benzoic acid, and acetylsalicylic acid (C). Parts A and B were determined at 135° with part C determined at 110° in order to have base line resolution of all the peaks. The ethereal extract of control serum sample (A) did not give any peak that interfered with the GLC analysis of acetylsalicylic acid, salicylic acid, and *p*-hydroxybenzoic acid. Similar results were obtained using samples of either rat or human plasma. The retention times of the trimethylsilyl derivatives of salicylic acid, *p*-hydroxybenzoic acid, and acetylsalicylic acid were 2.2, 4.4, and 5.4, respectively at 135°. Under these conditions, the retention times of the trimethylsilyl derivatives of methyl salicylate, ibuprofen, salicylamide, and acetaminophen were 1.9, 3.7, 10.8, and >30 min, respectively. Acetanilide and phenacetin did not form a trimethylsilyl derivative, but each compound produced a single peak with retention time values of 0.9 and 4.7 min, respectively, when chromatographed at 135°. Other nonsteroid anti-inflammatory drugs such as ketoprofen, indomethacin, and naproxen did not produce a peak when silylated under identical conditions. Complete separation between the peak of the trimethylsilyl derivatives of methylsalicylic acid and salicylic acid was obtained at 110° (Fig. 1C). Under these conditions, the retention time values of underivatized acetanilide and of the trimethylsilyl derivatives of methylsalicylic acid, salicylic acid, *p*-hydroxybenzoic acid, and acetylsalicylic acid were 2.0, 5.1, 7.1, 14.35, and 20.05 min, respectively. Salicyluric acid (2-hydroxyhippuric acid), the major metabolite of salicylic acid, did not give a peak under the analytical conditions used. The chromatographic conditions determined at 135° were suitable for the simultaneous analysis of acetylsalicylic acid and salicylic acid in serum samples. There was no interference from the commonly used analgesics often prescribed in combination with acetylsalicylic acid, except for phenacetin, which gave a peak of similar retention time value close to that of the trimethylsilyl derivative of *p*-hydroxybenzoic acid. In cases where phenacetin is also being administered, 4-chlorophenylacetic acid can be used as a reference standard since its trimethylsilyl derivative has a retention time value of 2.9 min.

Table II gives the coefficient of variation and the recovery of different concentrations of acetylsalicylic acid and salicylic acid added to the control serum. The coefficients of variation of triplicate analysis varied from 0.0 to 6.9% for acetylsalicylic acid and from 1.1 to 6.4% for salicylic acid. The mean values for the coefficient of variation of the eight concentrations of acetylsalicylic acid and salicylic acid used were 2.6 ± 2.6 and 3.7 ± 1.8 (*SD*%) respectively. The recovery of these two compounds was essentially quantitative. The overall recovery for acetylsalicylic acid was 104.0 ± 8.4 (*SD*)%, while that of salicylic acid was 98.2 ± 5.3 (*SD*)%. Calibration curves were obtained for acetylsalicylic acid ($y = 0.03 + 0.0474x$ and $r = 0.996$) and for salicylic acid ($y = 0.20 + 0.064x$ and $r = 0.997$) in the range of concentrations of 2.5–25 μg/0.1 ml of serum, *i.e.*, within the concentrations determined in pharmacokinetic studies. It should be pointed out that the hydrolysis of acetylsalicylic acid to salicylic in blood samples at 37° is rapid (9–11) and, therefore, it is necessary to extract the acetylsalicylic acid as soon as possible to avoid its breakdown in storage. There was no breakdown of acetylsalicylic acid to salicylic acid during the extraction, evaporation, and derivatization processes described herein.

Table II—Recovery of Different Amounts of Acetylsalicylic Acid and Salicylic Acid Added to Control Serum

	Acetylsalicylic Acid			Salicylic Acid		
Amount Added, μg/0.1 ml	Amount Recovered[a], μg/0.1 ml ± *SD*	*CV*, %[b]	Recovery, %	Amount Recovered[a], μg/0.1 ml ± SD	CV, %[b]	Recovery, %
2.5	2.25 ± 0.13	5.8	90.0	2.7 ± 0.1	3.3	108.0
7.5	9.1 ± 0.6	6.9	121.0	7.4 ± 0.3	5.3	98.7
10.0	10.5 ± 0.0	0.0	105.0	9.7 ± 0.3	3.2	97.0
12.5	12.9 ± 0.2	1.6	103.2	11.7 ± 0.55	4.7	93.6
15.0	15.6 ± 0.2	1.3	104.0	13.8 ± 0.6	4.5	92.0
17.5	17.9 ± 0.7	4.1	102.3	16.6 ± 0.2	1.4	94.8
20.0	21.1 ± 0.0	0.0	105.5	19.7 ± 0.2	1.1	98.5
25.0	25.3 ± 0.2	0.8	101.2	25.8 ± 1.6	6.4	103.2

[a] Mean value ± *SD* of triplicate analyses. [b] Coefficient of variation = *SD*/mean × 100.

The proposed GLC assay of acetylsalicylic acid and salicylic acid in serum is simpler and faster than other methods that have been published for the same purpose.

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Is Aspirin Phenylalanine Ethyl Ester a Prodrug for Aspirin?

Keyphrases □ Aspirin—high-performance liquid chromatography, phenylalanine ethyl ester as prodrug

To the Editor:

It is known that oral administration of aspirin induces gastric irritation and bleeding because of local irritation

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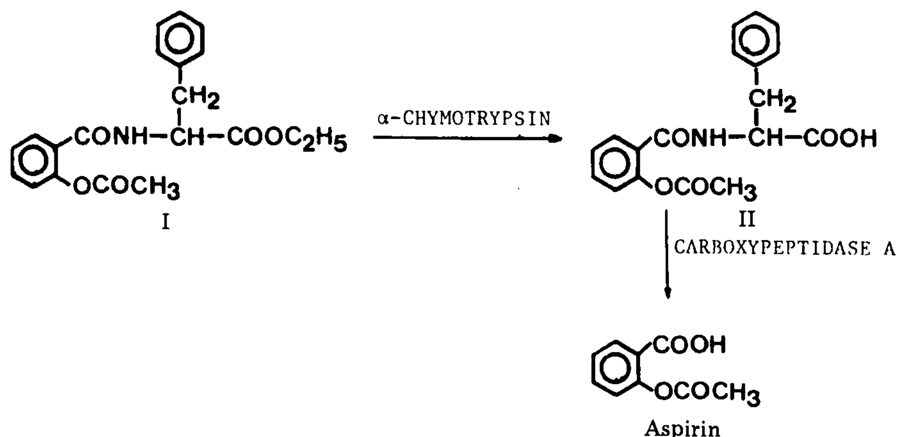


Scheme I

of the gastric mucosal membrane by the very acidic aspirin particles (1–3). One approach to minimizing this side effect is to mask the acidic carboxyl group of aspirin reversibly *via* a prodrug. Upon administration, the neutral derivative dissolves first, then hydrolyzes either in the GI tract or in the plasma, generating aspirin. It has been shown that classical esterification of the carboxyl group results in nonirritating but insoluble species which do not revert to aspirin but rather to the corresponding salicylate derivative (4). Thus, a prerequisite for any aspirin prodrug is that the masking group cleave much faster than the acetyl group. Prodrugs that cleave to aspirin have been reported (5–7). The compounds were aspirin derivatives in which the carboxyl group was masked *via* an acylal linkage.

Banerjee and Amidon (8–10) have recently proposed aspirin phenylalanine ethyl ester (I) as a possible prodrug for aspirin. It was rationalized that, because of the specificity of certain enzymes such as α-chymotrypsin and carboxypeptidase A, I would first be cleaved by α-chymotrypsin to aspirin phenylalanine (II) and subsequently to aspirin by carboxypeptidase A. To support their hypothesis, the authors studied the rate of hydrolysis of I at pH values of 7.5 and 8 in the presence of α-chymotrypsin and separately determined the rate of hydrolysis of II at pH 8.5 in the presence of carboxypeptidase A. In their kinetic studies, the reactions were followed by measuring the consumption of sodium hydroxide using a pH-stat titration. Aspirin was detected using TLC from a reaction mixture containing I and the two enzymes at pH 7.5. Based on this information, the authors proposed Scheme I for the stepwise enzymatic hydrolysis of I.

In this communication we report recent studies conducted using a specific high-performance liquid chromatographic (HPLC) assay and TLC assay which indicate that the work reported by these authors is in error. In disagreement with their conclusions, the new data indicate that (*a*) compound I does not generate aspirin in the presence of the two enzymes, (*b*) the hydrolysis of II is not catalyzed by carboxypeptidase A, and (*c*) compound I *does* hydrolyze to II in the presence of carboxypeptidase A.

Compound I was prepared according to the procedure of Banerjee and Amidon (8). Compound II was prepared from I using the reported procedure, but with slight modification; namely, treating I with α-chymotrypsin for longer than the reported 2 min to ensure complete conversion to II. The compounds were characterized by their melting points, NMR spectroscopy, and elemental analysis.

The activity of carboxypeptidase A[1] was redetermined in our laboratories and found to be 39.4 U/mg of protein, in close agreement with the labeled value of 41 U/mg of protein. The buffers used in our studies were 0.1 M phosphate adjusted to the desired pH values. The pH values of all the solutions were redetermined at the end of the experiments and found to be unchanged. An HPLC was equipped with a UV detector[2] set at 254 nm and 0.02–0.1 AUFS. Samples were analyzed on a 3.9-mm × 30-cm reverse-phase[3] column. The mobile phase was acetic acid–methanol–water (2:40:58), and the flow rate[4] was 2 ml/min. The injection volume[5] was 20 μl.

Figure 1 is a chromatogram of a mixture of equimolar concentrations (1.57×10^{-4} M) of I, II, aspirin, salicylic acid, salicyl phenylalanine, and salicyl phenylalanine ethyl ester. With this technique, the hydrolysis of I, even to the extent of 20%, would result in aspirin levels that are easily detectable. The enzymatic hydrolysis rates of I and II were examined using this HPLC method.

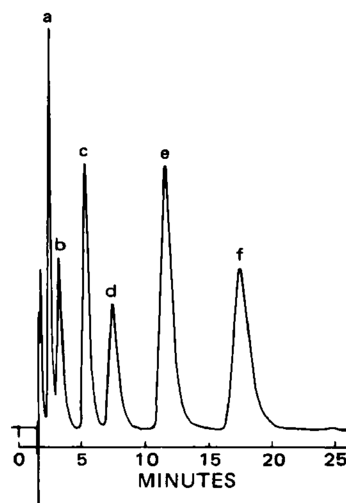


Figure 1—*HPLC chromatograms for* (a) *aspirin,* (b) *salicylic acid,* (c) *aspirin phenylalanine,* (d) *salicyl phenylalanine,* (e) *aspirin phenylalanine ethyl ester, and* (f) *salicyl phenylalanine ethyl ester.*

[1] Lot 12F-8185, Sigma Chemical Co., St. Louis, Mo.
[2] LDC III, Milton Roy Corp., Riviera Beach, Fla.
[3] μBondapak C_{18}, Waters Associates, Milford, Mass.
[4] Delivered by a Model 110A pump, Beckman Instruments, Inc., Irvine, Calif.
[5] Model 7125, Rheodyne Inc., Berkeley, Calif.

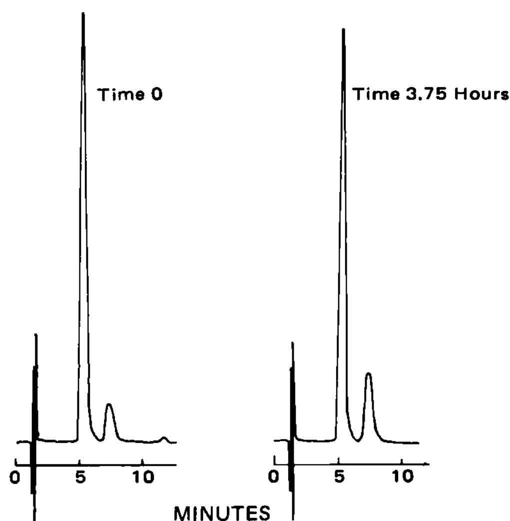


Figure 2—*HPLC chromatograms of aspirin phenylalanine (8.5 × 10^{-4} M) in the presence of carboxypeptidase A (1.0 × 10^{-5} M) at pH 8.5. Key: Aspirin phenylalanine, 5.0 min; Salicyl phenylalanine, 7.0 min.*

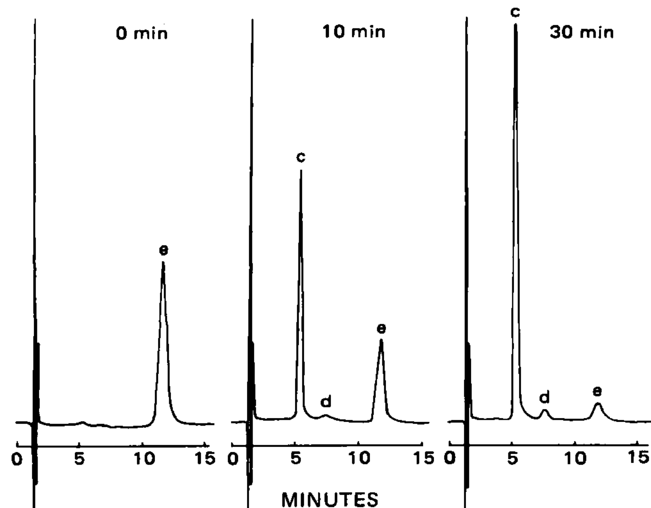


Figure 3—*HPLC chromatograms showing the disappearance of the prodrug and the appearance of aspirin phenylalanine as a function of time in the presence of 1 × 10^{-5} M carboxypeptidase A at pH 8.5. Key: (c) aspirin phenylalanine, (d) salicyl phenylalanine, and (e) prodrug.*

When a solution at pH 8 containing I (8 × 10^{-4} *M*) and α-chymotrypsin[6] was analyzed by HPLC, only two peaks were observed: a major peak corresponding to II and a minor peak corresponding to salicyl phenylalanine. This experiment confirms that I does indeed cleave to II in the presence of α-chymotrypsin (8). However, when a mixture of α-chymotrypsin (3.9 × 10^{-6} *M*) and carboxypeptidase A (1 × 10^{-5} *M*) was added to I (8 × 10^{-4} *M*) at pH values of 7.4 and 8.5 and the solution immediately analyzed by HPLC, the same two peaks were observed and no peak corresponding to aspirin was detected even after 30 min. Therefore, aspirin is not liberated from II in the presence of carboxypeptidase A. To support this conclusion, the hydrolysis of II itself (8.5 × 10^{-4} *M*) in the presence of carboxypeptidase A (1 × 10^{-5} *M*) at pH 8.5 (the pH used by the previous authors to study this reaction) was determined by HPLC. When the mixture was immediately injected into the HPLC, two peaks, corresponding to II and salicyl phenylalanine were observed. As shown in Fig. 2, no additional peaks were observed, even after 3.75 hr. The same result was observed when the carboxypeptidase A concentration was increased 20-fold.

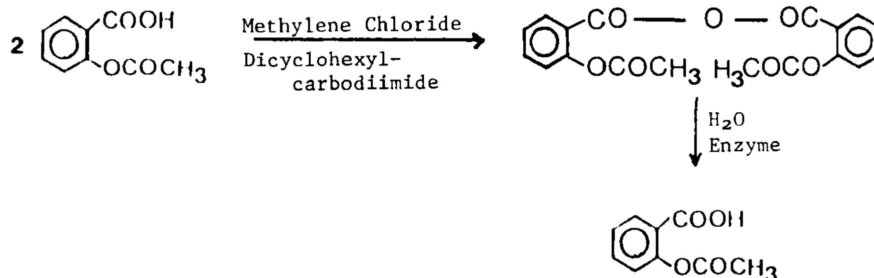
On the other hand, and contrary to the hypothesis of the authors, I was found to cleave to II in the presence of carboxypeptidase A. Figure 3 shows the time course for the appearance of II in a solution containing I (8 × 10^{-4} *M*) and carboxypeptidase A at pH 8.5.

To confirm the HPLC results, the same TLC system reported previously (8) was used to examine the mixture containing I and the two enzymes. Again, no aspirin spot was observed.

The results obtained by the authors which led to their conclusions appear to be in error for two reasons:

1. The aspirin spot appearing on the TLC plates (8) may have been the result of the hydrolysis of aspirin anhydride contaminating their prodrug. This is quite possible, since preparation of the prodrug involves the intermediate formation of aspirin anhydride, as shown in Scheme II. The half-life of the conversion of aspirin anhydride to aspirin is known to be ~8 min at pH 7.4 and 25°[7].

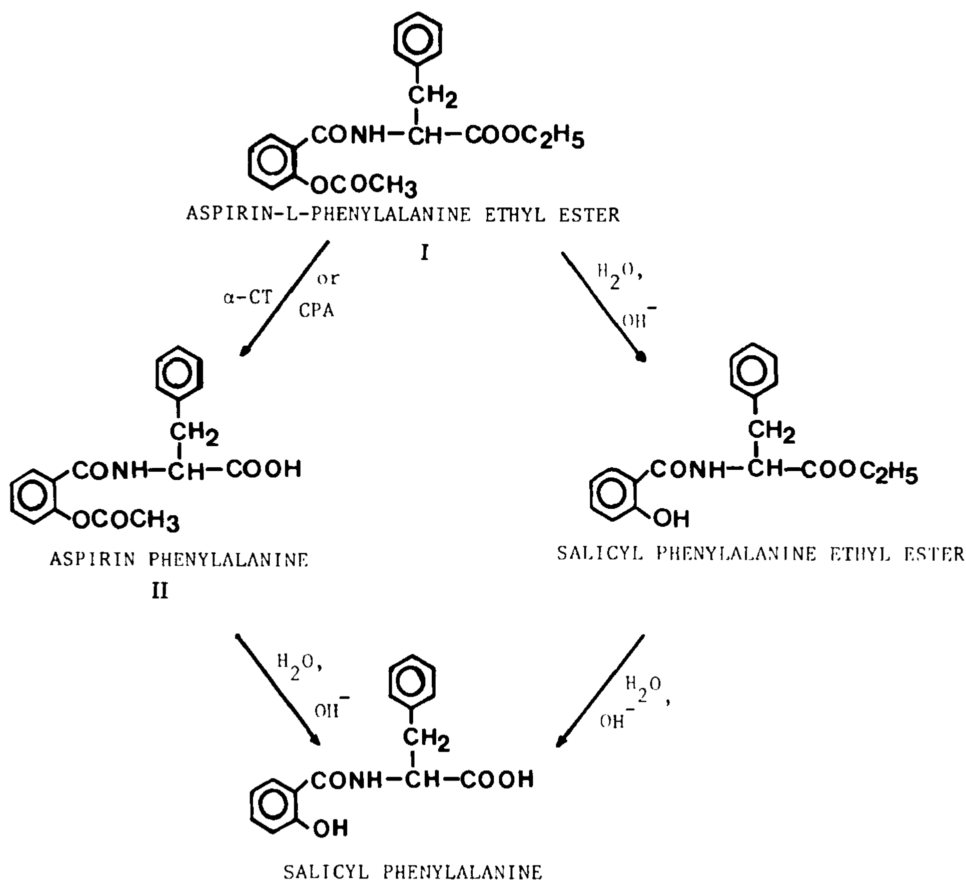
2. The consumption of sodium hydroxide observed previously when carboxypeptidase A was added to II may have been due to the hydrolysis of I, a very likely contaminant of the preparation. This is quite possible, since II was obtained by treating I with α-chymotrypsin for only 2 min and the purity of II was determined only by NMR (8). If this were the case, then I itself would, as shown above, hydrolyze in the carboxypeptidase A preparation. The hydrolysis of I by carboxypeptidase A follows Michaelis–Menten kinetics. Linear Lineweaver–Burk plots



Scheme II

[6] Lot 70F-8000, Sigma Chemical Co., St. Louis, Mo.

[7] M. N. Khawam, J. B. Bogardus, and A. A. Hussain, unpublished results.



Scheme III

at concentrations ranging from 4.4×10^{-4} to 1.2×10^{-3} M were obtained in the presence of 1×10^{-5} M enzyme. The details of these experiments will be published in a later paper.

Since I is hydrolyzed to II by α-chymotrypsin and II does not cleave to aspirin in the presence of carboxypeptidase A, I is not a prodrug for aspirin.

Based on the previous work (8–10) and the additional data obtained in this laboratory, Scheme III is proposed for the hydrolysis of aspirin phenylalanine ethyl ester.

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Comment on a Second-Degree Polynomial Mathematical Model for Tablet Friability and *In Vitro* Dissolution

Keyphrases □ Dissolution—*in vitro*, polynomial mathematical model, effect of moisture and crushing strength □ Friability—effect of moisture and crushing strength, mathematical model

To the Editor:

In a recent report, Chowhan *et al.* (1) have used a function of a two-variable model to describe the effect of moisture and crushing strength on tablet friability and *in vitro* dissolution. Elliptical shape and ridge contour curves were unfortunately not reproduced on a computer[1] using all published data. Further examination of the mathematical equation (Eq. 1) using SAS contour plot procedure[2] on the same computer showed indeed that Figs. 5 and 6 contour plots in the article did not agree with the analytical expression.

[1] IBM 3033.
[2] SAS Institute Inc., Cary, NC 27511.

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