Dapsone Assay Based on Schiff Base Formation[1]

Louis Levy and Lucy J. Higgins[2]

Because of increasing interest in the therapy of both malaria ([6, 10]) and leprosy ([2, 7, 8]) with dapsone in doses that result in extremely small tissue and blood concentrations of the drug, there is need for a more sensitive dapsone assay. A recent report ([4]) of a sensitive sulfonamide assay based on the phenomenon of Schiff base formation[3] led to the development of a dapsone assay, similarly based on Schiff base formation, which is somewhat more than twice as sensitive as the commonly used Bratton-Marshall method ([1]).

MATERIALS AND METHODS

Absorption spectra were recorded by means of a Beckman DK 2A ratio-recording spectrophotometer. Photometric measurements of the absorbance of the colored Schiff base solutions were made at the appropriate wave-length with a Beckman DU spectrophotometer using cells of 1 cm. light path or with a Hitachi-Perkin Elmer Model 139 UV-Vis spectrophotometer using cells of 2 cm. light path. Measurements of "total" and "free" dapsone concentrations were accomplished by Simpson's modification of the method of Bratton and Marshall ([9]).

Analysis by the Schiff base method. A 1 ml. sample of blood is made alkaline by the addition of 0.2 ml. of 0.25 M Na_3PO_4 and diluted with 1 ml. of distilled water in order to facilitate extraction. This solution is shaken for 15 minutes with 20 ml. of chloroform and centrifuged briefly in order to break the emulsion that results from shaking. Fifteen ml. of the chloroform layer are then removed and evaporated to dryness under an air jet at 37°C. The residue remaining after evaporation of the chloroform is dissolved in 5 ml. of ethanol, after which 1 ml. of 10 N H_2SO_4 and 4 ml. of a 0.4 M solution of an aromatic aldehyde[4] are added. A color appears instantaneously, the absorbance of which is measured in a spectrophotometer at the wavelength of the absorption maxima determined from a recording of the absorption spectrum of the Schiff base solution.

RESULTS

The aromatic aldehydes listed in Table 1 failed to yield a colored Schiff base solution with an ethanolic solution of dapsone. Other aromatic aldehydes, listed in Table 2, yielded intensely colored solutions. The color yielded by the reaction of 4-dimethylaminobenzaldehyde with dapsone is the most intense, followed by the reaction with dapsone of the very similar aldehyde, 4-diethylaminobenzaldehyde. The molar absorbance of the colored Schiff base resulting from the reaction of 4-dimethylaminobenzaldehyde with dapsone is 2.4 times that calculated for the chromophore resulting from the application of the Bratton-Marshall procedure to dapsone.

The Schiff base procedure, using 4-dimethylaminobenzaldehyde. and the Bratton-Marshall procedure were applied to the

[1]Received for publication May 9, 1966.

[2]Louis Levy, M.D., Ph.D., and Lucy J. Higgins, B.A., The Leprosy and Research Services, Public Health Service Hospital, 15th Avenue and Lake Street, San Francisco, California 94118.

[3]A Schiff base is defined ([5]) as the product of the condensation of an aldehyde with a primary amine that contains a carbon-nitrogen double bond.

[4]With the exception of pyridoxal and pyridoxal phosphate, purchased from the Sigma Chemical Company, St. Louis, Missouri, the aldehydes used were all purchased from the Eastman Kodak Company, Rochester, New York.

blood of a patient with lepromatous leprosy who was under treatment with 50 mgm. of dapsone orally daily. Blood was drawn before the daily dose of the drug, and again at 2, 4 and 6 hours after the dose was given. A comparison of the absorbances and calculated dapsone concentrations obtained by the two procedures is presented in Table 3. The data recorded demonstrate the close correspondence between the two methods for dapsone analysis. It is apparent from these data that the calculations of dapsone concentration from the Bratton-Marshall technic are based on

TABLE 1. *Aromatic aldehydes yielding a colorless solution with dapsone.*

3-aminobenzaldehyde	2-hydroxy-1-naphthaldehyde
benzaldehyde	2-hydroxy-3-nitrobenzaldehyde
1,2-benzenedicarboxaldehyde	2-hydroxy-5-nitrobenzaldehyde
1,4-benzenedicarboxaldehyde	4-isopropylbenzaldehyde
2-chlorobenzaldehyde	2-methoxybenzaldehyde
4-chlorobenzaldehyde	4-methoxybenzaldehyde
trans-cinnamaldehyde	4-methylbenzaldehyde
2,4-dichlorobenzaldehyde	1-naphthaldehyde
2,5-dihydroxybenzaldehyde	3-nitrobenzaldehyde
2-ethoxybenzaldehyde	4-nitrobenzaldehyde
2-hydroxybenzaldehyde	pyridoxal
2-hydroxy-5-chlorobenzaldehyde	pyridoxal phosphate

TABLE 2. *Aromatic aldehydes yielding intensely colored solutions with dapsone.*

	λ max.[a] (mμ)	A_M[b] x 10^{-4}
3,4-diethoxybenzaldehyde	410	3.7
4-diethylaminobenzaldehyde	470	9.7
2,4-dihydroxybenzaldehyde	395	5.0
3,4-dimethoxybenzaldehyde	405	1.2
4-dimethylaminobenzaldehyde	470	11.7
3-ethoxy-4-hydroxybenzaldehyde	410	3.3
4-hydroxybenzaldehyde	385	3.1
3-methoxy-4-hydroxybenzaldehyde	410	3.0
(Bratton-Marshall procedure)	550	4.9

[a] λ max. is that wavelength at which the absorbance of incident light is maximal.
[b] A_M is the molar absorbance, given for each compound at its absorption maximum. The molar absorbance is the absorbance of a solution of 1 M concentration per cm. of light path.

TABLE 3. *Comparison of the Bratton-Marshall and Schiff base methods.*

	Bratton-Marshall				Schiff base	
	"Total"		"Free"		"Free"	
Sample	A[a]	Dapsone[b]	A	Dapsone	A	Dapsone
0.5 μgm. dapsone standard	.009		.008		.032	
1.0 μgm. dapsone standard	.013		.014		.069	
5.0 μgm. dapsone standard	.101		.083		.367	
Baseline blood	.036	1.70	.017	1.10	.082	1.20
Blood 2 hours after dapsone	.074	3.43	.050	3.08	.211	2.92
Blood 4 hours after dapsone	.078	3.62	.050	3.08	.200	2.80
Blood 6 hours after dapsone	.066	3.13	.050	3.08	.180	2.50

[a] A is the measured absorbance, at 550 mμ for the Bratton-Marshall procedure, and at 470 mμ for the Schiff base procedure with 4-dimethylaminobenzaldehyde, employing cells of 2 cm. light path. All values represent the mean of duplicate determinations after subtraction of the blank absorbance.
[b] Dapsone is the concentration of dapsone calculated in μgm./ml. of the sample.

absorbance measurements that are much smaller than those from the Schiff base technic.

DISCUSSION

In their discussion of the Schiff base method for sulfonamide analysis, Colaizzi *et al.* ([4]) emphasized the importance of a maximal alcohol concentration, a high hydrogen ion concentration, and a large mole ratio of aldehyde to amine in obtaining the maximal yield of Schiff base. The method described here for dapsone was designed with these recommendations in mind. Thus, virtually a saturated solution in ethanol of the aldehyde was used. The volume and concentration of acid employed were selected to provide maximal color intensity. Extraction with chloroform followed by evaporation of the extract to dryness was undertaken as a means of limiting the water present to that in the 10 N H_2SO_4 and that contaminating the ethanol.

The need to limit the water in the system requires extraction of dapsone with a water-immiscible organic solvent from the alkalinized sample. This Schiff base method, therefore, measures only the dapsone that is "free," i.e., soluble in organic solvents. That which is conjugated with glucuronic acid ([3]) and thus made water-soluble, is not extracted by chloroform. This limitation is not severe, since the bulk of the dapsone measured by the Bratton-Marshall procedure is "free," and since the dapsone metabolites are probably not biologically active ([3]). The need to evaporate the chloroform extract to dryness results in a more cumbersome procedure than the Bratton-Marshall procedure for "total" dapsone, but a procedure that is no more difficult than the Bratton-Marshall technic for "free" dapsone. Chloroform was chosen as the solvent for extraction because of its low boiling point ,and its immiscibility with water. Ethyl acetate was found to yield a large blank absorbance, while extraction with ether, because of the great volatility of this solvent, was subject to such great volume changes as to make the measurement unreliable.

Despite these limitations, the Schiff base method using dimethylaminobenzaldehyde is inherently approximately 2.4 times as sensitive as the Bratton-Marshall technic, considering only the difference in the molar absorbance of the two chromophores. An additional factor of 1.5 is obtained because the entire 15 ml. of extract is used for the determination, following evaporation to dryness, rather than merely the 10 ml. used in the Bratton-Marshall procedure for "free" dapsone.

If one were satisfied with an absorbance of .020 (after subtraction of blank absorbance), this procedure might be modified so that a concentration of dapsone as small as 0.025 μgm./ml. might be measured. Thus, if 5 ml. of blood were extracted with 100 ml. of chloroform, the chloroform extract evaporated to dryness, the residue dissolved in 1.5 ml. of ethanol, and the volume made up to 3 ml. by the addition of 0.3 ml. of 10 N H_2SO_4 and 1.2 ml. of the ethanolic 4-dimethylaminobenzaldehyde solution, dapsone in an initial concentration of 0.025 μgm./ml. should yield an absorbance of .020. This degree of sensitivity might be achieved by analogous modifications of the Bratton-Marshall technic for "free" dapsone. But, because of the greater molar absorbance of the Schiff base, Schiff base formation would seem to be a more rewarding procedure.

Since so laborious a procedure would be very difficult to accomplish under field conditions, and since no amount of labor will increase the sensitivity if the sample size is limited (as, for example, when measurement of dapsone concentrations in skin biopsies is desired), better methods of dapsone analysis must yet be sought. Studies of a fluorometric method and a method involving the technic of isotope dilution are planned.

SUMMARY

An analytic method for the measurement of dapsone concentrations in blood has been developed, based on the phenomenon of Schiff base formation between 4-dimethylaminobenzaldehyde and dapsone. This method, which measures only that

dapsone that has not been metabolized, is proposed as an alternative to the measurement of "free" concentrations by the Bratton-Marshall technic. The Schiff base method is at least 2.4 times as sensitive as the Bratton-Marshall technic, but is somewhat more laborious. It is not yet the ideal sensitive assay for dapsone.

RESUMEN

Un método analítico para medir la concentración de dapsona en la sangre fué desarrollado, basándose en la formación base de Schiff, entre 4-dimethylaminobenzaldehydo y dapsona. Este método que mide solamente la dapsona que no ha sido metabolizada, se propone como una alternativa para medir las concentraciones "libres," por el método de Bratton-Marshall. El método base de Schiff es por lo menos 2.4 veces tan sensible como la técnica de Bratton-Marshall, pero exige mayor trabajo para ser ejecutado. No es todavía el método ideal de sensibilidad para la dapsona.

RÉSUMÉ

On a mis au point une méthode analytique destinée à mesurer les concentrations de dapsone dans le sang. Ce procédé est basé sur le phénomène de la formation de base de Schiff entre la 4-dimethylaminobenzaldehyde et la dapsone. Cette méthode, qui mesure seulement la dapsone qui n'a pas été métabolisée, est proposée comme solution de rechange pour la mesure de la concentration "libre" par la technique de Bratton-Marshall. La méthode à la base de Schiff est au moins 2.4 fois plus sensitive que la technique de Bratton-Marshall, mais elle exige plus de travail. Elle ne constitue pas encore l'epreuve dotée d'une sensibilité idéale pour le dosage de la dapsone.

Acknowledgments. This study was approved by the Division of Hospitals, Bureau of Medical Services, Public Health Service, and supported in part by Project Grant M66-17 from the Division of Hospitals and by Grant AI-06818 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. A supply of crystalline dapsone was generously furnished by Richard A. Carey, M.D., Ayerst Laboratories, New York.

REFERENCES

1. Bratton, A. C. and Marshall, E. K. A new coupling component for sulfanilamide determining. J. Biol. Chem. **128** (1939) 537-550.
2. Browne, S. G. Personal communication.
3. Bushby, S. R. M. The chemotherapy of leprosy. Pharmacol. Rev. **10** (1950) 1-42.
4. Colaizzi, J. L., Boenick, J. W., Martin, A. N. and Knevel, A. M. Schiff base formation in the development of spectrophotometric assay for sulfonamides. J. Pharm. Sci. **54** (1965) 564-568.
5. Conant, J. B. and Blatt, A. H. The chemistry of organic compounds. New York, The Macmillan Co., 1959, p. 456.
6. Elslager, E. F. and Worth, D. P. Repository antimalarial drugs: N,N-diacetyl-4,4'diaminodiphenylsulphone and related 4-acylaminodiphenylsulphones. Nature (London) **206** (1965) 630-631.
7. Pettit, J. H. S. Personal communication.
8. Shepard, C. C. Personal communication.
9. Simpson, I. A. Method of sulfone estimations. Internat. J. Leprosy **17** (1949) 208-210.
10. Thompson, P. E., Olszewski, B. and Waitz, J. A. Laboratory studies on the repository antimalarial activity of 4,4'diacetylaminodiphenylsulfone, alone and mixed with cycloguanil pamoate (CI-501). American J. Trop. Med. & Hyg. **14** (1965) 343-353.