of MAP did not interfere with the visual end-point determination in the titration of PAS. This is probably attributable to the large difference in pKa values for these compounds: 3.25 for PAS (15) and 9.71 for MAP (16). When the mixtures were titrated potentiometrically, two inflections in the titration curve were obtained, indicating the feasibility of a differentiating titration procedure. The titration curves for the series of mixtures are shown in Fig. 2, and the analysis data are listed in Table II. In the titration curves the first inflection is attributable to PAS, while the second end-point is due to the MAP. Although the differentiating titration may not be useful for determining the MAP content when present in low concentrations (*e.g.*, 10% or less), both the visual and the potentiometric titration procedures are specific for the PAS content.

The weak cation-exchange resin (Amberlite IRC-50) was found to be effective for converting the salts of PAS to the free acid. The PAS was eluted from the column with dimethylformamide and the eluate titrated with sodium methoxide. When mixtures of PAS (or its salt form) and MAP listed in Table II were passed through the resin column, both PAS and MAP appeared in the eluate. A differentiating titration yielded quantitative recoveries for both components. Apparently the PAS and MAP are too weakly basic to be retained by a carboxylic acid-type resin. When the strong cation-exchange resin (Dowex 50W-X8) was used in place of the weak resin, the MAP was retained by the column while the PAS passed through and was recovered quantitatively in the eluate. The percent recovery based on the potentiometric titration of seven samples was 100.22 ± 0.56%. A sulfonic acid-type resin (Dowex 50W-X8) is a sufficiently strong acid to extract the MAP from the mixture.

In preliminary studies, ethylenediamine, acetone, isopropyl alcohol, methyl isobutyl ketone, acetonitrile, and dimethylformamide were examined as solvents for the differentiating titration of PAS and MAP. Dimethylformamide was found to produce the most reproducible and clearly defined end-points. The sleeve-type calomel and platinum electrode system produced good differentiating titration curves where the conventional sleeve-type and glass electrode system was unsuccessful in differentiating PAS and MAP.

SUMMARY

The proposed assay procedure has advantage over the official assay in that the PAS content is determined by direct visual titration. MAP, if present, does not interfere with the end-point detection. Quantitative separation of MAP from PAS may be achieved by passing the mixture through a column of strong cation-exchange resin (Dowex 50W-X8). The MAP is retained by the column while the PAS appears in the eluate. Mixtures of MAP and PAS may be differentiated by potentiometric titration. While this procedure is not suitable for determining low concentrations of MAP, as is required for the official dosage forms (1% or less), it is useful for studying the kinetics of the decomposition of PAS in dosage forms, particularly solutions.

REFERENCES

(1) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, pp. 36, 37, 586, 587, 882.

(2) A. Tarnoky and B. A. Brews, *Biochem. J.*, **45**, 508(1949).

(3) M. Pesez, *Bull. Soc. Chim. Fr.*, **30**, 918(1949).

(4) M. Pesez, *Bull. Soc. Chim. Biol.*, **31**, 1369(1949).

(5) L. G. Chatten, in "Titrimetric Methods," D. S. Jackson, Ed., Plenum Press, New York, N. Y., 1969, pp. 146–154.

(6) J. L. Lach and J. Cohen, *Drug Stand.*, **28**, 65(1960).

(7) J. Kucharsky and L. Safarik, "Titrations in Nonaqueous Solvents," Elsevier, New York, N. Y., 1965, p. 182.

(8) L. G. Chatten, *J. Amer. Pharm. Ass., Sci. Ed.*, **45**, 556 (1956).

(9) A. Q. Butler and J. C. Ramsey, *ibid.*, **42**, 338(1953).

(10) J. R. Stockton and R. Zuckerman, *ibid.*, **43**, 273(1954).

(11) M. N. Das and S. R. Palit, *J. Ind. Chem. Soc.*, **31**, 34(1954).

(12) M. I. Blake, *J. Amer. Pharm. Ass., Sci. Ed.*, **46**, 287(1957).

(13) M. C. Vincent and M. I. Blake, *Drug Stand.*, **26**, 206(1958).

(14) M. I. Blake and D. A. Nona, *J. Pharm. Sci.*, **52**, 945(1963).

(15) "The Merck Index," 8th ed., Merck and Co., Rahway, N. J., 1968, p. 62.

(16) S. Diembeck, E. Grauer, and H. Jager, *Archiv Pharm.*, **301**, 628(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 17, 1969, from the *Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612*

Accepted for publication December 29, 1969.

The authors thank Dr. Walter F. Charnicki, Dorsey Laboratories, and Mr. James Tio, Hines Veterans Administration Hospital, for supplying samples for analysis.

Improved Differential Spectrophotometric Determination of Rifamycins

CARMINE R. PASQUALUCCI*, ARISTIDE VIGEVANI, PIETRO RADAELLI, and GIAN G. GALLO

Abstract □ A differential spectrophotometric method for the determination of rifamycin B, rifamide, rifamycin SV, and rifampin, based on the oxidation by $NaNO_2$ of the hydroquinone moiety of the compounds, is described. The application of the method to the determination of rifamycin B in fermentation broths and of rifampin in capsules and in syrup is reported. Precision and accuracy data are given.

Keyphrases □ Rifamycins, dosage forms—differential determination □ Fermentation broths—rifamycin determination □ Hydrolytic oxidation—rifamycin determination □ Colorimetric analysis—spectrophotometry

Rifamycin B (I) (1) is a metabolite produced by *S. mediterranei*, while rifamide (II) (2), rifamycin SV (III) (3), and rifampin[1] (IV) (4) are the derived semisynthetic antibiotics presently in therapeutic use.[2] All these rifamycins[3] possess a characteristic chromophoric group which permits their spectrophotometric determination (2, 5–7). Furthermore, based on the quinone–hydroquinone nature of the chromophore, a differential spectrophotometric method has been described for rifamycin B and SV (8). The subjects of the present

[1] The international nonproprietary name for this compound is rifampicin.

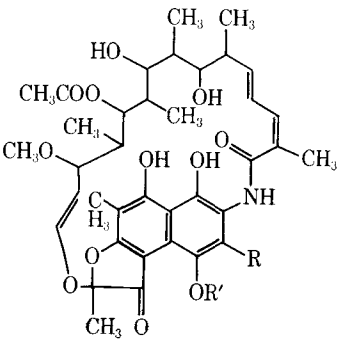
[2] Rifamide as Rifocin M; rifamycin SV as Rifocin; rifampin as Rifadin.

[3] Rifamycins LXIII (rifamycin LXII: G. C. Lancini, G. G. Gallo, G. Sartori, and P. Sensi, *J. Antibiot.* (Tokyo), **22**, 369(1969).

Table I—Differential Absorptivities of the Rifamycins as a Function of Reaction Time and of $NaNO_3$ Concentration, at the Given Wavelengths

Time, min.	$NaNO_2$, % 0.01	0.02	0.1	0.01	0.05	0.1	0.01	0.05	0.1	0.01	0.05	0.1
	Rifamycin B, 425 mμ			Rifamide, 424 mμ			Rifamycin SV, 447 mμ			Rifampin, 473 mμ		
2	15.81	17.90	21.00	1.18	12.90	14.00	2.31	15.19	15.60	0.95	7.76	14.29
5	18.12	21.30	21.51	—	14.00	13.90	—	15.60	15.58	5.72	14.63	14.70
10	20.35	21.48	21.47	10.30	13.97	14.06	14.28	15.67	15.65	—	14.71	14.69
15	21.02	21.50	21.50	12.43	14.02	13.97	15.52	15.55	15.57	11.84	14.67	14.71
20	21.47	—	21.50	13.53	13.98	14.00	15.58	—	15.60	13.34	14.70	14.70
30	—	21.52	20.90	13.92	14.00	13.94	15.60	15.58	15.55	14.16	14.73	—
40	21.51	21.39	20.40	14.03	—	13.98	15.62	15.60	15.60	14.43	14.65	14.68

paper are the modification of the differential spectrophotometric method, its extension to rifamide and rifampin, and its application to pharmaceutical preparations.



	R	R′
I,	H	CH_2COOH
II,	H	$CH_2CON(C_2H_5)_2$
III,	H	H
IV,	$CH{=}N{-}N$ ⟨piperazine⟩ $N{-}CH_3$	H

THEORY AND PROCEDURE

The differential spectrophotometric methods are suitable for the determination of substances in the presence of high concentrations of foreign-absorbing components. In the case of rifamycins the quinone–hydroquinone system, together with the position of the absorption maximum in the visible region, makes the method specific for this family of antibiotics. Thus, rifamycins can be determined in fermentation broths, in pharmaceutical preparations, and in biological fluids, all cases in which the presence of foreign components must be overcome. The authors have studied the differential method in the case of rifamycin B, rifamide, rifamycin SV, and rifampin, and they describe its application to the determination of rifamycin B in the fermentation broth and of rifampin in some pharmaceutical forms.

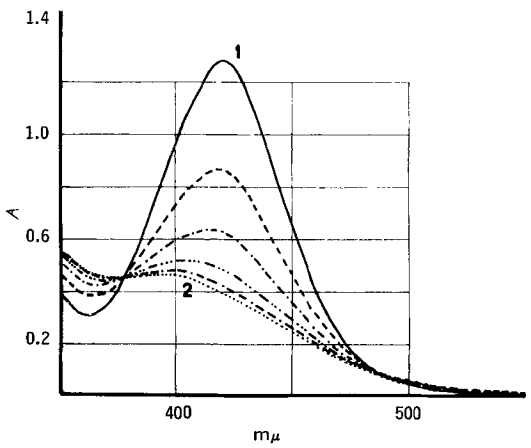


Figure 1—*Visible spectra of the hydrolytic oxidation of rifamide into rifamycin S (4.00 mcg./ml. of rifamide in pH 4.63 acetate buffer containing 0.01% w/v $NaNO_2$). 1: initial curve (rifamide), 2: final curve (rifamycin S); the curves were recorded at 5-min. intervals.*

Table II—Differential Absorptivities (*a*) of the Rifamycins

	$\lambda_{max.}$	*a*
Rifamycin B	425 mμ	21.5
Rifamide	424 mμ	14.0
Rifamycin SV	447 mμ	15.6
Rifampin	473 mμ	14.7

The previous differential spectrophotometric method (8) is not applicable for the determination of rifamycin B in the fermentation broth. In fact, the use of $HAuCl_4$ as the oxidizing agent and of alcoholic benzoate buffer causes the formation of precipitates, which hinder the photometric reading. The authors adopted as the oxidizing agent a solution of $NaNO_2$ in aqueous acetate buffer, pH 4.63. The same oxidizing agent was also chosen for the determination of rifamycin SV, rifampin, and rifamide. With rifamide it must be pointed out that a hydrolytic oxidation to rifamycin S occurs, with the splitting of the glycolic amide moiety. This was demonstrated by the visible spectrum of the reaction solution (Fig. 1) and by isolation and identification (TLC, IR, NMR, and UV) of the final product.

The effect of the concentration of the oxidizing agent on the reaction time was studied for all the rifamycins and the results are given in Table I. On the basis of these data, a reaction time of 5 min. and a $NaNO_2$ concentration of 0.1% were chosen. In fact, under these conditions the rifamycins are completely transformed to the oxidized forms. In Table II the wavelengths and the differential absorptivities of the examined rifamycins, determined on the pure compounds in the conditions of the method, are reported.

The general procedure of the differential determination is as follows: the rifamycin to be determined (as powder, suspension, or solution) is dissolved in a water-miscible solvent at a concentration of about 1 mg./ml. Two equal portions of this solution are separately diluted to a concentration of 20–40 mcg./ml., one with the pH 4.63

Table III—Precision Data of the Differential Method[a]

	Rifamycin B in Fermentation Broths, mcg./ml.	Rifampin Capsules, % w/w	Rifampin Syrup, % w/w
Values obtained	1251; 1251; 1260; 1256; 1256; 1237; 1242; 1256; 1257; 1237	78.9; 78.3; 78.2; 78.5; 78.6; 79.0; 78.5; 79.2; 77.6; 78.2	1.88; 1.89; 1.85; 1.90; 1.88; 1.87; 1.89; 1.85; 1.88; 1.88
Mean	1249.7	78.50	1.877
SD	8.25	0.464	0.0164
RSD	0.66	0.59	0.87

[a] Data for rifamide and rifamycin SV are not reported, but they are of the same order of magnitude as rifamycin B and rifampin.

Table IV—Accuracy Data of Differential Method for Rifamycin B in Fermentation Broth

Sample	Rifamycin B Broth, mcg./ml. Present[a]	Added	Total Content	Found	Error, mcg./ml.	$\frac{\text{Found}}{\text{Total Content}} \cdot 100$
A	777.3	804.3	1581.6	1545.5	−36.1	97.7
B	813.6	967.9	1781.5	1772.7	− 8.8	99.5
C	613.6	967.9	1581.5	1581.8	+ 0.3	100.0
Mean error, −14.87						
Relative error, − 0.90						

[a] Determined by this method.

Table V—Accuracy Data of Differential Method for Rifampin in Capsules and Syrup

Weighed, mcg./ml.	Found, mcg./ml.	Error, mcg./ml.	$\frac{\text{Found}}{\text{Weighed}} \cdot 100$
Rifampin in Capsules			
35.60	35.10	−0.5	98.6
32.86	32.53	−0.33	99.0
32.40	32.32	−0.08	99.8
35.74	35.59	−0.15	99.6
34.20	33.50	−0.7	98.0
33.56	33.02	−0.54	98.4
32.08	31.63	−0.45	98.6
33.20	32.67	−0.53	98.4
31.96	31.84	−0.12	99.6
31.90	31.28	−0.62	98.1
Mean error, −0.402			
Relative error, −1.21			
Rifampin in Syrup			
17.35	17.38	+0.03	100.2
18.65	18.84	+0.19	101.0
17.55	17.93	+0.38	102.2
19.00	19.05	+0.05	100.3
20.80	20.71	−0.09	99.6
27.75	27.32	−0.43	98.5
19.70	19.53	−0.17	99.1
24.00	23.49	−0.51	97.9
18.15	18.35	+0.20	101.1
18.40	18.49	+0.09	100.5
Mean error, −0.026			
Relative error, −0.13			

acetate buffer solution (Solution A) and the other one with the oxidizing pH 4.63 acetate buffer solution containing 0.1% w/v $NaNO_2$ (Solution B). The absorbance of the solution diluted with Solution A is determined against the solution diluted with Solution B with a spectrophotometer at the appropriate wavelength. The amount of rifamycin is calculated from the measured absorbance.

EXPERIMENTAL[4]

Reagents and Solutions—*Solution A*—Acetate buffer at pH 4.63, prepared according to *Reference 9*.

Solution B—Solution A containing 0.1% w/v of $NaNO_2$.

All the reagents and solvents were pure grade (obtained from C. Erba).

Procedures—*Determination of Rifamycin B in the Fermentation Broth*—Two 1-ml. portions of the broth in two 50-ml. volumetric flasks were made to volume, one with Solution A and the other with Solution B. The absorbance of the solution diluted with Solution A was determined at 425 mμ in a 1-cm. cell against the solution diluted with Solution B. The rifamycin B concentration in the fermentation broth was obtained by the formula:

$$\text{rifamycin B (mcg./ml.)} = \frac{A_{425} \cdot 50{,}000}{21.5} \qquad \text{(Eq. 1)}$$

where A_{425} is the absorbance at 425 mμ and 21.5 is the absorptivity of rifamycin B determined by this method. If the concentration of rifamycin B is higher than 1500 mcg./ml., a higher dilution of the broth is necessary. The precision and accuracy data are reported in Tables III and IV, respectively.

Determination of Rifampin in Capsules—The capsules were opened; about 30 mg. of the powder, accurately weighed, was placed in a 20-ml. volumetric flask and made to volume with methanol. Two 1-ml. portions of this solution were treated and examined at 473 mμ, as described for rifamycin B. The content of rifampin was obtained by the formula:

$$\text{rifampin \% (w/w)} = \frac{A_{473} \cdot 100}{14.7 \cdot p} \qquad \text{(Eq. 2)}$$

where A_{473} is the absorbance at 473 mμ, 14.7 is the absorptivity of rifampin determined by this method, and p is the weight of the powder expressed in grams.

Determination of Rifampin in Syrup—About 1.5 g. of syrup, exactly weighed, was placed in a 20-ml. volumetric flask and made to volume with methanol. Two 1-ml. portions of this solution were treated and examined at 473 mμ, as described for rifamycin B. The titer of rifampin was obtained by the formula:

$$\text{rifampin \% (w/w)} = \frac{A_{473} \cdot 100}{14.7 \cdot p} \qquad \text{(Eq. 3)}$$

where the symbols have the same meaning reported in the preceding method.

The precision and accuracy data of the rifampin determination are reported in Tables III and V, respectively.

[4] A Beckman DU spectrophotometer was used in the determinations.

REFERENCES

(1) P. Sensi, A. M. Greco, and R. Ballotta, "Antibiotics Annual 1959–1960," Medical Encyclopedia, Inc., New York, N. Y., 1960, p. 262.

(2) N. Maggi, G. G. Gallo, and P. Sensi, *Farmaco, Ed. Prat.*, **20,** 147(1965).

(3) P. Sensi, R. Ballotta, A. M. Greco, and G. G. Gallo, *Farmaco, Ed. Sci.*, **16,** 165(1961).

(4) N. Maggi, C. R. Pasqualucci, R. Ballotta, and P. Sensi, *Chemotherapia*, **11,** 285(1966).

(5) G. G. Gallo, P. Sensi, and P. Radaelli, *Farmaco, Ed. Prat.*, **15,** 283(1960).

(6) G. G. Gallo, C. R. Pasqualucci, and P. Radaelli, *ibid.*, **18,** 78(1963).

(7) C. R. Pasqualucci, A. Vigevani, P. Radaelli, and N. Maggi, *ibid.*, **24,** 46(1969).

(8) G. Sferruzza and R. Rangone, *ibid.*, **19,** 491(1964).

(9) H. T. S. Britton, "Hydrogen Ions," vol. I, Chapman and Hall Ltd., London, England, 1955, p. 357.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 8, 1969, from *Laboratori Ricerche Lepetit S.p.A., 20158 Milano, Italy.*

Accepted for publication January 6, 1970.

The authors are indebted to Dr. N. Maggi for helpful discussions.

* Present address: Archifar S.p.A., 20146 Milano, Italy.