was unrelated to the particle size. The size distributions for suspensions of Lot B prednisolone acetate with and without the presence of hydroxypropyl methylcellulose were almost identical (Fig. 8).

The dissolution profiles for Lot B prednisolone acetate suspensions are summarized in Fig. 9. The presence of hydroxypropyl methylcellulose in all concentrations and at all viscosities seemed to inhibit the dissolution of Lot B. This finding is similar to the results found in experiments with Lot A. As with Lot A, the degree of inhibition did not appear to depend on the original viscosity or concentration of hydroxypropyl methylcellulose used. The 0.1 and 0.5% hydroxypropyl methylcellulose 4000 cps and the 0.1 and 0.5% hydroxypropyl methylcellulose 50 cps all inhibited Lot B prednisolone acetate to approximately the same degree.

The agents studied are common suspending agents, and brand-to-brand variations in the dissolution of commercial prednisolone acetate products exist (5). Particle-size differences are not the only factors to consider when studying the variation of drug dissolution profiles. Other factors such as the method of manufacture (milling and order of milling and mixing) and aging may alter these results.

In conclusion, the common ionic suspending agent carboxymethylcellulose sodium acted in two ways. It seemed to alter the particle-size distribution by reducing the percentage of fine prednisolone acetate particles in suspension, and it enhanced prednisolone acetate dissolution. With Lot A prednisolone acetate, which contained a larger percentage of fine particles, the reduction of these fine particles was sufficient to exceed the concurrent dissolution rate increase. Therefore, the net result was decreased prednisolone acetate dissolution. But with Lot B, where the proportion of fine particles was small and there was no significant change in particle-size distribution, the dissolution enhancement properties of carboxymethylcellulose sodium predominated. Thus, the effects of carboxymethylcellulose sodium on prednisolone acetate dissolution were lot-to-lot dependent, with the main variable being the particle distribution of the powder.

Dissolution inhibition of the two lots by nonionic hydroxypropyl methylcellulose, which also can alter particle-size distribution, was independent of lot-to-lot variation. This inhibition might be due to the formation of a viscous diffusion layer similar to that described for methylcellulose (4). Since hydroxypropyl methylcellulose is used in many commercial prednisolone acetate suspensions, this finding should be considered by formulation scientists.

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Corticosteroid Determination in Skin Preparations by a Reaction Rate Method

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Received March 19, 1979, from the **Laboratory of Analytical Chemistry, University of Athens, Athens, Greece,* and the ‡*School of Chemical Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801.* Accepted for publication June 5, 1979.

Abstract □ A reaction rate method for the determination of betamethasone, betamethasone valerate, triamcinolone acetonide, and fluocinolone acetonide is described. The method is based on a modification of the widely accepted blue tetrazolium reaction. Analysis times of 30–70 sec are required. Relative standard deviations of 0.3–1.9% are obtained, and the analytical working curves are linear. Analysis of pharmaceutical skin preparations by the new method gave results that correlated well with the time-consuming standard equilibrium method. Analysis of betamethasone and betamethasone valerate mixtures by measuring absorbance values at two different times was performed also.

Keyphrases □ Corticosteroids—analysis, skin preparations, reaction rate method □ Betamethasone—analysis, skin preparations, reaction rate method □ Betamethasone valerate—analysis, skin preparations, reaction rate method □ Triamcinolone acetonide—analysis, skin preparations, reaction rate method □ Fluocinolone acetonide—analysis, skin preparations, reaction rate method □ Skin preparations—analysis, corticosteroids, reaction rate method

A widely used spectrophotometric method for the determination of corticosteroid purity and the potency of dosage forms containing such steroids is based on the blue tetrazolium reaction (1). Blue tetrazolium (I), 3,3′-(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis[2,5-diphenyl-2*H*-tetrazolium] dichloride, in an alcoholic solution of a strong base oxidizes the α-ketol group on the C_{17} side chain and is reduced quantitatively to red formazan, which is measured spectrophotometrically at 525 nm. This absorbance, measured a specified time after mixing of the sample with I and the base, is compared to the absorbances of a blank and of a standard solution to quantitate the steroid concentration in the sample. This procedure is the basis for the official NF (2) and USP (3) method.

BACKGROUND

Extensive investigations (4–13) of the reaction conditions established that the analytical procedure is subject to many variables such as temperature; solvent; concentrations of base, water, and tetrazolium; steric configuration of the corticosteroid molecule; light; and air oxygen. The effect of these variables is minimized by analyzing reagent blanks, standards, and samples concurrently. In addition to these problems, which decrease the precision and the accuracy of the official equilibrium method, long measurement times of 30–470 min are necessary for maximum absorbance (10).

Under the conditions usually employed in the assay, where the steroid

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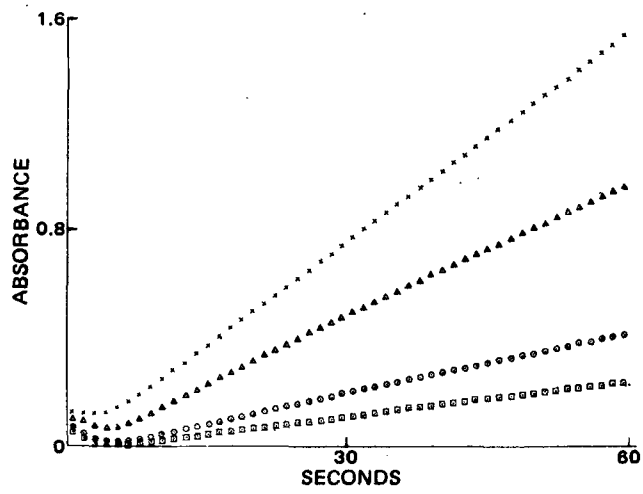

Figure 1—*Effect of tetramethylammonium hydroxide concentration (□, 0.5%; ○, 1%; △, 3%; and ×, 5%) on the reaction rate for 14 mg of betamethasone/100 ml.*

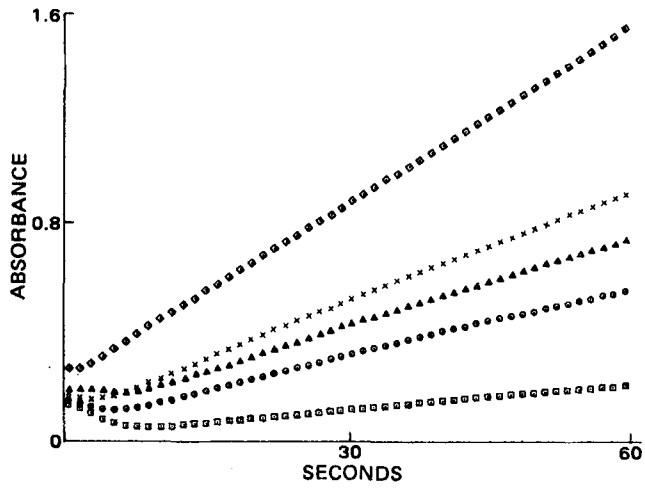

Figure 3—*Reaction rate curves for betamethasone concentrations of 2.00 (□), 6.00 (○), 8.00 (△), 10.0 (×), and 14.0 (◇) mg/100 ml using 5% tetramethylammonium hydroxide.*

is present at a much lower concentration than that of the base and the I reagent, the formazan appearance rate exhibits a first-order dependency on the steroid concentration (6). This behavior is of potential analytical utility because the reaction rate methods (14) are time saving and less sensitive to the reaction variables. Oteiza *et al.* (15), using the stopped-flow technique, developed a new reaction rate procedure for hydrocortisone determination which decreases the analysis time and increases the precision and accuracy considerably.

In this paper, the application of the reaction rate procedure is extended to betamethasone, betamethasone valerate, triamcinolone acetonide, and fluocinolone acetonide. Results obtained by the reaction rate method are compared with the USP–NF method for pharmaceutical skin preparations. The same technique was used to analyze betamethasone and betamethasone valerate mixtures.

EXPERIMENTAL

Apparatus—The apparatus used for the reaction rate method was the automated system described previously (16, 17). This system provided for automatic aliquoting and mixing of the sample and reagent and for delivery of the mixed solution into the measurement cell (2-cm path length, 60-μl volume) by a stopped-flow unit incorporated in a modular spectrophotometer. Automation was achieved by a minicomputer[1] with a 16 K memory. Associated peripheral devices were a teletype, a dual magnetic tape I/O, a linear printer, and an oscilloscope display.

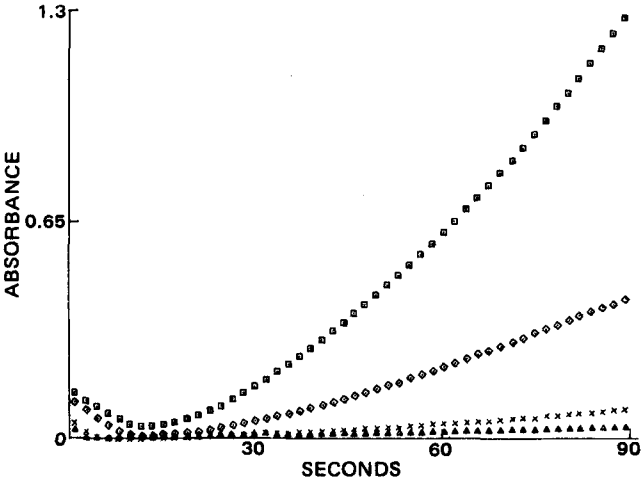

Figure 2—*Effect of tetramethylammonium hydroxide concentration (△, 0.5%; ×, 1%; ◇, 3%; and □, 5%) on the reaction rate for 30.0 mg of betamethasone valerate/100 ml.*

Several programs have been developed for the investigative or the routine operation mode. The investigative mode provides flexible data-taking options, oscilloscope display, data printing, and extraction of rate or equilibrium information from various portions of the absorbance–time curve. It generally is used for initial characterization or fundamental studies. The routine mode provides sequential analyses of standards and samples placed on a turntable and printing of a working curve and sample values. Either rate or equilibrium methods may be selected. An automated computer-controlled solution-handling system utilizing weights of solution can be used in conjunction with this system for the preparation of standards and samples (18). A ratio-recording spectrophotometer[2] was used for the equilibrium measurements.

Reagents—Corticosteroid stock solutions, 20 mg/100 ml, were prepared weekly by dissolving 20 mg of betamethasone[3], betamethasone valerate[3], triamcinolone acetonide[4], or fluocinolone acetonide[5] in 100 ml of 95% ethanol. A 0.5% blue tetrazolium[6] solution was prepared by dissolving 0.5 g of blue tetrazolium in 100 ml of absolute methanol. This solution was protected from light. A 5% solution of tetramethylammonium hydroxide was prepared by dissolving 2.5 g of tetramethylammonium hydroxide pentahydrate[6] in 50 ml of USP reagent quality absolute ethanol[7]. Different base concentrations were prepared from the 5% solution by appropriate dilution with absolute ethanol.

The standard corticosteroid solutions were prepared daily by adding 2 ml of the blue tetrazolium solution to an appropriate volume of the stock corticosteroid solution and diluting to 10 ml with 95% ethanol. Standard betamethasone and betamethasone valerate solutions were prepared similarly.

Sample Preparation—Samples were prepared from the pharmaceutical preparations—lotions, creams, and ointments—by the column chromatographic procedure of Graham *et al.* (19), in which the corticosteroid is held on the acetonitrile–diatomaceous earth column while decomposition products and interfering substances are removed by *n*-heptane. The corticosteroid was eluted from the column with chloroform. The eluate was evaporated carefully to dryness, and the residue was dissolved in 95% ethanol and diluted to 25 ml. A 5-ml aliquot was added to 2 ml of blue tetrazolium solution and diluted to 10 ml with 95% ethanol. This 10-ml solution is referred to here as the sample. The column could be readily modified to include acidic, basic, or neutral aqueous trap layers when necessary. Approximately 30 min was routinely required to prepare an interference-free sample for analysis.

Equilibrium Procedure—The equilibrium procedures were the official procedures given in USP XIX and NF XIV. The absorbance was measured at a specified time (45–90 min) after the standards and samples

[1] PDP 8/f, Digital Equipment Corp.

[2] Model 721, GCA/McPherson, Acton, MA 01720.
[3] Schering Co.
[4] Squibb Inc.
[5] Syntex Lab.
[6] Sigma Chemical Co., St. Louis, MO 63178.
[7] U.S. Industrial Chemicals Co., Tuscola, IL 61953.

Table I—Reaction Rates for Different Measurement Times for Triamcinolone Acetonide [a]

Measurement Time, sec	Rate, Δmamp/sec[b]	*RSD*, %
5.0	12.1	1.7
10.0	12.0	1.0
15.0	12.0	0.6
20.0	11.8	0.4
25.0	11.8	0.8

[a] Analysis of 5 mg/100-ml standard with 10-sec delay time. [b] Average of five determinations on a single sample.

Table II—Results Used for Corticosteroid Reaction Rate Working Curves

Corticosteroid	Concentration, mg/100 ml	Rate, Δmamp/sec[a]	*RSD*, %
Betamethasone[b]	2.00	7.7	1.5
	5.00	11.1	1.1
	10.0	16.3	1.6
	15.0	22.3	0.7
Betamethasone valerate[c]	10.0	6.9	1.2
	15.0	10.5	0.6
	20.0	14.5	0.9
	30.0	22.0	0.9
Triamcinolone acetonide[d]	1.00	2.3	1.4
	3.00	6.8	0.4
	5.00	11.4	0.7
	7.00	16.2	0.3
	10.0	23.6	0.7
Fluocinolone acetonide[e]	0.50	3.1	1.9
	1.00	6.2	1.7
	2.50	15.6	0.8
	3.50	22.5	0.8

[a] Average of five determinations on a single sample. [b] Working curve: slope = 1.11, intercept = 5.4, and r = 0.9991. [c] Working curve: slope = 0.758, intercept = −0.7, and r = 0.9998. [d] Working curve: slope = 2.37, intercept = −0.2, and r = 0.9996. [e] Working curve: slope = 6.44, intercept = −0.2, and r = 0.9995.

were mixed with the two reagents, and the mixtures were allowed to stand in the dark.

Reaction Rate Procedure—One hundred microliters each of the tetramethylammonium hydroxide solution and the appropriate standard or sample were sampled by the automatic syringes of the stopped-flow module (17). The syringes in the module then drove the solutions through the mixer and transferred the mixed solution to the observation cell. The change in absorbance was automatically monitored at 525 nm during the selected measurement time and used to construct a rate curve and a working curve or to provide quantitative concentration information for the pharmaceutical skin preparations.

The solutions and spectrophotometer were at ambient temperature in a temperature-controlled laboratory maintained at a nominal temperature of 25°.

RESULTS AND DISCUSSION

As shown in Fig. 1, the reaction rate was strongly dependent on the base concentration. The appropriate base strength could be chosen to provide the sensitivity needed at a minimum cost per analysis. Good sensitivities and precisions could be obtained in relatively short measurement times with the 5% base. The effect of base concentration was more critical for corticosteroid-17 ester reactions where the ester hydrolysis was a prerequisite. As shown in Fig. 2, in the betamethasone valerate reaction, the hydrolysis step appeared prior to the reaction process. Attempts to provide ester hydrolysis before the reaction with blue tetrazolium showed that both the corticosteroid and its esters were partially decomposed after contact with tetramethylammonium hydroxide.

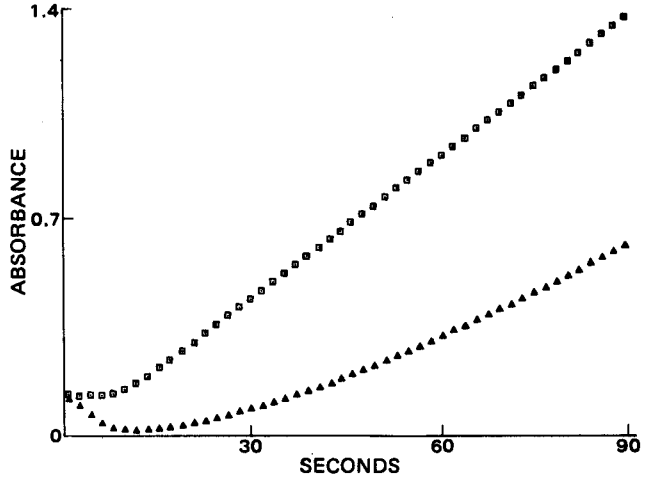

Figure 4—*Reaction rate curves for 10 mg of betamethasone/100 ml (□) and for 15 mg of betamethasone valerate/100 ml (△).*

The reaction rate curves for the four standards of betamethasone with 5% base over 60 sec are shown in Fig. 3. The reaction curves are sufficiently straight lines, as a result of the first-order conditions. A 10-sec delay after mixing was employed before the measurement period for betamethasone, triamcinolone acetonide, and fluocinolone acetonide. This time allowed for any nonreproducible behavior near the beginning of the reaction to terminate. For betamethasone valerate, a 40-sec delay was needed for completion of hydrolysis. The optimum measurement times were determined by using these delay times and varying the rate measurement over various periods. The results for triamcinolone acetonide are shown in Table I. The best reproducibility, ~0.4%, was obtained with a 20-sec measurement time. Similar results were obtained for betamethasone and fluocinolone acetonide. For betamethasone valerate, a 30-sec measurement time was optimum.

The results obtained for the working curves are shown in Table II. Good linearity was obtained for concentrations of 2–15 mg/100 ml for betamethasone, 10–30 mg/100 ml for betamethasone valerate, 1–10 mg/100 ml for triamcinolone acetonide, and 0.5–3.5 mg/100 ml for fluocinolone acetonide. The relative standard deviations varied from 0.3 to 1.9%. The working curves could be generated in ~8 min for triplicate analyses on each standard. This period is more than a factor of 10 less than the time required to prepare a working curve for the equilibrium method where single determinations on a standard, sample, and blank are generally performed (2, 3). Thus, the total analysis time, including sample prepa-

Table III—Corticosteroid Assay Results from Commercial Skin Preparations

Preparation		Concentration, %	Product Type	Assay, % of declared		
				Reaction Rate[a]	Equilibrium[b]	Difference[c]
Betamethasone valerate	1	0.1	Lotion	76.0	75.2	+0.8
	2	0.1	Lotion	74.8	75.5	−0.7
	3	0.01	Cream	94.2	95.0	−0.8
	4	0.01	Cream	90.8	90.0	+0.8
Triamcinolone acetonide	5	0.1	Ointment	90.9	91.8	−0.9
	6	0.5	Ointment	94.2	96.2	−2.0
	7	0.5	Cream	91.5	90.0	+1.5
	8	0.1	Cream	79.9	82.1	−2.2
Fluocinolone acetonide	9	0.025	Cream	97.4	98.9	−1.5
	10	0.025	Cream	95.2	95.0	+0.2
	11	0.025	Cream	95.1	94.6	+0.5
	12	0.01	Cream	97.8	98.5	−0.7

[a] Average of three determinations on a single sample. [b] Average of two determinations on a single sample. [c] Percent of reaction rate minus percent by equilibrium.

Table IV—Analysis of Betamethasone and Betamethasone Valerate Mixtures

Steroid Taken, mg/100 ml		Steroid Found[a], mg/100 ml	
Betamethasone	Betamethasone Valerate	Betamethasone	Betamethasone Valerate
4.00	30.0	4.41	27.5
6.00	25.0	6.13	24.4
8.00	20.0	7.61	21.8
10.0	15.0	9.30	17.5
12.0	5.0	12.5	4.3

[a] Average of three determinations on a single sample where $K_B = 4.494$ (intercept = −0.006 and $r = 0.9994$), $K_{BV} = 1.078$ (intercept = +0.02 and $r = 0.9998$), $K'_B = 8.443$ (intercept = −0.07 and $r = 0.9994$), and $K'_{BV} = 3.379$ (intercept = −0.04 and $r = 0.998$).

ration, can be reduced from over 2 hr using the equilibrium procedure to slightly over 30 min with the reaction rate procedure. The addition of acid to stop the very slow reaction, used in equilibrium procedures for betamethasone valerate and triamcinolone acetonide, is also unnecessary. Table III shows results obtained on a series of commercial lotions, creams, and ointments by the new reaction rate method and by the official method for steroid analysis. Good agreement exists between the two methods (differences of 0.2–2.2%).

The difference in the betamethasone and betamethasone valerate reaction rates can be used for analyzing mixtures of these closely related steroids. Betamethasone valerate, being an ester, has a relatively slow reaction rate due to the hydrolysis step. Figure 4 shows reaction curves for equivalent quantities of the two steroids. For the analysis of mixtures, the method of proportional equations (20) was employed. In a reacting mixture containing both steroids, a common product, the formazan, was produced by the two simultaneous processes. Although the kinetics of formazan generation in ester reaction are complex and not first order, absorbance at a constant reaction time is directly proportional to the initial ester concentration. Therefore, the method of proportional equations can be used to analyze mixtures of betamethasone and betamethasone valerate. The absorbance, which is specific for the product, is thus established at any time by:

$$A = K_B B + K_{BV} BV \qquad \text{(Eq. 1)}$$

where A is the absorbance at 525 nm; B and BV are the initial concentrations of betamethasone and betamethasone valerate, respectively; and K_B and K_{BV} are the experimental slopes of absorbance *versus* betamethasone and betamethasone valerate concentrations, respectively.

Measurement of absorbance values at a shorter time, t, and at a longer time, t', allows the formulation of two equations, which can be solved simultaneously to yield values for B and BV. The experimental factors K_B and K_{BV} for time t and K'_B and K'_{BV} for time t' were calculated by measuring absorbance values for standard steroid solutions. For this system, 30- and 50-sec times were appropriate. Analysis of binary mixtures containing 4–12 mg/100 ml of betamethasone and 30–5 mg/100 ml of betamethasone valerate were carried out (Table IV).

The method was also applied to an aged 0.1% betamethasone valerate lotion (a year after the expiration date). Only 67.5% of betamethasone valerate was present, and 8.3% was hydrolyzed to betamethasone. Decomposition of the rest must be assumed.

These results were obtained on an automatic stopped-flow spectrophotometric system with several features to ensure high reliability in its measurements. A beam splitter and a reference detector were employed to correct for light source fluctuations (16). The stopped-flow module provided precisions better than 0.1% *RSD* for the aliquoting, mixing, and transferring of solutions to the 2-cm observation cell. The entire system was controlled by a minicomputer, eliminating most manual steps required for investigative or routine use. Finally, the rapid analysis time and excellent results recommended this system for routine analytical applications.

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