3020 (aromatic), 2940, 2915, 1700 (CO), 1650, 1530, 1490, 1450, 1315, 1110, 1080, 1058, 860, and 748 cm^{-1}; NMR ($CDCl_3$): δ 7.1–7.42 (m, 4H, aromatic), 6.70–7.10 (broad s, 1H, NH), 3.66 (s, 3H, OCH_3), and 2.07 (s, 3H, CH_3) ppm.

Anal.—Calc. for $C_{11}H_{11}NO_3$: C, 64.39; H, 5.37; N, 6.83. Found: C, 64.28; H, 5.41; N, 6.97.

Compounds IV*b*–IV*g* were prepared similarly from III and the appropriate alcohols (Table I).

***p*-Chlorophenyl Benzo[*b*]-3-methyl-2-furancarbamate (IV*h*)**—A solution of III (2.01 g, 0.01 mole) and *p*-chlorophenol (1.28 g, 0.01 mole) in 30 ml of dry benzene was refluxed for 4 hr. The solvent was evaporated, and the residue was crystallized from benzene to give 1.65 g (55%) of the desired compound, mp 155–156°; IR (KBr): 3230 (NH), 1722 (CO), 1670, 1510, 1490, 1460, 1250, 1202, 1100, 1020, 1015, 855, and 752 cm^{-1}; NMR ($CDCl_3$): δ 7.7–7 (m, 8H, aromatic), 7–6.7 (broad s, 1H, NH), and 2.17 (s, 3H, CH_3) ppm.

Anal.—Calc. for $C_{16}H_{12}ClNO_3$: C, 63.68; H, 3.98; N, 4.64. Found: C, 63.79; H, 3.82; N, 4.73.

Compounds IV*i*–IV*n* were prepared similarly.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 27, 1975, from the *Department of Chemistry, College of Pharmacy, Tehran University, Tehran, Iran.*

Accepted for publication September 26, 1975.

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Simultaneous Analysis of Hydrocortisone and Hydrocortisone Phosphate by High-Pressure Liquid Chromatography: Reversed-Phase, Ion-Pairing Approach

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Abstract □ The reversed-phase, ion-pairing approach to high-pressure liquid chromatography was applied to the simultaneous analysis of hydrocortisone and its phosphate ester in laboratory-prepared samples and injectable solutions. Results of this technique were evaluated and compared with results of the official procedure.

Keyphrases □ Hydrocortisone—base and phosphate, simultaneous high-pressure liquid chromatographic analysis, prepared samples and injectable solutions □ High-pressure liquid chromatography—reversed-phase, ion-pairing approach, simultaneous analysis, hydrocortisone base and phosphate, prepared samples and injectable solutions □ Ion-pairing—application to high-pressure liquid chromatography, simultaneous analysis of hydrocortisone base and phosphate □ Glucocorticoids—hydrocortisone base and phosphate, simultaneous high-pressure liquid chromatographic analysis

For some time, these laboratories have been interested in the chromatographic applications of ion-pairing (1), and this interest has resulted in a unique approach to the rational separation of ionic compounds by high-pressure liquid chromatography (HPLC) (2). The technique involves the use of a lipophilic stationary phase and the addition of selected ionic compounds to the mobile phase. Ionic analytes injected into this chromatographic system are retained, apparently as a function of the lipophilicity of the ion-pair formed within the system. Therefore, the technique may be referred to as resulting from a reversed-phase, ion-pairing approach to HPLC.

The advantages of this technique over conventional ion-exchange HPLC were discussed previously, and the utility of the approach to the simultaneous analysis of several ionic substances was explored (2). In this investigation, the reversed-phase, ion-pairing approach was applied to the simultaneous analysis of nonionic and ionic compounds, as exemplified by hydrocortisone and hydrocortisone phosphate. These drugs were selected because they may be encountered together in commercial preparations (*e.g.*, injectable solutions) of the phosphate ester. Since free hydrocortisone is regarded as an impurity in these preparations and limited in concentration to less than 1%, procedures for the analysis of hydrocortisone and its ester are required.

Currently (3), the determination of hydrocortisone in the drug substance requires a number of manipulative steps prior to analysis and subsequent use of the enzyme alkaline phosphatase. Problems in the use of the enzyme were noted previously (4), and the complex workup makes the assay lengthy and the results subject to variation. Present methods for the analysis of hydrocortisone phosphate in injectable solutions (5) make no attempt to quantitate the free hydrocortisone present, so a procedure for the simultaneous analysis of the two drugs is of interest.

EXPERIMENTAL

Apparatus and Operating Conditions—A liquid chromatograph

Table I—Effect of Selected Salts on the Retention Volume of Hydrocortisone and Hydrocortisone Phosphate

Salt[a]	Hydrocortisone Phosphate Retention Volume, ml[b]	Hydrocortisone Retention Volume, ml
None	1.9	6.1
Tetramethylammonium hydroxide	4.5	6.1
Tetraethylammonium hydroxide	7.9	6.0
Tetrapropylammonium hydroxide	16.2	6.1
Tetrabutylammonium hydroxide	21.4	6.1
Tetrapentylammonium hydroxide	27.6	6.2
Tridecylammonium formate	39.4	6.0

[a] In a mobile phase of methanol–water (420:500). [b] Flow rate of 3.0 ml/min.

equipped with a 6000-psi pump, a high-pressure injector[1], and a 254-nm detector was used. Peak areas were determined with an electronic digital integrator[2].

Column—The column[3] (0.64 cm o.d. and 25 cm in length) was prepacked with a stationary phase of porous silica (average particle size of 10 μm) to which was bonded a monomolecular layer of octadecylsilane. The value of V_0 for the column was 1.9 ml.

Mobile Phase—A solution of tetrapentylammonium hydroxide (1×10^{-2} *M*) in methanol (100 ml) was prepared as described previously (6). An aliquot (20 ml) of this solution was mixed with distilled water (500 ml). The pH of the combined solution was adjusted to 7.5 with 1% formic acid solution, and the mixture was combined with methanol (400 ml). The flow rate used for the analysis was 3.0 ml/min.

Standard Solutions—Hydrocortisone phosphate[4] solutions in water were prepared to contain 4.0, 4.5, 5.0, 5.5, and 6.0 mg/ml (hydrocortisone equivalent); hydrocortisone[4] solutions in methanol contained 0.15, 0.10, 0.075, 0.05, and 0.025 mg/ml. Solutions of *p*-propenylanethole[5] in water (0.15 mg/ml) and benzophenone[6] in methanol (0.60 mg/ml) were prepared for use as internal standard solutions.

Calibration Curves—Aliquots (100 μl) of the hydrocortisone and hydrocortisone phosphate solutions were mixed with a like quantity of each internal standard solution, and a 15-μl portion of this mixture was injected into the chromatograph. The area ratio of each peak to its respective internal standard was determined, and calibration curves were constructed based on the area ratio of the drug peak to the concentration of drug in solution.

Analysis of Injectable Solutions—A portion of the solution was removed and diluted to a concentration of 5.0 mg/ml based on the labeled claim. An aliquot (100 μl) of this solution was mixed with 100 μl of each internal standard solution and of methanol, and 15 μl of this solution was injected. Peak area ratios were determined as already indicated, and the quantities of hydrocortisone and its phosphate ester were calculated by reference to the previously determined calibration curves.

RESULTS AND DISCUSSION

As noted in a recent discussion of the lineage of ion-pairing (7), the technique has been known since at least 1922. Analytical applications and other aspects of ion-pairing have been extensively reviewed (8, 9). However, the application of ion-pairing to HPLC has been of more recent origin; the approach has been to use cellulose as a support heavily loaded with aqueous solutions of the ion-pairing salt (10, 11). When using this approach, column lifetimes have been predictably short and the ability to use modern high-efficiency, small particle packings has been lost. These disadvantages are overcome by the addition of the ion-pairing agent to the mobile phase.

The process of selecting a mobile phase for an analysis using the ion-pairing approach may be divided into two stages. First, the customary procedures are employed to select a mobile phase in which the nonionic constituents are suitably separated. In this case, a solution of methanol–water (420:500) gave rapid separation of hydrocortisone from methylparaben and propylparaben. Second, quaternary ammonium compounds are added to the selected mobile phase, the retention characteristics of the ionic constituents are noted (Table I), and a single quaternary ammonium compound or a mixture is then chosen on the basis of the combined chromatographic characteristics of the ionic and nonionic constituents. Since the phosphate ester is normally present in quantities greatly in excess of free hydrocortisone, it was advisable to choose a reasonably lipophilic quaternary ammonium compound to retain the ester most strongly.

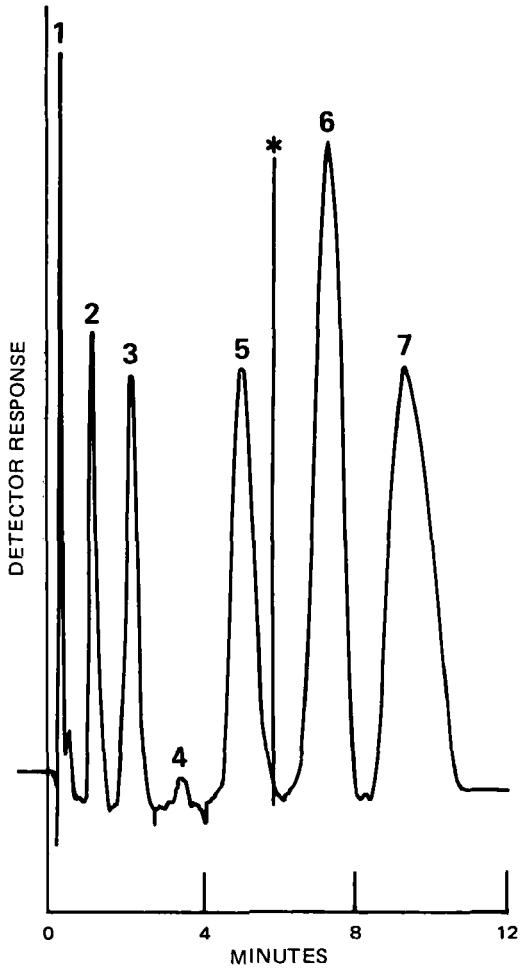

Figure 1—*Typical chromatographic trace of hydrocortisone phosphate injectable solution and internal standards. Key: 1, creatinine; 2, methylparaben; 3, hydrocortisone; 4, propylparaben; 5, benzophenone; 6, anethole; and 7, hydrocortisone phosphate.*

Figure 1 illustrates a typical chromatogram of constituents of the injectable hydrocortisone phosphate dosage form with internal standards. Since the amounts of hydrocortisone and its ester were quite different, it was deemed prudent to use a separate internal standard for each. The large difference in quantity also necessitated an attenuation change after elution of the first internal standard.

Standard curves were prepared daily for 10 days, and the standard deviation of slopes, average correlation coefficients, and average γ-intercept values were calculated. Values for hydrocortisone phosphate were 0.37%, 0.997, and −0.003, respectively; for hydrocortisone, they were 0.59%, 0.996, and 0.004, respectively. These values indicate that the procedure is amenable to use of a single-point standard for both compounds.

Data from laboratory-prepared samples (Table II) show close similarity between the currently official procedure and the HPLC procedure. Accuracy of the procedure in the analysis of hydrocortisone extends at least to the 0.5% level, and hydrocortisone is detectable at levels of 0.05%.

[1] ALC model 202 with model 6000 pump and U6K injector, Waters Associates, Milford, Mass.
[2] Model 450, Varian, Palo Alto, Calif.
[3] Microbondapak C_{18}, Waters Associates, Milford, Mass.
[4] USP reference standards.
[5] Baker Analyzed, J. T. Baker.
[6] Fisher Certified.

Table II—Results of the Analysis of Hydrocortisone and Hydrocortisone Phosphate in Laboratory-Prepared Samples

	Hydrocortisone Phosphate			Hydrocortisone		
		Found			Found	
Sample[a]	Added	UV[b]	HPLC	Added	UV	HPLC
1	50.0	49.45[c] (3.57)	49.64 (2.12)	5.0	4.54 (2.76)	4.68 (2.59)
2	50.0	49.57 (3.46)	49.76 (2.23)	0.25	0.23 (3.94)	0.24 (2.30)
3	60.0	57.56 (3.04)	58.31 (2.64)	0.5	0.47 (4.46)	0.47 (2.4)
4	40.0	39.89 (3.94)	40.44 (3.10)	5.0	4.62 (2.55)	4.74 (2.47)
5	40.0	39.46 (4.12)	40.16 (3.00)	0.25	0.24 (4.26)	0.25 (2.59)

[a] Expressed in milligrams per milliliter. [b] USP XIX. [c] Average (% *SD*), $n = 7$.

Table III—Analysis of Hydrocortisone and Hydrocortisone Phosphate in Injectable Solutions

	Hydrocortisone Phosphate Found[a]		Hydrocortisone Found[c]
Sample	Colorimetric[b]	HPLC	
1	103.42 (4.68)	102.94 (3.76)	0.76 (2.46)
2	98.50 (5.44)	97.64 (3.54)	1.18 (2.76)
3	96.64 (4.50)	97.76 (3.94)	2.02 (3.14)
4	105.74 (4.77)	104.68 (3.22)	0.46 (2.17)

[a] Expressed as percent of labeled claim (% *SD*). [b] USP XIX. [c] Expressed as percent of labeled claim of hydrocortisone phosphate (% *SD*).

Table III presents results of the analysis of these drugs in several lots of the injectable solution. Results of the two procedures are again comparable, and the standard deviations are again lower with the HPLC assay, reflecting the simplified sample workup procedure.

The HPLC procedure is simple in nature and easily mastered. Triplicate analyses of a sample require approximately 1 hr, and more than 400 injections were made with no discernible change in the characteristics of the chromatographic system. It appears, therefore, that the reversed-phase, ion-pairing approach is well suited to the simultaneous analysis of such nonionic and ionic drugs as hydrocortisone and its phosphate ester.

There is, however, a precaution regarding the use of this technique. The quaternary ammonium hydroxides are basic substances, and the pH of their aqueous solutions rapidly inactivates the permanently bonded, reversed-phase systems. As a result, it is necessary to adjust the pH of the aqueous solution of the salt to less than 8.0 prior to use.

In regard to other applications of the reversed-phase, ion-pairing approach, a corollary to the technique presented here is that the addition of lipophilic acid salts to the mobile phase would result in the retention of amines. Therefore, the technique should be of use in the analysis of amines. Also, it should not be necessary to restrict its use to the stationary phase used here. These additional aspects will be discussed later.

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 13, 1975, from **Waters Associates, Milford, MA 01757*, and the *‡School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO 64110*

Accepted for publication October 8, 1975.

The authors appreciate the technical assistance of Kathy Butler.

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