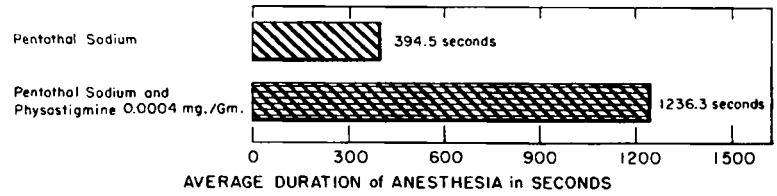


Fig. 6.—Effect of physostigmine on duration of anesthesia produced by 35 mg./Kg. of pentothal sodium intravenously in mice.

an average anesthetic time of 88.4 seconds and no prior medication showed an average anesthetic time of 88.4 seconds and 110.7 seconds until walking was recovered. These values were approximately the same as those for animals receiving DFP and pentothal.

Twenty physostigmine treated animals receiving 35 mg./Kg. of pentothal sodium gave an average anesthetic time of 1236.3 seconds (Fig. 6), contrasted with 394.5 seconds for the controls and 1412.7 seconds for recovery of walking ability compared to 471.9 seconds for the controls.

Potentiation of pentothal sodium by prior medication with physostigmine and neostigmine resulted in increasing the anesthetic activity of a non-anesthetic dose and prolonging the anesthetic time of an anesthetic dose. These potentiations by cholinesterase inhibitors could not be blocked by 0.1 mg./Gm. of atropine, and although acetylcholine enhanced the potentiation, it alone did not produce the potentiations equal to those of neostigmine and physostigmine.

The decreases in cholinesterase (Table I) activity brought about by the inhibitors used in this study correlate quite well with the increases in activity of the CNS stimulants and depressants. DFP showed the least inhibitory activity in the dose used and the least potentiating action, while neostigmine showed the most cholinesterase depression and the greatest potentiations.

SUMMARY

Physostigmine or neostigmine as prior medication caused potentiation in mice of the actions of strychnine and pentylenetetrazol. Subconvulsive doses were made convulsive and, in the case of strychnine, the subconvulsive dose was made lethal. In addition, the above cholinesterase inhibitors shortened the anesthetic lag time of intravenous phenobarbital sodium and increased the anesthetic activity of pentothal sodium, lengthening the anesthetic time. DFP was less effective in producing these potentiations.

Atropine sulfate (0.1 mg./Gm.) as prior medication did not block the potentiation; acetylcholine bromide (0.05 mg./Kg.) produced potentiations of much less magnitude than the reversible cholinesterase inhibitors. Thus, factors other than vasodilation must be of primary importance in these observed potentiations.

The per cent depression of cholinesterase activity by the inhibitors correlated with the degree of change in the activity of the CNS stimulants and depressants.

REFERENCES

(1) Koelle, G. B., *J. Pharmacol. Exptl. Therap.*, **100**, 158 (1950).
(2) Barbour, H. G., and Abel, J. J., *ibid.*, **2**, 167(1910).
(3) Greig, M. E., and Holland, W. C., *Science*, **110**, 237 (1949).
(4) Lewis, P. A., *J. Expll. Med.*, **23**, 669(1916).
(5) Slaughter, D., *J. Pharmacol. Exptl. Therap.*, **68**, 98 (1940).
(6) Green, V. A., Steber, M., McKenna, G. T., Davis, J. E., and Taylor, A., *Tex. J. Sci.*, **9**, 89(1957).
(7) Green, V. A., THIS JOURNAL, **52**, 227(1963).
(8) Greig, M. E., and Mayberry, T. C., *J. Pharmacol. Exptl. Therap.*, **102**, 1(1951).
(9) Gutierrez-Norregia, C., *Rev. Neurol. Psiquiat.*, **8**, 121 (1945).
(10) Schweitzer, A., Stedman, E., and Wright, S., *J. Physiol.*, **96**, 302(1939).
(11) Rosenberg, A., and Coon, J. M., *Proc. Soc. Exptl. Biol. Med.*, **98**, (No. 3650) (1958).
(12) Nachmansohn, D., *Compt. Rend. Soc. Biol.*, **129**, 941 (1938).

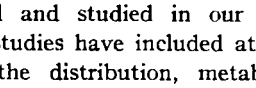
Determination of Sulfamylurea Hypoglycemic Agents and their Metabolites in Biological Fluids

By EDWARD H. WISEMAN, JOSEPHINE CHIAINI, and REX PINSON, JR.

Amine metabolites (basic) are separated from their parent sulfamylureas and sulfamide metabolites (both acidic) by extraction. Sulfamylureas are hydrolyzed by acid to the corresponding amine for assay, under which conditions sulfamides are unaffected. Sulfamides are similarly hydrolyzed to an amine under basic conditions, sulfamylureas being stable. Flourine-containing sulfamylureas were assayed by Schöniger combustion and determination of the resultant fluoride ion.

AS PART OF a continuing program on oral hypoglycemic agents, a series of sulfamylureas of the general formula (I) has been synthesized and studied in our laboratories (1). These studies have included attempts (2, 3) to relate the distribution, metabolism, and ex-

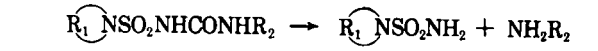
Received August 19, 1963, from the Medical Research Laboratories, Chas. Pfizer & Co., Inc., Groton, Conn.
Accepted for publication October 4, 1963.



I

cretion of these materials to various physical properties such as lipid/water distribution ratios, pKa, plasma binding, and solubility. This paper describes the development of analytical procedures required to determine these compounds and their metabolites in plasma and urine.

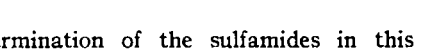
The major route of metabolism of the sulfamylureas is degradation to the sulfamide and a primary amine (2, 3)



Methods therefore had to be developed for the determination of the sulfamylurea, sulfamide, and amine either alone or in admixture with each other.

The sulfamylureas studied can be divided into two classes: those in which R_2 was either alkyl or cycloalkyl, and those in which R_2 was pentafluoropropyl. The former series were determined by a modification of the Spingler method (4). The sulfamylurea was extracted from the acidified biological sample into hexyl acetate and the hexyl acetate extract then heated to 130°. Under these conditions sulfamylureas are degraded to the corresponding cycloalkyl or alkyl amine, which can be determined as the yellow 2,4-dinitrophenyl derivative by reaction with 2,4-dinitrofluorobenzene (DNFB). The fluorine-containing sulfamylureas were extracted, and determined as fluoride ion after a Schöniger oxidation (5). There is no interference from metabolites in either assay procedure.

The sulfamylureas are stable in strongly basic solution at 100°, whereas the sulfamides are smoothly hydrolyzed to an amine



The determination of the sulfamides in this study was based on alkaline hydrolysis to the amine under conditions that did not affect the parent sulfamylurea. Interfering amines were first removed from the alkalinized biological sample by chloroform extraction. The aqueous solution was made strongly basic, and the sulfamide hydrolyzed to the corresponding secondary amine by heating at 100°. The amine produced was then determined by a methyl orange technique (6).

EXPERIMENTAL

Reagents

All organic solvents used were of analytical reagent grade. In addition, the following stock solutions are required: McIlvaine buffer, pH 5.0; 2:4-dinitrofluorobenzene (0.1%) solution in *n*-hexyl acetate (DNFB reagent) (the solution is stored in a brown bottle, and kept at 0–5°); methyl orange reagent (a solution of methyl orange (500 mg.) in water (100 ml.) is washed with chloroform. Before use, the aqueous solution is diluted 1:1 with saturated boric acid solution); and a solution of concentrated sulfuric acid (2%) in absolute ethanol.

Procedure

For Sulfamylureas Derived from Alkyl and Cycloalkyl Amines.—The biological sample (1 ml., either plasma or urine), in a glass-stoppered glass centrifuge tube is adjusted to pH 4–5 with 0.5 *N* hydrochloric acid and pH 5 buffer (1 ml.) added, followed by *n*-hexyl acetate (2 ml.). The mixture is mechanically shaken for 30 minutes, then centrifuged to separate the layers. A portion of the hexyl acetate layer (1 ml.) is transferred to a fresh glass-stoppered centrifuge tube containing the DNFB reagent (0.5 ml.). The tube is loosely stoppered and heated in an oil bath at 125–135° for 10 minutes. The solution is allowed to cool for 30 minutes, and the absorbance read at 398 mμ in a Beckman DU spectrophotometer against *n*-hexyl acetate as a blank.

For Sulfamylureas Derived from Pentafluoropropylamine.—The biological sample (1 ml.) is adjusted to *ca.* pH 4–5 with 0.5 *N* hydrochloric acid. The solution is buffered with pH 5 buffer (1 ml.), and extracted by shaking with ethyl acetate (2 ml.) for 30 minutes. A portion of the ethyl acetate layer (1 ml.) is carefully pipeted onto ashless filter paper, folded to contain a small ball of cotton (about 4 mg.), and the whole dried under an infrared lamp for 15 minutes. Potassium chlorate (about 1 mg.) is added, the paper carefully folded to contain this and the cotton ball, and placed in the platinum basket of a Schöniger flask (500 ml. capacity). The combustion is carried out in oxygen in the usual manner, and the gases absorbed in water (about 50 ml.). The flask and contents are allowed to stand for 20 minutes, and then the contents (together with washings) are transferred to a separator (250-ml. capacity), the final volume being about 150 ml. The fluoride ion concentration is then determined by the method of Johnson and Leonard (7).

For Sulfamides.—The biological sample (10 ml.) is made basic (pH 12) with sodium hydroxide solution and shaken with chloroform (10 ml.) for 15 minutes. The layers are separated by centrifugation, and a sample of the aqueous layer (5 ml.) transferred to a fresh tube containing 30% sodium hydroxide solution (1 ml.). The tube is stoppered, and the solution is heated on a steam bath for 20 minutes. After cooling, sodium chloride (*ca.* 3 Gm.) is added, and the solution shaken with benzene (20 ml.) for 30 minutes. The mixture is centrifuged, and a portion of the benzene layer (15 ml.) is washed with 0.1 *N* sodium hydroxide solution (5 ml.). Part of the benzene layer (8 ml.) is transferred to a tube containing isoamyl alcohol (0.4 ml.) and methyl orange reagent (0.6 ml.). The mixture is shaken for 10 minutes, centrifuged, and a portion of the benzene layer (6 ml.) transferred to a colorimeter tube containing 2% sulfuric acid in ethanol (1 ml.). The contents are mixed thoroughly and the absorbance read immediately in a Bausch & Lomb Spectronic 20 spectrophotometer at 540 mμ.

For Amines.—The biological sample (5 ml.) is alkalinized with 30% sodium hydroxide solution (1 ml.), sodium chloride (about 3 Gm.) is added, and the mixture shaken with benzene (20 ml.) for 30 minutes. A portion of the benzene layer (15 ml.) is separated and the sulfamide procedure followed from this point.

Calculations

All assays of unknown samples are related to standard curves prepared by submitting to the entire assay, pooled control urine or plasma samples, to which known amounts of the compound to be assayed had been added. The sulfamylurea assay obeys Beer's law over the range 10–200 mcg./ml. The sulfamylureas investigated varied widely in oil-water partition coefficients [0.03– <4 in the system cyclohexane/0.1 *N* hydrochloric acid (3)], and this affected the percentage recoveries from plasma and urine. However, in all cases, recovery was between 70–100%. The absorbance at 398 mμ of the 2,4-dinitrophenylamine derivative corresponding to an initial sulfamylurea concentration of 100 mcg./ml. fell in the range 0.410 to 0.560. The sulfamide assay follows Beer's law over the range 5–100 mcg./ml. and is subject to variations similar to those of the sulfamylurea assay. Sulfamylureas do not interfere in the sulfamide assay, but amines must be removed prior to hydrolysis. The amine assay has a range of 1–15 mcg./ml. Neither sulfamylurea nor sulfamide interfere in this assay.

Determination of Wavelength for Measurement of Dinitrophenyl Derivatives

The dinitrophenyl derivatives of all amines studied (cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, *n*-propyl, *n*-butyl) exhibit a plateau in the ultraviolet at 390–410 mμ, together with a peak at a lower wavelength (*ca.* 350 mμ), in agreement with previously reported observations (4, 8–11). At the higher wavelength blank values are considerably reduced, and errors arising from the absorbance of 2:4-dinitrophenol are also minimized (12). Accordingly, the higher wavelength is utilized in the assay procedure.

Stability Studies

Sulfamylureas (at concentrations *ca.* 100 mcg./ml.) could be recovered after heating at 100° for 20 minutes with 30% sodium hydroxide essentially quantitatively (92–100%). At concentrations up to 200 mcg./ml., sulfamylureas cause no interference in the sulfamide assay.

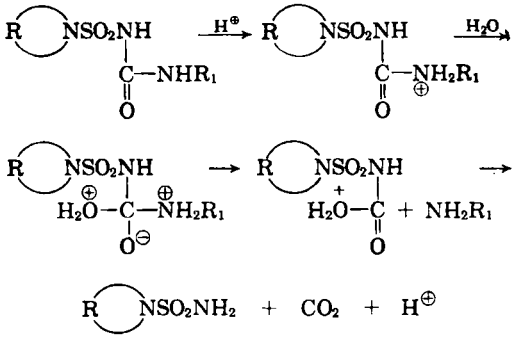
Sulfamides (at concentrations of *ca.* 60 mcg./ml.) could be recovered after 30 minutes at 100° in aqueous acid (pH 2) to the extent of 70–90%. Under assay conditions, concentrations of sulfamide in excess of 100 mcg./ml. cause no interference in the sulfamylurea assay.

Both sulfamylureas and sulfamides could be recovered essentially quantitatively (90–100%) after 48 hours at 37° from urine (pH 5–8).

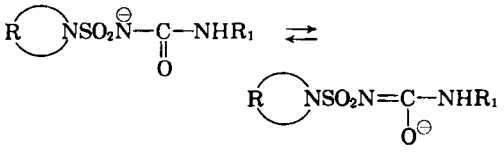
DISCUSSION

The determination of a substituted urea by the DNFB procedure was first reported by Spingler (4) who applied it to tolbutamide. Carmichael (8) extended the procedure to the determination of chlorpropamide. The present method is a modification of the original and incorporates an improvement since high blanks due to hemolyzed plasma samples have been eliminated. This has been achieved by buffering the plasma to be extracted to pH 5, at which pH the color from hemolyzed samples is not extracted by hexyl acetate. In addition, in selecting the higher absorption wavelength of the dinitrophenyl derivatives for assay use, the reliability of the assay has been further increased.

The development of assays capable of differentiating between two closely related compounds such as a sulfamylurea and its sulfamide metabolite is a reflection of the different stabilities of these two classes of compounds under hydrolytic conditions. By analogy with the proposed mechanism for the hydrolysis of amides (13), the hydrolysis of the sulfamylureas in acid solution may be envisioned as

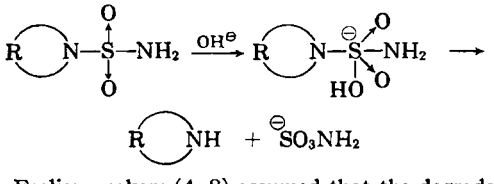


The initial (and most probably, rate determining) step will be the protonation of the sulfamylurea. If the group R_1 is of sufficient electronegativity, the availability of electrons at the nitrogen, and thus protonation, will be limited. The high electronegativity of the pentafluoropropyl group presumably restricts electron availability at the nitrogen to such an extent that the series of sulfamylureas containing that group was resistant to hydrolysis. For this reason, an alternative assay procedure for these compounds was developed. In alkaline solution, the sulfamylureas will exist in the salt form



The high electron density at the carbonyl group of this form which hinders attack by OH^-, accounts for the observed stability of the sulfamylureas in basic solution.

The hydrolytic behavior of the sulfamide metabolites can be explained on similar grounds. Much weaker acids than their sulfamylurea counterparts, they resist protonation and are therefore stable under acidic conditions. In basic solution however, the hydrolysis probably proceeds by



Earlier workers (4, 8) assumed that the degradation of the substituted urea occurred upon heating at 130°, the solvent being inert. We have found,

however, that both the sulfonyl and sulfamylureas are stable when heated alone in pure hexyl acetate but that prior washing of the hexyl acetate with dilute hydrochloric acid causes the reaction to proceed smoothly. The requirement of both acid and water becomes clear from the preceding discussion of the reactions involved.

The analytical method described for the fluorine containing sulfamylureas is novel because direct use is made of the fluorine substituent in the molecule. The electronegativity of the pentafluoropropyl group increases the acid strength of the series, evidenced by a decrease in pKa [from 7.5 to 8.3 in the cycloalkyl series to 6.5 in the pentafluoropropyl series (3)], enhancing the acid stability of these compounds to the point where they are resistant to hydrolysis under the assay conditions described for the sulfamylureas of the cycloalkyl series. The plasma and urine blank values in the combustion assay are low (*ca.* 1 mcg./ml.), being limited more by the cleanliness of the analytical apparatus than by biological contaminants.

The DNFB assay has been most extensively studied in the sulfamylurea series, but is also applicable to the sulfonylureas (tolbutamide, chlorpropamide,[1] acetohexamide, and cycloheptolamide). Plasma and urine blanks are low (about 5 mcg./ml.) and the assay has a wide range (10–200 mcg./ml.).

SUMMARY

Assays for sulfamylurea hypoglycemic agents and their metabolites in blood plasma and urine have been described. Sulfamylureas were determined by degradation to the corresponding primary amine which was determined spectrophotometrically after reaction with 2:4-dinitrofluorobenzene. The compounds in a series of fluorine-containing sulfamylureas were determined as fluoride ion after Schöniger combustion. One class of metabolites, the sulfamides, was hydrolyzed under basic conditions to give a secondary amine, which was then determined by the methyl-orange-dye–complex technique. The remaining amine metabolites were assayed directly by the methyl orange procedure. Although the assay has been primarily studied for the sulfamylureas, it is equally applicable to the determination of the hypoglycemic sulfonylureas. The high blanks caused by hemolyzed plasma samples in currently used sulfonylurea assays have been eliminated in the assay described.

REFERENCES

(1) McManus, J. M., McFarland, J. W., Gerber, C. F., Pereira, J. N., Finger, K. F., McLamore, W. M., and Laubach, G. D., in preparation.
(2) Pinson, R., Jr., "Abstracts of the American Chemical Society, 142nd Annual Meeting," Atlantic City, N. J., September 1962, p. 97.
(3) Wiseman, E. H., and Pinson, R., Jr., in preparation.
(4) Spingler, H., *Klin. Wochschr.*, **35**, 533(1957).
(5) Schöniger, W., *Mikrochim. Acta*, **1**, 123(1955).
(6) Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, **158**, 705(1945).
(7) Johnson, C. A., and Leonard, M. A., *J. Pharm. Pharmacol.*, **13**, 164T(1961).
(8) Carmichael, R. H., *Clin. Chem.*, **5**, 597(1959).
(9) Ekladius, L., and King, H. K., *Biochem. J.*, **65**, 128 (1957).
(10) McIntire, F. C., Clements, L. M., and Sproull, M., *Anal. Chem.*, **25**, 1757(1953).
(11) Dubin, D. T., *J. Biol. Chem.*, **235**, 783(1960).
(12) Kolbezen, M. J., Eckert, J. W., and Bretschneider, B. F., *Anal. Chem.*, **34**, 583(1962).
(13) Ingold, C. K., "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953

[1] Considerable early work on the application of the Spingler assay (4) to chlorpropamide was carried out by Dr. R. L. Wagner of these laboratories. The authors gratefully acknowledge the help given in this study by Dr. Wagner's initial investigations.

Relation of pH to Preservative Effectiveness II

Neutral and Basic Media

By BILLIE WICKLIFFE and DURWARD N. ENTREKIN

Liquid trypticase soy broth media was buffered with Prideaux and Ward's universal buffer to neutral and basic pH levels (pH 7 to 10 inclusive). Preservative solutions of various concentrations were added to tubes containing the media. After inoculation of the tubes with a slurry obtained from soil samples suspended in water, these preparations were examined for preservative effectiveness over a 6-month period. In most cases preservative effectiveness varied with pH alteration. The preservative activity ranged from negligible with cinnamic acid and some of its derivatives, the amides of bromal and dichloroacetaldehyde, sorbic acid and dehydroacetic acid-sodium, to fair in the case of parabens and salicylanilide, to good with cetrimide, chlorophenesin, vanillic acid esters, hexylresorcinol, hexachlorophene, and phenylethanol.

THE DEGREE of dissociation and the pH have been shown to influence the ability of preservatives to act in a chemical or physical way to prevent multiplication of organisms (1–3). The acidity or alkalinity of a product is just one of the many factors involved in the selection of effective preservatives for pharmaceutical preparations (1, 4–6). Antiseptics are said to be effective

Received May 4, 1963, from the School of Pharmacy, University of Georgia, Athens.
Accepted for publication November 9, 1963.
Abstracted from a thesis submitted by Billie Wickliffe in partial fulfillment of Master of Science degree requirements.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.