c_i = free (unbound) molar concentration of ith compound
$\mathbf{c} = (c_1, c_2, \ldots, c_M)^T$ (see c_i)
$[c_i]$ = total (free + bound) molar concentration of ith compound
$[\mathbf{c}] = ([c_1], [c_2], \ldots, [c_M])^T$ (see $[c_i]$)
$(c_b)_i$ = "membrane bound concentration" of ith compound
D = diag$(1/K_1, 1/K_2, \ldots, 1/K_M)$ = diagonal matrix containing reciprocals of dialysis rate constants
f_i = function describing relationship between $[c_i]$ and $\mathbf{c}$ or $[c_i]$ and $\mathbf{s}$
$\mathbf{f} = (f_1, f_2, \ldots, f_M)^T$ (see f_i)
g_i = function describing relationship between $(c_b)_i$ and s_i
$\mathbf{g} = (g_1, g_2, \ldots, g_M)^T$ (see g_i)
i = subscript ($i = 1, 2, \ldots$)
j = subscript ($j = 1, 2, \ldots$)
$\mathbf{J}\left(\frac{\mathbf{f}}{\mathbf{s}}\right)$ = Jacobian matrix of $\mathbf{f}$ with respect to $\mathbf{s}$
J_{ij} = ijth element of Jacobian matrix
k_{ij} = association constant for ith compound's binding to jth class of binding sites on macromolecule
k_i = k_{i1} (see k_{ij})
k_i^* = membrane binding "association constant" of ith compound
K_m = dialysis rate constant for mth compound
m = subscript ($m = 1, 2, \ldots$)
M = number of compounds
n_j = number of binding sites in jth class of sites
n_i^* = mathematical analog to n_i in Langmuir-type membrane "binding" model
n = n_1 (see n_j)
N = number of binding classes
$\bar{\nu}_i$ = number of moles of ith compound ("ligand") bound per mole of macromolecule
$\bar{\nu}_i^*$ = amount of ith compound bound per amount of available membrane material
P = total molar concentration of macromolecular compound
θ = parameter vector containing elements such as n_j, k_{ij}, and P or other parameters used in the particular mathematical model $\mathbf{f}$ describing binding kinetics
r = correlation coefficient
s_i = absolute value of slope in a $[c_i]$ *versus* t plot at time t. This quantity is used as a parameter variable in the parametric representation of the variables $[c_i]$ and t
$\mathbf{s} = (s_1, s_2, \ldots, s_M)^T$ (see s_i)
$\mathbf{s}_0$ = $\mathbf{s}$ evaluated at $t = 0$
t = time
T = transpose
u_i = molecular weight of ith compound
υ = subscript ($\upsilon = 1, 2, \ldots$)
V = volume of dialysis compartment
w = weight of available membrane material
y_i = ith dependent variable in transformed set of linear regression equations (Eq. A2) used to obtain initial estimates of the binding parameters (n's and k's) by multiple linear regression technique

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Analysis of Steroid Phosphates by High-Pressure Liquid Chromatography: Betamethasone Sodium Phosphate

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Abstract □ A sensitive, automatable high-pressure liquid chromatographic procedure is presented for the determination of steroid phosphates. Quantitation is described for betamethasone sodium phosphate in dosage forms in the presence of polar excipients. The separation of a multicomponent mixture of steroid phosphates also is reported.

Keyphrases □ Betamethasone sodium phosphate—high-pressure liquid chromatographic analysis in dosage forms □ High-pressure liquid chromatography—analysis, betamethasone sodium phosphate in dosage forms □ Glucocorticoids—betamethasone sodium phosphate, high-pressure liquid chromatographic analysis in dosage forms □ Steroid phosphates, various—high-pressure liquid chromatographic analysis in dosage forms

Steroid phosphates are highly effective anti-inflammatory agents produced in ophthalmic, injectable, and solid dosage forms. Reported analyses utilize spectrophotometric procedures preceded by extraction and/or reaction methods (1–3). These procedures are time consuming and relatively difficult. Two chromatographic methods have been reported: TLC of the methyl ester and ion chromatography (4, 5). This laboratory previously used

0022-3549/78/0700/0913$01.00/0

Table I—Retention Times for Betamethasone Sodium Phosphate, Its Decomposition Products, and Common Preservatives Used in Formulations

Compound	Retention Time[a], min
Decomposition product	2.5–3.5
Phenol	4.0
Betamethasone sodium phosphate	5.0
Decomposition product	5.5
Betamethasone alcohol	8.0
Decomposition product	11.0
Butylparaben	12.0
Methandrostenolone	15.0

[a] Mobile phase: methanol–0.09 *M* KH_2PO_4 (1:1).

paper chromatography followed by elution of the steroid phosphate and quantitative measurement by colorimetric assay or by an enzymatic–colorimetric analysis.

The present study was undertaken to establish a rapid, reliable, stability-indicating high-pressure liquid chromatographic (HPLC) procedure for steroid phosphates, specifically for betamethasone sodium phosphate in the presence of compounds typically present in formulations. Quantitation of betamethasone sodium phosphate and other steroid phosphates in various formulations was achieved.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] with a UV absorption detector (254 nm) and a stainless steel column, 30 cm × 4 mm i.d., was used. The column packing[2] was porous silica particles permanently bonded to a monomolecular layer of organosilane. The column was operated at 1200 psi, resulting in a flow rate of 1 ml/min. Each analysis was performed at ambient temperature. Samples were injected with a 5-μl high-pressure syringe[3].

Materials—Acetone[4], methanol[4], butylparaben[5], and methandrostenolone[5] were used as received. The samples of betamethasone sodium phosphate, prednisolone sodium phosphate, dichlorisone sodium phosphate, testosterone sodium phosphate, and 16β-methylprednisone sodium phosphate were obtained in pure form[5].

Mobile Phase—The mobile phase was degassed methanol–0.09 *M* KH_2PO_4[6] (60:40) (Fig. 1). Multicomponent steroid phosphates were separated using methanol–0.09 *M* KH_2PO_4 (1:1) (Fig. 2).

Internal Standard, Reference Standard, and Sample Solutions—The internal standard solution of butylparaben was prepared by dissolving an accurately weighed (~150 mg) quantity of material in acetone in a 50-ml volumetric flask. The reference standard, about 35 mg of betamethasone sodium phosphate, was weighed accurately into each of two 10-ml volumetric flasks and dissolved in water. A response factor solution was prepared by adding 3.0 ml of reference standard solution, 2.0 ml of internal standard solution, and 3.0 ml of water to a stoppered flask. The remaining quantity of reference standard solution was saved for the corrected reference standard weight determination described under *Determination of Corrected Standard Weight*.

The sample solution consisted of an aliquot of sample equivalent to about 10 mg of betamethasone sodium phosphate[7], 2.0 ml of internal standard solution, and water (total of 8.0 ml)[8].

Chromatography—Each of two analyses consisted of an injection of 5 μl of sample solution followed by a 5-μl injection of a reference standard solution into an equilibrated column, using a detector attenuation of 0.64 absorbance unit. Phenol eluted immediately following the solvent. Betamethasone sodium phosphate eluted after 5 min, followed by butylparaben at 12 min (Fig. 1).

[1] Model 841, DuPont, Wilmington, Del.
[2] μBondapak C_{18}, Waters Associates, Milford, Mass.
[3] Model HP-305, Hamilton, Reno, Nev.
[4] Analytical reagent, Mallinckrodt.
[5] Reference standards, Schering Corp., Bloomfield, N.J.
[6] Reagent, Matheson, Coleman and Bell.
[7] Celestone Phosphate Injection, Schering. Label claim of 2.63, 3.95, or 5.26 mg of anhydrous betamethasone sodium phosphate/ml.
[8] The reference standard must be corrected for moisture content using a standard absorptivity figure.

Table II—Method Reproducibility[a]

Sample	Betamethasone Sodium Phosphate[b], mg/ml Assay I	Assay II
1	3.8	3.9
2	3.8	3.9
3	3.7	3.7
4	3.7	3.8
5	3.8	3.8
6	3.7	3.7

[a] Mobile phase: methanol–0.09 *M* KH_2PO_4 (60:40). [b] Each assay result is an average of two injections.

The peak height ratios were used to quantitate the resulting chromatograms. Equation 1 was used to calculate the response factor (*RF*) for the reference standard *versus* the internal standard:

$$RF = \left(\frac{PH_1}{PH_2}\right)\left(\frac{W_{is}}{W_s}\right)(0.133) \qquad \text{(Eq. 1)}$$

where PH_1 is the peak height or area of betamethasone sodium phosphate in the response factor standard solution, PH_2 is the peak height or area of butylparaben in the response factor standard solution, W_{is} is the weight (in milligrams) of butylparaben in the internal standard solution, W_s is the corrected weight (in milligrams) of standard in the reference standard solution, and 0.133 is the combined dilution factor of sample and internal standard solutions ($^1/_{50} \times {}^{10}/_1 \times {}^2/_8 \times {}^8/_3$).

This calculation is repeated for each determination, and an average response factor is used to derive the milligrams of betamethasone sodium phosphate per milliliter of original sample using:

$$\text{mg/ml} = \left(\frac{PH_3}{PH_4}\right)\left(\frac{W_{is}}{RF}\right)\left(\frac{1}{V}\right)(0.04) \qquad \text{(Eq. 2)}$$

where PH_3 is the peak height or area of betamethasone sodium phosphate in the sample solution, PH_4 is the peak height or area of butylparaben in the sample solution, *RF* is the average response factor, *V* is the volume of sample used in the preparation of the sample solution, and 0.04 is the combined dilution factor of sample and internal standard solutions ($^1/_{50} \times {}^2/_8 \times {}^8/_1$).

Determination of Corrected Standard Weight, W_s[8]—Reference standard solution (2.0 ml) is diluted with water to 200 ml in a volumetric flask. The absorbance (A_s) is read at 240 nm; W_s, in milligrams, is calculated in accordance with:

$$W_s = (A_s)(33.67) \qquad \text{(Eq. 3)}$$

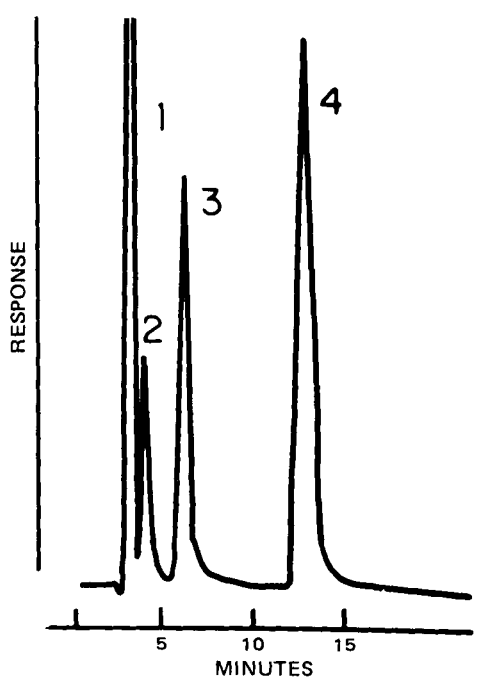

Figure 1—*Chromatogram of betamethasone sodium phosphate formulation. Key: 1, solvent; 2, phenol; 3, betamethasone sodium phosphate; and 4, butylparaben.*

Table III—Comparative Analyses Using Paper Chromatography and HPLC [a]

Sample	Betamethasone Sodium Phosphate, mg/ml	
	HPLC	Paper Chromatography
1	4.2	4.0
2	4.3	4.2
3	4.0	4.0
4	4.2	4.0
5	4.0	4.0
6	4.2	4.2
7	5.4	5.3
8	5.7	5.6
9	5.3	5.3
10	5.4	5.5

[a] Mobile phase: methanol–0.09 *M* KH_2PO_4 (60:40).

where A_s is the absorbance of the sample solution, and 33.67 equals 1000 divided by the absorptivity of betamethasone sodium phosphate which is 29.7.

RESULTS AND DISCUSSION

The polarity of the steroid phosphates necessitated the use of a nonpolar column. The high efficiency of the reversed-phase, small particle-size column enabled resolution of betamethasone sodium phosphate from all of its degradation products and most of the common excipients present in the formulation[7]. Mixtures of several steroid phosphates also were separated readily using the mobile phase indicated previously [methanol–0.09 *M* KH_2PO_4 (1:1)].

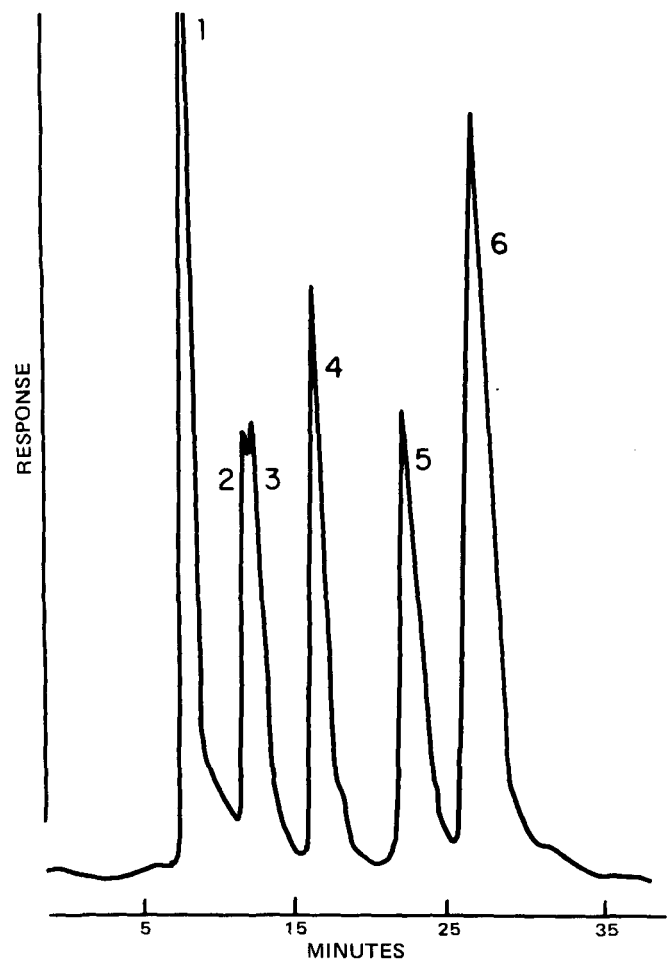

Figure 2—*Chromatogram of a mixture of steroid phosphates. Key: 1, prednisone sodium phosphate; 2, prednisolone sodium phosphate; 3, 16β-methylprednisone sodium phosphate; 4, betamethasone sodium phosphate; 5, testosterone sodium phosphate; and 6, dichlorisone sodium phosphate.*

Table IV—Analysis of Related Formulations

Sample	Percent of Label Strength
	Betamethasone Phosphate[a]
1	97
2	98
3	99
4	100
5	100
	Prednisolone Phosphate[b]
1	100
2	100
3	100
4	102
5	101
	Betamethasone Phosphate with Betamethasone Acetate[c]
1	101
2	100
3	101
4	100
5	103

[a] Ophthalmic solution (0.1%), Schering. [b] Metreton Ophthalmic and Optimyd Ophthalmic, Schering. [c] Celestone Soluspan, Schering.

An acidic mobile phase was utilized to keep the steroid phosphates in the nonionized state. The addition of small quantities of the aqueous component increased both the retention time and the separation of the materials. The optimum resolution and retention times were obtained with a solvent ratio of 13:8 for methanol–0.09 *M* KH_2PO_4.

The internal standard method for the quantitative calculations was chosen to minimize errors resulting from both the apparatus and the injection procedure.

After surveying several compounds, either butylparaben or methandrostenolone was found to be suitable as an internal standard. Each was easily resolved from betamethasone sodium phosphate and was separated from degradation products and excipients. Retention times are presented in Table I.

The plot of the peak height response *versus* the amount of betamethasone sodium phosphate was linear, thus demonstrating that the method can be used for quantitation. This conclusion was obtained after five samples, varying in the quantity of betamethasone sodium phosphate but with a constant concentration of internal standard, were analyzed and did not deviate from the results expected if Beer's law were valid.

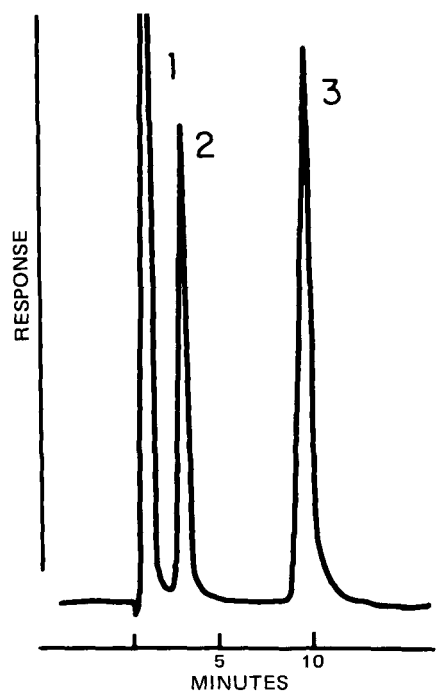

Figure 3—*Chromatogram of a prednisolone sodium phosphate formulation. Key: 1, solvent; 2, prednisolone sodium phosphate; and 3, butylparaben.*

(Similar results were obtained from aged samples of betamethasone sodium phosphate formulations.)

To ensure the usefulness of this technique, pure betamethasone sodium phosphate was degraded by heat, alkalinity, and acidity and the decomposed material was chromatographed. The chromatographic system separated all degradation products and possible manufacturing impurities. The decrease in the peak height upon degradation was not compensated for by an increase in the size of the decomposition peak. Therefore, the primary product, presumed to be the 17-ketone (6), was not detected in this system and was probably retained. Constant amounts of reference standard and internal standard were added to varying amounts of placebo and aged placebo. Recoveries were consistently in the 100% range.

The instrument precision was good at the attenuation used. Duplicate assays were performed for the injection formulation over several days, and the results indicate high reproducibility (Table II) and agreed well with the manufacturer label claims. Comparison of these results with the results obtained with a paper chromatographic assay shows the methods to be equivalent (Table III).

This method can be applied to other steroid phosphate formulations (Fig. 3 and Table IV). HPLC shortens analysis time and is accurate and easy to perform. In addition, it can be easily automated with automatic samplers.

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ACKNOWLEDGMENTS

The authors express their appreciation to Mr. S. Gruber and Ms. V. Bole for assistance.

Theophylline Bioavailability following Chronic Dosing of an Elixir and Two Solid Dosage Forms

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Received May 26, 1977, from the *Departments of Medicine and Pharmacology, Clinical Pharmacology–Toxicology Center, Unive sity of Kansas Medical Center, College of Health Sciences and Hospital, Kansas City, KS 66103.* Accepted for publication October 19, 1977.

Abstract □ Theophylline bioavailability following chronic dosing of an elixir and two commercial tablet formulations (I and II) relative to an acute dose of elixir was evaluated in healthy volunteers. Both tablet formulations contained ephedrine. In addition, Tablet I contained hydroxyzine hydrochloride, and Tablet II contained phenobarbital. The mean area under the serum concentration–time curve (*AUC*) calculated either from time $0 \rightarrow \infty$ for a single dose or over one dosing interval after repetitive doses was the highest after chronic administration of the elixir. The *AUC* after chronic elixir, in fact, was statistically different from the values after acute elixir ($p < 0.05$) and Tablet II ($p < 0.05$). There was, however, a large variation in the elimination half-life among the four theophylline treatments. The mean $t_{1/2}$ was the longest after chronic elixir followed by Tablet I, Tablet II, and acute elixir. The *AUC* values for the four treatments, when corrected for differences in $t_{1/2}$, were no longer significantly different, indicating that the extent of theophylline absorption was essentially the same from all three tested products. The time to peak and the peak serum concentration also did not differ among treatments. The prolongation in $t_{1/2}$ following chronic treatment with the elixir and its subsequent shortening during tablet administration suggest an initial inhibition followed by induction of theophylline metabolism. These changes may be due to the prolonged treatment with theophylline itself or the other drug ingredients in the dosage form.

Keyphrases □ Theophylline—bioavailability of commercial elixir and two tablets compared, chronic dosing in humans □ Bioavailability—theophylline, commercial elixir and two tablets compared, chronic dosing in humans □ Relaxants, smooth muscle—theophylline, bioavailability of commercial elixir and two tablets compared, chronic dosing in humans

Literature reports (1, 2) suggested that incomplete bioavailability of theophylline tablets, singly or in combination with ephedrine and sedatives such as phenobarbital or hydroxyzine, may frequently cause therapeutic failures. Theophylline bioavailability after a single oral dose of an elixir and two different combination tablets was essentially complete as compared to intrave. ous aminophylline (3). However, it is not known whether differences in bioavailability for these theophylline formulations occur when given chronically.

Several factors might affect the serum theophylline concentration when it is administered over an extended period. In humans, theophylline is eliminated largely by oxidative microsomal metabolism (4). Drugs frequently combined with theophylline, such as phenobarbital and hydroxyzine, may alter its pharmacokinetics. Cigarette smoking significantly increased the serum clearance of theophylline (5, 6), presumably due to microsomal enzyme induction by the polycyclic aromatic hydrocarbon constituents in the smoke. Theophylline induced its own metabolism in the rat (7), and a similar action could occur in humans.

The present study was designed to ascertain the chronic oral availability of theophylline from previously studied lots of elixir and combination tablet preparations relative to an acute dose of the elixir. In addition, the effects of long-term administration of theophylline and the other drugs in the combination tablets on the disposition kinetics of theophylline were observed.

EXPERIMENTAL

Subjects—Twelve healthy volunteers between 22 and 40 years of age and weighing between 46.2 and 82.4 kg participated. Of the 12 subjects (five men and seven women), only two were habitual smokers, each averaging fewer than 20 cigarettes/day. Written informed consent, a history, a physical examination, and laboratory tests (complete blood count, urinalysis, long-lead II ECG, serum thyroxine, bilirubin, creatinine, glutamic-oxaloacetic transaminase, and alkaline phosphatase) were

0022-3549/78/0700/0916$01.00/0