Application of the method to plasma level determination of γ-hydroxyphenylbutazone by GLC is shown in Fig. 4. This profile was assembled following multiple doses of phenylbutazone to this subject (15 × 100 mg). Following single doses of phenylbutazone (up to 400 mg) in six subjects, γ-hydroxyphenylbutazone could not be detected.

The described GLC method is sensitive and specific for γ-hydroxyphenylbutazone. It offers the advantage that both metabolites (γ-hydroxyphenylbutazone and oxyphenbutazone) can be determined simultaneously following phenylbutazone administration.

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Simultaneous GLC Estimation of Salicylic Acid and Aspirin in Plasma

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Abstract □ Plasma salicylates, salicylic acid and acetylsalicylic acid (aspirin), were estimated as their respective trimethylsilyl derivatives by GLC. The method involved the use of fluoride and cooling to minimize the hydrolysis of aspirin in whole blood. Following solvent extraction, internal standards were added to each plasma extract. The extracts were evaporated to dryness and the residues were trimethylsilylated with hexamethyldisilazane in acetone. Small portions of the reaction mixture were chromatographed and the salicylates were quantitated. The components eluting from the chromatograph were identified by IR spectrophotometry and mass spectrometry.

Keyphrases □ Salicylic acid and aspirin—simultaneous GLC determination in plasma □ Aspirin and salicylic acid—simultaneous GLC determination in plasma □ GLC—analysis, simultaneous, salicylic acid and aspirin in plasma

Numerous methods have been described for the determination of salicylic acid and aspirin in pharmaceuticals and in biological media. Of these published techniques, surprisingly few have been developed for the simultaneous and direct determination of aspirin and salicylic acid in a single sample.

Among the techniques employed in the past is a large group of colorimetric estimations involving complexation with ferric ion (1–9). Other colorimetric assays involve diazotization with *p*-nitroaniline and nitrous acid (10), use of Folin and Ciocalteu phenol reagent (11), complexation with cupric ion in nitrous acid (13–15), or estimation as the nitro derivative (16).

Collectively, colorimetric assays are quite nonspecific. Plasma blank values are often high; blanks equivalent to 240 μg of salicylic acid/ml have been reported (11). Common plasma constituents such as salicyluric acid (1, 12), gentisic acid, uric acid, tyrosine, and tryptophan (12) interfere with colorimetric assays.

Furthermore, this entire group of assays determines salicylic acid only; to estimate aspirin, hydrolysis to salicylic acid and a second determination are required. The difference between the two salicylic acid concentrations is assumed to be due to aspirin.

A second analytical method is the group of UV assays. By these techniques, either salicylic acid alone or salicylic acid plus aspirin may be determined. Salicylic acid has been estimated, either in combination with aspirin (17) or individually following a physical separation step (18–21).

Both salicylates have been determined simultaneously by use of the pH-dependent shift in their individual absorption spectra (22–24). This hypsochromic shift technique results in some overlap of the absorption spectra of salicylic acid and aspirin, and corrections may be required for these spectral interferences (23, 24). The individual determination of salicylic acid is subject to interference from aspirin, gentisic acid, and 2,6-dihydroxybenzoic acid (19).

Salicylates have also been estimated fluorometrically. Aspirin may be estimated directly (25) or, more commonly, as salicylic acid. This has been accomplished by direct hydrolysis (26) or after a separation step followed by hydrolysis (27–30). Fluorometric assays generally require a hydrolytic procedure and a dual determination to estimate aspirin. Another problem is the high and variable background fluores-

cence, which may be minimized by use of blank plasma from each individual subject (26).

The most specific quantitative methods employed to date are chromatographic estimations. TLC (31, 32) and liquid chromatography (33) have been used, but most quantitative chromatographic procedures for salicylates involve GLC. Estimation of aspirin and salicylic acid as the free acids by this technique (34–36) is difficult due to their low vapor pressures and the presence of polar functional groups which cause absorption and tailing. Masking the functional groups by derivatization makes these molecules much less polar, more volatile, and, consequently, more amenable to GLC analysis. Among the derivatives that have been prepared quantitatively are the methyl ester or methyl ether derivatives (37–39) and the trimethylsilyl derivatives (40–46).

The most promising of the mentioned methods appears to be GLC because:

1. It permits the estimation of both compounds simultaneously (35, 37–39).
2. GLC assays are more rapid than are the colorimetric, UV, and fluorometric assays because of the elimination of the hydrolytic step and dual determination. As little as 3 min is required to estimate both salicylates (38).
3. It is the most specific of the methods mentioned. Even similar compounds such as benzoic acid (41), 3-hydroxybenzoic acid (41), 4-hydroxybenzoic acid (41), 2,5-dihydroxybenzoic acid (38), 2,3-dihydroxybenzoic acid (38), 2,3,4-trihydroxybenzoic acid (38), and phenacetin (34) exhibit different retention times from the salicylates and, consequently, do not affect their estimation.
4. GLC methods are quite sensitive and compare favorably with fluorometric procedures. For example, 0.4 μg of aspirin/ml may be estimated in the presence of a vast excess of salicylic acid (43).

The generation and use of methylating agents may be hazardous. For this reason and to provide an alternative method of analysis, it was decided to develop a procedure for the simultaneous GLC estimation of salicylic acid and aspirin as their trimethylsilyl derivatives.

EXPERIMENTAL

Materials—The following were used: salicylic acid[1], *n*-butyl benzoate[1], acetone[1], aspirin[2] (recrystallized from benzene prior to use), ethyl anisate[3], chloroform[4], and hexamethyldisilazane and other silylating reagents[5].

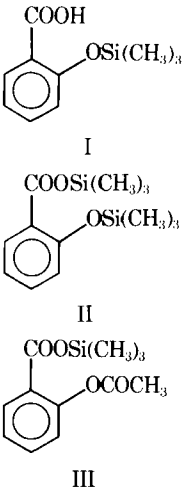
Apparatus—All routine salicylate analyses were conducted on a gas chromatograph[6] equipped with dual flame-ionization detectors. The signal from the detectors was fed into a strip-chart recorder[7], with a 1.0-mv full-scale response. Dual coiled glass columns, 1.8 m (6 ft) in length and 4 mm (i.d.), containing 3% OV-25 coated onto 60–80-mesh Chromosorb G (regular), were used.

The operating temperatures were: injectors, 240°; oven, 160° (isothermal); and detectors, 240°. The gas flow rates were: helium, 55 ml/min; hydrogen, 40 ml/min; and air, approximately 600 ml/min.

For the IR study, a gas chromatograph[8] equipped with thermal conductivity detectors was used to separate and collect the salicylate derivatives. These fractions were incorporated into micro- (1.5 mm diameter) potassium bromide pellets and scanned on a grating IR spectrophotometer[9].

A gas chromatograph[10] coupled to a mass spectrometer[11] was used in the mass spectrometric study.

In both the IR and mass spectrometric studies, the columns and operating conditions were identical to those mentioned previously.



RESULTS AND DISCUSSION

Silylation Studies—The structure of salicylic acid suggests that it should be possible to obtain up to three individual trimeth-

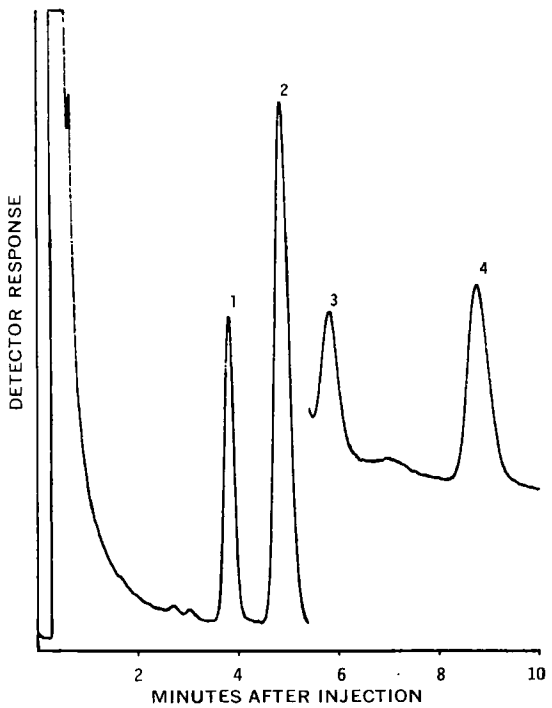


Figure 1—*Typical chromatogram. Key: 1,* n*-butyl benzoate; 2, trimethylsilyl 2-trimethylsiloxybenzoate (II); 3, ethyl anisate; and 4, trimethylsilyl 2-acetoxybenzoate (III).*

[1] Fisher Scientific Co.
[2] British Drug Houses.
[3] Eastman Organic Chemicals.
[4] Matheson, Coleman and Bell.
[5] Pierce Chemical Co.
[6] Perkin-Elmer model F-11.
[7] Hewlett-Packard model 7127A.
[8] Hewlett Packard model 5700A.
[9] Perkin-Elmer model 627.
[10] Varian model 1200.
[11] AEI-MS2.

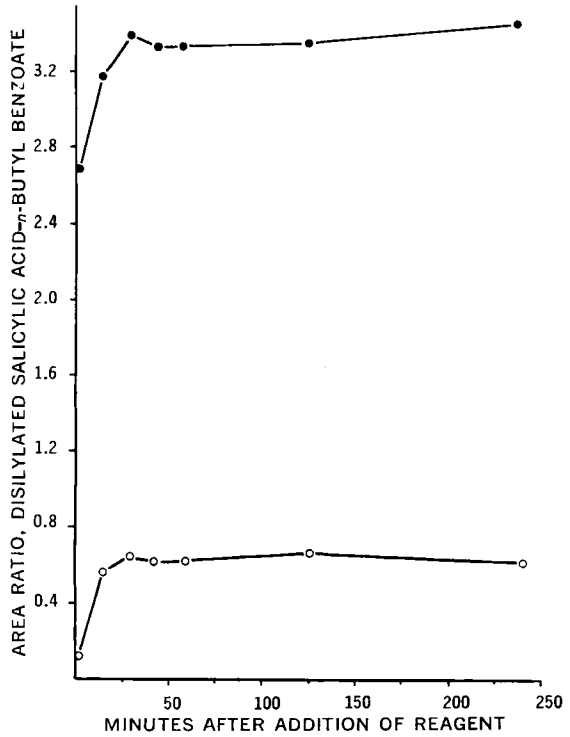


Figure 2—*Time required for disilylation of salicylic acid. Key:* ●, *150 μg of salicylic acid and 25 μg of* n-*butyl benzoate; and* O, *25 μg of salicylic acid and 25 μg of* n-*butyl benzoate.*

ylsilylated (or silylated) derivatives. Multiple GLC peaks have been reported from attempts to silylate salicylic acid (39, 42). Furthermore, two of the three possible structures, namely 2-trimethylsiloxybenzoic acid (I) and trimethylsilyl 2-trimethylsiloxybenzoate (II), were isolated and characterized (44–46). In contrast, aspirin can only produce one silylated derivative, trimethylsilyl 2-acetoxybenzoate (III). The molecular weights and chromatographic retention times of these silylated derivatives are as follows: I (mol. wt. = 210; retention time = 2.1 min), II (mol. wt. = 282; retention time = 4.9 min), and III (mol. wt. = 252; retention time = 8.6 min).

A typical chromatogram is depicted in Fig. 1. Peak 2 is II, and peak 1 is its internal standard, *n*-butyl benzoate. The break in the trace between peaks 2 and 3 is due to a change of attenuation required to increase the size of the subsequent peaks. Peak 3 is ethyl anisate, the internal standard for peak 4 (III).

Time for Reaction—To check for completeness of silylation, the disilylation of salicylic acid and the monosilylation of aspirin were monitored. Two concentrations of salicylic acid (25 and 150 μg) and of aspirin (5 and 25 μg) were studied for 250 min after the addition of silylating reagent (Figs. 2 and 3). It is apparent from Figs. 2 and 3 that the silylation reactions run to completion in approximately 50 min since there are no appreciable increases in peak area ratios after that time.

Furthermore, both II and III synthesized in this fashion are stable in the reaction mixture for over 4 hr after the addition of reagents. In subsequent studies, 60 min was allowed to elapse between the addition of reagent and injection of a portion of the mixture into the chromatograph.

Silylating Reagent—Several common silylating reagents and reagent mixtures were investigated to determine the most useful: *(a)* 100 μl of hexamethyldisilazane, *(b)* 100 μl of *N,O*-bis(trimethylsilyl)acetamide, *(c)* 100 μl of trimethylchlorosilane, *(d)* 50 μl of hexamethyldisilazane and 50 μl of trimethylchlorosilane, and *(e)* 80 μl of hexamethyldisilazane and 20 μl of trimethylchlorosilane.

Fixed quantities of salicylic acid (150 μg) and of aspirin (25 μg) were silylated with each reagent at room temperature. Neither *N,O*-bis(trimethylsilyl)acetamide nor trimethylchlorosilane produced as large a peak area ratio for salicylic acid in 60 min as did hexamethyldisilazane. Mixtures of hexamethyldisilazane and trimethylchlorosilane showed no advantage over hexamethyldisilazane alone, and the extent of silylation appeared to be dependent upon the amount of hexamethyldisilazane present. Consequently, hexamethyldisilazane was selected as the silylating reagent.

Solvent for Silylation—Choice of solvents for silylation reactions and subsequent GLC is limited to those that are inert to silylation and elute rapidly from the column. The latter stipulation excludes most tertiary amines, even those of low molecular weight such as trimethylamine and triethylamine.

Using fixed quantities of salicylic acid (150 μg) and of aspirin (25 μg) and 100 μl of hexamethyldisilazane, various solvents were investigated. Dioxane, chloroform, *n*-hexane, carbon disulfide, and ether all produced less than a quantitative synthesis of II, as evidenced by the presence of a I peak after 60 min. Dimethylformamide, pyridine, and acetone catalyzed quantitative synthesis of both II and III, but the first two solvents tailed badly on chromatographing; consequently, acetone was selected as the solvent.

Presence of Moisture—The effects of moisture on the silylation of 150 μg of salicylic acid and 25 μg of aspirin in 100 μl of acetone were investigated. Salicylic acid tolerated about 1.5% moisture, while aspirin was more sensitive and decreased peak area ratios were observed with 1.0% moisture. Water in the reaction mixture was slow to elute off the column.

Preliminary experiments suggested that 25 μl of hexamethyldisilazane was sufficient to silylate a mixture of 150 μg of salicylic acid and 25 μg of aspirin in 100 μl of acetone. On this basis, 100 μl of hexamethyldisilazane should silylate 600 μg of salicylic acid plus 100 μg of aspirin.

IR Studies—The silylated salicylates were separated and collected for these studies by preparative GLC. The GLC effluent was condensed in small glass capillaries, and the capillary contents were incorporated into a potassium bromide microdisk (1.5 mm diameter). When used with a beam condenser, reliable spectra were obtained from these disks.

These spectra were consistent with those reported earlier for I

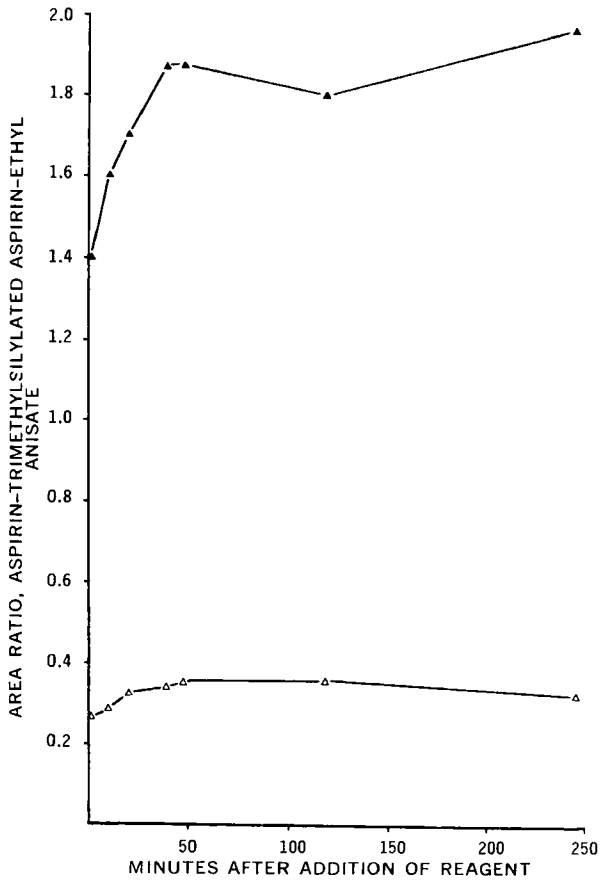


Figure 3—*Time required for monosilylation of aspirin. Key:* ▲, *25 μg of aspirin and 0.5 μg of ethyl anisate; and* △, *5 μg of aspirin and 0.5 μg of ethyl anisate.*

Table I—Effects of Fluoride and Cooling on the Hydrolysis of Aspirin in Plasma

Conditions		Salicylic Acid, μg Present	Aspirin Hydrolyzed, μg	Percent of Added Aspirin Hydrolyzed
Potassium Fluoride	Temperature			
−	37°	6.07	7.92	31.7
−	1°	3.35	4.37	17.5
+	37°	3.62	4.72	18.9
+	1°	2.46	3.21	12.8

and II (45, 46). Some difficulty was encountered with the II spectra. This compound appeared to decompose to I during collection and disk manufacture, as evidenced by the gradual diminution of the peaks at 1710, 1077, and 920 cm^{-1} and by the appearance of a peak at 3240 cm^{-1}. In fact, within 30 min of collection of supposedly pure II, the IR spectrum was indistinguishable from that of I.

GLC–Mass Spectrometry—Mass spectral data, from combined GLC–mass spectrometry, were obtained at both 20 and 70 ev. The mass spectra of I were consistent with the structure proposed here. A 20-ev spectrum showed characteristic ions at *m/e* 210 (5.4%, M^+), 195 (17.3%, M − 15$^+$), 151 (5.5%, M − 15 − CO_2^+), and 75 [12.5%, $(CH_3)_2Si{=}O^+H$], and 73 [8.7%, $(CH_3)_3Si^+$]. In addition, the 70-ev spectrum of this compound had an ion at *m/e* 45 (30.8%), shown by accurate mass measurement to be $COOH^+$.

Both the 20- and 70-ev scans of II showed the following characteristic ions: *m/e* 267 (77.4%, M − 15$^+$), 209 [5.5%, M − $Si(CH_3)_3^+$], 193 [4.9%, M − $OSi(CH_3)_3^+$], and 73 [100.0%, $(CH_3)_3Si^+$]. These ions are consistent with a previously published spectrum of the disilylated derivative of salicylic acid (44) and support the proposed structure.

The mass spectrum of III at 20 ev showed ions of *m/e* 193 (23.0%, M − $OCOCH_3^+$) and 73 [100.0%, $(CH_3)_3Si^+$]. In addition, the corresponding 70-ev spectrum had the following ions: *m/e* 210 [15.2%, $(M - CH_2{=}C{=}O)^+$], 195 (44.8%, M − 42 − 15$^+$), and 135 [20.0%, M − $COOSi(CH_3)_3^+$]. The loss of $CH_2{=}C{=}O$ (42 amu) is characteristic of acetates.

Plasma Extraction Studies—*Acidifying Agent*—Unacidified 1-ml plasma samples containing known quantities of salicylic acid and aspirin yielded approximately 4.0% of the salicylic acid and 6.0% of the aspirin present when extracted into an organic solvent. These extremely low recoveries clearly indicated the need to lower the pH of the plasma to facilitate partitioning into the organic phase. Potassium bisulfate solutions have been employed successfully (43) as acidifying agents, and several concentrations were investigated here. The optimal concentration of potassium bisulfate in terms of recovery of added salicylates was 10% (w/v). In subsequent experiments, 1.0-ml plasma samples were acidified with 1.0 ml of 10% potassium bisulfate solution. The pH of this acidified plasma was approximately 1.0.

Extraction Solvent—Several solvents were screened in an attempt to obtain quantitative recovery of added salicylates. A single extraction procedure was selected to minimize handling and the time required for analysis. The solvents investigated were ether, methylene chloride, ethylene dichloride, and chloroform. The highest and most consistent recoveries of salicylate added to blank plasma were obtained with chloroform and ethylene dichloride.

Chloroform was chosen over ethylene dichloride on the basis of its higher volatility and ease of removal by flash evaporation. Although ether was relatively easy to remove, it was difficult to work with due to its volatility. It also extracted appreciable quantities of moisture from plasma. Chloroform offered the best compromise between volatility and extraction of moisture.

Subsequently, all 1.0-ml plasma samples were acidified with 1.0 ml of 10% potassium bisulfate solution in 15-ml glass-stoppered centrifuge tubes. Five milliliters of chloroform was added to each tube, and these were then agitated on a mechanical shaker for 15 min. The resulting emulsions were centrifuged, and 4.0 ml of the chloroform solution was uniformly recovered. These extracts were transferred into tubes containing 25 μg of *n*-butyl benzoate and 0.5 μg of ethyl anisate as internal standards. The contents of each tube were carefully flash evaporated to dryness and then silylated for 60 min at room temperature with 100 μl of hexamethyldisilazane in 100 μl of acetone.

Standard Calibration Curves—Calibration curves were constructed for both salicylic acid (*versus n*-butyl benzoate) and aspirin (*versus* ethyl anisate) by adding known and varying amounts of each salicylate to 1.0-ml portions of plasma. The salicylates were then extracted as outlined, and fixed quantities of internal standards were added. After evaporation and silylation, the chromatographic peak area ratios of II–*n*-butyl benzoate and III–ethyl anisate were plotted against the known weight ratios.

Aspirin Hydrolysis—It had previously been demonstrated that the presence of fluoride in whole blood samples and the cooling of the samples after drawing the blood resulted in considerable reduction of the hydrolysis of aspirin in the samples (43). The effects of fluoride and cooling were investigated here as follows.

Whole blood samples were drawn from normal human volunteers into glass tubes containing 50 units of heparin, free from preservatives. Common preservatives such as benzyl alcohol may be extracted, silylated, and subsequently chromatographed. The blood was immediately centrifuged, and the plasma was pipetted off and pooled at room temperature.

Four 10-ml aliquots of the pooled plasma were withdrawn. Two of the aliquots of the pooled plasma were warmed to 37° and the other two were cooled to 1° on ice. Then 50 μl of a potassium fluoride solution (25 mg of potassium fluoride) was added to one warmed and one cooled aliquot. Subsequently 250 μg of aspirin was added in ether to each 10-ml aliquot. The ether layer was gently evaporated and the samples were mixed and frozen immediately on dry ice. The total time that elapsed between the addition of the aspirin and freezing of the samples was 10 min.

For the analysis, six 1.0-ml aliquots were assayed for aspirin and salicylic acid. The results (Table I) show that the presence of fluoride and the cooling of the samples each reduced the extent of hydrolysis, but that the combination of fluoride plus cooling was best. When these results are combined with those given below, it is evident that approximately 4.3% of the aspirin hydrolysis observed occurred during the freezing, storage, and thawing process.

Storage of Samples—Previous experimenters observed that plasma samples containing aspirin could be stored on dry ice for as long as 1 month without loss of salicylic acid or aspirin (43). In this experiment, 35 ml of pooled heparinized plasma containing fluoride was used. The plasma was cooled to 1°, and 25 μg of aspirin/ml of plasma was added. The plasma was well mixed and divided into four equal portions; one was analyzed immediately and the others were rapidly frozen and stored on dry ice. Individual por-

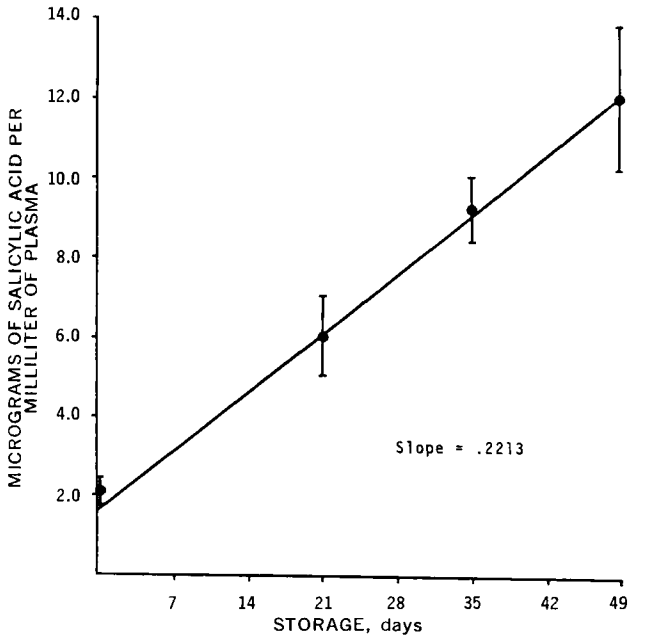


Figure 4—*Effects of storage on decomposition of aspirin. Each point on the curve represents the mean of seven determinations with standard deviation.*

tions were thawed and analyzed for salicylic acid and aspirin after 21, 35, and 49 days of storage.

The results of this experiment (Fig. 4) showed that approximately 8.5% of the aspirin was hydrolyzed during the extraction and handling procedure prior to estimation. The half-life of aspirin in frozen plasma, assuming aspirin decomposition to salicylic acid, was calculated to be 23.5 days and the corresponding rate constant was 0.02949 day^{-1}.

The conclusion to be drawn from this experiment is that plasma samples containing aspirin should be assayed as soon as possible after drawing.

CONCLUSIONS

The method presented enables the simultaneous estimation of salicylic acid and aspirin in a single sample. One-milliliter plasma samples are conveniently assayed, although the procedure is adaptable to smaller volumes. The limits of detection are approximately 1.0 μg of salicylic acid/ml plasma and 0.5 μg of aspirin/ml plasma. A correction should be made to allow for aspirin hydrolyzed during storage and extraction.

This method is currently being applied to the determination of the comparative bioavailabilities of various dosage forms of aspirin in arthritic patients.

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