filtration. The results of this procedure have been shown to be in good agreement with those obtained by equilibrium dialysis (6). However, this technique will not determine accurately the binding parameters of any rapidly equilibrating system; under such circumstances, an overestimation of binding is observed.

In the present studies, it was possible to separate the detergent bound to the enzyme from that unbound and to estimate the amount of interaction between the two moieties. The enzyme, at a 4–5% concentration dissolved in a 0.9% solution of radioiodinated detergent, was chromatographed on Sephadex G-75. It was found that 1.4×10^{-5} mmole (mol. wt. 744) of the detergent was associated with 825 μg of the enzyme. This result indicates that 17 moles of detergent bound per mole of enzyme, calculated on the latter having a molecular weight of 1×10^6. This ratio of detergent to enzyme nearly corresponds to one binding site per 55,000 molecular weight subunit of the enzyme (7). This calculation presupposes detergent that is bound rather than in the micellar form. Such binding has occurred when ligand and protein were present in relatively low concentrations (8–10).

One interesting aspect of the interactions between proteins and detergents is the inclusion of several protein molecules in one detergent micelle (11). To test whether the enzyme could be included in detergent micelles, solutions of the radioiodinated enzyme in detergent were prepared and passed through a column of Bio-Gel A-15m. The results indicated that the enzyme could not be aggregated in detergent micelles, because no sample of L-glutamic acid dehydrogenase in detergent had an elution volume less than the native enzyme, even when the eluting buffer contained 1% by volume of the detergent.

While this experiment on agarose showed that the enzyme was not measurably aggregated in detergent micelles, it was not clear whether the enzyme was dissociating into its 55,000 subunits (7). Therefore, gel filtration on Sephadex G-200 was performed. The enzyme buffer only eluted as a single peak, whereas the enzyme in detergent gave a chromatograph of two peaks. In this latter case, the first peak eluted with the void volume whereas the position of the second peak indicated a molecular weight of 55,000. However, the amount of dissociation into these small subunits was never more than 5% of the total amount applied to the column.

The results indicate that the nonionic detergent can bind to the enzyme L-glutamic acid dehydrogenase. This binding is only in the order of approximately 17 moles of detergent/mole of enzyme, hardly sufficient to allow the enzyme to be included in a detergent micelle. However, it obviously is sufficient to cause enhancement of the activity of this enzyme (1).

These results suggest a possible avenue for enhancement of the biological activities of other proteins and drugs.

REFERENCES

(1) D. H. Kempner and B. J. Johnson, *J. Pharm. Sci.*, **65**, 1799 (1976).

(2) M. W. Bitensky, K. L. Yielding, and G. M. Tomkins, *J. Biol. Chem.*, **240**, 668 (1965).

(3) S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and W. Weigle, *Science*, **178**, 871 (1972).

(4) J. J. Marchalonis, *Biochem. J.*, **113**, 299 (1969).

(5) G. S. David and R. A. Reisfeld, *Biochemistry*, **13**, 1014 (1974).

(6) P. F. Cooper and G. C. Wood, *J. Pharm. Pharmacol., Suppl.*, **20**, 150S (1968).

(7) N. Tatal, G. M. Tomkins, J. F. Muchinski, and K. L. Yielding, *J. Mol. Biol.*, **8**, 46 (1964).

(8) S. Makino, J. A. Reynolds, and C. Tanford, *J. Biol. Chem.*, **248**, 4926 (1973).

(9) J. A. Reynolds and C. Tanford, *Proc. Nat. Acad. Sci. USA*, **66**, 1002 (1970).

(10) Y. Nozaki, J. A. Reynolds, and C. Tanford, *J. Biol. Chem.*, **249**, 4452 (1974).

(11) J. N. Umbreit and J. L. Strominger, *ibid.*, **248**, 6759 (1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received April 7, 1976, from the *Department of Microbiology, University of Alabama Medical School, Birmingham, AL 35294.*

Accepted for publication July 28, 1976.

Supported in part by Grant AI 11970 from the National Institutes of Health.

ˣ To whom inquiries should be directed.

Separation and Quantitative Determination of 2,4-Disulfamyl-5-trifluoromethylaniline in Hydroflumethiazide Using High-Pressure Liquid Chromatography

B. J. OWEN ˣ and S. V. WILKIE

Abstract ☐ A high-pressure liquid chromatographic method was developed to quantify 2,4-disulfamyl-5-trifluoromethylaniline in hydroflumethiazide using a bonded pellicular packing. An internal standard was added to the samples to ensure reproducibility. The results were compared with methods currently in use.

Keyphrases ☐ 2,4-Disulfamyl-5-trifluoromethylaniline—high-pressure liquid chromatographic analysis in hydroflumethiazide samples and tablets ☐ Hydroflumethiazide—prepared samples and tablets, high-pressure liquid chromatographic analysis of its precursor 2,4-disulfamyl-5-trifluoromethylaniline ☐ High-pressure liquid chromatography—analysis, 2,4-disulfamyl-5-trifluoromethylaniline in hydroflumethiazide prepared samples and tablets ☐ Antihypertensives—hydroflumethiazide prepared samples and tablets, high-pressure liquid chromatographic analysis of its precursor 2,4-disulfamyl-5-trifluoromethylaniline

The hydroflumethiazide monographs in the National Formulary (1) and the British Pharmacopoeia (2) include assays for free amine in the form of its precursor 2,4-disulfamyl-5-trifluoromethylaniline (I) (3–6). In the BP method, the free amine in the sample is compared visually with a standard of I using TLC for the separation. A diazotization is performed in the NF method, and the solution absorbance is read spectrophotometrically and compared with a standard preparation.

This paper describes a high-pressure liquid chromatographic (HPLC) method for the separation of I from hydroflumethiazide, and the results are compared with those from the compendial methods.

EXPERIMENTAL

Materials—Standard solutions containing 0.02, 0.04, 0.06, and 0.08 mg of I[1]/ml of reagent grade methanol were prepared. Hydroflumethia-

[1] E. R. Squibb standard substance.

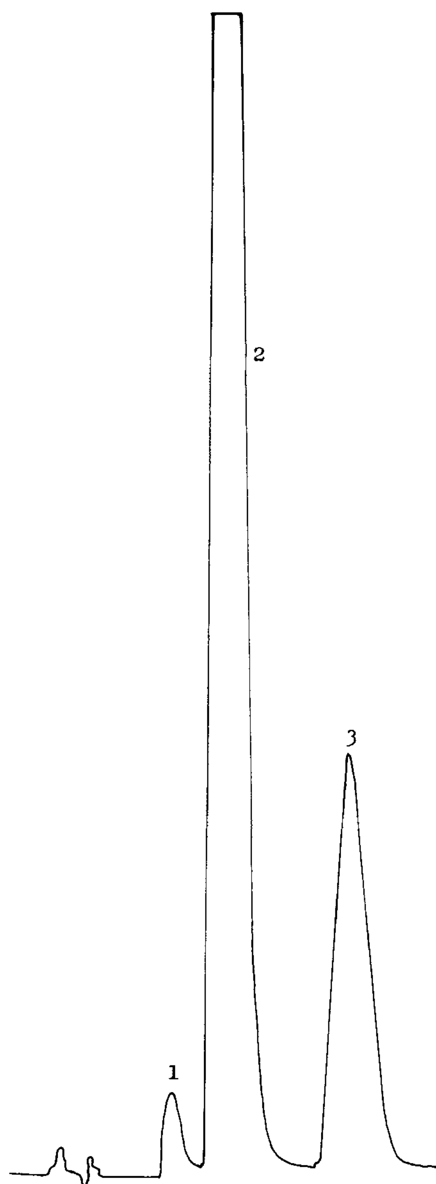

Figure 1—*HPLC separation of I (1), hydroflumethiazide (2), and sulfathiazole (3).*

zide samples were diluted to 4 mg/ml in methanol. Hydroflumethiazide tablets were made up to the same concentration; the solutions were filtered before injection. Sulfathiazole, 0.4 mg/ml, was added to both standard and sample solutions as an internal standard.

Apparatus—A high-pressure liquid chromatograph[2] with a double-beam UV detector operating at 254 nm was used in conjunction with a 10-mv recorder. The columns were 0.5 m long stainless steel (2.6 mm i.d.) packed with an octadecyl hydrocarbon bonded to a deactivated silica[3].

Separation Conditions—The eluting solvent was 5% methanol (reagent grade) in distilled water flowing at 1.5 ml/min, and the temperature of the column oven was 35°. Three microliters of each solution was chromatographed, and the time for complete elution of the sample was approximately 15 min. Each sample was run once, and the calculations were based on peak height. The response for I in the sample was compared with that of the standard solutions. The separation is shown in Fig. 1.

[2] Perkin-Elmer, Norwalk, Conn.
[3] O.D.S. Sil-X-I, Perkin-Elmer, Norwalk, Conn.

Table I—Comparison of Assay Methods for the Estimation of Free Amine in Hydroflumethiazide

Sample	HPLC, %	TLC, %	Diazotizable Substances Assay, %
1	0.97	~1	1.14
2	0.83	<1	0.88
3	0.55	<1	0.83
4	0.30	<1	0.51
5	0.45	<1	0.57
6	0.29	<1	0.57
7	0.55	<1	0.74
8	0.56	<1	0.57
9	0.51	<1	0.55
10	0.58	<1	0.70
11	0.71	<1	0.69
Tablet 1	0.44	<1	—
Tablet 2	0.46	<1	—
Tablet 3	0.40	<1	—
Tablet 4	0.46	<1	—
Tablet 5	0.41	<1	—

Table II—Recovery of Free Amine Added to 4.000-mg/ml Samples of Hydroflumethiazide

Weight of Free Amine Added, mg/ml	Free Amine Added, %	Free Amine Determined, %	Free Amine Recovered, %
0.000	0.00	0.495	—
0.008	0.2	0.696	0.201
0.016	0.4	0.897	0.402
0.024	0.6	1.125	0.630
0.032	0.8	1.304	0.809

RESULTS AND DISCUSSION

The primary goal in developing this assay was to provide a fast precise method for the estimation of I in hydroflumethiazide and hydroflumethiazide tablets. The assay described met these criteria. This HPLC assay gave better precision than TLC and similar results to the diazotizable substance assay (Table I). The linearity of the detector response for 0.02, 0.04, 0.06, and 0.08 mg of I/ml of methanol was satisfactory. From one sample of hydroflumethiazide, 10 weighings produced assays giving a mean result for free amine content of 0.971 ± 0.0281% (*SD*). The recovery of free amine added to a sample of hydroflumethiazide was also checked (Table II).

REFERENCES

(1) "The National Formulary," 14th ed., Mack Publishing Co., Easton, Pa., 1975, p. 329.

(2) "The British Pharmacopoeia," Pharmaceutical Press, London, England, 1973, p. 232.

(3) C. T. Holdrege, R. B. Babel, and L. C. Cheney, *J. Am. Chem. Soc.*, **81**, 4807 (1959).

(4) W. J. Close, L. R. Swett, L. E. Brady, J. H. Short, and M. Vernsten, *ibid.*, **82**, 1132 (1960).

(5) H. L. Yale, K. Losee, and J. Bernstein, *ibid.*, **82**, 2042 (1960).

(6) F. C. Novello, S. C. Bell, L. A. Abrams, C. Ziegler, and J. M. Sprague, *J. Org. Chem.*, **25**, 970 (1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 2, 1976, from *Linson Limited, Swords, Dublin, Ireland.*

Accepted for publication July 28, 1976.

[x] To whom inquiries should be directed.