Table IV—Predicted (Extrapolated) and Experimental (Actual) Degradation Rate Constants at 25° for Formulations I–IV

Formulation	Degradation Rate Constants, $k \times 10^{-4}$	
	Experimental[a]	Predicted
I	0.28	0.29
II	0.54	0.58
III	0.72	0.79
IV	3.49	2.33

[a] Ambient room temperature, 25 ± 2°.

responded with the slight increases in pH on dilution with water (Table III).

A fair correlation was evident between the predicted and experimental degradation rate constants, $k_{25°}$ (Table IV), thus supporting the predicted stability values (Table II). Preliminary IR studies showed a carbonyl band shift from 1595 cm^{-1} of L-methionine[7], where no bonding takes place, to 1640 cm^{-1} when the amino acid was dissolved in propylene glycol, indicating weak hydrogen bonding. Similarly, weak hydrogen bonding was indicated between aspirin and propylene glycol because of a slight carbonyl band shift to a higher frequency.

[7] Kaydol, Ruger Chemical Co., Irvington, N.J.

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Radiochemical Plasma Salicylamide Assay Using Ring-Labeled Tritiated Salicylamide

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Abstract □ A rat plasma salicylamide assay was developed using ring-labeled tritiated salicylamide, synthesized by reacting salicylamide with tritium oxide in the presence of heptafluorobutyric acid. The reaction yielded ^3H-salicylamide of specific activity up to 8.41 mCi/mmole, 60% yield. Plasma containing ^3H-salicylamide and its metabolites was extracted with a toluene-based scintillation fluid, which was subsequently counted. Specificity for free salicylamide was demonstrated by radiochemical and standard fluorescence plasma salicylamide level–time curves. Specificity resulted from nonextraction of the salicylamide sulfate and glucuronide metabolites. Sulfatase and β-glucuronidase treatment allowed the analysis of plasma sulfate and glucuronide conjugates as free salicylamide. This procedure should be effective for the analysis of salicylamide and its metabolites in the presence of similar phenolic compounds.

Keyphrases □ Salicylamide—radiochemical analysis, from plasma, compared to fluorescence assay, rats □ Radiochemistry—analysis, salicylamide in plasma, compared to fluorescence assay, rats □ Fluorometry—analysis, salicylamide in plasma, compared to radiochemical assay, rats

In a study of the effects of alternate substrates on their ability to block temporarily the first-pass metabolism (1, 2) of phenolic drugs, the oral administration of salicylamide to rats was chosen as a suitable animal model because of the similarity of salicylamide's metabolic behavior in rats (3–7) and humans (8, 9). Numerous assays for salicylamide have been proposed (9–11). The fluorometric assay of Barr and Riegelman (9) is the most extensively used procedure for the analysis of salicylamide and its metabolites in biological fluids. This sensitive and specific fluorometric assay, along with a few modifications, has been the assay of choice in numerous studies (3, 9, 12).

Since the long-term objective of this project was to screen the effects of various alternate substrates on their ability to block temporarily the sulfation–glucuronidation first-pass effect of a model phenolic compound, salicylamide, many of the alternate substrates would most likely be other phenolic compounds. Some of these phenolic alternate substrates have physical, chemical, and fluorescense properties so similar to salicylamide that they interfere with the fluorometric procedure (9). For this reason, an assay for salicylamide and its metabolites was developed using ring-labeled tritiated salicylamide. The assay selectivity was due to the separation of salicylamide from its sulfate and glucuronide metabolites by a relatively nonpolar organic solvent.

EXPERIMENTAL

Reagents and Materials—All reagents were analytical grade, and all aqueous solutions were prepared using glass-distilled, deionized water. The scintillation fluid was prepared by stirring overnight 1000 ml of toluene[1] with 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene[2] and 4 g of 2,5-diphenyloxazole[2].

[1] Scintillation grade, Research Products International Corp., Grove Village, Ill.

[2] Amersham/Searle Corp., Arlington Heights, Ill.

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Tritiated Salicylamide Synthesis—A screw-capped Pyrex glass tube containing 300 mg of salicylamide[3], 20 μl of tritium oxide[4], and 200 μl of heptafluorobutyric acid[5] was maintained in a sealed metal container at 200–210° for 4 days. After being cooled to room temperature, the solidified contents were dissolved in a minimal amount of 0.25 *N* NaOH (~15 ml).

The solution pH was adjusted to 6 with 1 *N* HCl, and this solution was then extracted with 30 ml of ethyl acetate. The ethyl acetate layer was separated, washed twice with 30 ml of saturated sodium bicarbonate solution, and dried over anhydrous magnesium sulfate. The ethyl acetate was evaporated on a rotary evaporator[6].

The solid white material obtained was purified by vacuum sublimation at 10 mm Hg and 118–122°. The isolated salicylamide specific activity varied from 5.24 to 8.41 mCi/mmole. Labile tritium was assayed by standard methods and was negligible[7]. The radiochemical purity was determined by TLC[8]. Chemical purity, checked qualitatively by melting points and quantitatively by fluorescence spectrophotometry, was 99–99.5%. The ^{3}H-salicylamide final yield was 180–200 mg.

Animal Studies—Sixteen male Sprague–Dawley rats[9] (250–275 g) were used. The rats were fed standard rat chow and water *ad libitum*. Prior to oral salicylamide administration, the rats were fasted overnight.

The oral salicylamide dose, 500 mg/kg, was prepared by dissolving the appropriate salicylamide and ^{3}H-salicylamide amounts in 1 *N* NaOH[10] and adjusting the pH to 10 with dropwise addition of 1 *N* HCl. For example, a 250-g rat received 4.2 mg of ^{3}H-salicylamide (8.4 mCi/mmole) and 120.8 mg of salicylamide. This aqueous salicylamide solution (1.5–2 ml depending on the weight of the animal) was administered orally by intubation. After drug administration, the animals were placed in metabolism cages.

Five minutes prior to predetermined times (15, 30, 60, or 90 min postdosing), each rat was anesthetized with ether, and 5-ml blood samples were removed from the inferior vena cava (after an abdominal incision) into an edetate sodium pretreated syringe. After centrifugation, duplicate 50-μl plasma samples were assayed for free salicylamide, salicylamide sulfate, and salicylamide glucuronide by the fluorescence and radiochemical assays.

Radiochemical Assay—Free plasma salicylamide was determined by adding 100 μl of a pH 7.0 phosphate buffer (0.2 *M*) to 50 μl of plasma in a 50-ml screw-capped centrifuge tube[11] and extracting with 16 ml of the toluene-based scintillation fluid. After agitation for 2 min and centrifugation, 15 ml of the upper toluene layer was transferred by disposable pipet to a 20-ml scintillation vial[12]. The vial was stored in a dark place for 12–24 hr prior to counting.

Radioactivity was determined by liquid scintillation counting[13] using the ^{3}H optimized window. A blank was prepared from predosed tail vein plasma. Free salicylamide was determined from a previously prepared plasma standard curve. Correction for counting efficiency was not necessary because the external standard channel ratio remained essentially constant and equal for the standard curve and the measured plasma samples.

Plasma salicylamide glucuronide was determined as free salicylamide obtained after salicylamide glucuronide hydrolysis with β-glucuronidase[14]. β-Glucuronidase (25 μl, 125 units) and 0.5 ml of 0.1 *M* pH 5.0 acetate buffer were added to the 50-μl plasma sample and incubated in a constant-temperature shaking water bath[15] at 37° for 16 hr. The incubation mixture was assayed for free salicylamide as described.

Salicylamide sulfate was determined as free salicylamide after hydrolysis of a 50-μl plasma sample with sulfatase (175 units), β-glucuronidase (875 units)[16], and 100 μl of 0.1 *M* pH 5.2 acetate buffer. Salicylamide glucuronide and salicylamide sulfate concentrations were calculated as described previously (5).

Fluorometric Assay—The salicylamide fluorometric assay was described previously (5, 9, 12). The plasma salicylamide conjugates were determined as free salicylamide after sulfatase and β-glucuronidase treatment.

RESULTS AND DISCUSSION

The fluorometric salicylamide assay is selective for salicylamide in the presence of its metabolites and can detect salicylamide levels as low as 2 μg/ml from 50-μl plasma samples. However, this assay is prone to interferences from other fluorescent or fluorescence quenching phenolic compounds.

The two major metabolites of salicylamide are the highly polar and charged sulfate and glucuronide conjugates (5). Polarity differences between salicylamide and these metabolites make possible a specific radiochemical salicylamide assay. Similar assays were reported for other radiolabeled phenolic compounds (13, 14).

As stated under *Experimental*, ring-labeled tritiated salicylamide was prepared by the reaction of salicylamide with tritium oxide in the presence of heptafluorobutyric acid. The salicylamide hydroxy group is electron donating and tends to promote electrophilic substitution reactions such as the acid-catalyzed tritium–hydrogen exchange. The carboxamide group tends to be deactivating (electron withdrawing). For this reason, the conditions necessary for the acid-catalyzed tritium incorporation were severe and had to be optimized for this particular system. Heptafluorobutyric acid was more useful than trifluoroacetic acid because of its lower volatility (15). The conditions required for equilibrium tritium incorporation into salicylamide (ring substitution[17]) were worked out using deuterium incorporation, substituting deuterium oxide for tritium oxide. Deuterium incorporation was followed by mass spectral analysis of the P, P + 1, and P + 2 peaks. The high temperature and extended heating period finally used were necessary for maximum tritium incorporation with minimal product decomposition.

The white crystalline material obtained from the final sublimation showed only one spot, corresponding to salicylamide, by TLC. Spectrofluorometric analysis showed the product to be >99–99.5% pure. It was necessary, for maximum purification, to collect small fractions (~50 mg) of the sublimed material from the sublimation apparatus cold finger to prevent excess accumulation, which produced material that was only ~90% pure. This enclosed sublimation system was found to be the most efficient both in terms of the purification and recovery of ^{3}H-salicylamide. Once the synthesis procedure was standardized, ^{3}H-salicylamide of >99% purity and 5.24–8.4 mCi/mmole specific activity was consistently obtained.

The radiochemical assay for free salicylamide involved a one-step extraction. The detection limit (100 cpm above background) was 0.002 μg (0.04 μg/ml from 50-μl plasma samples)[18]. No chemiluminescence problems occurred, although routinely the samples were stored in the dark for 12–24 hr prior to counting. The overall mean ± *SD* (3.1–25 μg/ml, n = 17) toluene-based scintillation fluid ^{3}H-salicylamide extraction efficiency was 89.7 ± 0.8% (3.1 μg/ml, n = 6, 89.9 ± 0.7%; 6.25 μg/ml, n = 3, 89.5 ± 1.1%; 12.5 μg/ml, n = 4, 89.1 ± 0.5%; 25 μg/ml, n = 4, 90.1 ± 0.7%).

Salicylamide (^{3}H-salicylamide to salicylamide ratio of 1:29) was administered at 500 mg/kg to rats as an oral solution (pH ~10) to minimize problems associated with administering different crystalline forms and particle sizes. In initial studies, salicylamide in suspension in methylcellulose gave spurious results because the larger ^{3}H-salicylamide crystals dissolved more slowly than the salicylamide powder in the rat GI tract.

The fluorescent and radiochemical plasma level–time curves for salicylamide and its major metabolites are shown in Fig. 1. Each point is the mean of four animals. The areas under the curve for the fluorometric *versus* radiochemical assay (in microgram minutes per milliliter) calculated *via* the trapezoidal method for 0–90 min were 4363 and 4512 for free salicylamide, 1548 and 1570 for salicylamide sulfate, and 1405 and 1425 for salicylamide glucuronide, respectively. Plots of plasma salicylamide,

[3] Eastman Kodak Co., Rochester, N.Y.
[4] Specific activity of 5 Ci/ml, Amersham/Searle Corp., Arlington Heights, Ill.
[5] Aldrich Chemical Co., Milwaukee, Wis.
[6] Buchi Rotavapor, Brinkmann Instruments, Westbury, N.Y.
[7] Approximately 5 mg of ^{3}H-salicylamide was dissolved in 10 ml of methanol and allowed to stand for 2 hr. The methanol was evaporated on a rotary evaporator, and the total number of counts in the collected methanol was determined. Labile tritium represented <0.01% of total counts per minute. Repetition of this procedure showed no further labile tritium.
[8] Run on Polygram Sil G precoated plates for TLC, Brinkmann Instruments, Westbury, N.Y. Solvents were ethyl acetate–ammonium hydroxide (9:1, R_f 0.47) and isopropanol–chloroform–ammonium hydroxide (3:1.5:0.5, R_f 0.58).
[9] ARA/Sprague–Dawley, Madison, Wis.
[10] Fisher Scientific Co., Fair Lawn, N.J.
[11] Pyrex tubes 8062.
[12] Scientific Products, Kansas City, Mo.
[13] Beckman LS-3133T, Fullerton, Calif.
[14] Glucurase, β-glucuronidase solution from bovine liver, Sigma Chemical Co., St. Louis, Mo.
[15] American Optical Corp., Buffalo, N.Y.
[16] Glusulase, Endo Laboratories, Garden City, N.Y.
[17] Ring substitution is assumed because tritium incorporated in the hydroxyl group and carboxamide group readily exchanged. The only position for stable tritium to be incorporated into the molecule is on the phenyl ring.
[18] This limit is dependent on the specific activity of the ^{3}H-salicylamide batch.

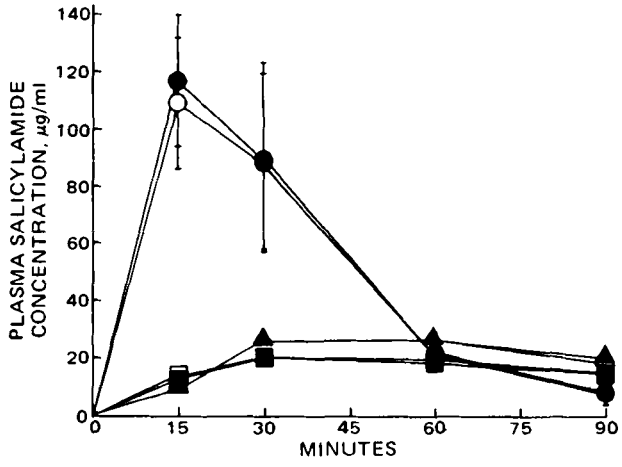

Figure 1—*Plasma concentration* ± SEM versus *time profiles for salicylamide (○, fluorescence assay; ●, radiochemical assay), salicylamide sulfate (□, fluorescence assay; ■, radiochemical assay), and salicylamide glucuronide (△, fluorescence assay; ▲, radiochemical assay) after oral administration of 500 mg of salicylamide/kg to rats. Each point is the mean of four animals. If no error bars are noted, the standard error of the mean was within the size of the symbol.*

salicylamide sulfate, and salicylamide glucuronide assayed radiochemically *versus* levels determined fluorometrically had slopes (±*SEM*) of 1.06 ± 0.02 (r = 0.99), 1.11 ± 0.14 (r = 0.81), and 0.96 ± 0.07 (r = 0.98), respectively. An overall plot combining all 48 data points gave a 1.06 ± 0.01 slope (r = 0.99). The new assay gives ~6% higher plasma salicylamide analysis than does the fluorescence assay, probably due to a slight total plasma radioactivity carryover into the extraction medium. The poorer correlation coefficients for plasma sulfate and glucuronide analyses were probably due to concentration calculation by differences (5) rather than to errors in the assay.

The radiochemical assay gave levels of salicylamide and its conjugates that were statistically equivalent ($p < 0.05$) to those determined by the fluorescence assay (Fig. 1). The large standard errors for the salicylamide levels at 15 and 30 min (Fig. 1) were due to animal differences and not to assay variations. The plasma free salicylamide levels generated in this study were considerably higher than the free salicylamide levels reported previously (3) for rats administered 500 mg of salicylamide suspension/kg. Reported glucuronide levels were larger than those generated in this study. These differences are probably attributable to the more efficient metabolism of salicylamide from the suspension compared to the solution. Similar effects were seen in a human study (12), which showed that a sodium salicylamide solution produced significantly higher free salicylamide bioavailability than salicylamide tablets. These differences were due to more efficient first-pass salicylamide metabolism from the slowly dissolving tablets. Sodium salicylamide partially saturated the enzymes responsible for the first-pass effect, which allowed more unmetabolized drug to enter the general circulation (12).

In conclusion, the advantages of the radiochemical assay just described are: (*a*) its ability to determine specifically salicylamide and its conjugates in the presence of other phenolic substances capable of exhibiting fluorescence or of quenching the fluorescence of salicylamide and (*b*) the low level of detection it can afford. This last point, of course, will be dependent on the specific activity of ^{3}H-salicylamide that can be synthesized. Another important advantage is that once the salicylamide has been extracted into the scintillant fluid, the vials can be counted at the investigator's convenience. In the fluorometric assay, the fluoresence intensity of extracted salicylamide (in 0.25 *N* NaOH) did deteriorate with time. The single-step extraction directly into the scintillant fluid used in the radiochemical assay increased its ruggedness by minimizing technical errors while speeding up the analysis. Balanced against these advantages were the problems of synthesis and handling of the ^{3}H-salicylamide.

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