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Determination of Related Compounds in Aspirin by Liquid Chromatography

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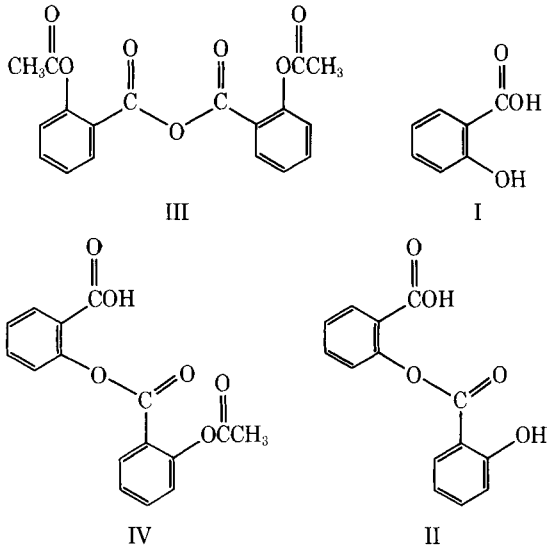
Abstract □ A rapid liquid chromatographic procedure has been validated for the determination of salicylic acid, salsalate, acetylsalicylsalicylic acid, and acetylsalicylic anhydride in aspirin. Samples are dissolved in methylene chloride and analyzed directly by adsorption chromatography in a 7-min separation using an isocratic mobile phase. Recoveries averaged 99% over a 200–10,000 ppm concentration range with standard deviations of <4% for the four compounds of interest. Detection limits ranged from 5 to 36 ppm. Compared to a recently published reversed-phase liquid chromatographic procedure for analyzing aspirin, this method is twice as fast, more sensitive, and avoids the use of hydroxylic solvents which lead to degradation of aspirin and acetylsalicylic anhydride.

Keyphrases □ Aspirin—determination of salicylic acid and related compounds by liquid chromatography □ Liquid chromatography—determination of salicylic acid and related compounds in aspirin □ Salicylic acid—determination in aspirin by liquid chromatography, related compounds

Several recent papers (1–5) have discussed the possible immunological response to the presence of low levels of related compounds in aspirin. Methods, too numerous to discuss, employing gas chromatography, spectrophotometry, liquid chromatography, *etc.*, have been published describing the determination of salicylic acid (I), salsalate (II), acetylsalicylic anhydride (III), and acetylsalicylsalicylic acid (IV) in aspirin. Liquid chromatography (LC) appears to be the most useful approach with respect to specificity, speed, and sensitivity. Various LC methods have appeared in the literature employing adsorption, polar bonded phase, as well as reversed-phase column packings.

After considering the various LC methods, it appeared that the methods employing adsorption chromatography are most appropriate for the determination of related compounds in aspirin on a routine basis. Reversed-phase methods are not desirable because III and aspirin are not stable in the mixed aqueous–organic eluents used in that



form of LC (5). In addition, the selectivity of the reversed-phase system is such that I elutes from the column immediately following aspirin and a poor detection limit is found for I because the larger aspirin peak tails into the peak for I. This difficulty can be avoided by using fluorescence detection (6) to selectively detect I, but this requires the use of dual detectors which increases the cost and complexity of the LC system.

Several normal-phase LC systems have been published for these analyses. A silica gel support containing perchloric acid as a stationary phase for the determination of I, III, and IV in aspirin has been used (7). In another study (8) a polar bonded phase[1] column has been used for the separation of II, III, IV, and other compounds. However,

[1] CYANO.

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Table I—Recovery Data for Related Compounds

Level Spiked	Percent Recovery I	II	III	IV
10,000 ppm	100.9	96.8	95.9	98.3
10,000 ppm	98.7	95.1	96.1	98.7
5,000 ppm	100.0	96.6	95.5	98.8
5,000 ppm	101.0	97.5	96.2	96.8
1,000 ppm	98.8	98.2	95.2	98.8
1,000 ppm	96.6	98.5	97.7	98.4
500 ppm	103.1	101.1	98.2	98.2
500 ppm	100.0	102.1	96.6	96.3
200 ppm	93.8	99.5	90.2	94.1
200 ppm	104.2	106.4	106.3	98.3
200 ppm	108.4	105.9	101.0	108.4
Average	100.5	99.79	97.17	98.65
SD	3.87	3.72	3.98	3.53

the separation on silica gel described previously (9) appears to be the most practical for use on a routine basis, and modification of this approach was used in this current work.

EXPERIMENTAL

Reagents—Hexane, chloroform, and methylene chloride of distilled-in-glass quality were used[2,3]. Glacial acetic acid of reagent grade[4] and 2,2-dimethoxypropane (98%)[5] were used as purchased.

Standards—Salicylic acid[6], salsalate[7], and acetylsalicylic anhydride[8] were used as purchased. The acetylsalicylsalicylic acid was synthesized[9] and characterized utilizing spectroscopic and chromatographic techniques to confirm its identity and purity.

A standard solution containing I, II, III, and IV was accurately prepared by weight in methylene chloride with each component at ~800 μg/ml. This concentrated standard solution was accurately diluted to yield a standard at the ~40-μg/ml level, and was used to calibrate the LC system. It was observed that the standard solutions were somewhat unstable and, therefore, all standards were prepared fresh whenever the system was recalibrated.

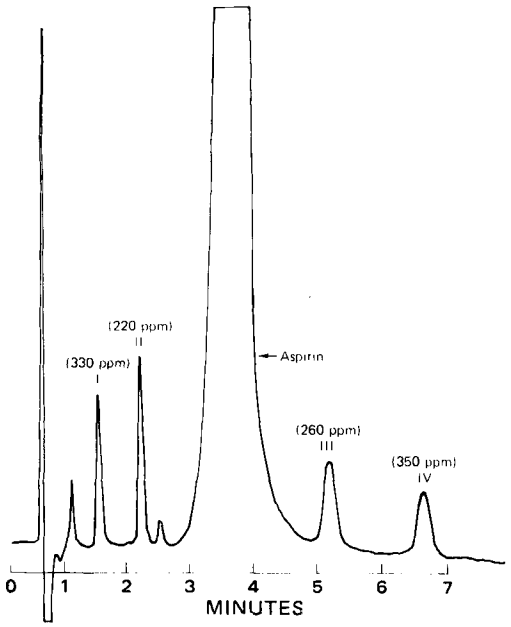

Figure 1—*Analysis of spiked aspirin. Column, 4.6 × 150 mm, Zorbax-SIL; flow rate, 3.0 ml/min; injection, 10 μl; detector, 0.016 aufs at 254 nm; mobile phase, hexane–chloroform–acetic acid (80:19:3).*

[2] Burdick and Jackson Laboratories, Inc., Muskegon, Mich.
[3] MCB Manufacturing Chemists, Inc., Cincinnati, Ohio.
[4] J. T. Baker Co., Phillipsburg, N.J.
[5] Aldrich Chemical Co., Milwaukee, Wis.
[6] United States Pharmacopeial Convention, Inc., Rockville, Md.
[7] Pfaltz & Bauer, Inc., Stamford, Conn.
[8] Tridom Chemical, Inc., Hauppauge, N.Y.
[9] Dow Chemical Co.

Apparatus—The liquid chromatograph was a modular system consisting of a pump[10], sample injection valve[11], and a single wavelength (254 nm) UV detector[12]. A computing integrator[13] was used for measuring peak areas and calculating results of analyses.

The microparticulate silica gel column[14] (4.6 × 150 mm) was purchased prepacked from the supplier. Prior to use, the column was activated using 2,2-dimethoxypropane (10). Ten column volumes of a solution consisting of methylene chloride–acetic acid–2,2-dimethoxypropane, 96:2:2 (v/v/v) were pumped through the column at a flow rate of 3.0 ml/min to condition and activate the packing. The column was then equilibrated by pumping mobile phase, hexane–chloroform–acetic acid, 80:19:3 (v/v/v), at a flow rate of 3.0 ml/min until a flat base line was obtained, ~15 min. The specific LC conditions used for the analysis were: mobile phase—hexane–chloroform–acetic acid, 80:19:3 (v/v/v); temperature ambient; detector wavelength, 254 nm, 0.016 aufs; injection, 10 μl.

Sample Preparation—Samples of aspirin crystals were dissolved in methylene chloride (20 mg/ml) and analyzed directly by LC. The sample solutions were injected as soon as dissolution had occurred because it was observed that aspirin hydrolyzed to I in methylene chloride solution. Therefore, artificially high results were obtained for I unless sample solutions were analyzed immediately.

Samples of aspirin tablets were finely ground and dissolved in methylene chloride (20 mg/ml). The resulting solutions were rapidly filtered[15] to remove insoluble materials, and the clear filtrate was immediately analyzed by LC. This entire dissolution–filtering operation was completed in less than 2 min to minimize formation of I.

RESULTS AND DISCUSSION

Validation—A typical chromatogram of a spiked aspirin sample is shown in Fig. 1. The linearity of the LC system was demonstrated by

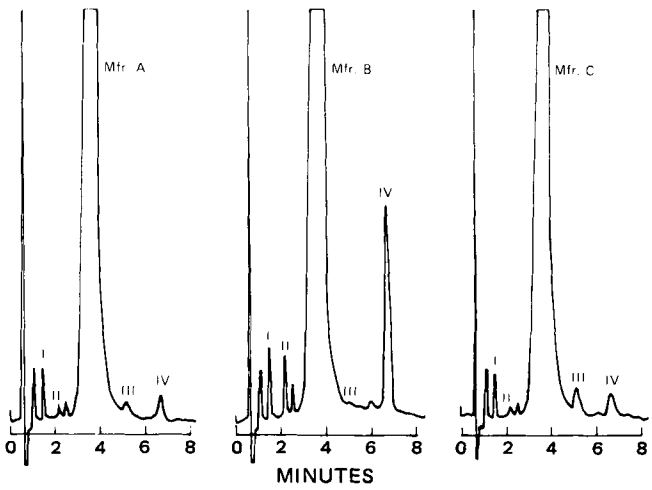

Figure 2—*Comparison of aspirin crystals. Same conditions as Fig. 1.*

[10] Model M-45, Waters Associates, Milford, Mass.
[11] Model 7125, Rheodyne, Inc., Berkeley, Calif.
[12] Model 1203, Laboratory Data Control, Riviera Beach, Fla.
[13] System I, Spectra Physics, Santa Clara, Calif.
[14] Zorbax SIL, 6μ, DuPont Co., Wilmington, Del.
[15] 0.45 μ Fluropore Filter, Sample Clarification Kit, Waters Associates, Milford, Mass.

Table II—Precision Study for Analysis of Aspirin

Component	Compound I	II	III	IV
Day 1	103[a]	195	758	1455
	105	188	750	1421
	101	179	702	1388
	103	179	701	1348
	99	181	722	1394
Day 2	112	171	678	1309
	101	173	695	1361
	103	179	699	1363
	98	177	706	1386
	106	179	701	1356
Average	103.1	180.1	711.2	1378.1
SD	3.98	6.93	13.86	40.6

[a] Measured in parts per million.

Table III—Detection Limits for Aspirin-Related Compounds

Compound	Peak Area, ppm	Peak Height, ppm
I	14	15
II	5	6
III	6	19
IV	11	36

chromatographing a series of standard solutions containing varying levels of the four compounds of interest. Plots of peak area against concentration yield straight lines going through the origin for up to the equivalent of 1% of each of the related compounds in the samples. The peak height response was also linear up to 1200 ppm at 0.016 aufs.

For the recovery study, a sample of aspirin was analyzed in triplicate using the external standard technique to determine the native levels of the related compounds. Recovery data were generated by spiking the control sample at levels in a 200–10,000 ppm range with the components of interest and analyzing those components by the external standard technique. The results were corrected for native levels of each component before calculating the recovery values. The results are summarized in Table I. The data demonstrated that relative standard deviations of 4% were obtained for the four compounds of interest.

Precision data were generated by analyzing a single sample of aspirin 5 times on each of two consecutive days. The results are summarized in Table II. The precision obtained would have been predicted from the recovery study, further demonstrating the validity of the method.

The detection limits of the method for the four components of interest for both peak area and peak height are summarized in Table III. The minimum detectable quantity was defined as a peak <2.5 times the short-term peak-to-peak baseline noise. The retention times for several other aspirin related compounds on this LC system are listed in Table IV. Chromatograms of typical bulk aspirin samples are shown in Fig. 2; results are summarized in Table V.

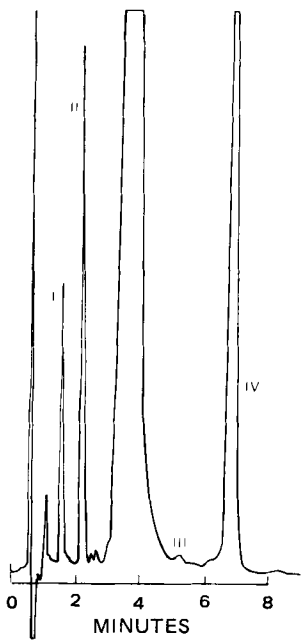

Figure 3—*Analysis of Aspirin Tablet. Same conditions as Fig. 1.*

Table IV—Retention Times of Aspirin-Related Compounds

Compound	Retention Time, min
Salicylic Acid	1.6
Salsalate	2.2
p-Acetoxybenzoic Acid	2.5
Acetylsalicylic Acid	3.6
Acetylsalicylic Anhydride	5.2
p-Hydroxyisophthalic Acid	6.3
Acetylsalicylsalicylic Acid	6.7
p-Acetoxyisophthalic Acid	13.0
p-Hydroxybenzoic Acid	15.5

Table V—Analysis of Bulk Aspirin

Manufacturer	I	II	III	IV
A	190[a]	16	81	230
B	260	100	*ND*[b]	2000
C	160	16	110	190

[a] Measured in parts per million. [b] Not detected at a detection limit of 6 ppm.

Table VI—Analysis of Aspirin Tablets

Brand	I	II	III	IV
A	640[a]	650	ND[b]	4300
B	440	650	ND	4800
C	240	*ND*[c]	ND	650
D	460	690	ND	4400
E	330	20	ND	530
F	540	700	ND	4500

[a] Measured in parts per million. [b] Not detected at a detection limit of 6 ppm. [c] Not detected at a detection limit of 5 ppm.

Mobile Phase—During this investigation, it was found that the aspirin separation was somewhat dependent on the particular brand of chloroform used. Various suppliers use different preservatives to inhibit degradation, and it was found in particular that ethanol used as a preservative had a significant effect on retention times.

The separation between III and aspirin is most critically affected. Using a ratio of hexane–chloroform–acetic acid of 80:19:3 (v/v/v), with a nonpolar hydrocarbon stabilizer in the chloroform, the relative retention of III to aspirin was 1.59. With 80:19:3 and 1% ethanol in the chloroform, the relative retention was 1.36. Thus, when ethanol is used as a preservative in chloroform, the relative amount of chloroform in the mobile phase should be reduced to adequately resolve III and aspirin.

Analysis of Tablets—While this method was developed and validated for the specific determination of related compounds in bulk aspirin, it is also applicable to the analysis of aspirin tablets. A complicating factor in the analysis of tablets is the presence of starch and other insoluble excipients in the sample matrix which must be removed prior to the LC separation. However, this was readily accomplished *via* filtration using the filter described previously. A small, 4.6 × 50-mm guard column packed with pellicular silica gel[16] was used to protect the analytical column when analyzing tablets.

The method was not formally validated for the analysis of tablets. However, the LC separation was evaluated for this application by analyzing six commercial brands of tablets. A typical chromatogram is shown in Fig. 3 and the results are summarized in Table VI. It is interesting to note that no III was detected in any of the samples analyzed. At this time, it is not known whether these particular brands were formulated from bulk aspirin not containing III, or if III is not stable in the starch matrix since the starch may contain up to 14% water.

CONCLUSIONS

A new, rapid method has been validated for the determination of related compounds in bulk aspirin. Recoveries averaged 99% with a standard deviation of <4% for the four compounds of interest over a 200–10,000 ppm concentration range. For a single determination, results may be expected to be <7.7% relative error at the 95% confidence level.

This method has three significant advantages compared to the re-

[16] Corasil II, Waters Associates, Milford, Mass.

versed-phase LC procedure recently published by the United States Food and Drug Administration (11, 12). In addition to being twice as fast, the selectivity of the adsorption LC system is such that I and II elute prior to aspirin, permitting a low limit of detection for I. The normal phase LC method avoids the use of hydroxylic solvents, which lead to degradation of aspirin and III preventing accurate determination of III by reversed-phase LC. This method uses an inexpensive fixed wavelength (254 nm) UV detector and has a lower limit of detection than any previously published normal phase LC procedure.

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Soft Drugs V: Thiazolidine-Type Derivatives of Progesterone and Testosterone

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Received April 8, 1981, from the **Department of Medicinal Chemistry, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610* and the ‡*Inter$_x$ Research Corporation, Lawrence, KS 66044.* Accepted for publication August 5, 1981.

Abstract □ Progesterone and testosterone are natural soft drugs, but to be used as drugs, their fast and facile metabolism must be prevented and their delivery controlled. A prodrug–soft drug combination can serve this purpose. Thiazolidines of testosterone, testosterone 17-propionate and progesterone were synthesized from the reaction of cysteine alkyl esters, *N*-methylaminoethanethiol, and mercaptamine and their hydrochlorides with the appropriate steroids. The thiazolidines function as bioreversible derivatives of the parent steroids.

Keyphrases □ Soft drugs—thiazolidine-type derivatives of progesterone and testosterone □ Prodrugs—thiazolidine-type derivatives of progesterone and testosterone □ Progesterone—thiazolidine-type derivatives, prodrugs □ Testosterone—thiazolidine-type derivatives, prodrugs

Oral contraception is accomplished currently by products containing synthetic hormones, mostly as fixed combinations of synthetic estrogens and progestins (1). The contraceptive action of these products is mediated primarily by inhibition of ovulation through specific macromolecular receptors for each hormone. It is clear that if the natural hormones, *e.g.*, progesterone and estradiol, were delivered to the receptors, they would elicit the same contraceptive effect as the synthetic analogs. The advantage of this method is that the natural hormone might decrease or eliminate the side effects accompanying synthetic contraceptive agents. Some of these side effects are the result of oxidative metabolism (2), such as the one involving the 17α-ethinyl group in norethindrone (17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one) or norgestrel (13-ethyl-17β-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one), which leads to destruction of cytochrome P-450 (3).

Natural hormones such as progesterone, estradiol, and testosterone are natural soft drugs (4, 5); that is, due to their efficient and nontoxic metabolic disposition, they will not cause unexpected toxicity at concentrations close to their natural levels. On the other hand, natural hormones administered as drugs suffer from low physiological availability because, as natural substances, the body has developed efficient mechanisms for their metabolism and excretion. For example, the α,β-unsaturated ketone and the 20-ketone in progesterone are reduced in the liver to give pregnane-3α,20-diol, which can then be conjugated and excreted (1). In addition, the natural hormones are virtually insoluble in water which precludes their efficient dissolution and absorption from most formulations. Thus, to utilize progesterone as a contraceptive drug component, solutions to the problems of slow dissolution (water solubility) and rapid metabolism must be found.

In the case of hydrocortisone (4), the preferred approach to solving the described problems is through chemical modification, *i.e.*, a prodrug. However, progesterone is a difficult candidate for such an approach because the only functional groups available for reversible modification are the 3- and 20-ketones. Therefore, an extension of the spirothiazolidine approach (4) seemed the most attractive, as thiazolidines are unique examples of a bioreversible steroidal ketone derivative. The spontaneous S_N1 cleavage of the thiazolidine (6) to a β-thioethylene imine is followed by hydrolysis of the imine [possibly through a hydration–disassociation mechanism (7)] to regenerate the parent carbonyl compound (4). In addition to their potential bioreversibility, thiazolidines were attractive as prodrugs because they could be prepared from cysteine. Consequently, the hydrolysis products (steroid and cysteine) of the prodrugs would not present any unusual metabolic burden to the body. The carboxylic group of cysteine could also be easily esterified, thus providing a convenient method for changing the lipophilicity/hydrophilicity of the derivatives. In addition to progesterone, testosterone thiazolidines were also investigated[1].

[1] Part 4 of this series (N. Bodor, K. B. Sloan, R. J. Little, S. H. Selk, and L. Caldwell, *Int. J. Pharm;* in press).

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