gelatin. Flunisolide tritiated at the 1,2-positions with a specific activity of 39.9 Ci/mmole, the 21-hemisuccinate of flunisolide (II), and the flunisolide–bovine serum albumin conjugate were all synthesized in-house[3]. The 6β-hydroxy metabolite of flunisolide (6β,11β,16α,17α,21-pentahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) (III) used in the cross-reactivity experiment also was synthesized in-house[3].

Antiserum Production—The flunisolide–bovine serum albumin conjugate used for immunization was prepared by coupling II to bovine serum albumin with a water-soluble carbodiimide coupling reagent (5). The reaction mixture was dialyzed exhaustively against normal saline. The extent of conjugation was determined by measuring the protein concentration by the method of Lowry *et al.* (6), and the flunisolide residues were determined by UV absorption. The molar ratio was 57 moles of flunisolide/mole of bovine serum albumin.

An emulsion of the conjugate in saline was prepared by mixing with an equal volume of Freund's complete adjuvant. The bovine serum albumin concentration was 100 μg/ml, and 0.25 ml was injected subcutaneously into four sites in New Zealand White rabbits. Six weeks after the initial injection, all animals were placed on a regimen of weekly booster shots; the conjugate was prepared in incomplete Freund's adjuvant. After 6–8 months, antiserum dilutions of 1:10,000 to 1:30,000 yielded 50% binding or more. At this stage, assay development was begun.

Radioimmunoassay—Flunisolide standards for the standard curve were obtained by dilution of a stock solution of 10.0 mg of flunisolide in 10.0 ml of ethanol. A series of standard solutions, 20, 50, 100, 200, 300, 500, and 600 pg/0.1 ml, was prepared in tris(hydroxymethyl)aminomethane–hydrochloric acid buffer, pH 7.2, 0.05 *M*, without gelatin in 10.0-ml volumes and stored in the refrigerator. Gelatin was withheld from the buffer in the standards to give them a longer shelflife.



I: R = F, R_1 = OH
II: R = F, R_1 = $CO_2(CH_2)_2COOH$
III: R = OH, R_1 = OH

An ethanolic solution of the tritiated flunisolide was diluted with tris(hydroxymethyl)aminomethane–hydrochloric acid buffer, pH 7.2, 0.05 *M*, with 0.1% gelatin such that a 0.1-ml aliquot contained 8000–10,000 cpm. The solution was prepared in a volume sufficient for one assay and then was discarded. Radioisotope purity checks were carried out periodically on silica gel TLC plates[4] with chloroform–methanol–water (40:9:1) as the solvent system. To identify the radioactive band, cold reference standards were cospotted and identified under UV light. The R_f values were ~0.4–0.5.

The antiserums were diluted in tris(hydroxymethyl)aminomethane–hydrochloric acid buffer, pH 7.2, 0.05 *M*, with 0.1% gelatin to yield a total binding of between 35 and 60%. Depending on the quality of the antiserum, this binding was achieved by diluting 1:10,000, 1:20,000, or 1:30,000; a 0.1-ml aliquot was added to each tube.

The charcoal stock suspension (1% in water) was diluted as required with tris(hydroxymethyl)aminomethane–hydrochloric acid, pH 7.2, 0.05 *M*, with 0.1% gelatin immediately before use. A 0.2-ml aliquot of the diluted charcoal suspension (0.4%) was added to blocks of 30–36 tubes at a time. The tubes were vortexed, and the 30-min time cycle was started. Midway through this cycle, charcoal was added to a second block of tubes and they were started on their 30-min cycle. This staggered array procedure was repeated until all tubes were processed. At the end of the 30-min cycle, each block of tubes was centrifuged immediately and sampled for counting.

The centrifuge used was refrigerated with a swingout head[5]. Liquid scintillation counting was done in a scintillation spectrometer[6], using commercially available scintillation fluid[7].

A typical radioimmunoassay protocol is given in Table I, and a stepwise procedure for setting up the assay is as follows. Buffer, tritiated flunisolide, standards, unknowns, antiserums, *etc.*, were added to the appropriate tubes (12 × 75-mm disposable culture tubes). After all additions had been made, the sides of the tubes were washed down by briefly vortexing (1–3 sec). The tubes were covered with sheets of waxed film[8] cut to fit the test tube racks and were incubated overnight at room temperature.

After overnight incubation, the tubes were placed in an ice bath for 2–3 hr. Then 0.2 ml of the charcoal suspension was added to blocks of 30–36 tubes. The tubes were vortexed for 6 sec, and the 30-min time cycle was started. After 30 min, the tubes were centrifuged at 2500 rpm for 4 min and immediately 0.5 ml of the supernate was transferred into scintillation vials. Then 10.0 ml of scintillation fluid was added, and the solutions were counted for 10 min.

Data Processing—Data processing was done with the aid of the Rodbard and LeWald Model I computer program (7), a logit–log trans-

[1] Difco Laboratories, Detroit, Mich.
[2] Norit A, Matheson, Coleman and Bell, Norwood, Ohio.
[3] Syntex Research, Palo Alto, Calif.
[4] Silica gel 60 F-254 plates, EM Laboratories, Elmsford, NY 10523.
[5] IEC model DPR-6000.
[6] Packard Tri-Carb model 3330.
[7] Aquasol or Oxifluor-H_2O, New England Nuclear.
[8] Parafilm.

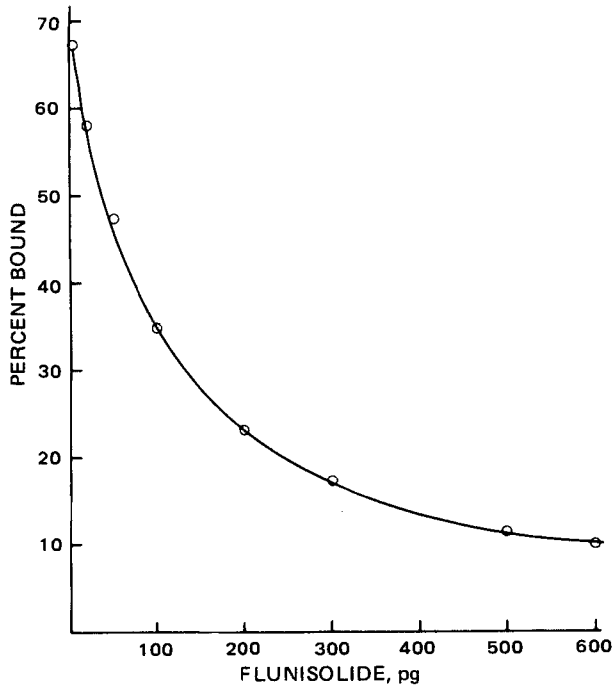


Figure 1—*Plot of a typical displacement curve (or standard curve). The antiserum dilution was 1:20,000. The binding–displacement reaction was carried out overnight at room temperature.*

formation that linearizes the sigmoid curve normally obtained in a displacement curve. However, the percent bound *versus* the flunisolide concentration can be plotted manually and the unknowns can be determined by interpolation. One advantage of this computer system is that it eliminates operator variation in plotting the standard curve and estimating the unknowns. It also increases the speed with which the computations can be made, especially if a teletype punched-tape system is coupled to the program.

Accuracy and Precision—Known amounts of flunisolide were added to plasma and serum, and then these samples were analyzed in triplicate.

RESULTS AND DISCUSSION

The described method can be used to analyze flunisolide from human plasma directly without prior extraction or purification. This approach was possible because an aliquot of control plasma was added to all points in the standard curve, thereby making it chemically equivalent to that of the unknown samples. A typical standard curve is shown in Fig. 1. Since the control plasma was obtained from each subject prior to flunisolide dosing, it did not contain any endogenous flunisolide.

Pooled plasma from different subjects could be used, but the lower level of sensitivity was not achieved. This observed difference could be due to differences in plasma lipid content or other endogenous components. The exact nature of the component effecting this difference is not known. The effect of this difference is that the lower limit of sensitivity may have to be changed from 20 to 50 or 100 pg. If 50 pg becomes the lowest point on the standard curve that can be measured reliably, then for a 0.2-ml plasma aliquot the lower limit of sensitivity would change from 100 to 250 pg/ml; for a 0.1-ml plasma aliquot, it would change from 200 to 500 pg/ml.

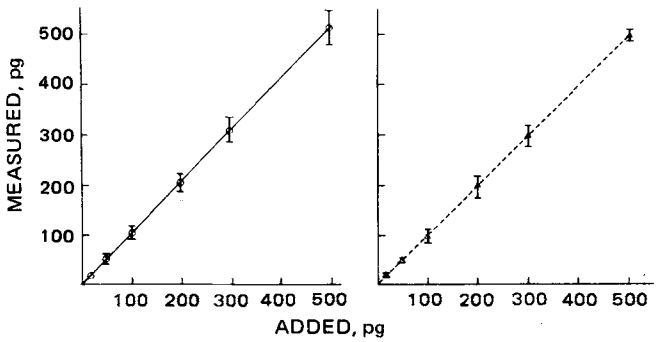


Figure 2—*Plots of accuracy measurements using 0.1- (left) and 0.2- (right) ml plasma aliquots. The regression line for the 0.1-ml curve was* y = *1.06*x, r = *1.00; for the 0.2-ml curve, it was* y = *0.99*x, r = *0.99.*

To obtain maximal sensitivity in a radioimmunoassay, certain assay conditions must be optimized. The first parameter to be optimized was the amount of charcoal required to effect the optimum separation of bound from free. This value was established by adding fixed amounts of tritiated flunisolide to a series of tubes containing buffer only and to another series of tubes containing buffer and 0.1 ml of plasma. Graded amounts of charcoal then were added in a constant-volume aliquot (0.2 ml) until the amount of free radioactivity remaining behind, *i.e.*, the nonspecific bound, was 2–4% for tubes containing buffer only and 4–8% for tubes containing an aliquot of plasma. In this laboratory, these conditions were met by using a 0.4% charcoal suspension.

Method sensitivity depends on the size of the aliquot subjected to analysis. If a 0.1-ml aliquot was used, then the lower limit of sensitivity corresponded to 200 pg/ml; for a 0.2-ml aliquot, it was 100 pg/ml. These limits were determined by the lowest concentration on the standard curve that could be reliably measured. With the present method, the lowest concentration that can be reliably measured is 20 pg/0.1-ml aliquot.

Method accuracy was determined by adding known amounts of flunisolide to blank plasma and then assaying these samples using 0.1- and 0.2-ml plasma aliquots. Accuracy measurements were carried out at six different concentrations and covered the entire range of the standard curve. The results were then averaged for each concentration and plotted as measured amounts *versus* added amounts (Fig. 2). The linearity of the plots and the fact that the slopes are essentially equal to unity with no intercept indicate that the measurements can be made accurately over

Table II—Accuracy Experiments Carried Out in 0.1- and 0.2-ml Plasma Aliquots Showing the Ratios of Measured to Added Flunisolide

Flunisolide Added, pg/plasma aliquot (ml)	Flunisolide Measured[a], pg/plasma aliquot (ml)	Ratio $\frac{\text{Measured}}{\text{Added}}$	Group *CV*, %
20/0.1	22.7/0.1	1.14	4.0
20/0.1	20.7/0.1	1.04	
20/0.1	21.6/0.1	1.08	
20/0.1	21.2/0.1	1.06	
100/0.1	95.7/0.1	0.96	4.1
100/0.1	100.2/0.1	1.00	
100/0.1	91.4/0.1	0.91	
100/0.1	97.5/0.1	0.98	
300/0.1	289.2/0.1	0.96	6.7
300/0.1	330.4/0.1	1.10	
300/0.1	292.0/0.1	0.97	
300/0.1	290.7/0.1	0.97	
600/0.1	626.1/0.1	1.04	5.5
600/0.1	646.7/0.1	1.08	
600/0.1	690.4/0.1	1.15	
600/0.1	605.2/0.1	1.01	
20/0.2	15.9/0.2	0.84	15.5
20/0.2	19.3/0.2	0.97	
20/0.2	20.2/0.2	1.00	
20/0.2	24.4/0.2	1.22	
50/0.2	54.0/0.2	1.08	10.1
50/0.2	42.0/0.2	0.84	
50/0.2	49.0/0.2	0.98	
50/0.2	49.9/0.2	1.00	
100/0.2	92.3/0.2	0.92	3.7
100/0.2	90.4/0.2	0.90	
100/0.2	94.4/0.2	0.94	
100/0.2	86.0/0.2	0.86	
300/0.2	294.2/0.2	0.98	2.2
300/0.2	279.4/0.2	0.93	
300/0.2	285.2/0.2	0.95	
300/0.2	284.0/0.2	0.95	
500/0.2	498.8/0.2	1.00	3.5
500/0.2	499.0/0.2	1.00	
500/0.2	508.0/0.2	1.02	
500/0.2	470.9/0.2	0.94	

[a] Mean of triplicate determinations.

Table III—Quality Control Experiments Carried Out with Each Experiment over 3 Months of Analysis

Sample	Plasma Aliquot, ml	Flunisolide Added: 100 pg/Plasma Aliquot, Picograms Measured ± *SD*	100 pg/Plasma Aliquot, Ratio Measured / Added	300 pg/Plasma Aliquot, Picograms Measured ± *SD*	300 pg/Plasma Aliquot, Ratio Measured / Added
1	0.20	92.4 ± 4.95	0.92	281.9 ± 3.04	0.94
2	0.20	98.2 ± 2.33	0.98	297.8 ± 12.66	0.99
3	0.20	95.8 ± 4.10	0.96	293.0 ± 12.94	0.98
4	0.10	113.5 ± 3.47	1.13	329.3 ± 11.29	1.10
5	0.10	94.0 ± 4.78	0.94	292.1 ± 10.75	0.97
6	0.10	93.0 ± 7.78	0.93	271.7 ± 6.43	0.91
7	0.10	102.4 ± 0.99	1.02	323.0 ± 8.06	1.08
8	0.10	103.1 ± 7.40	1.03	294.3 ± 11.10	0.98
9	0.10	91.4 ± 1.41	0.91	298.7 ± 17.68	1.00
10	0.10	104.9 ± 0.64	1.05	316.8 ± 16.60	1.06
11	0.10	93.5 ± 3.04	0.93	310.2 ± 7.99	1.03
12	0.10	97.3 ± 6.15	0.97	305.6 ± 2.40	1.02
13	0.10	94.2 ± 2.76	0.94	285.3 ± 10.04	0.95
14	0.10	102.2 ± 4.03	1.02	276.0 ± 9.69	0.92
15	0.20	92.7 ± 0.42	0.93	300.6 ± 2.33	1.00
16	0.20	92.6 ± 1.63	0.93	282.1 ± 1.63	0.94
17	0.20	84.2 ± 4.67	0.84	277.4 ± 11.24	0.92
18	0.20	95.5 ± 6.08	0.95	281.5 ± 11.17	0.94
19	0.20	91.2 ± 1.56	0.91	286.1 ± 9.26	0.95
20	0.20	84.9 ± 4.10	0.85	286.2 ± 14.50	0.95
21	0.20	90.3 ± 5.52	0.90	287.4 ± 3.25	0.96

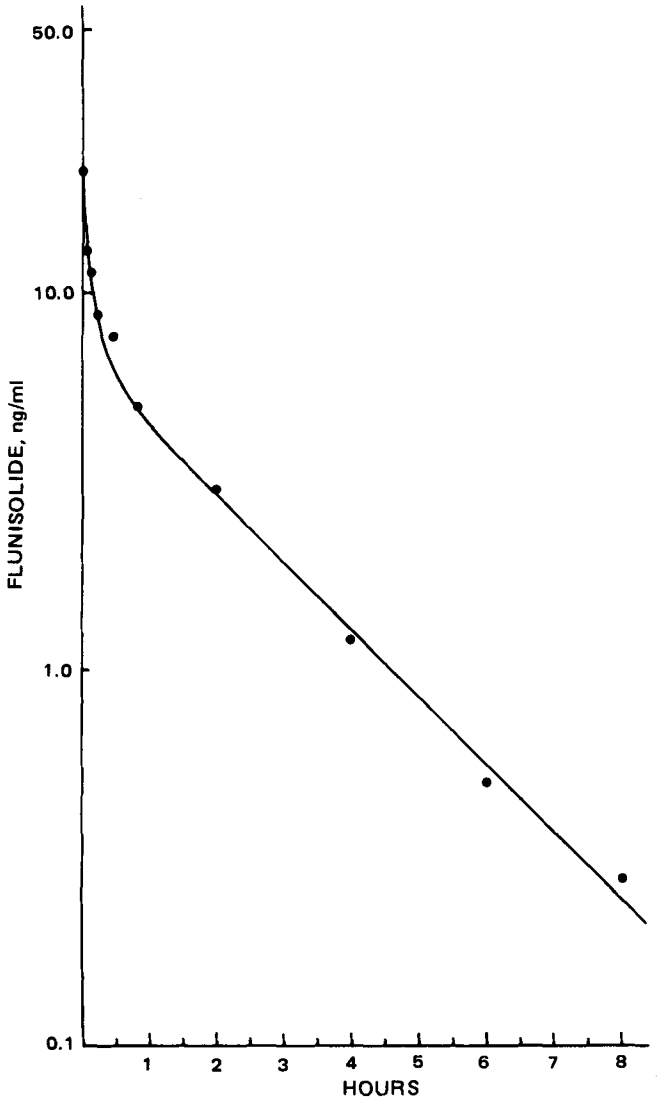


Figure 3—*Plasma concentration–time profile in human subjects after administration of a single 1.0-mg iv dose. Data are the average of 12 male volunteers.*

the concentration range tested. The results listed in Table II indicate that the method is capable of measuring within ±15%. The results of quality control experiments carried out during 3 months of routine analyses are given in Table III. The ratios of measured to added flunisolide show that an accuracy of ±15% is possible and can be maintained over time.

Assay validity also was tested by comparing plasma flunisolide levels determined by this method to the levels found by a carbon 14 radiochemical assay (8). A human male volunteer was given a 2.0-mg iv dose of flunisolide containing ~70 μCi of ^{14}C-labeled flunisolide. Blood samples were drawn at 0.5, 1.0, 3.0, 4.0, and 6.0 hr. The plasma was extracted with ethyl acetate, the extracts were evaporated to dryness, and the residue was subjected to TLC. The TLC plates were divided into 1-cm segments, and each segment was assayed for radioactivity. This procedure separated flunisolide from its metabolites. The plasma levels measured at each respective time point were 8.15, 6.07, 1.99, 1.27, and 0.47 ng/ml by radioimmunoassay and 8.26, 5.42, 2.02, 1.19, and 0.55 ng/ml by the radiochemical assay. The inherent variability of the radioimmunoassay is ±15%, and that of the radiochemical method combined with TLC can be as high as ±20%. Therefore, an absolute correlation by the two methods is not possible.

As in any radioimmunoassay, structurally related compounds can interfere. Cortisol is present endogenously at relatively high levels and is structurally related; the 6β-hydroxy compound is structurally related and is also the major metabolite of flunisolide. These compounds were chosen for cross-reactivity measurements. Cross-reactivities were determined from displacement curves generated for cortisol and the 6β-hydroxy metabolite of flunisolide, and these curves were compared to the flunisolide displacement curve. The concentration of each compound that corresponded to the 50% inhibition point on the flunisolide curve was then used to calculate the percent cross-reactivity. Cortisol and the 6β-hydroxy metabolite of flunisolide showed cross-reactivities of 0.02 and 0.06%, respectively.

The developed method was utilized to establish plasma concentration–time profiles in human subjects after the administration of a single 1.0-mg iv dose (8). Twelve male volunteers were used. Blood samples were taken at 2, 5, 10, and 30 min and at 1, 2, 4, 6, 8, and 24 hr and were analyzed in duplicate. The means, determined at each time point, were averaged for all subjects and plotted as concentration *versus* time (Fig. 3). The profile indicated that the method has sufficient sensitivity to be useful in various dosage form experiments.

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Fluoride Remineralization of Demineralized Bovine Tooth Enamel and Hydroxyapatite Pellets

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Received June 30, 1980, from the **College of Pharmacy, University of Michigan, Ann Arbor, MI 48109*, and the ‡*American Dental Association, Chicago, IL 60611.* Accepted for publication January 26, 1981.

Abstract □ Both bovine enamel and hydroxyapatite pellets were remineralized in a fluoride-containing remineralization solution after prior demineralization for various lengths of time. In both the enamel and pellet systems, the degree of remineralization attainable was directly related to the extent of prior demineralization, although the demineralized material was never 100% recovered in remineralization. In some cases, fluoride levels up to several thousand parts per million were found at depths as great as 50 μm from the surface. The stoichiometry of the remineralized material and electron microprobe examination were consistent with the formation of fluoridated hydroxyapatite rather than calcium fluoride.

Keyphrases □ Fluoride—remineralization of demineralized bovine tooth enamel and hydroxyapatite pellets □ Remineralization—of demineralized bovine tooth enamel and hydroxyapatite pellets □ Hydroxyapatite pellets—remineralization along with demineralized bovine tooth enamel □ Teeth, bovine—remineralization of demineralized tooth enamel and hydroxyapatite pellets

Previous studies performed in these laboratories (1, 2) led to the development of a model for hydroxyapatite dissolution involving two distinct crystalline dissolution sites (3). This model is consistent with dissolution kinetic data and explains the observed morphology of hydroxyapatite dissolution at both the single-crystal level (4) and at the level of a compressed hydroxyapatite pellet or a block of tooth enamel (3).

In studying demineralization of remineralized enamel, it was noted (5, 6) that when enamel was demineralized under conditions where the medium was partially saturated with respect to hydroxyapatite, subsequent remineralization was successful; but when prior demineralization was carried out under more severe conditions, remineralization attempts gave poor results.

Since successful remineralization occurred under conditions where dissolution *via* only Site 1 would be expected, the hypothesis follows that this site might be the principal one for successful remineralization. This remineralization might consist chiefly of the filling of *c*-axis holes.

The present experiments were designed to test for the existence of a relationship between the amount of demineralization *via* Site 1 and the subsequent degree of remineralization attainable.

EXPERIMENTAL

Materials—*Bovine Teeth*—Bovine incisors were used. Labial surfaces were ground with fine sandpaper to remove the pellicle.

Hydroxyapatite Pellets—Synthetic hydroxyapatite crystals, prepared using the procedure developed by Moreno *et al.* (7), were used in the preparation of pellets. About 50 mg of hydroxyapatite, preequilibrated in a humidity chamber containing saturated potassium nitrate aqueous solution to maintain the humidity at ~67%, was compressed in a die (0.62-cm diameter) under a force of 10,000 lb using a laboratory press.

Buffer Solutions—A solution 16% saturated (on a molar basis) with respect to the thermodynamic solubility of hydroxyapatite was used for demineralization. The solution was a 0.1 *M* acetate buffer containing 3.5 m*M* each of total calcium and total phosphate. The pH was adjusted to 4.5 with sodium hydroxide, and the ionic strength was adjusted to 0.5 with sodium chloride. For remineralization, a 0.1 *M* acetate buffer (pH 4.5) containing 12 m*M* of total calcium (including calcium 45 activity of 0.2 mCi/liter of solution), 12 m*M* total phosphate, 10 ppm of fluoride, and sodium chloride to adjust the ionic strength to 0.5 was used.

The ionic activity of hydroxyapatite, K_{HAP} ($\equiv a_{Ca}^{10} a_{PO_4}^{6} a_{OH}^{2}$), of this buffer was 10^{-120}. Although not saturated with respect to hydroxyapatite, this solution has a high enough degree of partial saturation so that hydroxyapatite dissolution would be expected to proceed slowly, if at all, based on previous work with solutions approaching this level of calcium and phosphate concentrations (3, 8).

Demineralization—A bovine tooth was covered with inlay wax except for 0.25 cm^2 of the labial surface. In the pellet experiments, a hydroxyapatite pellet with a surface area of 0.58 cm^2 was fixed on a small glass plate by the wax. Either the tooth or pellet then was demineralized in the 16% partially saturated buffer solution for 0.5–6 hr. The solution was shaken gently with a wrist-action shaker[1] and kept at 30°. The volume of the buffer solution was 10 ml/0.25 cm^2 of the sample surface.

Remineralization—The demineralized tooth or pellet then was given a remineralization treatment for 24–72 hr in 50 ml of the remineralization buffer solution. The solution was shaken gently and kept at 30° as remineralization proceeded.

Etching and Scraping—The remineralized sample then was etched in 1 ml of 0.5 *M* $HClO_4$ for successive periods of 15, 15, 30, 60, 120, 200, and 200 sec; the surface was washed with 1 ml of water after each etching step. The depths of the etched layers were estimated from the amounts of phosphate etched. Some hydroxyapatite pellets were scraped evenly

[1] Burrell Co.

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