scopic inspection of these three suspensions were used to confirm the authors' findings with the apparatus.

Therefore, a physically stable suspension of the drug was developed using a calculated amount of buffer to maintain pH control and insure complete floc formation.

Flocculation with Anti-D Sera Using Purified Silica.—The final example is drawn from experiences with a biological application in determining the flocculating efficiency of several anti-D sera, available as diagnostic reagents for Rh blood typing. It was felt that a suitable product could be developed with the proper ZP for agglutination testing. Purified silica was chosen as a substitute for red blood cells (RBC's) because of the difficulties involved in working with blood dispersions (21).

Approximately 500 ml. of a 0.01% purified silica dispersion in 0.45% sodium chloride solution was prepared. The ZP of such a system (approximately −30 mv.) is comparable to the value for human blood dispersions in saline solution (22). Increments of a $^1/_{100}$ dilution of anti-D serum in 0.45% sodium chloride solution were added to each purified silica dispersion. The EM of the system was determined after each addition of reagent. After a total of 0.02% serum reagent had been added, undiluted portions of serum were used to complete each ZP titration.

The results of this study are presented in Fig. 12. The flocculating efficiency of the improved formula *A* was found to be greater than a control system *B* or the original formula *C*. Recommendations for product improvement were made on the basis of a previous study of the original formula *C*. The amount of anti-D serum required to obtain complete flocculation was based upon an extrapolation of linear portions of each test curve to the isoelectric point (zero ZP). The length of the concentration-induction period before flocculation began was also related to the efficiency of the diagnostic reagent. The ranking of the three anti-D sera according to flocculating efficiency for purified silica correlated very well with results of blood-test agglutination with human RBC's.

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Notes

Method for the Direct Measurement of Acetylsalicylic Acid in Human Blood

By V. F. COTTY and H. M. EDERMA

A procedure was developed for directly determining acetylsalicylic acid in human blood specimens. The method instantly stops enzymatic hydrolysis, removes salicylic acid and conjugates of salicylic acid by reaction with ceric ammonium nitrate, automatically hydrolyzes acetylsalicylic acid, and determines the resulting salicylic acid fluorometrically. It sensitively measures acetylsalicylic acid in the presence of salicylic acid, salicylamide, salicyluric acid and salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide).

SEVERAL recent publications have reported acetylsalicylic acid (ASA) concentrations in the blood of humans following the ingestion of ASA-containing proprietary preparations (1–3). In each case, ASA was calculated as the difference between the level of salicylic acid (SA) in the hydrolyzed and unhydrolyzed serum (1) or in the blood extracts (2, 3). "Difference" procedures can be characterized by

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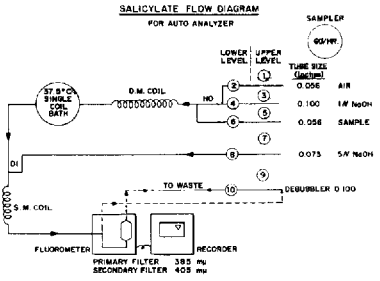

Fig. 1.—System for the analysis of acetylsalicylic acid in extracts of blood.

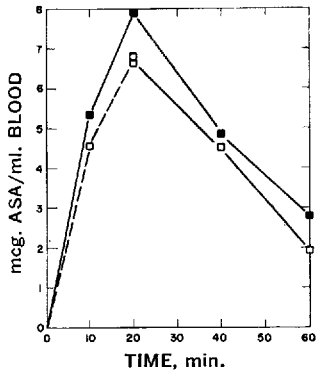

Fig. 2.—Blood levels of ASA obtained by the difference method (□) and by the direct method (■) following the ingestion of ASA. In each of the three studies, analytical blood specimens were drawn immediately before and at the indicated times after each subject ingested 2 tablets (10 gr.) of buffered aspirin. Analyses were performed by either the difference or the direct procedure.

TABLE I.—RECOVERY OF ASA[a]

ASA Added	ASA Found	Other Compd. Added
0.5	0.5	...
0.8	0.7	...
1.3	1.4	30 SA
1.3	1.0	50 SA
1.8	2.1	...
2.1	2.5	...
2.6	3.4	20 SA
2.6	3.6	40 SA
3.9	3.9	10 SA
4.4	4.3	...
6.0	5.7	...
7.0	7.0	...
9.1	9.1	...
9.1	10.0	70 SA
9.6	9.5	60 SA
11.7	11.6	...
0	0	100 SA
0	0	100 SA
0	0	100 SA
0	0	100 SA
0	0.1	100 SA
0	0.1	100 SA
0	0.0	70.7 Salicyluric acid
0	0.0	70.7 Salicyluric acid
0	0.1	141.3 Salicyluric acid
0	0.0	141.3 Salicyluric acid
0	0.0	49.7 Salicylamide
0	0.0	49.7 Salicylamide
0	0.3	99.3 Salicylamide
0	0.0	99.3 Salicylamide
1.3	1.4	141.3 Salicyluric acid
2.6	2.4	141.3 Salicyluric acid
5.2	5.1	141.3 Salicyluric acid
10.4	10.3	141.3 Salicyluric acid
1.3	1.6	99.3 Salicylamide
2.6	2.5	99.3 Salicylamide
5.2	5.5	99.3 Salicylamide
10.4	10.6	99.3 Salicylamide
0	0	227.5 Salicylic acid ether glucuronide
0	0	227.5 Salicylic acid ether glucuronide
1.3	1.6	227.5 Salicylic acid ether glucuronide
2.6	2.5	227.5 Salicylic acid ether glucuronide
5.2	5.4	227.5 Salicylic acid ether glucuronide
10.4	10.3	227.5 Salicylic acid ether glucuronide

[a] ASA and related compounds were added to whole pooled human blood and ASA was then determined. Concentrations in mcg/ml.

accumulated errors which may become particularly serious when a small difference exists between large analytical values. Thus, it is difficult to measure low concentrations of ASA in the presence of relatively high concentrations of SA by these methods. A second problem is that the difference method may not distinguish between ASA and conjugates of SA. The purpose of the present communication is to present a semiautomated direct procedure for the determination of ASA in human blood which is not interfered with by SA, salicylamide, salicyluric acid, or salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide).

EXPERIMENTAL

Determination of ASA.—Blood specimens are initially inactivated and extracted as described by Cotty *et al.* (3) in the following manner. Five milliliters of whole blood, immediately upon withdrawal, are measured into a bottle containing 0.5 ml. of 6 *N* HCl and 50 ml. of ethylene dichloride (EDC). The bottle is capped with a polyethylene snap cap and then immediately shaken vigorously by hand until the blood becomes a brown color. The bottle is then shaken on a mechanical shaker for 10 min. The EDC is separated from the precipitated blood by filtering through dry filter paper (Whatman No. 42). The EDC extract is placed in a bottle containing 0.1 ml. of an aqueous solution of 40% ceric ammonium nitrate and 5.9 ml. of distilled water. The bottle is then capped and shaken on a mechanical shaker for 1 hr. The EDC is separated from the aqueous layer in a separator and filtered through a dry Whatman No. 42 filter paper. Thirty milliliters of the filtered EDC is transferred to a bottle containing 4 ml. of 1% $NaHCO_3$. This bottle is capped and shaken vigorously on a mechanical shaker for 10 min. Upon separation, the aqueous layer is transferred into a polystyrene sample cup which is placed in the sampler of a system[1] consisting of a special salicylate manifold, proportioning pump, heating bath, fluorometer, and recorder. (See Fig. 1.) As it traverses this system, the sample is mixed

[1] AutoAnalyzer, Technicon Instruments Corp., Chauncey, N. Y.

TABLE II.—COMPARISON OF DIFFERENCE AND DIRECT METHODS OF ANALYSIS WHEN EMPLOYED IN CLINICAL STUDIES[a]

Time, min.	Blood Levels, mcg. ASA/ml. — Difference Method						Direct Method		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
10	4.6	0.51	38				5.3	0.77	39
20	6.8	0.44	38	6.3	1.24	39	7.9	0.66	40
40				4.6	1.28	38	4.8	0.27	40
60				1.9	1.40	39	2.7	0.17	40

[a] Each subject ingested 2 tablets of proprietory buffered ASA (10 gr.) after the initial (blank) blood specimen was drawn. Other specimens were drawn for analysis at the time intervals indicated. The results of two studies employing the difference method and one the direct procedure are shown in the table.

with air and 1 N NaOH in a double mixing coil. The sample then passes through a heating bath at 37.5° where aspirin hydrolysis is completed. The analytical sample is then mixed with 5 N NaOH and passed through the fluorometer flow cell. The range selector of the fluorometer is set at 30X with a No. 4 light shield slit and interference filters of 385 and 405 mμ. The results are recorded on a linear recorder. A reagent baseline is established at about 15% transmission with a 1% $NaHCO_3$ solution in the place of the sample. The sampler is set at 60 samples per hour and samples are placed in alternate holes on the turntable. Ethylene dichloride (EDC) used for the extractions was specially prepared by the Fisher Scientific Corp., Fairlawn, N. J. Ceric Ammonium Nitrate was manufactured by the G. F. Smith Co., Columbus, Ohio. Pure salicyluric acid (4) and salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide) (5, 6) were synthesized.[2] These compounds yielded satisfactory elemental analyses and produced single spots when subjected to thin-layer chromatography. Salicylamide and aspirin were U.S.P., and all other chemicals were of reagent grade. Standard curves are prepared by adding 1 to 10 mcg. of aspirin per ml. of freshly drawn blood and then carrying out the procedure as described above. ASA concentrations are plotted against peak heights.

Results.—Table I lists the amount of ASA found when analyses were performed upon blood to which known amounts of ASA had been added. The addition of related substances (salicylic acid, salicylamide, salicyluric acid, and salicylic acid ether glucuronide) does not affect precision because the discrepancies between added and found amounts of ASA are small and not increased over those obtained when ASA alone is present.

A practical comparison of the difference and direct methods was made in the following manner. Data collected from two absorption studies in which the difference method (3) was used were compared with a study in which the direct method was employed.

Each subject received two tablets (total 10 gr. ASA) of proprietary aspirin[3] and blood specimens were collected immediately before and at 10, 20, 40, and 60 min. after administration.

One study employing the "difference" method measured ASA blood levels at 10 and 20 min. has been reported previously (3). The second measured ASA blood levels at 20, 40, and 60 min. The single study employing the direct procedure measured ASA levels at 10, 20, 40, and 60 min. Thirty-eight to 40 persons were employed in each study. In most cases the subjects were the same for all studies. The analytical data of all studies are plotted in Fig. 2. The arithmetic means and standard errors are tabulated in Table II. Note the general agreement between results obtained with the two different analytical procedures. There are no significant differences between corresponding values.

DISCUSSION

Previous attempts to develop chemical procedures for the direct determination of ASA have been frustrated by its lack of a suitable characteristic group which easily reacts with appropriate reagents. The present methodology makes use of this situation, for it employs a reagent, ceric ammonium nitrate, which reacts with compounds possessing the phenolic or aliphatic hydroxyl group, as do the metabolites of ASA. The use of this reagent resulted from a screening of a number of reagents which were known to react with phenols. The formation of complexes with phenols and their subsequent oxidation by ceric salts has been described by Duke and Smith (8) and Sharma and Mehrotra (7). Thus, acetylation "protects" the phenolic group in ASA and this substance remains in solution in the organic solvent.

The high sensitivity and specificity of the procedure described above should make it a useful tool in investigations into the nature of the pharmacological responses produced by the ingestion of ASA as well as other salicylates. Much present day research on salicylates involves the relative roles of ASA and SA in the analgesic, antipyretic, and anti-inflammatory responses to salicylate therapy. These can be tested more reliably with the use of the procedure described above.

SUMMARY

A direct, semiautomated method for the determination of ASA in human blood has been described which is sensitive, reproducible, and specific. Results obtained during absorption studies of ASA in which this method was employed yielded results similar to those obtained in studies employing a "difference" method.

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[2] The assistance of Mr. Leonard Weintraub and Mr. Stanley Oles in performing the syntheses is acknowledged.
[3] Marketed as Bufferin by Bristol-Myers Products, Hillside, N. J.