Isolation of Salicylsalicylic Acid, Acetylsalicylsalicylic Acid, and Acetylsalicylic Anhydride from Aspirin Tablets by Extraction and High-Pressure Liquid Chromatography

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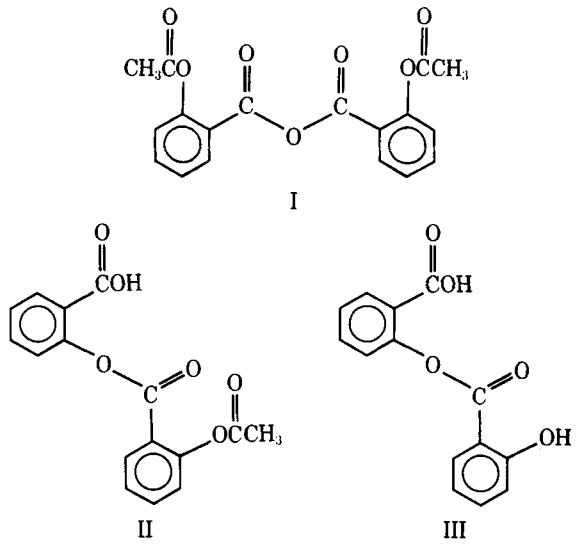
Abstract □ Aspirin and four salicylate impurities of aspirin (salicylic acid, acetylsalicylsalicylic acid, acetylsalicylic anhydride, and salicylsalicylic acid) were resolved by silica gel TLC and by high-pressure liquid chromatography (HPLC) on a reversed-phase C_{18} column. Care was necessary in the choice of a column because a similar column failed to resolve these five compounds. Salicylsalicylic acid was isolated from aspirin tablets by extraction, followed by reversed-phase C_{18} HPLC. The structure of this compound was confirmed by comparison with an authentic sample of salicylsalicylic acid by HPLC, TLC, IR and UV spectrophotometry, and mass spectrometry. Two other compounds, acetylsalicylic anhydride and acetylsalicylsalicylic acid, which had been previously identified by chromatography as impurities in aspirin, were isolated and further characterized.

Keyphrases □ Aspirin—impurities in tablets, salicylsalicylic acid, acetylsalicylsalicylic acid, acetylsalicylic anhydride, isolation by extraction and high-pressure liquid chromatography □ Aspirin, derivatives—isolated as impurities from tablets, extraction, high-pressure liquid chromatography □ Analgesics—aspirin, isolation of derivatives from tablets, extraction, high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, aspirin derivatives in tablets

In recent years, two salicylate derivatives, acetylsalicylic anhydride (I) (1) and acetylsalicylsalicylic acid (II) (2), have been detected as impurities in aspirin tablets. These two compounds were reported (1, 3, 4) to be immunogenic substances capable of inducing the formation of acetylsalicyloyl- or salicyloyl-specific antibodies. According to these reports, allergic reactions to commercial aspirin preparations are due to these antibodies and, therefore, are caused by the aspirin impurities rather than by aspirin itself[1]. The clinical significance of these *in vivo* findings has been disputed (6). Demonstration of the potential immunogenic activity of these salicylate derivatives has emphasized the significance of their presence in pharmaceutical aspirin preparations even at low concentrations. Two papers (7, 8) reported the use of high-pressure liquid chromatography (HPLC) in the determination of salicylic acid, I, and II in aspirin.

A third salicylate derivative, salicylsalicylic acid (III), was found to undergo aminolysis to *N*-salicyloyl derivatives by reaction with amino acids (4) and to be an intermediate in the aminolysis of II, an alleged immunogenic agent (3). *In vivo* tests demonstrated the immunogenicity of I–III in guinea pigs (4). Although Bundgaard and DeWeck (4) did not find III in various aspirin samples, III was detected in most of the commercial aspirin tablets analyzed by HPLC in the present studies.

This paper reports the detection and identification of III as an impurity in aspirin and aspirin tablets. Methods for separation of aspirin and four salicylate impurities by TLC and HPLC are described. The isolation of I–III from aspirin tablets by extraction and HPLC and the possible confusion of III with I in reversed-phase HPLC are discussed.



I

II

III

EXPERIMENTAL

Instruments—All samples for IR spectral analysis were prepared as potassium bromide pellets, and the spectra were recorded on a grating IR spectrophotometer[2]. Melting points were taken in a capillary melting-point apparatus[3] and are uncorrected. NMR spectra were recorded on a 60-MHz spectrometer[4] with tetramethylsilane as an internal reference standard. UV spectra were recorded on a double-beam UV–visible spectrophotometer[5]. A high-pressure liquid chromatograph[6] with a UV detector set at 254 nm was used.

Reagents and Chemicals—Acetylsalicylic anhydride was synthesized according to a literature procedure (9). Recrystallization (95% ethanol) provided a white crystalline solid, mp 83.5–84.5°; IR: ν 1790, 1765, and 1730 cm^{-1} (ester and anhydride carbonyl stretching frequencies), identical to a published I spectrum (10); NMR ($CDCl_3$): δ 8.05 (d of d, J = 7.5 and 2 Hz, 2H, protons *ortho* to each anhydride carbonyl), 7.83–7.02 (m, 6H, aromatic protons other than the protons *ortho* to the anhydride carbonyl), and 2.27 (s, 6H, methyl protons). This compound is referred to throughout as reference standard I.

Acetylsalicylsalicylic acid was prepared by acetylation of salicylsalicylic acid[7] with acetic anhydride in cold aqueous sodium hydroxide according to a literature procedure (11). Recrystallization (twice) from aqueous ethanol (charcoal) gave white crystals, mp 163.5–164.5°; IR: ν 1748 with a shoulder at 1752 (two ester carbonyl stretching frequencies) and 1690 (acid carbonyl stretching frequency) cm^{-1}; NMR (CD_3OD): δ 8.36–7.84 (m, 2H, aromatic protons *ortho* to the carbonyl groups), 7.84–7.02 (m, 6H, aromatic protons other than those *ortho* to the carbonyl groups), and 2.22 (s, 3H, methyl protons). This compound is referred to throughout as reference standard II.

[1] An alternative theory is that aspirin itself is responsible for the formation of an antigenic determinant by initially undergoing an intramolecular rearrangement to a mixed anhydride, which, in turn, reacts with a protein amino function (5).

[2] Model 337, Perkin-Elmer Corp., Norwalk, Conn.
[3] Thomas-Hoover, Arthur H. Thomas Co., Philadelphia, Pa.
[4] Model T-60A, Varian Associates, Palo Alto, Calif.
[5] Cary model 219, Varian Associates, Palo Alto, Calif.
[6] Chromatronix model 3500B, Spectra-Physics, Santa Clara, Calif.
[7] K & K Laboratories, Plainview, N.Y.

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Acetylsalicylic acid[8], salicylic acid[8], salicylsalicylic acid[7], and methylacetylsalicylate[9] were used as obtained. Glass-distilled benzene[10], methanol[11], and cyclohexane[11] were used. All other chemicals and reagents were USP, ACS, or chromatographic grade. Deionized water was used for preparation of aqueous solutions.

Phosphate buffer (0.5 *M*, pH 8) was prepared from 2 *N* NaOH (120 ml), monobasic potassium phosphate (34 g), and water (to 500 ml). Phosphate buffer (0.5 *M*, pH 11.3) was prepared from 2 *N* NaOH (380 ml), monobasic potassium phosphate (68 g), and water (to 1000 ml).

α-Benzamidocinnamate–pyridine reagent, used in the spectrophotometric analysis of I, was prepared according to Bundgaard and Bundgaard (9). Sodium α-benzamidocinnamate was synthesized by the following route: benzaldehyde + hippuric acid → 4-benzylidene-2-phenyloxazoline-5-one (12) → α-benzamidocinnamic acid (12) → sodium α-benzamidocinnamate (9).

HPLC Parameters—The analytical HPLC system was operated with a flow rate of 1.6 ml/min and a chart speed of 30.48 cm (12 in.)/hr. The mobile phase was prepared by adding 12.5 ml of acetic acid to 450 ml of water and diluting with methanol to 1000 ml. This solvent mixture was filtered through a 0.6-μm filter[12] before use. Two different analytical reversed-phase C_{18} columns, A[13] and B[14], were used.

For the isolation of aspirin impurities, a semipreparative HPLC system was used with the following parameters: an 8-mm (i.d.) × 250-mm (length) stainless steel column packed with a reversed-phase C_{18} stationary phase[15]; a 1-ml loop injector; a chart speed of 30.48 cm/hr; a flow rate of 7 ml/min; and a mobile phase of acetic acid (11.5 ml), water (540 ml), and methanol (to 1000 ml).

Qualitative TLC—A single TLC system, one that has been employed successfully in separating I from aspirin (1, 13), separated aspirin and its four salicylate impurities. Samples were spotted on silica gel plates containing a fluorescent indicator[16] and developed in 2-butanone–cyclohexane (1:1) by ascending chromatography. Due to the instability of I on silica gel, the chromatogram was developed immediately after this compound was spotted. The compounds were detected by a UV hand lamp (254 nm). The R_f values for salicylic acid, aspirin, II, III, and I were 0.27, 0.34, 0.38, 0.44, and 0.51, respectively. Salicylic acid was fluorescent.

Isolation of I–III from Aspirin Tablets—One hundred 324-mg (5 gr) aspirin tablets were triturated in a mortar and pestle, and the powder was added to a cold biphasic mixture of benzene (130 ml) and phosphate buffer solution (650 ml, 0.5 *M*, pH 11.3). The mixture was stirred with a magnetic stirrer for 5 min and poured into a separator. The benzene layer was separated, dried over magnesium sulfate, and saved for isolation of I. The aqueous solution, pH 8 following neutralization of the original buffer solution with aspirin, was acidified with 2 *N* HCl (~300 ml) to pH 1–2 and filtered to remove the excipients and precipitated aspirin. The filtrate was extracted with 300 ml of benzene.

At this point, after all of the salicylates were in benzene solution, attention was directed toward the final isolation of I, since this compound undergoes decomposition. The first benzene extract (containing I) was filtered and concentrated to ~3 ml under reduced pressure at a bath temperature not exceeding 35°. Since TLC showed the presence of aspirin as well as I, the benzene solution was extracted with an equal amount of phosphate buffer (0.5 *M*, pH 8). The benzene solution, which now showed only I, was dried over magnesium sulfate, filtered, and evaporated under a dry nitrogen stream. This isolated I sample was compared to the reference standard I by UV (cyclohexane), TLC, and the α-benzamidocinnamate–pyridine test (9).

The second benzene extract, containing all salicylates except I, was extracted with four 100-ml portions of 0.1 *N* HCl to concentrate the remaining salicylate impurities relative to aspirin. Solvent benzene was removed under reduced pressure. The residue was dissolved in 4 ml of methanol, and an equal volume of water containing 1% acetic acid was added. This solution was passed through a 0.6-μm filter[12] before injection onto the semipreparative HPLC column. Fractions corresponding in retention times to II and III were collected. Solvents were evaporated under reduced pressure at 35°, with last traces being removed with a vacuum pump. Benzene (1 ml) was added to the isolated II sample; the solution was filtered and evaporated. The isolated II and III samples were compared to their reference standards by HPLC, IR, UV (95% ethanol), and TLC.

To demonstrate that III did not result from hydrolysis of II during the extraction procedure, reference standard II was subjected to the described extraction process; no evidence of III was found.

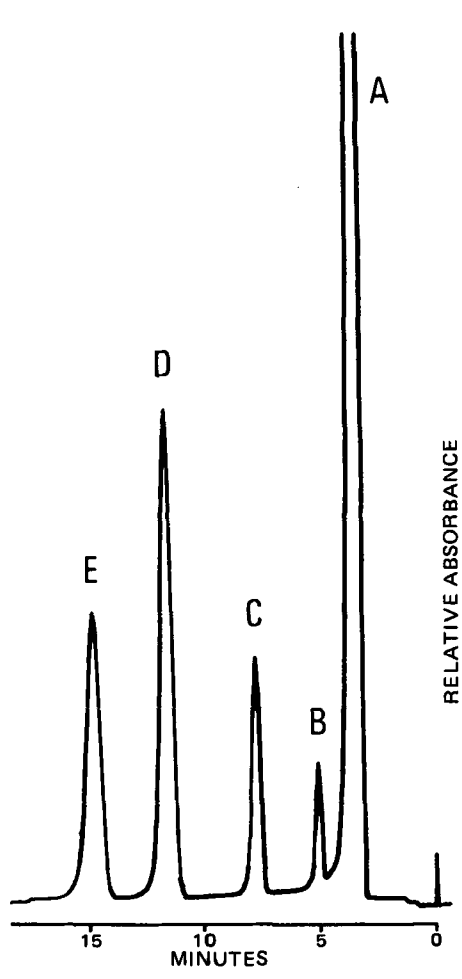

Figure 1—*HPLC on Column A of a synthetic mixture of aspirin (A) containing 1% each of salicylic acid (B), acetylsalicylsalicylic acid (C), acetylsalicylic anhydride (D), and salicylsalicylic acid (E). For the mobile phase, 12.5 ml of acetic acid was added to 450 ml of water and diluted to 1000 ml with methanol.*

RESULTS AND DISCUSSION

Aspirin and four salicylate impurities were adequately resolved by HPLC on Column A (Fig. 1). However, on Column B, another reversed-phase C_{18} column, I and III had almost identical retention times. Changes in the methanol–water ratio of the mobile phase or changes in the flow rate did not significantly change the difference between the retention times of these two compounds on Column B. The lack of resolution of these two compounds on one column emphasizes the precautions that must be taken in the HPLC analysis of aspirin for these compounds and the uncertainty of identifying them by HPLC alone.

HPLC of solutions obtained by extraction of aspirin or aspirin tablets with the mobile phase showed II, III, and salicylic acid in most samples tested. Although I generally was present in the aspirin samples and could be quantitated by the α-benzamidocinnamate–pyridine reagent[17] (9),

[8] Sigma Chemical Co., St. Louis, Mo.
[9] Aldrich Chemical Co., Milwaukee, Wis.
[10] Nanograde, Mallinckrodt, St. Louis, Mo.
[11] Burdick & Jackson Laboratories, Muskegon, Mich.
[12] Type BD, 0.6-μm pore size, Millipore Corp., Bedford, Mass.
[13] LiChrosorb RP-18, 10-μm particle size, 250 mm (length) × 4.6 mm (i.d.), EM Laboratories Inc., Elmsford, N.Y.
[14] μBondapak C_{18}, 10-μm particle size, 30 cm (length) × 3.9 mm (i.d.), Waters Associates, Milford, Mass.
[15] Corasil, 37–50 μm, Waters Associates, Milford, Mass.
[16] Precoated TLC plates, silica gel 60 F-254, EM Laboratories Inc., Elmsford, N.Y.

[17] We have determined that aspirin itself will react with the α-benzamidocinnamate–pyridine reagent, although at a slower rate than I. When present in the benzene extract at a concentration even as low as that of I, aspirin will interfere seriously with the analysis of I. The presence of interfering amounts of aspirin in this analysis can be determined by following the reaction over time. Absorbance resulting from I reaches a plateau in 20–30 min, whereas it continues to show a gradual increase past 30 min when aspirin is present.

detection and quantitation of I by HPLC was difficult because of its instability in the mobile phase and its presence in low concentration.

One feature that distinguishes I from III and the other salicylates mentioned is its high susceptibility to solvolysis. When reference standard I was dissolved in mobile phase (1 mg/ml) and examined by successive injection on Column A, the compound underwent solvolysis at room temperature to give rise to two new compounds with retention times corresponding to aspirin and methyl acetylsalicylate[18]. After 4 hr, <5% of the anhydride remained. The observed rate constant for the anhydride hydrolysis in 10% dioxane–90% water containing 0.57 *M* acetic acid at pH 2.59 and 25.1° has been reported as 6.0×10^{-4} sec^{-1} (14), from which the half-life is calculated to be 19 min. This rate was much faster than the rate of aspirin hydrolysis and was in general agreement with our observations. The salicylates other than acetylsalicylic anhydride were relatively stable in the mobile phase and showed no decomposition under the analytical conditions.

In a recent report (15) on aspirin impurities, it was tentatively assumed that III was present as an impurity in some aspirin samples based on its retention time by silica gel HPLC. The present study confirmed the presence of III in aspirin samples by isolation and identification of the compound; it is recognized that this compound is a commonly occurring impurity in aspirin and commercial aspirin preparations. The isolation of this compound from aspirin tablets by extraction and HPLC, as described, provided a sample that was identical to the reference standard III[7] by TLC and by HPLC on Columns A and B.

The UV spectrum (λ_{max}, relative absorbance) in 95% ethanol of the isolated III sample (234 nm, 1; 308 nm, 0.313) was comparable to that of reference standard III (234 nm, 1; 308 nm, 0.310). The IR spectrum of the isolated III showed maximum absorbance at frequencies of 3455 (phenolic OH stretching) and 1740 and 1690 (carbonyl stretching) cm^{-1}; this spectrum, including the fingerprint region, was identical to an IR spectrum of the reference standard. The structure was confirmed by an electron-impact mass spectrum[19], which provided a parent ion (*m/e* 258) and gave a breakdown pattern similar to that of the reference standard III.

Two other salicylate impurities found in aspirin, I and II, whose characterization by chromatographic methods was previously reported (1, 2, 7, 8), were isolated by extraction and HPLC and were characterized further. The isolated II sample was identical to its reference standard by HPLC, TLC, IR, and UV (95% ethanol). The isolated I gave a positive test for I with the α-benzamidocinnamate–pyridine reagent. The UV (cyclohexane) and TLC characteristics were the same as those for the reference standard I. When dissolved in mobile phase and monitored by HPLC on Column A, the isolated anhydride, like the reference standard, underwent hydrolysis and methanolysis at ambient temperature to give aspirin and methyl acetylsalicylate.

In the analysis of 176 samples of commercial aspirin tablets, the III concentration varied from below detectable limits (0.001%) to 0.25% of the amount of aspirin present. Of the samples tested, 70% contained levels that were detectable by HPLC (≥0.001%). Details of the quantitative analysis of aspirin impurities in aspirin and aspirin tablets will be the subject of a future report.

[18] The retention time of methyl acetylsalicylate was similar to that of salicylic acid under these conditions. To differentiate between these two compounds, the mobile phase was changed to one consisting of methanol (46 ml), acetic acid (1.2 ml), and water (to 100 ml) after I had decreased to about 5% of its original concentration.

[19] Varian MAT 311 mass spectrometer, Varian Associates, Palo Alto, Calif.

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