Liquid Chromatography in Pharmaceutical Analysis II: Determination of a Reserpine–Chlorothiazide Mixture

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Abstract ◻ Operating conditions are described for the qualitative and quantitative analysis of a reserpine–chlorothiazide mixture in two steps by high-pressure liquid chromatography. Each compound was chromatographed on an octadecyl column using a 55:45 mixture (pH 5.6) of absolute methanol–aqueous 0.5% ammonium chloride at a flow rate of 2.0 ml/min (2000 psig). Each determination can be achieved in approximately 60 min with an accuracy of 3–5%.

Keyphrases ◻ Reserpine–chlorothiazide—high-pressure liquid chromatography analysis ◻ Chlorothiazide–reserpine—high-pressure liquid chromatography analysis ◻ High-pressure liquid chromatography—analysis, reserpine–chlorothiazide mixture ◻ Liquid chromatography, high pressure—analysis, reserpine–chlorothiazide mixture

The use of commercially available high-pressure liquid chromatography (HPLC) systems in the analysis of multicomponent pharmaceutical dosage forms has been the subject of investigations in this laboratory. The successful separation and quantification of drugs in cough–cold mixtures by HPLC have been reported recently (1). In a continuation of the applicability of liquid chromatography in pharmaceutical analysis, an in-depth study of the separability of antihypertensive agents (2) was undertaken. A direct result of this work led to the investigation of the single-dose analysis of the antihypertensive combination, reserpine–chlorothiazide, by HPLC. Some analytical problems associated with this mixture include instability of reserpine in solution and a 2000-fold difference in concentration of the ingredients.

Urbanyi and O'Connell (3) reviewed the problems involved in the analysis of reserpine in combination with hydralazine and chlorothiazide or hydrochlorothiazide, and they used a fluorometric procedure based on oxidation of reserpine with vanadium pentoxide. Other fluorescence (4, 5) and colorimetric (6, 7) methods have been reported, but these techniques are subject to interferences from components present in the samples. Chu (8) used an ion-exchange separation followed by spectrophotometry to analyze chlorothiazide in the presence of other drugs such as methyldopa. Other reported methods for chlorothiazide mixtures use bromometric or bromochlorometric titrimetry (9), nonaqueous titrimetry (10, 11), and spectrophotometry (12).

The determination of reserpine–chlorothiazide mixtures by HPLC overcomes or circumvents many shortcomings in the reported methods. This paper reports a two-step liquid chromatographic analysis of reserpine–chlorothiazide, and a quantitative study is described for a typical mixture. The preparation of samples is simple and rapid, with the analysis time requiring less than 20 min for each component. The overall analysis time for both components in the simulated dosage form including the extraction procedure is approximately 60 min.

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of reserpine[2] and chlorothiazide[3] were used in the analytical procedure and in preparation of standard curves. All other chemicals and solvents were the highest grade of the commercially available materials.

Mobile Phase—The mobile phase, consisting of 55:45 absolute methanol–0.5% aqueous ammonium chloride, was prepared fresh daily. The pH of the solution was 5.6.

Internal Standard Solutions—Stock internal standard solutions consisting of 1 mg/ml of phenanthrene in chloroform and 4.996 mg/ml of phenanthrene in absolute methanol were prepared. Phenanthrene powder, mp 98–99°, was purified before use by Method 1 of Phillips (13).

Standard Solutions for Calibration Curves—A stock solution of reserpine (3.125 mg/ml) was prepared in chloroform. Volumes of 1.0, 2.0, and 4.0 ml of the stock solution were each placed in 100-ml volumetric flasks. One milliliter of the internal standard stock solution in chloroform was pipetted into each flask, and the solution was diluted to volume with chloroform. These solutions were made and used within 24 hr. If the solutions were stored overnight, they were kept at 4°.

Into individual 250-ml volumetric flasks were added accurately weighed quantities of 125, 250, and 375 mg of chlorothiazide powder. Five milliliters of internal standard stock solution in absolute methanol and 2.0 ml of internal standard solution in chloroform were pipetted into each flask and the solution was diluted to volume with absolute methanol.

The three concentrations of each drug were subjected to a linear regression analysis, and the slope and intercept were calculated (Table I).

Procedure for Simulated Dosage Form—The simulated dosage form was assayed in two steps. Into a 12-ml centrifuge tube containing 250 mg of chlorothiazide powder was added 2.0 ml of chloroform, which contained 0.125 mg of reserpine and 0.02 mg of internal standard. The mixture was centrifuged for 5 min at 10,000 rpm and the supernatant liquid was analyzed for reserpine.

In the second step, the mixture was resuspended and transferred to a 250-ml volumetric flask with 10 10-ml washings of absolute methanol. To this was added 5.0 ml of internal standard stock solution in methanol, and the solution was diluted to volume with absolute methanol.

[1] A Waters Associates liquid chromatograph (model ALC 202), equipped with an M-6000 pump, a UV monitor, an Infotronics integrator (model CRS-204) with digital printout, and Waters packed columns, 1.22 m long × 2.3 mm i.d., was used.
[2] Ciba Pharmaceutical Co., Summit, N.J.
[3] Merck Sharp and Dohme, Rahway, N.J.

Table I—Calibration Data for Standard Drug Solutions

Compound	Final Concentration, mg	D/IS Ratio[a]	Slope	Intercept	$r \pm s$
Reserpine	0.0625[b]	0.2290 ± 0.018[c]	3.2281	0.0303	0.9999 ±0.004
	0.1250	0.4384 ± 0.044			
	0.2500	0.8358 ± 0.036			
Chlorothiazide	125.0[d]	0.1925 ± 0.0069	0.00152	0.0042	0.9999 ±0.002
	250.0	0.3874 ± 0.0144			
	375.0	0.5727 ± 0.0447			

[a] Data represent five to eight replicate injections of standard solutions; D/IS is the ratio of the integrated area of the drug at some concentration divided by the integrated area of phenanthrene at concentrations of 0.01 and 0.1 mg/ml for reserpine and chlorothiazide, respectively. [b] Total mg/2-ml sample. [c] Confidence limits at $p = 0.05$. [d] Total mg/250-ml sample.

Conditions for Chromatographic Separation and Quantification—The degassed mobile phase was pumped through a column containing a monomolecular layer of octadecyltrichlorosilane (C_{18}), chemically bonded to a high efficiency pellicular packing consisting of solid glass cores with a porous silica surface[4], at a flow rate of 2.0 ml/min (2000 psig) at room temperature until a stable baseline was obtained. For the standard solutions and the analysis of chlorothiazide in the congeneric dosage form, replicate 20-μl injections of each solution were made using a 25-μl syringe[5]. For the analyses of reserpine in the dosage form, replicate 50-μl injections were made using a 100-μl syringe[5,6]. The chart recorder provided a record of the elution of the drugs from the column as peaks on a chromatogram. In all cases, the solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The analysis of the two-component dosage form, reserpine–chlorothiazide, necessitated the examination of three separate analysis problems: *(a)* the development of a set of operating parameters of HPLC that would separate the two components without peak overlap, *(b)* the detection and quantification of each component at the level found in the dosage form, and *(c)* the development of a series of analytical steps in which the detector response remains linear over the concentration range of each compound in the dosage form.

The operating parameters studied showed that the compounds were separated best on an octadecyl column using a 55:45 mixture (pH 5.6) of absolute methanol–aqueous 0.5% ammonium chloride (2). A flow rate of 2.0 ml/min, which resulted in a pressure of 2000 psig at room temperature, was the most satisfactory since separations could be obtained in less than 20 min. UV detection was employed since both reserpine and chlorothiazide are strong absorbers and the detector provided maximum sensitivity for the low concentration levels of reserpine in the dosage form.

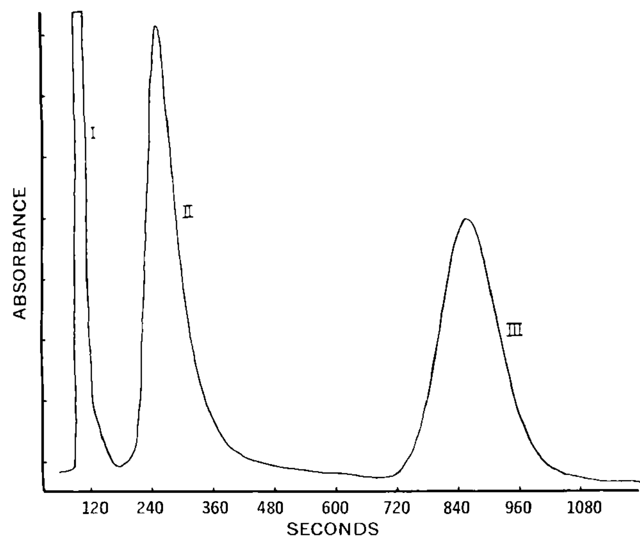

Figure 1—*Liquid chromatogram of reserpine analysis in first step of simulated dosage form on octadecyl column with 55:45 absolute methanol–aqueous 0.5% ammonium chloride. Key: I, chloroform; II, reserpine; and III, phenanthrene.*

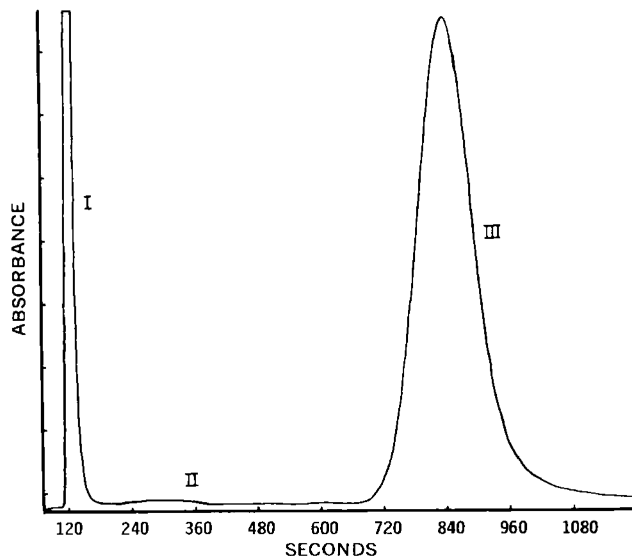

Figure 2—*Liquid chromatogram of chlorothiazide analysis in second step of simulated dosage form on octadecyl column with 55:45 absolute methanol–aqueous 0.5% ammonium chloride. Key: I, chlorothiazide; II, reserpine; and III, phenanthrene.*

Various concentrations of standard solutions of reserpine and chlorothiazide were chromatographed using the octadecyl column (Figs. 1 and 2). Phenanthrene was added to each solution as the internal standard. The area under the curve for each peak on the chromatograms was integrated digitally. The ratio of each peak area to the area of the internal standard was calculated for each chromatogram. A linear regression line of these data at three concentrations of each drug gave the slope, intercept, and correlation coefficient for each calibration curve (Table I).

A simulated dosage form containing quantities of each drug similar to those found in commercial products was analyzed sequentially in two steps due to the 2000-fold difference in concentration of the two ingredients. In the initial step, 2 ml of chloroform containing the internal standard was added to solubilize the reserpine. The mixture was centrifuged for 5 min and the supernatant liquid was analyzed for reserpine (Fig. 1). The shoulder shown on the chloroform peak is thought to be due to minute quantities of chlorothiazide soluble in the chloroform.

In the second step of the simulated dosage form assay, the reserpine–chlorothiazide mixture was quantitatively transferred with absolute methanol. Additional internal standard solution in methanol was added and the final solution was analyzed for chlorothiazide (Fig. 2).

Attempts to use a single solvent for the simultaneous assay of

[4] Corasil/C_{18}, 37–50 μm, Waters Associates, Milford, Mass.
[5] Model B-110, Precision Sampling Corp., Baton Rouge, La.
[6] To improve precision of analysis of reserpine in the dosage form, it was necessay to increase the total quantity of drug in each analysis; therefore, 50-μl injections of reserpine were employed.

Table II—Analysis of Reserpine–Chlorothiazide in Known Mixture

Mixture	Added, mg	Amount Found[a], mg	Accuracy, %
Reserpine	0.125	0.120 ± 0.013[b]	3.8
Chlorothiazide	250.000	260.600 ± 4.886	4.2

[a] Based on five replicate determinations of known mixture. [b] Confidence limits at $p = 0.05$.

both drugs in the simulated dosage form were unsuccessful. Solvents such as acetonitrile and methanol solubilized both reserpine and chlorothiazide, but the ratio of chlorothiazide to reserpine was so large that the two components could not be resolved completely. This difficulty was resolved by the sequential use of the two solvents, chloroform and methanol, in the analytical scheme. Reserpine was very soluble in chloroform whereas chlorothiazide was essentially insoluble. Peak overlap of the two drugs was avoided, thus enabling determination of microgram quantities of reserpine in the presence of milligram concentrations of chlorothiazide. In addition, the volume of chloroform could be reduced, thus enhancing the detectability of reserpine in a more concentrated solution. Methanol was then added to solubilize the undissolved chlorothiazide and the analysis was completed.

The ratios of drug peak areas/internal standard peak areas (D/IS) were calculated for each drug. The constants (slope and intercept) for the linear regression equation shown in Table I were used to solve for drug concentration [D/IS = slope (concentration) + intercept]. The calculations were performed on a programmable calculator[7].

The data in Table II demonstrate the quantitative results obtained for the simulated dosage form. The utility of HPLC in the analysis of the reserpine–chlorothiazide mixture is clearly demonstrated, with an accuracy of 3–5% (14).

[7] Olivetti-Underwood programma 101.

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Ion-Pair Extraction and Precipitation Methods for Ethambutol Determination

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Abstract □ A sensitive method for determining ethambutol (20–100 μg) in aqueous solutions and tablets is described, using bromthymol blue as a complex-forming agent. Extraction of the complex in chloroform as well as in methylene chloride or ethylene dichloride is accomplished readily at an optimum pH of 7. A stoichiometric relationship of 1:2 between ethambutol and the acid dye is proved. Cycloserine, isoniazid, and sodium aminosalicylate do not interfere with the assay. The reineckate precipitation method for ethambutol determination is compared and its direineckate derivative is identified.

Keyphrases □ Ethambutol hydrochloride—analysis, ion-pair extraction and precipitation methods □ Ion-pair extraction—bromthymol blue–ethambutol complex, precipitation and analysis □ Acid-dye technique—ion-pair extraction of ethambutol–bromthymol blue complex, precipitation and analysis □ Bromthymol blue—ion-pair extraction of ethambutol and analysis

Oral administration of ethambutol hydrochloride, (+)-2,2′-(ethylenediimino)-di-1-butanol dihydrochloride, currently marketed as the dextro form, is used in the treatment of tuberculosis (1, 2). Colorimetric methods for determination of ethambutol as the copper complex (3, 4) and as the reineckate derivative (5) were reported previously. The chelation of ethambutol with copper required either a nonaqueous medium or a critical amount of excess alkali to produce a stable color with low absorptivity, and small amounts could not be determined with sufficient accuracy. In the present study the acid-dye technique formerly adopted for the microdetermination of various pharmaceutical amines (6–12) was applied to assay ethambutol and compared with the reineckate assay method after being optimized.