drug causing the elevated tail to lay flat in 50% of the animals) were obtained by the method of Litchfield and Wilcoxon (10).

LD_{50} in Mice—The test compounds were administered perorally in 0.5% methylcellulose to groups of unfasted male mice[3] (n = 10/group). The animals were held in cages for 72 hr, and the deaths were recorded. The LD_{50} values (drug dose causing death in 50% of the animals) were calculated using the method of Litchfield and Wilcoxon (10).

RESULTS AND DISCUSSION

The synthesized compounds (6) exhibited a better absorption and solubility profile than the parent compound, dantrolene. Eleven compounds were synthesized and evaluated for skeletal muscle relaxant activity (Table II). In the pithed rat gastrocnemius muscle preparation, all compounds were effective intravenous skeletal muscle relaxants (>50% inhibition of the electrically induced twitch contraction) (Table II).

Compounds III*a* and III*f* were also potent inhibitors of the gastrocnemius twitch tension. These compounds were the most effective in that the maximal inhibition of the twitch response was 86–87% whereas dantrolene sodium and clodanolene sodium only yielded a maximal inhibition of 80%.

Gross observation in mice identified nine of the 11 compounds as orally effective muscle relaxants. Among these nine (muscle relaxant score of >1 at doses up to 800 mg/kg), III*a* and III*e* were the most effective, producing muscle relaxant scores of 4. Compounds III*b*, III*g*, III*j*, and III*k* were slightly less effective, producing muscle relaxant scores of 3; III*b* produced a 3 level of muscle relaxation at 200 mg/kg.

Lowering of the Straub tail previously was shown (11) to result from relaxation of the sacrococcygeal muscles at the base of the tail. Several different drugs can produce this muscle relaxation (11). However, the muscle relaxant properties of a drug are useful only when they can be separated in a dose-dependent manner from its neurotoxic properties (motor incoordination). Motor incoordination is an unacceptable consequence of skeletal muscle relaxant therapy and, therefore, is a limiting factor in determining muscle relaxant efficacy. In the Straub tail model, the muscle relaxant dose is identified as St ED_{50}; the motor incoordination dose is identified with the rotarod test (Rr ED_{50}). Comparing these two doses as a ratio, ED_{50} rotarod:ED_{50} Straub tail, gives an indication of the separation between the two dose ranges (*i.e.*, the greater the ratio, the more separation between the two doses).

Compound III*a* yielded a muscle relaxant efficacy index of 2.0, and all other compounds produced muscle relaxant efficacy indexes of <1.0. Additionally, when III*a* was evaluated for acute toxicity, no deaths were seen up to 4000 mg/kg, and the resultant therapeutic index was >225.

The difference in muscle relaxant efficacy indexes between III*a* and other muscle relaxants of this type (dantrolene sodium and clodanolene sodium), including the compounds in Table II, is small but consistent. Compound III*a* is a potent, direct-acting skeletal muscle contraction antagonist (inhibition of the directly induced gastrocnemius twitch tension). The muscle relaxant efficacy index and acute therapeutic index values reflect a high degree of efficacy and safety.

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Formation of Methyl Ester of Salicyluric Acid during Quantitation of Salicyluric Acid in Urine by High-Pressure Liquid Chromatography

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Abstract □ The formation of the methyl ester of salicyluric acid was observed during the quantitation of salicyluric acid and other salicylate metabolites in urine by high-pressure liquid chromatography. This methyl ester formation caused artificially low values for salicyluric acid and high values for salicylic acid.

Keyphrases □ Salicyluric acid—methylation during high-pressure liquid chromatography □ Methylation—salicyluric acid in urine, high-pressure liquid chromatography □ High-pressure liquid chromatography—salicyluric acid in urine, methylation

Several high-pressure liquid chromatographic (HPLC) assays for salicylic acid and its metabolites, salicyluric acid and gentisic acid, in serum and urine were reported recently (1–8). Review of these methods indicates that certain precautions must be taken to prevent sublimation losses of salicylic acid during evaporation (6) and decom-

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position of salicyluric acid to salicylic acid by microorganisms (3, 4). There are no reports of the methylation of salicyluric acid during its quantitation in biological fluids.

In measuring salicylic acid and its metabolites in urine by HPLC, it was observed that salicyluric acid was methylated as a consequence of sample preparation. Because similar alteration of the salicyluric acid may occur in other laboratories during quantitation, the methylated product was identified by GLC–mass spectrometry. The conditions under which this product is formed and methods to prevent its formation are described.

EXPERIMENTAL

Reagents—Methanol[1], ether[2], *o*-methoxybenzoic acid[3] (*o*-anisic acid), sodium salicylate[3], salicyluric acid[3] (*o*-hydroxyhippuric acid), and gentisic acid[3] were used.

Stock standard solutions of sodium salicylate (5 mg/ml), salicyluric acid (10 mg/ml), and gentisic acid (2 mg/ml) were prepared by dissolving the compounds in small volumes of ethanol and bringing to volume with pH 7 phosphate buffer (0.1 *M*). The stock solutions were kept frozen at 0° until required. Working standards of sodium salicylate (500, 250, and 50 μg/ml), salicyluric acid (1000, 500, and 100 μg/ml), and gentisic acid (200, 100, and 20 μg/ml) were made in fresh, blank urine.

Procedures—Urine (1 ml), to which 1 ml of a standard solution of *o*-methoxybenzoic acid (100 μg/ml) in pH 7 phosphate buffer (0.1 *M*) had been added, was acidified with 0.6 *M* HCl (1 ml) and extracted into 5 ml of ether by shaking for 5 min. Following centrifugation, the aqueous layer was frozen in acetone–dry ice, and the ether layer was collected. The ether was evaporated to dryness at 10° with air or nitrogen. The residue was resuspended in 0.75 ml of anhydrous methanol or a mixture of 85% water containing 1.6% acetic acid and 15% methanol. Samples were placed in 1-ml capped vials[4] and injected into the HPLC system *via* an automatic sampler[5] fitted with a 20-μl injection loop. The flush time was 30 sec, and the run time was 20 min. A 15-cm × 4.6-mm i.d. reversed-phase column[6] and a 5-cm × 2.1-mm i.d. precolumn[7] were used to separate salicylic acid and its metabolites. The mobile phase of 85% water (containing 1.6% acetic acid) and 15% methanol was pumped[8] at 2.5 ml/min. Peaks were detected by a UV detector[9] (310 nm) coupled to a recorder[10] and peak integrator[11].

To determine the conditions under which salicyluric acid was altered, salicyluric acid dissolved in water was taken through the described sample preparation but with the following changes: (*a*) the evaporated ether extract was dissolved in methanol for up to 3 weeks before injection onto the column; (*b*) the ether extract was blown to dryness with air or nitrogen; and (*c*) the residue following ether evaporation was resuspended either in methanol or 85% water (containing 1.6% acetic acid) and 15% methanol.

Aliquots of water and urine samples were extracted with ether in the presence and absence of hydrochloric acid, and the ether extracts were added to test tubes containing salicyluric acid prior to drying with nitrogen or air. The resulting residues then were redissolved in methanol and allowed to stand for up to 2 weeks before analysis.

The structure of the altered salicyluric acid was identified by GLC and mass spectrometry following repeated collection and lyophilization of the unknown chromatographic peak. The trimethylsilyl derivative of this sample was prepared by dissolving the residue in an excess (0.5 ml) of pyridine–*N*,*O*-bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (10:10:1). The mixture then was warmed for 5 min at 90°. An aliquot of the trimethylsilyl derivative was injected onto a GLC column (2 m × 2 mm i.d.), packed with 4% OV-17 on Gas Chrom Q[12] (100–120 mesh), programmed from 80 to 300° at 10.0°/min. The altered product of salicyluric acid then was identified by mass spectrometry[13].

Preparation of the methyl esters of salicyluric acid, gentisic acid, *o*-methoxybenzoic acid, and salicylic acid was attempted by bubbling hydrogen chloride through methanolic solutions of these chemicals for 5 min. The acidified methanolic solutions then were taken to dryness, and the residues were redissolved in 1.0 ml of methanol and subjected to HPLC using the described conditions. The methyl ester of salicylic acid was not formed under these conditions; complete esterification of *o*-methoxybenzoic acid and salicyluric acid was achieved, but gentisic acid esterification was only partially complete.

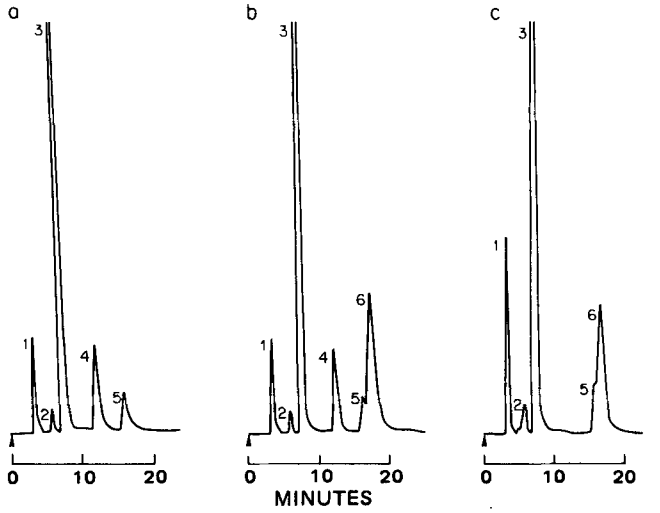


Figure 1—*Chromatograms of salicylic acid and its metabolites in urine under different experimental conditions: urine extract in methanol (a) immediately after the addition of methanol, (b) after allowing the methanolic extract to stand at room temperature for 8 hr, and (c) after the evaporation of ether with air. Key: 1, gentisic acid; 2, glucuronide of salicylic acid; 3, salicyluric acid; 4,o-methoxybenzoic acid; 5, salicylic acid; and 6, methyl salicylurate. The arrows indicate the points of injection.*

RESULTS AND DISCUSSION

Quantitation of salicylic acid and its metabolites in urine gave accurate and reproducible results. Nitrogen was used to remove the ether at 10°, and the methanolic residue was immediately injected into the chromatograph. The sensitivity of the method was 5 μg/ml for salicylic acid, 5 μg/ml for salicyluric acid, and 2 μg/ml for gentisic acid. The coefficients of variation for repeated measurements of salicylic acid at 50 μg/ml, for salicyluric acid at 100 μg/ml, and for gentisic acid at 20 μg/ml were 2.6, 0.7, and 1.5%, respectively. These figures are similar to those previously reported (1, 4).

A typical HPLC tracing is shown in Fig. 1*a*. The retention times for gentisic acid, salicyluric acid, *o*-methoxybenzoic acid, and salicylic acid were 4, 8.4, 13, and 17.5 min, respectively.

The small peak that eluted between the gentisic acid and salicyluric acid peaks was probably one of the salicyl glucuronides since it disappeared following incubation of the urine with β-glucuronidase.

Occasionally, a peak that interfered with the salicylic acid peak was observed (Figs. 1*b* and 1*c*). This effect became apparent when extracted urine samples were prepared for HPLC (by the addition of methanol) well in advance of their actual injection onto the column *via* the automatic sample injector accessory. Interference also was observed if the ethereal extracts of urine were evaporated to dryness with air. The appearance and extent of this interference were variable. Sequential HPLC analysis of the salicylic acid, salicyluric acid, and gentisic acid standards in urine or water indicated that this peak was due to changes in the salicyluric acid standard.

When the salicyluric acid standard was extracted into ether from urine or water, interference occurred under the following experimental conditions: (*a*) if air or nitrogen was used to evaporate the ether; (*b*) if methanol was used to resuspend the residue from the ether evaporation; (*c*) if the residue from the ether evaporation stood in methanol for more

[1] Burdick & Jackson Laboratories, Muskegon, Mich.
[2] Nanograde, Mallinckrodt, St. Louis, Mo.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Wheaton Scientific, Millville, N.J.
[5] Model 500, Altex Scientific, Berkeley, Calif.
[6] Ultrasphere-ODS, Altex Scientific, Berkeley, Calif.
[7] Perisorb RP-18, 30–40 μm, Merck, Darmstadt, West Germany.
[8] Model 110A, Altex Scientific, Berkeley, Calif.
[9] Model 100-10 spectrophotometer, Hitachi Scientific Instruments, Mountain View, Calif.
[10] Model 250-2, two channel, Curken Scientific, Danbury, Conn.
[11] Model 485, Varian Instrument Division, Palo Alto, Calif.
[12] Supelco Inc., Bellefonte, Pa.
[13] Model 3300 with 6000 data system, Finnigan Corp., Sunnyvale, Calif.

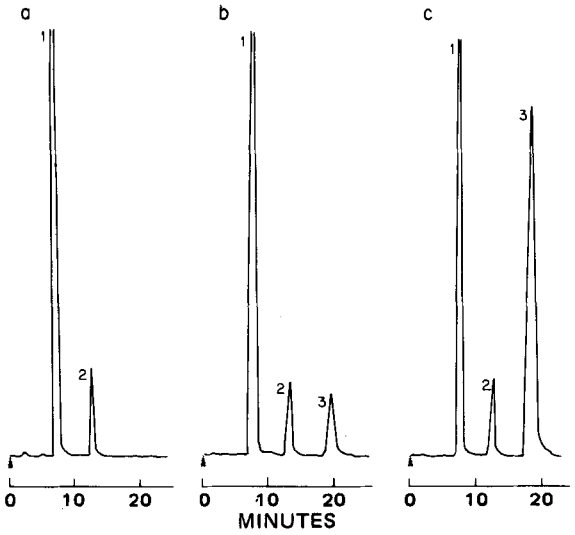


Figure 2—*Chromatograms of the salicyluric acid standard (1 mg/ml) under different experimental conditions: ether extract of salicyluric acid standard after standing in (a) 85% water containing 1.6% acetic acid–15% methanol for 10 days, (b) methanol for 6 hr, and (c) methanol for 21 days. The salicyluric acid concentration decreased 47%. Key: 1, salicyluric acid; 2, o-methoxybenzoic acid; and 3, methyl salicylurate. The arrows indicate the points of injection.*

than 2 hr; or (*d*) if hydrochloric acid was used to acidify the ester extract.

The time course and extent of changes in the standards were quite variable (Fig. 2*a*), but salicyluric acid levels decreased ~20% after the samples had stood in methanol for 6 hr (Fig. 2*b*). The maximum observed decrease in salicyluric acid levels was 47% after the salicyluric acid ether extract (1000 μg/ml) had stood in methanol for 21 days (Fig. 2*c*). However, there was no alteration of the salicyluric acid standard dissolved in methanol for up to 3 weeks.

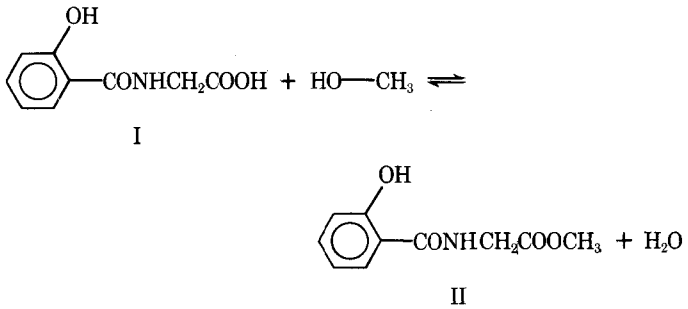
The interfering peak was collected after repeated injection and subjected to GLC–mass spectrometry for identification. Examination of the electron-impact mass spectra revealed that this peak was the methyl ester of salicyluric acid.

Like many esterification reactions, the conversion of salicyluric acid to methyl salicylurate is catalyzed by small amounts of acid. In addition, dehydration of the residue upon ether evaporation also may promote the methyl salicylurate formation. Dehydration by ether secondary to the formation of an azeotrope with water is well known. These effects favor ester formation between the carboxyl group of glycine and methanol under anhydrous conditions (Scheme I). In the ether extract, methanol is in large excess and the equilibrium is driven to the right (*i.e.*, methyl salicylurate is formed).

The formation of the ester can be prevented by adding water (which shifts the equilibrium to the left) or by adding another carbonyl group (*e.g.*, acetic acid), which competes with salicyluric acid for ester formation. Use of nitrogen gas and a limited time of exposure of salicyluric acid to methanol also help to control the ester formation with salicyluric acid.

Alternatively, salicyluric acid esterification can be circumvented if diluted urine (after centrifugation and filtration) is injected directly onto the column. However, with the HPLC column and solvent system employed, resolution of the gentisic acid peak from other endogenous substances in urine was not satisfactory without extraction.

Although some previous reports discussed precautions to prevent sublimation losses of salicylic acid and decomposition of salicyluric acid to salicylic acid by microorganisms (3, 6), there are no reports on the formation of methyl ester of salicyluric acid.



Scheme I—Mechanism of the formation of methyl salicylurate (II) from salicyluric acid (I).

One method (1) employed ether for urine extraction, but the ethereal residue was dissolved in methanol–water–acetic acid (50:60:1), which inhibits formation of methyl salicylurate.

Esterification of gentisic acid, salicyluric acid, and *o*-methoxybenzoic acid occurred when hydrogen chloride gas was bubbled through methanolic solutions of these compounds. The retention times of these esters were 17.3, 17.4, and 41.4 min, respectively. The methyl ester of salicylic acid was not formed under these conditions.

However, the absence of interference peaks other than the peak corresponding to methyl salicylurate and the lack of change of the gentisic acid and salicylic acid standards during the ethereal extraction of standards in urine or water indicate that esterification of gentisic acid and *o*-methoxybenzoic acid did not occur.

It is obvious from the chromatograms in which the formation of methyl salicylurate was not inhibited (Figs. 1*b* and 1*c*) that an underestimation of salicyluric acid and an overestimation of salicylic acid are possible since large amounts of methyl salicylurate can appear as a single peak of salicylic acid.

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