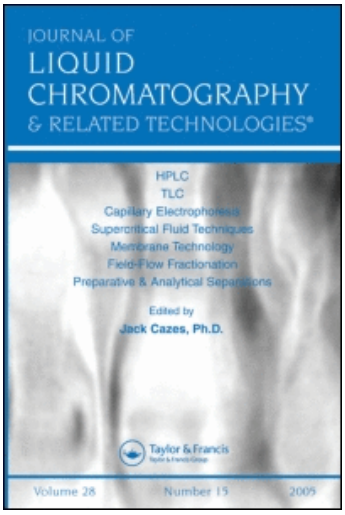

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Determination of Reserpine in Pharmaceutical Formulations by High Performance Liquid Chromatography

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DETERMINATION OF RESERPINE IN PHARMACEUTICAL FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography was employed in the assay of reserpine in tablet formulations. A reverse-phase RP-8 column was used and reserpine was separated from its major degradation products and quantitated by peak-height measurement using ultraviolet detection at 254 nm and fluorescence at 330 nm excitation. Tablets analysed were within the official limits even after more than ten years following manufacture.

INTRODUCTION

The official method (1) for the determination of reserpine in tablets involves reaction of the chromatographically extracted reserpine with nitrous acid to form a colored solution which is then measured spectrophotometrically in the visible region. The major source of light absorption by the sample solution is due to the formation of the major solid-state degradation product of reserpine, 3,4-dehydroreserpine[1]. 3,4-dehydroreserpine (apple-green fluorescent), 3,4,5,6-tetradehydroreserpine (bright-blue fluorescent) and 3-isoreserpine (non-fluorescent) have been identified by Wright and Tang (2) in U.V. irradiated solutions of reserpine in methanol and chloroform. These authors also claim to have identified 3,4-dehydro-

reserpine and 3-isoreserpine (but not 3, 4, 5, 6-tetradehydroreserpine) in all commercially available brands of reserpine tablets examined by solvent extraction and T.L.C. analysis. The procedure incorporates a blank to compensate for absorbance due to degradation products present in the sample.

Prior to the advent of high performance liquid chromatography (HPLC), analysis of reserpine formulations was based either on direct U.V. determination or oxidation of reserpine followed by U.V. or fluorimetric estimation (3-8).

Horriberg, Stewart, Smith and Hester (9) have applied reverse phase HPLC successfully to the analysis of reserpine in multicomponent dosage forms but the retention times were quite long and gave considerable band spreading in some instances. It was also noted that reserpine was very sensitive to the pH (>8) of the solvent system.

Butterfield et al. (10) used a forward phase HPLC system to analyse reserpine and hydrochlorothiazide in two-component formulations and suggested that their method could be applied to single component formulations. However their method does not permit detection of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine as these compounds were retained on the column. As well, the identification and presence of 3-isoreserpine was ascertained solely on the basis of retention behaviour in one system. Elution of both 3-isoreserpine and reserpine is very rapid as supported by very low k' values. Furthermore, monitoring by the HPLC system employed in the present study, a reserpine solution in tetrahydrofuran at room temperature for more than 5 days under laboratory light showed no 3-isoreserpine. The formation of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine could be easily followed with time; initially, 3,4-dehydroreserpine is the major product of decomposition but 3,4,5,6-tetradehydroreserpine eventually gains ascendancy. Although the possibility of other factors relating to the other drug component or excipients may be of relevance to this issue, the efficacy of the identification of 3-isoreserpine based solely upon retention time in one system must be viewed with suspicion.

EXPERIMENTAL

Materials: Reserpine was obtained from two sources 1) U.S.P. Reference Standard 2) Working standard was from the Aldrich Chemical Co. Sodium phosphate, certified A.C.S. (Fisher Scientific, Fair Lawn, N.J., U.S.A.). Methanol and ethyl acetate were HPLC grade.

3-Isoreserpine: 3-Isoreserpine was prepared according to MacPhillamy et al. (11)

3,4-Dehydroreserpine and 3,4,5,6-Tetradehydroreserpine: These compounds were prepared by the procedure of Wright and Tang (2).

Extraction solvents[2]: The extraction solvent system consisted of water saturated with ethyl acetate and ethyl acetate saturated with water. Saturation was achieved by mixing in a large separatory funnel approximately equal amounts of both solvents, shaking for 10-15 minutes and allowing the system to stand until both layers became clear (preferably overnight).

Solutions:

a) Internal standard solution: A solution containing approximately 20 mg (roughly equivalent to 1 drop) of propiophenone in 500 ml of water-saturated ethyl acetate was used; at this concentration, a peak corresponding to 60% full scale deflection was obtained. Although this solution was found to be stable over a period of 1 month, a fresh solution was made weekly.

b) Reserpine standard solution: Approximately 25 mg of reserpine bulk drug (Aldrich) was weighed accurately and dissolved in 100 ml of the internal standard stock solution. This solution was prepared fresh daily.

Chromatographic parameters:

Apparatus: A Laboratory Data Control HPL Chromatograph (LDC, Division of Milton Roy Co., Riviera Beach, Florida) fitted with a Valco

valve and a 20 µl injection loop was used. An LDC (range 0.01 aufs) variable wavelength detector (set at 254 nm) was connected in series with a Schoeffel FS970 fluorescence detector (set at 330 nm, 0.05 µA range, sensitivity 6.8, time constant 0.5 sec) fitted with a Corning 7-51 excitation pre-filter and a 470 nm cut-off emission filter.

Column: The 4.6 mm i.d. x 25 cm stainless steel columns used contained 10 µm Lichrosorb sorbant coated with chemically bonded octylsilane (RP8, Brownlee Laboratories, Santa Clara, Calif., U.S.A.).

Recorders: Chromatograms were recorded on a Honeywell Electronik 196 and a Pharmacia Fine Chemicals model 410.

Integrator: Calculations of peak height were done manually or by using a Minigrator (Spectra-Physics, Santa Clara, Calif., U.S.A.) capable of measuring peak heights, set at attenuation 4.

Mobile phase: A 50:50 V/V mixture of methanol and sodium phosphate monobasic 0.05M in water (6.9 g of the phosphate dissolved in 1 liter of water, pH 4.5) was used. The solvent was filtered through a millipore filtering apparatus using glass fiber filters (Reeve-Angel, Whatman, Inc. Claxton, N.J. U.S.A.). The flow of the mobile phase was 2.0 ml/min at a pressure of approximately 2000 psi.

Analysis of Pharmaceuticals: Tablet-composite samples were prepared by grinding manually using a mortar and pestle, 25 tablets for a 0.25 mg dosage and 50 tablets for a 0.10 mg dosage, mixing until a uniform powder was obtained. An aliquot equivalent to .25 mg or .10 mg of reserpine was accurately weighed (equivalent to the amount of reserpine in 1 tablet) and the powder transfered to a 13 x 100 mm culture tube (Canadian Laboratory Supplies, Montréal, Québec, Canada) fitted with a teflon-lined screw cap. 1.0 ml of water (ethyl acetate saturated) and 1.0 ml of the internal standard solution were added to each individual sample. Samples were then extracted using an Evapo-Mix apparatus (Buchler Instruments, Fort Lee, N.J., U.S.A.) for 20 minutes and centrifuged for 2 minutes at 2000 rpm

(Safety Centrifuge, Fisher Scientific). Ten microliters of the ethyl acetate supernatant was injected after a small portion of this layer was transfered with a Pasteur pipette to a suitable container. Quantitation was by the peak height ratio between the sample and the internal standard. Fig. 1.

Calibration of the working standard: In order to establish the purity and integrity of the reserpine bulk drug, a working standard solution was compared with similar solutions of reserpine using the U.S.P. reference standard. Triplicate weighings of approximately 50 mg of reserpine bulk drug and the U.S.P. reference standard (dried 3hrs at 60°) were made up in 25 ml ethyl acetate containing the internal standard. From these solutions 1 ml aliquots were taken and diluted to 10 ml with internal standard solution. Duplicate 10 µl injections and subsequent ratio comparison of peak heights for reserpine and the internal standard in both of these prepared solutions gave a percentage value of 97.75%. Relative standard deviations of the peak height ratios for the Official Standard and working standard were ±1.4% and .8% respectively.

Calculations:

% found =

$$\frac{PHR_{SPL}}{PHR_{STD}} \times \text{Wt of STD} \times \frac{\text{Wt. tab}}{\text{Wt of SPL}} \times \frac{1}{\text{label claim (mg)}} \times \text{STD purity}$$

where:

PHR_{SPL} = peak height ratio of sample to internal standard.

PHR_{STD} = peak height ratio of standard to internal standard.

Wt of STD = weight of the standard in mg.

Wt tab. = average weight of tablet analysed in mg.

Wt of SPL = weight of the sample in mg.

STD purity = percentage value of the working standard as determined by calibration of the working standard.

RESULTS AND DISCUSSION

Reserpine formulations were assayed using a reverse phase mode and intact reserpine measured directly by ultra violet absorbance detection.

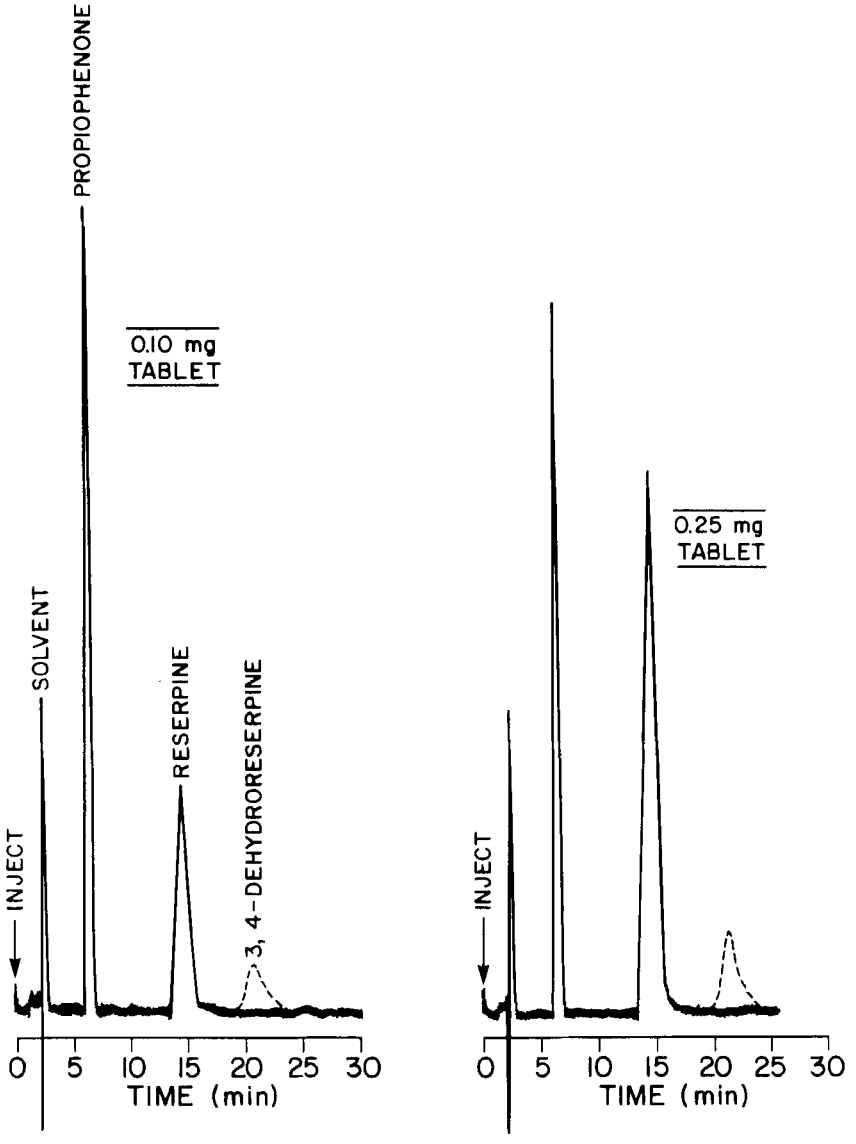

FIGURE 1

Typical chromatograms obtained when the equivalent of one tablet is extracted using water saturated ethyl acetate and 10 ul of supernatant injected on RP-8 column. (————) UV detection at 254 nm. (---------) fluorescence detection, excitation wavelength set at 330 nm, and 470 nm cut-off filter was used.

The use of a buffered water:methanol system in reverse phase obviates the necessity of a very complex solvent system (such as employed by Butterfield et al.) (10) which renders the latter procedure less easily applicable to routine use. In addition, the HPLC assay here presented permits direct observation of reserpine degradation products, as well as 3-isoreserpine. Fig. 2.

Reserpine tablets from various manufacturers were analysed and results compared with analytical data obtained by the official method. The sample lots consisted of three different age groups, namely, very old (>10 years), old (>3 years) and recent (<1 year).

The HPLC method for reserpine assay is shown to be rapid, precise and accurate. In addition, no interference from formulation excipients is observed. The relative standard deviations for representative samples assayed five times were between 1 and 2 percent (Table 1).

The reproducibility of the chromatographic system was assessed by injecting six 10 µl aliquots of an extracted composite sample; the relative standard deviation based on peak height ratio was 0.8%.

Linear response was determined using a stock solution of reserpine (Aldrich Chem. Co.) in 100 ml of water, saturated with ethyl acetate containing the internal standard, propiophenone. Volumes of 0.25, 0.50, 1.0 1.5, 2.0 and 4.0 ml were made up to volume with internal standard solution in a 10 ml volumetric flask. 10 µl of each solution was injected onto the column. Linearity was obtained for a concentration range of 0.250 µg to 4.0 µg/10 µl when peak height ratios were plotted against concentrations. The coefficient of correlation,(R^2) for 7 determinations, was 0.9998 (Y = 30.967 x + .242).

Extraction time was determined by extracting two weighed samples of a representative formulation for 20 minutes and 40 minutes, respectively. The peak height ratio gave a relative standard deviation of 0.8%, in accordance with the reproducibility of the system; maximum extraction efficiency was attained within 20 minutes. Also, injection of the aqueous layer of extracted samples gave no U.V. light absorption. Initially a Roto-Rack (Fisher Scientific Model 343) was used to tumble the sample tubes for various lengths of time. It was found, however, in some instances that

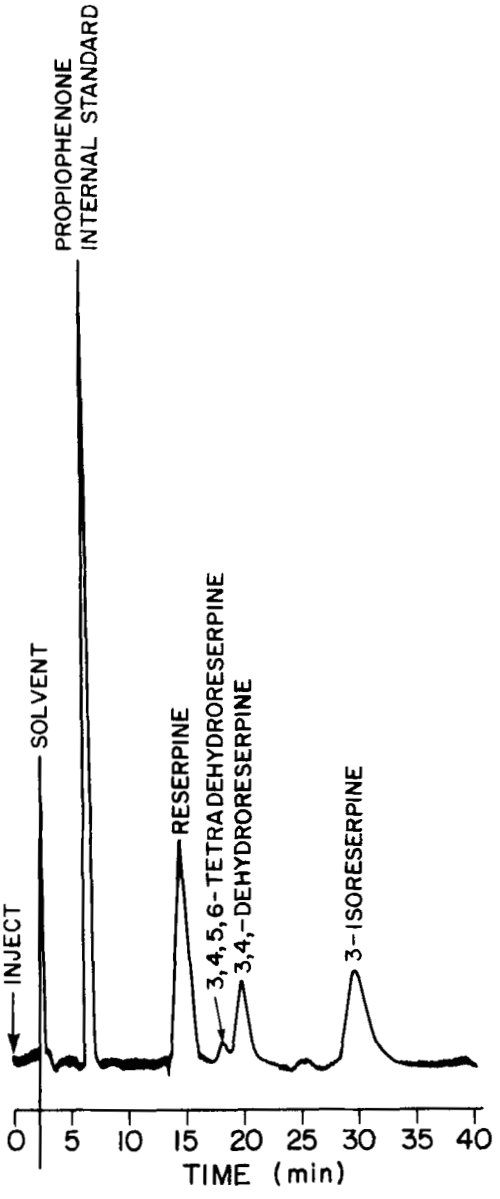

FIGURE 2

Separation of Reserpine and its degradation products on an RP-8 column using methanol:.05M aqueous phosphate buffer 50:50 and a flow rate of 2.0 ml/min. Detection is at 254 nm.

TABLE 1.
Results of Reserpine Formulation Assay

Reserpine Sample Formulation	Age (years)	Label Claim (mg)	% Label Claim USP ASSAY	% Label Claim HPLC ASSAY	RSD, %*
1 new	<1	.25	97.4	97.9	1.04
2 new	<1	.10	105.0	105.8	1.58
3 old	>3	.25	97.1	96.9	1.07
4 old	>3	.10	97.3	96.5	1.52
5 very old	>10	.25	94.4	93.0	2.05

*Calculated on the basis of 5 determinations for each sample.

time of extraction was not the only significant factor in extraction efficiency. Erratic extraction efficiency may indicate the need for more vigorous agitation of the extraction medium. The use of a Vortex or Evapo-Mix apparatus -instead of the Roto-Rack- for agitation of some samples resolved this problem.

The addition of water in the first step of the extraction procedure is a significant aid; failure to do so often produced cloudy solutions and led to erratic results when such solutions were analysed. Water facilitates disintegration of the solid and promotes extraction of water-soluble dyes and excipients[3]. Also maximum extraction of reserpine into the ethyl acetate layer and minimum carry-over of water-soluble material was ensured by the use of a mutually pre-saturated ethyl acetate -water extraction solvent.

Adjustment of the mobile phase by addition of a third solvent permitted greater separation between 3,4,-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine in some cases, but reserpine was invariably not as well resolved in such instances; it was therefore decided that optimal separation of degradation products would be sacrificed in favour of better resolution of reserpine. Use of short connecting tubes between the U.V. detector and the fluorescence detector permitted simultaneous determination of reserpine and 3-isoreserpine, by U.V. and 3,4,-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine, by fluorescence. Even though 3,4-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine are well separated

TABLE 2.
Retention Time and k' Values*

Compound	Retention time in seconds	* k'
Propiophenone	400	3.9
Reserpine	910	10.2
3,4,5,6-Tetradehydroreserpine	1081	12.5
3,4-Dehydroreserpine	1200	14.0
3-Isoreserpine	1777	21.0

*Similar k' values were obtained on two other RP-8 columns.

from reserpine, the amounts present in the formulations examined gave little or no U.V. absorbance; the presence of these degradation products was therefore monitored by fluorescence at 330 nm, the excitation peak maximum wavelength for 3, 4, 5, 6-tetradehydroreserpine.

Precise individual determination of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine is not yet possible but efforts are continuing to determine the chromatographic characteristics of these compounds and in particular the assessment of the extent of 3,4-dehydroreserpine oxidation to 3,4,5,6-tetradehydroreserpine during formulation work up and chromatography; the objective is to establish a fluorimetric assay method for low levels of reserpine degradation products. The results of this continuing effort will be reported in due course. It is recommended that in order to ensure reliability of the separation and HPLC system when analysing tablets on a routine basis when only a U.V. detector is available, that a mixture of reserpine and lumireserpine be injected occasionally to verify k' values, and the methanol content of the mobile phase adjusted accordingly.

The results herein reported indicate that the reserpine content in a variety of tablets on the Canadian market is still within acceptable official limits even after more than ten years following manufacture.

FOOTNOTES

1 Thin-layer chromatography (TLC) has shown that the reaction product contains a number of minor components (so far unidentified) in addition to 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine (lumireserpine).

2 The use of chloroform as solvent (as in the U.S.P. procedure) was avoided because reserpine was observed to undergo ready oxidation in chloroform solution unless care is taken to exclude light and oxygen. Also, its use would necessitate the pipetting of a subnatant solution in separation of the extract from the aqueous layer.

3 It was found that in one case that it was necessary to filter the supernatant through a small pledget of glass wool to obtain a clear upper layer.

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