Table II—Screening Data Summary

Compound	Dose Number	Dosage, mg/kg	T/C[a], %	NSC Number
Cyclophosphamide	1	500	168	26271
	2	250	310	
	3	135	201	
	4	62.5	144	
I	1	750	129	252152
	2	500	124	
	3	330	123	
	4	250	106	
	5	220	105	
	6	62.5	99	
II	1	500	130[b]	266053
	2	500	118	
	3	500	110	
	4	333	106	
	5	333	111	
	6	250	114	
	7	222	108	
	8	222	111	
	9	62.5	108	

[a] Ratio of median survival time of tested animal to median survival time of control animals; expressed as percent. [b] Could not be confirmed.

(35.4%) of I as an oil. The product was eluted by benzene followed by methanol. It showed IR bands in mineral oil at 5.75, 7.75–8.30, 8.85–9.1, and 13.9 μm.

Compound II—Approximately 3.0 g (0.016 mole) of DL-homoserine ethyl ester hydrochloride and 4.14 g (0.016 mole) of III were suspended in tetrahydrofuran and heated. Triethylamine, 6.60 ml (0.047 mole), in tetrahydrofuran was added slowly. After addition was complete, the reaction mixture was kept overnight at room temperature.

The reaction was worked up as previously described to give 1.26 g (23.1%) of II as an oil. It showed IR bands in mineral oil at 5.75 and 13.9 μm. Other IR bands appeared but were broad and not well resolved.

RESULTS

Compounds I and II showed minimum activity relative to that demonstrated by cyclophosphamide. Generally speaking, an increase in survival of treated animals compared to control animals resulting in a test/control percent value of 125 or more is required to merit further investigation[1]. An attempt to increase the antitumor activity of cyclophosphamide by testing II against L-1210 lymphoid leukemia proved to be negative. The same can be said for I.

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Simple and Rapid High-Pressure Liquid Chromatographic Simultaneous Determination of Aspirin, Salicylic Acid, and Salicyluric Acid in Plasma

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Abstract □ A rapid and sensitive high-pressure liquid chromatographic assay was developed for aspirin, salicylic acid, and salicyluric acid in plasma. The procedure involves the solvent extraction of these compounds from plasma and separation using a reversed-phase column eluted by acidified aqueous acetonitrile. Small quantities of aspirin can be assayed directly in the presence of a large quantity of salicylic acid. The assay is also free from blank interference.

Keyphrases □ Aspirin—high-pressure liquid chromatographic analysis in plasma simultaneously with salicylic acid and salicyluric acid □ Salicylic acid—high-pressure liquid chromatographic analysis in plasma simultaneously with aspirin and salicyluric acid □ Salicyluric acid—high-pressure liquid chromatographic analysis in plasma simultaneously with aspirin and salicylic acid □ High-pressure liquid chromatography—analyses, aspirin, salicylic acid, and salicyluric acid simultaneously in plasma □ Analgesics—aspirin, salicylic acid, and salicyluric acid, simultaneous high-pressure liquid chromatographic analyses in plasma

Many procedures have been reported for quantifying salicylates in dosage forms and biological media because of the frequent and popular use of these drugs and their various pharmacological properties. The standard colorimetric (1) and fluorometric (2) determinations of aspirin and salicylic acid are not specific and measure aspirin only by difference after hydrolysis to salicylic acid. The differential spectrophotometric assay of the two salicylates based on the pH-dependent shift in their UV absorbances may require corrections because of overlap of their absorption spectra (3). Furthermore, when applied to biological samples, such as plasma, these procedures may yield high and variable blank values.

Several GLC determinations have been reported for aspirin and salicylic acid in dosage forms (4–6) and biological fluids (7–9). These methods are specific and sensitive and permit the assay of both compounds simultaneously without conversion of aspirin to salicylic acid. However, chemical derivatizations, such as silylations, are necessary to make these compounds suitable for GLC (7, 10). The chemical derivatizations are inherently time consuming (requiring up to 60 min) and can be complicated by hydrolysis of aspirin to salicylic acid (10) and multiple-product formation (ester and/or ether) for the latter (7).

TLC (11) and liquid chromatographic (12) methods for salicylates also were reported. Recently, an automated high-pressure liquid chromatographic (HPLC) analysis of aspirin, phenacetin, and caffeine in dosage forms was

Table I—Linearity of Calibration Curves for Salicyluric Acid, Aspirin, and Salicylic Acid

Internal Standard in Plasma, μg/ml	Drug in Plasma, μg/ml	Peak Area Ratio[a]			Peak Area Ratio[a]/ Weight Ratio[b]		
		Salicyluric Acid[c,d]	Aspirin[d,e]	Salicylic Acid[d,f]	Salicyluric Acid	Aspirin	Salicylic Acid
5.0	0.5	0.156	0.119	0.186	1.56	1.19	1.86
5.0	2.0	0.614	0.412	0.668	1.54	1.03	1.72
5.0	5.0	1.56	1.065	1.721	1.56	1.07	1.72
5.0	10.0	3.03	2.16	3.53	1.52	1.08	1.77
160.0	50.0	0.495	0.355	0.611	1.58	1.14	1.96
160.0	100.0	0.971	0.689	1.11	1.55	1.10	1.78
160.0	200.0	2.00	1.42	2.31	1.60	1.14	1.85
160.0	300.0	3.00	2.15	3.68	1.60	1.15	1.96
				Average	1.56	1.11	1.83
				SD	0.041	0.074	0.097

[a] Area of drug/area of internal standard. [b] Weight of drug/weight of internal standard. [c] Linear regression line: $y = 0.320x - 0.144$, $r = 1.000$. [d] For linear regression analysis, the peak area ratios obtained using the 160-μg/ml internal standard were corrected to an internal standard concentration of 5 μg/ml by a factor of 32. [e] Linear regression line: $y = 0.229x - 0.141$, $r = 0.9999$. [f] Linear regression line: $y = 0.386x - 0.524$, $r = 0.9992$.

reported (13). This paper describes a simple and fast HPLC method for the simultaneous determination of salicylic acid, aspirin, and salicyluric acid in plasma, using only small quantities of samples.

EXPERIMENTAL

Reagents—Benzene[1], ethyl acetate[1], acetonitrile[2], phosphoric acid[1], phthalic acid[1], aspirin USP[3], salicylic acid USP[4], and salicyluric acid[5] were used.

Apparatus—A high-pressure liquid chromatograph, equipped with a column oven[6] and a valve injector[7], was employed. A microparticulate reversed-phase column[8] and a variable wavelength spectrophotometer[9] were used for separation and detection. A potentiometric recorder[10] and an integrator[11] were used for chromatogram recording and quantitation of the peak areas.

Procedures—An aliquot of 0.1 ml of plasma in a 13 × 100-mm conical tube was spiked with phthalic acid (160 or 5 μg/ml as the internal standard) and acidified with 1 drop of 85% phosphoric acid. The mixture was vortexed with 0.5 ml of benzene–ethyl acetate (1:1 v/v) for 30 sec and centrifuged for 1 min at about 2000 rpm. The organic layer was aspirated into another tube, and the solvent was evaporated under a stream of air or nitrogen while the tube was placed in an ice water bath.

The residue was dissolved in 50 μl of the mobile phase, consisting of 30% (v/v) acetonitrile in diluted phosphoric acid (0.05%, pH 2.5 ± 0.1), and 45 μl of the solution was injected into the column through the injector. The mobile phase flow rate was 1 ml/min. The quantitation was achieved by monitoring the UV absorbance of the column effluent at 237 nm. The column, used for all separations, was housed in an oven maintained at 50°. The ratios of the peak areas of the sample components to those of the internal standard were used to calculate concentrations of each component, based on calibration curves prepared from spiked plasma for the individual components.

To show the applicability of the proposed method to determine concentrations of aspirin and the two metabolites after dosing, a series of plasma samples from a 4-kg albino rabbit, treated intravenously with 200 mg of aspirin in 5 ml of polyethylene glycol 400 and saline solution, was analyzed similarly.

RESULTS AND DISCUSSION

It is difficult to measure accurately small concentrations of aspirin in the presence of large concentrations of salicylic acid in the same plasma sample (14). This difficulty can be overcome by the present HPLC method. Figure 1 shows the typical chromatograms of phthalic acid, salicyluric acid, aspirin, and salicylic acid from human and rabbit plasma samples. The retention times of these compounds were 3.7, 4.6, 5.3, and 7 min, respectively.

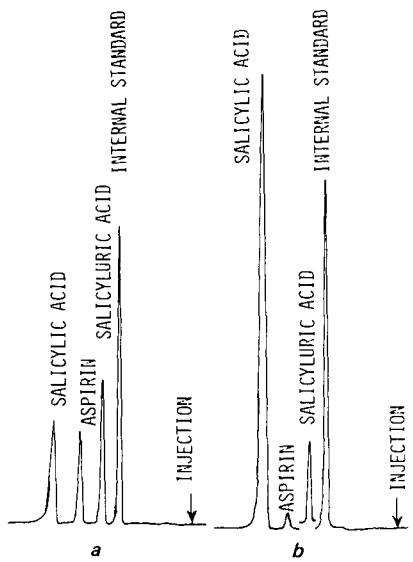

Figure 1—*Chromatograms of salicylates (5 mg %) in spiked human plasma (a) and in rabbit plasma samples at 30 min after administration of 50 mg of aspirin/kg iv (b). The salicyluric acid in* b *was recorded at 10 times greater sensitivity.*

The complete separation of the components in the chromatograms allows accurate measurements of a small quantity of aspirin or salicyluric acid in the presence of a large quantity of salicylic acid. The column separations were carried out at an elevated temperature to shorten the retention times and to increase the resolution. The chromatograms of human and rabbit plasma blanks showed no peaks that would interfere with the assay. The blank value was essentially zero by this method as compared to the high and variable blank values by spectrophotometric and fluorometric methods (7).

Table I illustrates the linearity of the calibration curves for aspirin, salicylic acid, and salicyluric acid in plasma in concentrations ranging from 0.5 to 300 μg/ml. The corrected response factors (peak area ratio/weight ratio) were fairly constant for the salicylates at all concentrations studied, indicating good linearity. Two concentrations of internal stan-

Table II—Assay Precision and Reproducibility

Compound	Concentration in Plasma, μg/ml	*n*	Concentration Found, μg/ml		*CV*, %
			Mean ± *SD*	Range	
Salicyluric acid	50.0	10	50.27 ± 0.93	49.02–51.96	1.85
	200.0	6	200.7 ± 6.69	196.08–203.73	3.33
Aspirin	50.0	10	49.98 ± 0.64	47.62–52.34	1.28
	200.0	6	201.36 ± 6.38	191.84–209.52	3.17
Salicylic acid	100.00	2	98.69 ± 1.86	97.37–100.00	1.88
	200.00	6	201.32 ± 7.70	190.35–210.52	3.82

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Burdick & Jackson Laboratories, Muskegan, Mich.
[3] J.T. Baker Chemical Co., Phillipsburg, N.J.
[4] Merck & Co., West Point, Pa.
[5] Fox Chemical Co., Los Angeles, Calif.
[6] Model 601, Perkin-Elmer Corp., Norwalk, Conn.
[7] Glenco Scientific, Houston, Tex.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Model LC-55, Perkin-Elmer Corp.
[10] Laboratory Data Control, Riviera Beach, Fla.
[11] Minigrator, Spectra-Physics, Santa Clara, Calif.

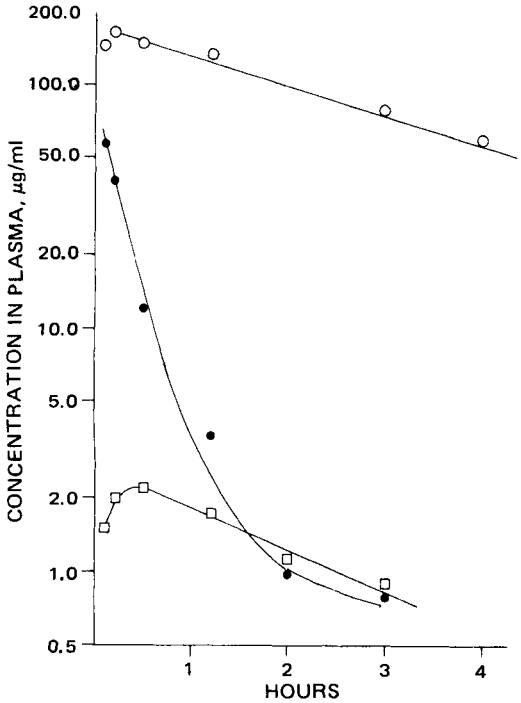

Figure 2—*Concentrations of aspirin (●), salicylic acid (○), and salicyluric acid (□) in rabbit plasma after administration of 50 mg of aspirin/kg iv.*

dard were used to improve accuracy. For least-squares linear regression analysis, the peak area ratios obtained using the 160-μg/ml internal standard were corrected to an internal standard concentration of 5 μg/ml by multiplying by a factor of 32. The assay precision and reproducibility are summarized in Table II. The coefficient of variation of these results was less than 4% in all concentrations investigated.

The time course of the concentrations of salicylic acid, aspirin, and salicyluric acid in plasma of the rabbit treated with 50 mg of aspirin/kg iv is shown in Fig. 2. The aspirin concentrations declined very rapidly in the first 2 hr. The salicylic acid concentrations were very high in the plasma shortly after drug administration and declined exponentially with a half-life of 2.42 hr. The salicyluric acid levels were relatively low, and the decay curve 0.5 hr after dosing appeared to be parallel to that of salicylic acid. No special effort was made to prevent aspirin hydrolysis, so the plasma concentration–time profiles might be different when such precautions are taken (7). These results indicate the feasibility of accurate assay of a small quantity of aspirin and salicyluric acid in the presence of a large quantity of salicylic acid.

The present method employed a mixture of ethyl acetate and benzene for extraction of the drug and internal standard from the acidified plasma samples. This solvent combination gave good recovery of these compounds (about 89–94%) while yielding essentially no interference from the plasma. The other solvent, such as ether, gave better recovery of these compounds but also extracted much material from the plasma that would interfere with the assay. The acidification of the plasma samples enhanced the recovery by solvent extraction because of the acidic nature of these compounds (13).

When the ethyl acetate–benzene extracts of the plasma samples were evaporated at ambient or higher temperature, erratic results, usually underestimations, were often obtained for salicylic acid. However, the results for salicylic acid became reproducible when the evaporation was carried out at lower temperatures by cooling the tubes in an ice water bath. Therefore, the erratic results were probably due to salicylic acid loss through sublimation. Although salicylic acid sublimes at 76° (15), salicylic acid sublimation during analysis manipulation has not been reported. Aspirin, salicyluric acid, and phthalic acid did not appear to sublime.

By simple solvent extraction on small samples, this method offers high sensitivity with essentially zero blank values. The capability to separate and measure salicyluric acid should make this method applicable also for salicyluric acid (a major metabolite) assay in urine samples. With 0.1-ml plasma samples, the present HPLC method can accurately and simultaneously measure salicyluric acid, salicylic acid, and aspirin in the same sample to concentrations as low as 0.5 μg/ml. The proposed method also can be used in pharmacokinetic and/or plasma level monitoring studies of aspirin or salicylates.

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