stimulated at high frequencies could reflect either a deficiency of acetylcholine release or that the end plate receptors are already occupied. Observation that the blockade is reversed by acetylcholine injected close-arterially during stimulation indicates that the latter situation does not obtain. Therefore, since the receptors can still respond to administered acetylcholine, the evidence is in favor of a presynaptic, rather than a postsynaptic, mechanism of action.

NP-HC-3 may be assumed to act, at least in part, by decreasing the presynaptic supply of acetylcholine. The exact mechanism by which this is caused has not been elucidated. Replacement of the biphenyl by the monophenyl nucleus has not altered the hemicholinium-like activity, although there is a decrease in the toxicity.

REFERENCES

(1) Schueler, F. W., *J. Pharmacol.*, **115**, 127(1955).
(2) Marshall, F. N., and Long, J. P., *ibid.*, **127**, 236 (1959).
(3) Powers, M. F., Kruger, S., and Schueler, F. W., *J. Pharm. Sci.*, **51**, 27(1962).
(4) Thomas, J., and Marlow, W., *J. Med. Chem.*, **7**, 75(1964).
(5) Long, J. P., *Federation Proc.*, **20**, 583(1961).
(6) Schueler, F. W., *ibid.*, **20**, 561(1961).
(7) Krohnke, F., and Vogt, I., *Chem. Ber.*, **86**, 1132(1953).
(8) Miller, L. C., and Tainter, M. L., *Proc. Soc. Exptl. Biol., N. Y.*, **57**, 261(1944).
(9) Koster, R., Anderson, M., and de Beer, E. J., *Federation Proc.*, **18**, 1626(1959).
(10) D'Amour, F. E., and Smith, D. L., *J. Pharmacol.*, **72**, 74(1941).
(11) Schueler, F. W., *Intern. Rev. Neurobiol.*, **2**, 77(1960).
(12) Bush, G. H., and Baraka, A., *Brit. J. Anaesthesia*, **36**, 356(1964).
(13) Levy, G., *Nature*, **206**, 517(1965).
(14) Volle, R. L., personal communication.
(15) Stovner, J., *Acta Pharmacol. Toxicol.*, **15**, 55(1958).
(16) Bhatnagar, S. P., Lam, A., and McColl, J. D., *Biochem. Pharmacol.*, **14**, 421(1965).

Fluorometric Determination of Acetylsalicylic Acid and Salicylic Acid in Blood

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A useful micro-method has been developed for the paper chromatographic separation of acetylsalicylic acid and salicylic acid with subsequent determination of each compound by fluorometry from a 0.1-ml. sample of capillary blood. This method permits frequent determinations of both acetylsalicylic acid and salicylate blood levels on the same subject. The accuracy and precision of the micro-method has been studied. *In vivo* studies utilizing the method emphasized the validity of results obtained by a rapid micro-method of analysis.

MANY METHODS for the determination of salicylates in blood and in plasma have been described in the literature. The generally accepted methods of Brodie (1) and Routh (2) involve the extraction of the salicylate from the blood sample and the determination of the concentration colorimetrically by measuring the absorbance of an iron complex. However, Saltzman (3) and Chirigos (4) have described the determination of salicylate in biological tissues by measurement of the characteristic fluorescence of the salicylate ion on exposure to ultraviolet light.

The quantity of acetylsalicylic acid in the biological tissues was estimated from the difference between "free" salicylate and "total" salicylate, and conjugated salicylate being considered to be acetylsalicylic acid (5). Mandel (6) has reported a paper chromatographic procedure for the separation of acetylsalicylic acid from salicylic acid in a plasma sample, and their separate determination fluorometrically. Recently, Nikelly (7) described the gas chromatographic determination of acetylsalicylic acid in the presence of salicylic acid.

Although the information obtained from any of the above methods is valuable, there does not seem to be general agreement among investigators about the levels of acetylsalicylic acid in the blood after taking aspirin. Since the time span of the analgesic effect of aspirin (2–4 hr.) appears to be more closely related to the time that the acetylsalicylic acid persists in the blood, the blood level of acetylsalicylic acid would seem to be a critical measure of the potential analgesic effectiveness of an aspirin formulation. Thus, for the *in vivo* study of acetylsalicylic acid blood levels it is desirable to have a micro-method requiring small volumes of blood for the repeated sampling required to observe simultaneously sustained blood levels of acetylsalicylic acid and salicylic acid. The method should also be fast, practical, and accurate.

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METHODS AND PROCEDURES

The fluorometric assay described below is based on the characteristic chromatographic separation of acetylsalicylic acid and salicylic acid and the ultraviolet emission of alkaline salicylate ion at 415 mμ when activated at 325 mμ.

Blood Sampling.—To 100 μl. of blood in a 3-ml. centrifuge tube was added 250 μl. of ethylene dichloride (redistilled). The sample was stirred vigorously with a glass rod for 10 sec., 50 μl. of 6 *N* hydrochloric acid was added, and the mixture thoroughly mixed for 1 min. The sample was then centrifuged at 6000 r.p.m. for 15 sec., thoroughly mixed for 1 min., and centrifuged again for 1 min. It was found that for reproducible results the finger before puncture had to be pretreated with isopropyl alcohol and the blood sample representative of the capillary circulation.

Paper Chromatography.—The ethylene dichloride layer in the centrifuge tubes was withdrawn by means of a 250-μl. transfer pipet. The contents of the pipet were carefully streaked in a narrow band across a $1^1/_2 \times 15^5/_8$ in. strip of S & S 589 White Ribbon chromatography paper. The strips were dried and developed by ascending technique in chromatographic tanks containing a 0.75% nitric acid developing solution. The strips were developed at 25° until the solvent front reached 21 cm. (about 1 hr.), and then were removed from the tanks and air dried. Two 4-cm. wide rectangular pieces were cut from the strips at R_f 0.6 and 0.8 corresponding to the salicylic acid and acetylsalicylic acid, respectively. The pieces from the chromatographic strip were placed in 50-ml. conical flasks and exactly 5.0 ml. of 5 *N* sodium hydroxide was added. The alkali was allowed to remain for exactly 8 min. in contact with the papers. The flasks were gently swirled during the hydrolysis and extraction period.

Fluorescent Measurements.—The alkaline solutions were poured into 12 × 75 mm. cells and read in a fluorometer (Turner model 110) containing a "sandwich" excitation filter consisting of two 7-54 filters and a Wratten No. 34A filter; for the emission filter a half-thickness Corning No. 5-58 filter was used. A 4-cm. rectangle cut from the lower portion of a developed chromatograph strip was treated with exactly 5 ml. of 5 *N* sodium hydroxide to give an instrument blank.

Preparation of Standard Curves.—Known amounts of acetylsalicylic acid in the range of 0–1.0 mcg./100 μl. and 0–100 mcg./10.0 μl. of salicylic acid were added to whole citrated blood. These samples were carried through the complete chromatographic separation and fluorometric assay. The meter readings from the fluorometer were plotted against concentration to give standard curves for the salicylic acid and acetylsalicylic acid. To provide assurance of the continuing validity of the standard curves, standard samples were carried through the complete procedure periodically. So long as precautions were taken to control the temperature and drafts of the chromatographic room, the standard deviation reported in the discussion was not exceeded.

The drug concentration in plasma can be estimated from the whole blood concentration either by assuming a 50% hematocrit and using a factor of 2, or by using the equation 1/1 − (hematocrit × 0.01) if the subject's hematocrit has been determined. A comparison of results as calculated by the 2 methods is shown in Table I. The failure to consider the actual hematocrit or binding of the drug by plasma proteins has no effect on the validity of results comparing different dosages or different dosage forms in the same group of individuals.

TABLE I.—COMPARISON OF SALICYLIC ACID BLOOD LEVELS BY THE MICRO-METHOD AND THE BRODIE PROCEDURE

Sampling	Salicylic Acid, mg. %: Micro-Method, Subj. A[a]	Micro-Method, Subj. B[b]	Brodie, Subj. A	Brodie, Subj. B
Whole blood (venipuncture)	1.8	6.7	2.0	8.5
Whole blood (fingertip)	1.8	6.6	...	...
Plasma	3.3	12.5	4.2	15.8
Plasma (from whole blood)[c]	3.5	12.7	...	...
Plasma (from whole blood)[d]	3.6	13.2	...	...

[a] Dosage was 10-gr. aspirin; blood samples taken 2 hr. after dosage; hematocrit, 49%. [b] Dosage was 10 gr. aspirin every 4 hr. for a total of 70 gr.; blood samples taken 2 hr. after last dose; hematocrit, 48%. [c] Calculated from whole blood by applying a correction for hematocrit as follows: mg. % plasma = mg. % whole blood × 1/1 − (hematocrit × 0.01). [d] Calculated from whole blood by applying a correction factor of 2.

Comments on Analytical Procedures.—All of the procedures in the assay were studied to eliminate unnecessary steps and variables and to reduce background fluorescence to a minimum. All glassware was scrupulously clean, cells were selected to have low fluorescence readings, and only reagents with low native fluorescence were used. Special care was taken to prevent contamination by dust that might produce fluorescence. Variability of subjects was reduced by requiring the dose of medication to be taken in a fasting condition with a fixed quantity of water. When repeated dosage studies were conducted the subsequent doses were taken at fixed times in relation to meals. Subjects were cautioned regarding the use of topical preparations because of the possibility of their containing salicylates or ultraviolet absorbers.

The time required for acidification and extraction of the blood samples was found to be critical as is well known in order that hydrolysis of the acetylsalicylic acid might be minimized and that the extraction could be as complete as possible. The method of application of the sample to the chromatographic paper by streaking was critical because of the large volume to be spotted and the need for well-separated sharply defined developed zones of acetylsalicylic acid and salicylic acid. The spotting of the large volume of sample was facilitated by passing a current of cool air over the paper during the operation to speed the volatilization of the ethylene dichloride. The selection of the chromatographic paper was dependent on its purity and separation characteristics. Whatman No. 1 paper gave the same separation of acetylsalicylic acid and salicylic acid as the paper used, but had to be pretreated with dilute nitric acid solution due to a high and variable background fluorescence. With the

S & S paper the R_f for the salicylic acid was approximately 0.6 while the acetylsalicylic acid R_f was 0.8 at room temperature which agreed with the values reported by Mandel (6). The R_f values were established by visual examination of a paper strip containing a reasonable concentration of salicylic acid and acetylsalicylic acid under ultraviolet light. The salicylic acid appears as a blue fluorescent spot while the acetylsalicylic does not fluoresce. However, its R_f was determined by its hydrolysis to salicylic acid on the paper strip in the presence of ammonia vapors. Hydrolysis of the acetylsalicylic acid on the paper strips with alkali or ammonia vapors was found to be unnecessary. Kinetic studies showed complete conversion of the acetylsalicylic acid to salicylic acid and complete elution from the paper in from 5–8 min. in the 5 *N* sodium hydroxide solution.

The fluorescence of salicylic acid and acetylsalicylic acid was somewhat greater in solutions of higher sodium hydroxide concentration, but solutions stronger than 5 *N* disintegrated the paper substrate too rapidly and gave solutions difficult to handle due to their high viscosity. The filter combination used produced excitation at 318–327 mμ and emission fluorescence in the range of 350–480 mμ (maximum at 410 mμ). Other filter combinations were investigated but in most cases these combinations (*i.e.*, primary, Corning No. 7-54 and Wratten No. 34A; secondary, Corning No. 7-54) permitted some overlap of transmitted wavelengths. Fluorescence was found to be linearly proportional to the concentration of acetylsalicylic acid and salicylic acid up to 12 mg. % in 5 *N* sodium hydroxide and reproducible for a period of at least 1 hr.

In the preparation of standard curves for the salicylic acid and acetylsalicylic acid it was found that the recovery of either drug was higher from an aqueous solution than from whole blood. Thus, if aqueous solutions were used to prepare the standard curves one would obtain low concentration values during actual *in vivo* blood level studies. In a check of the accuracy and precision of the method it was found that 90 to 110% of acetylsalicylic acid and salicylic acid was recovered from whole citrated blood to which known amounts of the drugs had been added in the following ranges: acetylsalicylic acid—0.20, 0.50, and 1.20 mg. %; salicylic acid—2.00, 5.00, and 12.00 mg. %. In these same ranges, pairs of duplicate determinations made by the same analyst showed standard deviations of the pairs to be 0.015 to 0.021 mg. % for acetylsalicylic acid, and 0.07 to 0.14 mg. % for salicylic acid.

The micro-method was also studied in a comparison with the procedure of Brodie (1). Two subjects were given aspirin in different doses, and blood samples were assayed for salicylic acid by the 2 methods. Because the Brodie procedure is proposed for plasma separated from a sample of venous blood while the micro-method uses a sample of whole blood, subjects were sampled for both and each method was employed to estimate salicylic acid in both whole blood and in the separated plasma. The results are summarized in Table I. The data for the micro-method show good agreement between the venous whole blood and the capillary whole blood in both subjects. Also by applying a correction value for the hematocrit in each case the plasma salicylate values when calculated from whole blood agree well with that actually recovered from plasma by the micro-method. The higher results obtained by the Brodie method, which determines only salicylate, are possibly due to acetylsalicylic acid which hydrolyzed during the assay to salicylic acid.

APPLICATION OF THE PROCEDURE

This technique is not suitable for routine clinical use because of the critical time factor between collection of the blood sample and extraction of the acetylsalicylic acid. No more than 4 min. should elapse before completion of the extraction procedure, and no more than 6 min. should elapse before the extracted sample is ready for chromatography.

Past methods of analysis have, in general, depended on making the assumption that the difference between "total" and "free" salicylates in the plasma constituted the acetylsalicylic acid. Because of failure by some investigators to take into account the very rapid hydrolysis of acetylsalicylic acid by the serum esterase, investigators rarely agreed about the plasma levels. As a consequence, they were misled in drawing conclusions about the mechanism of absorption.

The authors have observed that absorption of acetylsalicylic acid is very rapid from the stomach, and therefore it can survive long enough to be absorbed into the blood stream. The authors have observed that absorption of acetylsalicylic acid from the intestines is much slower, and that, therefore, the rate of entry into the blood is too slow to permit an appreciable level to be built up. These observations are consistent with the conclusions of Schanker (8) and Smith (9). They both have shown that an increase in pH reduces the rate of absorption of acetylsalicylic acid and salicylic acid to a lesser extent.

TABLE II.—PLASMA SALICYLATE LEVELS[a]

Subj.	Drug Detd.	Dose Given	0.5	1	2	4	4.5	5	6	8
Av. (15 males)	ASA[b]	20r[c]	1.27	0.69	0.17	0.13	1.10	0.79	0.08	0.02
Av. (20 females)	ASA	20r[c]	1.20	0.76	0.28	0.17	1.61	0.89	0.14	0.03
Av. (15 males)	TS[d]	20r[c]	3.8	4.7	4.8	3.4	6.1	7.5	7.3	5.8
Av. (20 females)	TS	20r[c]	3.9	5.3	6.3	5.0	9.2	10.4	10.3	7.9
Av. (11 males)	ASA	20[e]	2.17	1.30	0.29	0.07	...	...	0.04	0.01
Av. (13 females)	ASA	20[e]	2.30	1.42	0.55	0.14	...	...	0.09	0.01
Av. (11 males)	TS	20[e]	6.4	8.6	9.5	8.4	...	...	6.8	6.4
Av. (13 females)	TS	20[e]	6.6	8.5	11.7	10.1	...	...	8.0	6.9

[a] mg. % in plasma was calculated from whole blood with assumed hematocrit of 50% in all cases. [b] ASA = acetylsalicylic acid. [c] 20r = two 5-gr. aspirin tablets at time zero and two 5-gr. tablets again at 4 hr. [d] TS = total salicylate. [e] 20 = four 5-gr. aspirin tablets taken at time zero.

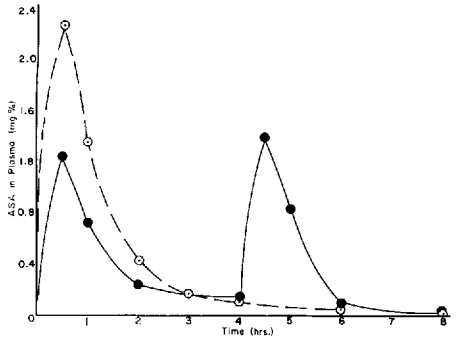

Fig. 1.—Comparison of average plasma levels of acetylsalicylic acid (ASA) after taking single and divided doses of aspirin at equal total dose of 20 gr. Key: ⊙, aspirin, single dose (4 × 5 gr.) 20-gr. dose at time 0; ●, aspirin, divided dose (2 × 5 gr.) 10 gr. at time zero and (2 × 5 gr.) 10 gr. after 4 hr. for a total 20-gr. dose.

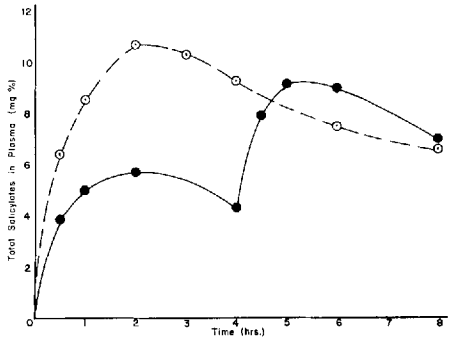

Fig. 2.—Comparison of average plasma levels of salicylates after taking single and divided doses of aspirin at equal total dose of 20 gr. Key: ⊙, aspirin, single dose (4 × 5 gr.) 20-gr. dose at time zero; ●, aspirin, divided dose (2 × 5 gr.) 10 gr. at time zero and (2 × 5 gr.) 10 gr. after 4 hr. for a total 20-gr. dose.

To provide a picture of the levels of acetylsalicylic acid and total salicylate in the blood after normal doses of aspirin, 35 subjects took 10 gr. of aspirin at time zero (8:00 a.m.) and a second 10 gr. exactly 4 hr. later. Blood samples were taken at various time intervals and assayed for acetylsalicylic acid and salicylic acid. Twenty-four of the above panel, at a later date, also took single 20-gr. doses of aspirin. The results of the 2 studies are summarized in Table II. Figure 1 is a graphic comparison of average plasma levels of acetylsalicylic acid after taking 10 gr. of aspirin repeated after 4 hr. for a total of 20 gr., and after taking 20 gr. of aspirin in a single dose. Figure 2 compares average total salicylate plasma levels after the same 2 dosage regimens.

The levels of acetylsalicylic acid and of the total salicylates in the plasma resulting from typical dosage regimens for aspirin provide a framework of reference for this new method of analysis of the content of drug and its principal degradation product in the blood. The peak values provide guidance in product formulation with respect to what levels in the plasma may be considered safe for a new aspirin dosage form. They also provide information which may be considered related to effectiveness. For instance it can be seen that a 20-gr. dose of aspirin, compared with a 10-gr. dose, gives a peak plasma level of acetylsalicylic acid almost twice as great (2.24 *versus* 1.25 mg. %). However, doubling the dose only slightly prolongs the plasma level of acetylsalicylic acid.

It will also be noted from Table II that differences are apparent in the average plasma levels for males and females. These are especially prominent in the total salicylate levels. A portion of this difference can be accounted for by the assumption made in calculating plasma levels that the hematocrits were all 50%. In the case of the females this would generally lead to a 9% high result. Also no consideration was given to differences in blood volumes of the individual subjects. This also on the average would show female plasma levels to be about 11% high.

A more detailed discussion of the clinical aspects of the micro-method and its implications in regard to the pharmacology of aspirin will be published at a later date.

REFERENCES

(1) Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol. Exptl. Therap.*, **80**, 114(1944).
(2) Routh, J. I., *et al.*, *Clin. Chem.*, **2**, 432(1956).
(3) Saltzman, A., *J. Biol. Chem.*, **174**, 399(1948).
(4) Chirigos, M. A., and Udenfriend, S., *J. Lab. Clin. Med.*, **54**, 769(1959).
(5) Lester, D., Lolli, G., and Greenberg, L. A., *J. Pharmacol. Exptl. Therap.*, **87**, 329(1946).
(6) Mandel, H. G., Cambosos, N. M., and Smith, P. K., *ibid.*, **112**, 495(1954).
(7) Nikelly, J. G., *Anal. Chem.*, **36**, 2248(1964).
(8) Schanker, L. S., *Am. Assoc. Coll. Pharm. Teachers Seminar*, **13**, 134(1961).
(9) Smith, P. K., *Ann. N.Y. Acad. Sci.*, **86**, 38(1960).