Stability-Indicating Methods for Quantitative Determination of Spironolactone Using High-Pressure Liquid Chromatography and Blue Tetrazolium Reaction

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Received March 17, 1977, from the *College of Pharmacy, University of Houston, Houston, TX 77004.* Accepted for publication October 17, 1977.

Abstract □ Stability-indicating methods for the quantitative determination of spironolactone were developed. The methods are based on high-pressure liquid chromatography and a reaction with blue tetrazolium. Both methods are accurate, precise, and sensitive and gave excellent results with commercial tablets, including those containing hydrochlorothiazide in addition to spironolactone. The blue tetrazolium method cannot be used in the presence of high concentrations of either polyethylene glycols or water. Spironolactone decomposition in water or polyethylene glycol appears to follow pseudo-first-order kinetics. The decomposition constant at 65° was 0.0253/day in water *versus* 0.115/day in polyethylene glycol ointment base USP.

Keyphrases □ Spironolactone—high-pressure liquid chromatographic and colorimetric analyses in tablets □ High-pressure liquid chromatography—analysis, spironolactone in tablets □ Colorimetry—analysis based on blue tetrazolium reaction, spironolactone in tablets □ Diuretics—spironolactone, high-pressure liquid chromatographic and colorimetric analyses in tablets

Spironolactone (I) is extensively used as a diuretic. The USP procedure (1) for the quantitative determination of spironolactone in tablets, based on UV absorption, is not a stability-indicating method since the decomposition products of spironolactone absorb at the same wavelength. A fluorometric procedure was reported for the quantitative determination of spironolactone in biological fluids (2), but this method is tedious and time consuming. Some steroids were separated using acetonitrile–diatomaceous earth (3). *n*-Heptane was used for elution, and spironolactone in the eluate was assayed using the isoniazid reaction.

This article reports stability-indicating methods for the quantitative determination of spironolactone. The methods investigated are based on high-pressure liquid chromatography (HPLC) and a reaction with blue tetrazolium similar to that of corticosteroids.

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were ACS, USP, or NF grade and were used without further purification.

Apparatus—A high-pressure liquid chromatograph[1] equipped with a UV detector (254 nm), a recorder[2], and an integrator[3] was used.

Column—A column[4] (30 cm × 4 mm i.d.) of a semipolar packing material, consisting of a monomolecular layer of cyanopropylsilane permanently bonded to silica, was used.

Chromatographic Conditions—The chromatographic solvent was 0.02 *M* KH_2PO_4 in water containing 40% (v/v) ethanol. The temperature was ambient, and the flow rate was 2.4 ml/min. The detector was set at a sensitivity of 0.08 absorbance unit full scale, and the chart speed was 30.5 cm/hr.

[1] ALC 202, equipped with U6K universal chromatograph injector, Waters Associates, Milford, Mass.
[2] Omniscribe 5213-12, Houston Instruments, Austin, Tex.
[3] Autolab Minigrator, Spectra-Physics, Santa Clara, Calif.
[4] μBondapak CN, Catalog No. 84042, Waters Associates, Milford, Mass.

Table I—Data on Concentrations of Spironolactone *versus* Areas or Absorbance Values

Quantity of Spironolactone, μg	Method	Areas or Absorbance Values
0.25	HPLC	14,147
0.5	HPLC	28,160
1.0	HPLC	56,218
125	Blue tetrazolium	0.262
250	Blue tetrazolium	0.521
375	Blue tetrazolium	0.785

Preparation of Solutions—A stock solution of spironolactone was prepared by dissolving 25.0 mg of I in enough alcohol to make 100.0 ml.

Standard Solution in Alcohol—A standard solution of I in alcohol was prepared by diluting 10.0 ml of the stock solution to 100.0 ml with alcohol.

Standard Solution in Water—A standard solution in water was prepared by diluting 10.0 ml of the stock solution to 100.0 ml with water.

HPLC Assay of Tablets—Grind one tablet or transfer powder equivalent to one tablet or 25 mg of I (if a synthetic mixture) in a mortar and mix with 40 ml of alcohol with a pestle. Transfer the powder to a 100-ml volumetric flask with the aid of more alcohol to bring to volume. Filter and reject the first 20 ml of the filtrate and then dilute 10.0 ml of the clear filtrate to 100.0 ml with either water or alcohol, depending on the standard solution to be used for comparison purposes. Inject 20 μl into the chromatograph. As a control, inject 20 μl of the standard solution after the assay sample is eluted.

Since preliminary investigations indicated (Table I) that the spironolactone peak area was related directly to the concentration, the results were calculated using:

$$\frac{A_a}{A_s} \times 100 = \% \text{ of label claim} \qquad \text{(Eq. 1)}$$

where A_a is the peak area of the assay sample and A_s is the peak area of the standard sample. The results on commercial dosage forms are presented in Table II, and sample chromatograms are presented in Fig. 1.

Blue Tetrazolium Assay—The same procedure is used as reported

Table II—Assay Results on Commercial Tablets

Number	Commercial Sample (Claim)	Synthetic Mixture (Composition)	Percent[a] of Claim Found HPLC[b]	Percent[a] of Claim Found Blue Tetrazolium
1	25 mg of I	—	99.8	99.4
2	25 mg of I	—	97.8	97.2
3	25 mg each of I and II	—	101.7	101.8
4	—	25 mg of I and 50 mg of II	100.2	99.4
5	—	25 mg each of I and II	99.6	99.8
6	—	25 mg of I and 10 mg of II	99.8	101.2

[a] Average of two results. Maximum variation between two readings was 1.2%. [b] Based on five injections of the same solution. Standard deviation was ±0.98%. Results on different lots of solutions on different days showed similar deviations.

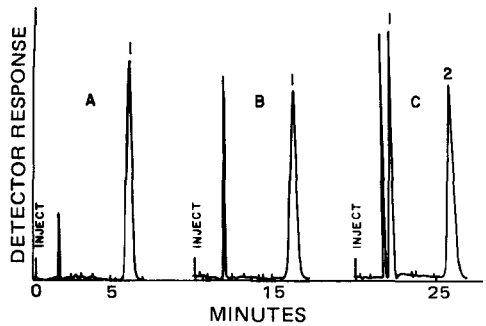

Figure 1—*Sample chromatograms. Key: A, from a standard solution in water; B, from a standard solution in alcohol; C, from a tablet containing 25 mg each of I and II; Peak 1, from II; and Peak 2, from I.*

for HPLC for the preparation of assay sample, except that the last dilution is made with alcohol only. Pipet 10.0 ml each of the standard solution in alcohol and the assay sample into separate glass-stoppered 50-ml conical flasks. Dilute each solution to 20.0 ml with alcohol. In a third flask, pipet 20.0 ml of alcohol to serve as the blank and follow the USP procedure (4) for steroids, starting with " . . . add 2.0 ml of a solution . . . ," except that it should be allowed to stand for 150 instead of 90 min (150 min is required for the reaction to go to completion).

If the dosage form or the synthetic mixture contained hydrochlorothiazide (II), an equivalent amount (based on the label claim) was added to the reagent blank also. An appropriate quantity of the alcoholic solution of II was transferred to the flask and then brought to 20.0 ml with alcohol.

Effect of Water on Blue Tetrazolium Reaction—To determine the effect of water on the blue tetrazolium method, the method described was followed using the standard solution. Before diluting the standard to 20.0 ml with alcohol, an appropriate quantity of water (2.0, 4.0, and 6.0 ml) was added. Water was also added to the reagent blank. The results, based on the standard with no water, were 12.3, 0.41, and 0% for 2.0, 4.0, and 6.0 ml of water added, respectively.

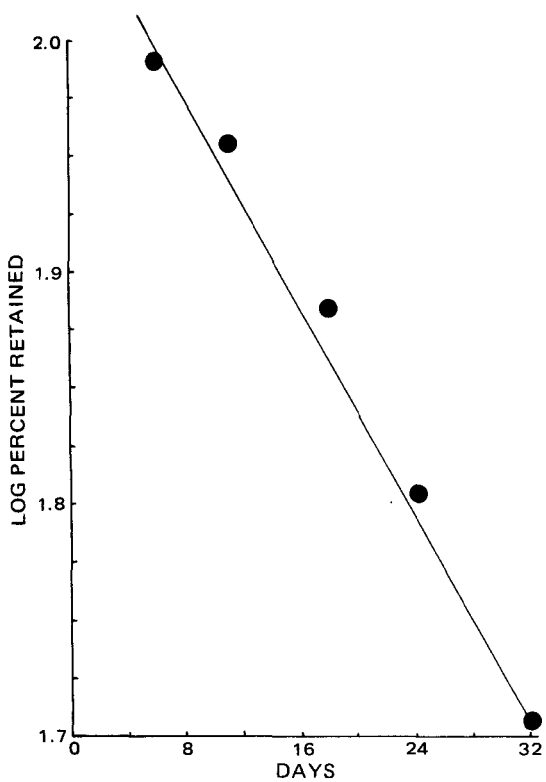

Figure 2—*Pseudo first-order plot of log percent retained* versus *time when spironolactone in water (initial concentration of 0.0025%) was stored at 65°.*

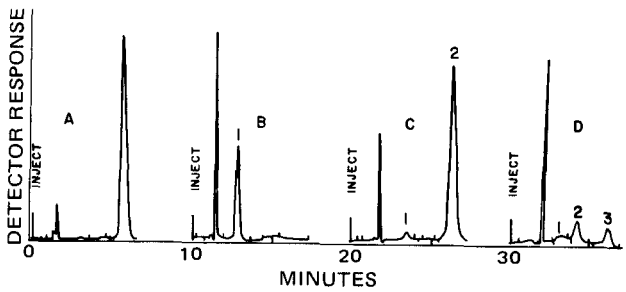

Figure 3—*Sample chromatograms from decomposed solutions. Key: A, from a solution decomposed with 0.1 N HCl (no decomposition was noticed); B, from a solution decomposed with 0.1 N NaOH (all was decomposed); C, from a standard solution in water decomposed for 14 days at 65°; Peak 1, from the decomposition product; Peak 2, from spironolactone; D, from a standard solution (0.0025%) in polyethylene glycol ointment base decomposed for 20 days at 65°; Peak 3, from spironolactone; and all other peaks, from the decomposition products.*

Since preliminary investigations indicated (Table I) that Beer's law was followed, the results were calculated by direct comparison of readings[5] of the assay and standard solution (Table II).

Stability Tests—A standard solution in water was stored at 65° and assayed frequently using the HPLC method (Figs. 2 and 3C).

A 10.0-ml quantity of the stock solution was mixed with 10 g of polyethylene glycol ointment base USP (5), stored at 65°, and assayed frequently using HPLC (Figs. 3D and 4).

A 25.0-ml quantity of the standard solution in alcohol was mixed with 5.0 ml of 0.1 *N* H_2SO_4 and boiled until the volume was reduced to approximately 10 ml. The mixture was cooled, neutralized with 0.1 *N* NaOH, brought to volume (25.0 ml) with alcohol, and assayed using HPLC (Fig. 3A). No I decomposition was noticed.

A 25.0-ml quantity of the standard solution in alcohol was mixed with 5.0 ml of 0.1 *N* NaOH and boiled until the volume was reduced to approximately 10 ml. The mixture was cooled, neutralized with 1 *N* HCl, brought to volume (25.0 ml) with alcohol, and assayed using all three methods. The results were 0, 4, and 33.4% by the HPLC, blue tetrazolium, and UV absorption methods, respectively. The maximum was at about 239 nm. The chromatogram is presented in Fig. 3B.

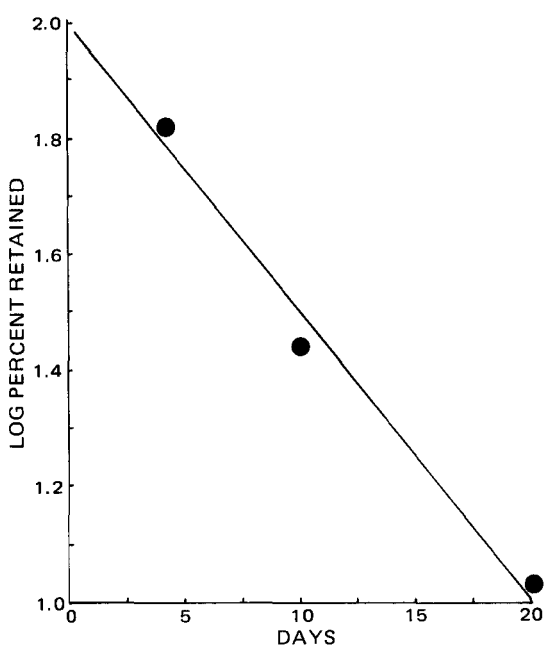

Figure 4—*Pseudo-first-order plot of log percent retained* versus *time when spironolactone in polyethylene glycol ointment base (initial concentration of 0.0025%) was stored at 65°.*

[5] Bausch & Lomb Spectronic 20.

DISCUSSION

The USP method (1) for the quantitative determination of spironolactone in tablets does not appear to be stability indicating since an almost completely decomposed sample indicated that 33.4% of the drug was still intact. The HPLC method showed a potency of 0% (Fig. 3B). The blue tetrazolium method, which indicated a potency of 4%, cannot be reliable because of the presence of a small amount of water in this system; water interferes with this reaction (see *Experimental* and Ref. 6). At lower concentrations (less than 5% of I), clearly identifiable peaks were obtained by the HPLC method. The quantitation of the results was not very accurate.

A high concentration of polyethylene glycol ointment base (above 100 mg in the reaction flask) strongly interfered with a blue tetrazolium assay. In the presence of polyethylene glycols and/or water, the HPLC method should be preferred. The HPLC method is also more sensitive (Table I). There was no interference from hydrochlorothiazide in the HPLC method (Table II and Fig. 1C). In the blue tetrazolium method, an equivalent amount of hydrochlorothiazide must be added to the reagent blank for more accurate results.

The HPLC method is sensitive, accurate, and precise (the standard deviation based on five readings was ±0.98%). The area of the peak was directly related to the concentration range tested (0.25–1.0 μg of I). In water, the peak was sharper than in alcohol (Figs. 1A and 1B). With the UV absorption technique, the standard solution in alcohol absorbed no light at 288 nm (maximum was at 239 nm) while the completely decomposed sample (with 0.1 *N* NaOH) showed maximum absorption at 288 nm. It may be possible to develop a stability-indicating assay based on two readings at two different wavelengths. The study was not pursued further.

Spironolactone decomposition, both in water and polyethylene glycol ointment base USP (5), appears to follow pseudo-first-order reaction kinetics (Figs. 2 and 4). The *K* values at 65° were estimated to be 0.0253/day in water *versus* 0.115/day in polyethylene glycol ointment base.

Whenever new prepacked HPLC columns are purchased, the flow rate or the percentage of alcohol may have to be changed to obtain the same retention time.

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ACKNOWLEDGMENTS

The authors thank Searle Laboratories, Chicago, Ill., for the generous supply of spironolactone powder.

COMMUNICATIONS

Plasma Protein Binding of Diazepam after a Single Dose of Sodium Oleate

Keyphrases □ Diazepam—plasma protein binding, effect of sodium oleate in rats □ Binding, plasma protein—diazepam, effect of sodium oleate in rats □ Sodium oleate—effect on plasma protein binding of diazepam in rats □ Sedatives—diazepam, plasma protein binding, effect of sodium oleate in rats

To the Editor:

Competitive displacement of drugs from plasma protein binding sites is well known, but most information has been derived from *in vitro* studies. Accordingly, little is known regarding the time course of such drug interactions. Specifically, we have been studying whether the decreased plasma protein binding of drugs after the administration of a displacing agent can be accounted for by the concentration of the displacing agent in the plasma. The unusually prolonged impairment of the plasma protein binding of diazepam after a single intravenous dose of sodium oleate is the subject of this communication.

In the first experiment, six male Sprague–Dawley rats were infused (0.6 ml/hr iv) for 1 hr with a buffered (phosphate) aqueous solution (pH 7.2) of sodium ^{3}H-oleate (7 mEq/liter). Blood samples were obtained during and for 14 days after intravenous infusion. The rats were randomly paired into three groups, and the blood was pooled for each pair to ensure adequate amounts of plasma. Three control rats were infused with saline (0.6 ml) for 1 hr, and blood was drawn periodically for 14 days.

Plasma oleate concentrations in treated rats were determined using the modified Dole extraction method (1, 2) in conjunction with liquid scintillation counting. Plasma binding of ^{14}C-diazepam (initial concentration of 400 ng/ml) was determined by equilibrium dialysis against an equal volume of phosphate buffer (0.1 *M*, pH 7.2) at 37° for 18 hr, followed by liquid scintillation counting of buffer and plasma.

The second experiment consisted of administering a bolus dose of sodium oleate (0.6 ml, 7 mEq/liter, premixed with 1 ml of blood) to two rats or of saline (0.6 ml) to two rats. Blood samples were taken immediately before, 5–10 min after, and 24 hr after the intravenous dose. Diazepam binding in plasma was determined by equilibrium dialysis as described.

Plasma binding of diazepam in saline-treated rats was essentially constant over 14 days (Fig. 1). In contrast, the free fraction of diazepam was elevated significantly in

Table I—Free Fraction of Diazepam in Rat Plasma Obtained before and after Intravenous Administration of Sodium Oleate or Saline

	Saline		Sodium Oleate	
Sample	Rat 1	Rat 2	Rat 1	Rat 2
Before dosing	0.100	0.112	0.106	0.121
5–10 min after dosing	0.099	0.112	0.176	0.194
24 hr after dosing	0.096	0.114	0.157	0.146