Table VII—Percent of Lots Tested that Fail Acceptance Criteria for ≥10- and ≥25-μm Particle Ranges Simultaneously

Product Type	UQL, %	Maximum Allowable Particle Count[a] LVP	C_1	C_2
Aqueous solution	5	62	8	4
	10	58	6	4
	25	37	4	2
Freeze-dried product	5	71	29	13
	10	61	23	10
	25	42	6	3
Oil solution	5	100	13	7
	10	93	0	0
	25	67	0	0
Average	5	70	15	7
	10	64	10	7
	25	43	4	2

[a] LVP = NMT 50 ≥10-μm and NMT 5 ≥25-μm particles, C_1 = NMR 200 ≥10-μm and NMT 50 ≥25-μm particles, and C_2 = NMT 420 ≥10-μm and NMT 230 ≥25-μm particles.

for the ≥10- and ≥25-μm ranges, respectively. Table VII presents the overall rejection rate when both particulate ranges are considered simultaneously. The overall reject rates for product samples at or beyond their expiration date are not significantly different from those reported in Table VII. Thus, the direct application of the USP large-volume parenteral limits to the present data resulted in an overall rejection rate of 43–70%, depending upon the UQL. If the present data are indeed representative of the current industrial technology, adoption of the large-volume parenteral limits for small-volume parenterals would cause extreme difficulty.

Guidelines in Establishing Small-Volume Parenterals—The medical consequences of subvisual-size particulate matter in parenteral formulations are believed to be dependent on the total number and nature of particles that a patient receives from injectables. The standard criteria for particulate matter in a small-volume parenteral could be established based on the concept of the maximum injectable dose.

The USP requirement for particulate matter in large-volume parenterals allows up to 50 and 5 particles for the ≥10- and ≥25-μm size ranges, respectively. Therefore, a patient could receive up to 5000 particles ≥10 μm and 500 particles ≥25 μm from a dose of a 100-ml large-volume parenteral (the minimum size for large-volume parenterals). Infusion from 1 liter of parenteral solution could subject a patient with as many as 50,000 and 5000 particles in the respective size ranges. Therefore, any proposal limiting the particulate matter in small-volume parenteral products could be established based on the concept of the maximum injectable dose and on the statistical acceptance criteria.

This paper represents only the beginning of an evaluation of the quantitative aspects of particulate level methodology. More quantitative data together with the investigation of large numbers of lots and wide varieties of products are needed prior to the establishment of particulate limits in small-volume parenterals. In view of the inevitability that particles of various sizes will be generated by manipulations necessary prior to injection, *e.g.*, breaking a glass ampul and piercing a rubber septum, an in-line final filter is recommended as an efficient means of eliminating particulate introduction into a patient.

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Chemical Constituents of Gentianaceae XXIII: Tetraoxygenated and Pentaoxygenated Xanthones and Xanthone *O*-Glucosides of *Swertia angustifolia* Buch.-Ham.

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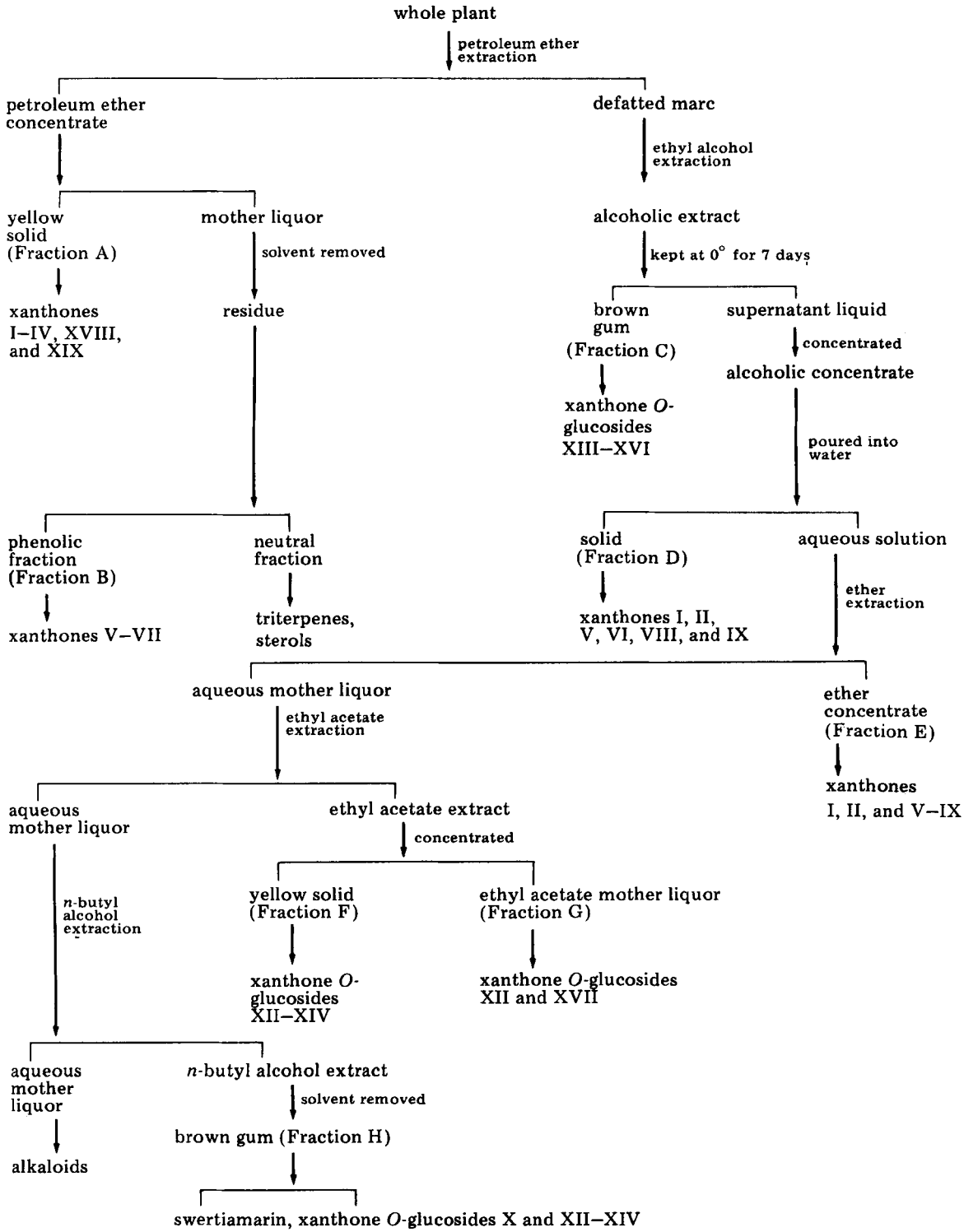
Abstract □ The whole plant extract of *Swertia angustifolia* Buch.-Ham., collected at different stages of growth, contained 14 tetraoxygenated and five pentaoxygenated xanthones and xanthone 1-*O*-glucosides. Of the eight xanthone 1-*O*-glucosides isolated, five were previously unreported in nature. The xanthones are broadly based on 1,3,5,8- and 1,3,7,8-oxygenated systems, with an added oxygen function at C-4 in some compounds, and represent a number of methoxylated patterns. The content and relative abundance of the free xanthones and their 1-*O*-glucosides changed with plant growth. These results are the first demonstration of the variation in chemical characters in the different parts of a *Swertia* species during its ontogeny. The biological significance of these results is appraised.

Keyphrases □ Xanthones and *O*-glucosides—isolated and identified in *Swertia angustifolia*, whole plant extract, various growth stages compared □ *Swertia angustifolia*—whole plant extract, various xanthones and *O*-glucosides isolated and identified, various growth stages compared

Swertia angustifolia (var. *angustifolia*) Buch.-Ham., native to the subtropical Himalayas from the Chenub to Bhutan, 304.8–1828.8 m (1000–6000 ft), is a small flowering species. It is used as a substitute for the Indian pharmacopeial drug *S. chirata* Buch.-Ham. Extracts of this plant are used as a bitter tonic, as a febrifuge, in epilepsy, and



Scheme I

in certain mental disorders (1).

Work in this laboratory previously demonstrated the chemotaxonomic significance of the xanthonic constituents of members of the genus *Swertia* and of related genera (2–4). The present investigation, with previously unexplored *S. angustifolia*, complements the earlier studies. Additionally, it provides information concerning the content and relative abundance of the free xanthones and xanthone *O*-glucosides in the different parts of this species during its vegetation.

EXPERIMENTAL[1]

Extraction of *S. angustifolia*[2]—The isolation of xanthones and xanthone *O*-glucosides from the whole plant, harvested at three different stages of growth (1–2 weeks, 4–6 weeks, and with fruits), was accomplished following the general procedure shown in Scheme I. The changes

[1] The general directions were reported previously (5).

[2] The plant materials were collected by Mr. V. K. Lal, Central Council of Research in Indian Medicine and Homeopathy, Ranikhet, Almora, India. Voucher specimens are available at the Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Banaras Hindu University, Varanasi-5, India.

in the content and patterns of the xanthones are recorded in Table I.

In a typical experiment, dried and milled aerial parts with fruits (about 200 g) were batch extracted, at ordinary temperature, in a percolator with petroleum ether (bp 60–80°) and then with ethyl alcohol (4 weeks each). The two extracts were separately processed.

Isolation of Xanthones from Petroleum Extract—The petroleum ether extract was concentrated (about 100 ml), and the concentrate was kept at room temperature (24 ± 8°) overnight. A yellow solid (1.5 g, Fraction A) separated. The solid was collected by filtration, and the mother liquor was evaporated to dryness (2.2 g). A portion (0.2 g) of this residue was dissolved in ether, and the phenolic and nonphenolic constitutents were separated by extraction with an aqueous solution of sodium hydroxide (2%, 3 × 30 ml). The alkaline solution after the usual workup afforded a brown solid (24 mg, Fraction B).

The neutral fraction was column chromatographed according to a previously described procedure (6). TLC and mass spectrometry of the eluted fractions suggested the presence of *n*-alkanes (C_{27}–C_{31}), alkanols (C_{26}–C_{32}), phytosterols (a typical mixture of sitosterol, stigmasterol, and campesterol), and two triterpenes (friedelin and β-amyrin).

Separation of Xanthones from Fraction A—A portion of the solid (0.15 g) was mixed with silica gel[3] (about 1 g) and placed in a 25 × 1.8-cm silica gel column. Elution was carried out with petroleum ether (1 liter), benzene (2 liters), chloroform (1 liter), and chloroform–methanol (90:10, 1 liter). Fractions (500 ml) were collected. The presence of xanthonic constituents in the eluates was monitored by analytical TLC, using silica gel G[4] as the adsorbent.

The petroleum ether eluates gave only a small quantity of an intractable gum, and the gum was not processed further.

Xanthone I (1,5,8-Trihydroxy-3-methoxyxanthone)—The first benzene fraction was concentrated, and the concentrate was subjected to preparative TLC on silica gel G[4] plates (2-mm thickness), developed with benzene–acetic acid (100:2). The upper pale-yellow zone, R_f 0.35, was eluted with chloroform–methanol (10:1). The solution was evaporated to give a dull-yellow solid (3 mg), mp 270–271°. Its melting point, mixed melting point, R_f value, and UV and IR absorption spectra were identical with those of 1,5,8-trihydroxy-3-methoxyxanthone (2).

Xanthone II (1,7,8-Trihydroxy-3-methoxyxanthone)—The third and fourth benzene eluates were combined and concentrated to give a yellow solid (47 mg). This solid crystallized from ethyl alcohol as yellowish-orange needles, mp 218°. Its melting point, mixed melting point, R_f value, and UV and PMR spectra were identical with those of 1,7,8-trihydroxy-3-methoxyxanthone (3). The 7,8-dimethyl ether of the xanthone, prepared with ethereal diazomethane, crystallized from ethyl alcohol as yellow needles; the melting point and mixed melting point with decussatin (2) were 149–150°.

The chloroform eluates also were combined and concentrated. The concentrate showed two major and one minor ferric positive spots on analytic TLC. The components, however, could not be separated by preparative TLC. Therefore, the mixture was methylated with dimethyl sulfate and potassium carbonate in anhydrous acetone under reflux (45 hr). The permethyl ethers were separated by preparative TLC.

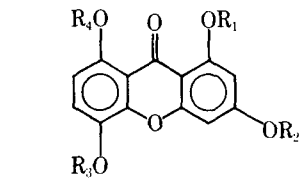
Xanthone XVIII (1,3,4,5,8-Pentamethoxyxanthone)—The upper pale-yellow zone, R_f 0.5 (chloroform–acetic acid, 100:1), which also showed a strong fluorescence under shortwave UV light, was eluted with chloroform. The residue (1.8 mg) from the chloroform solution showed an R_f value and UV absorption spectrum that indicated it was 1,3,4,5,8-pentamethoxyxanthone (7). The mass spectrum of the compound, *m/e* 346 (M^+, relative intensity 100%), 331 (30), 329 (7), 317 (18), 316 (18), 303 (42), 275 (12), and 260 (5), was also consistent with this conclusion.

Xanthone XIX (1,3,4,7,8-Pentamethoxyxanthone)—The lower yellow zone, R_f 0.32, was eluted with chloroform. The residue (2.5 mg) from the chloroform solution showed an R_f value, UV absorption spectrum, and mass spectrum (M^+, 346) identical with those of 1,3,4,7,8-pentamethoxyxanthone (7).

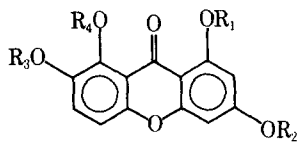
When the chloroform–methanol (90:10) eluates were combined and concentrated, a triterpene (22 mg), mp 300–302°, was obtained. The melting point, mixed melting point, and IR and PMR spectra of the compound established its identity as oleanolic acid. The mother liquor showed the presence of two strongly polar xanthones on analytical TLC. The R_f values of the two compounds corresponded with 1,3,5,8- and 1,3,7,8-tetrahydroxyxanthones (2).

The mixture of xanthones was acetylated with acetic anhydride and pyridine. Workup in the usual fashion afforded the corresponding tetraacetate derivatives, which were separated by column chromatography over silica gel. Petroleum ether, benzene, and benzene–chloroform (1:1), 1 liter each, were used as the eluents. Fractions (100 ml) were collected, and each fraction was monitored by analytical TLC.

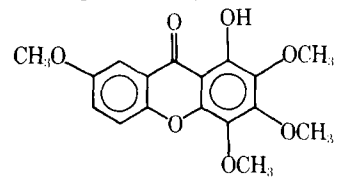
Xanthone III (1,3,5,8-Tetrahydroxyxanthone)—The early benzene



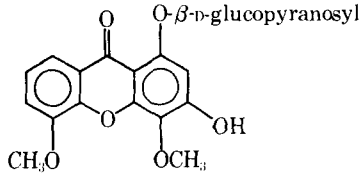
I: $R_1 = R_3 = R_4 = H$, $R_2 = CH_3$
III: $R_1 = R_2 = R_3 = R_4 = H$
VI: $R_1 = R_4 = H$, $R_2 = R_3 = CH_3$
X: R_1 = β-D-glucopyranosyl, $R_2 = CH_3$, $R_3 = R_4 = H$
XI: R_1 = β-D-glucopyranosyl, $R_2 = R_3 = CH_3$, $R_4 = H$
XIII: R_1 = β-D-glucopyranosyl, $R_2 = R_3 = R_4 = H$



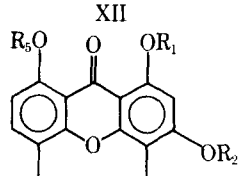
II: $R_1 = R_3 = R_4 = H$, $R_2 = CH_3$
IV: $R_1 = R_2 = R_3 = R_4 = H$
VII: $R_1 = R_4 = H$, $R_2 = R_3 = CH_3$
VIII: $R_1 = H$, $R_2 = R_3 = R_4 = CH_3$
IX: $R_1 = R_2 = R_4 = H$, $R_3 = CH_3$
XIV: R_1 = β-D-glucopyranosyl, $R_2 = R_3 = R_4 = H$
XVII: R_1 = β-D-glucopyranosyl, $R_2 = CH_3$, $R_3 = R_4 = H$



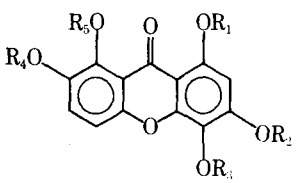
V



XII



XV: R_1 = β-D-glucopyranosyl, $R_2 = R_3 = R_4 = R_5 = H$
XVIII: $R_1 = R_2 = R_3 = R_4 = R_5 = CH_3$



XVI: R_1 = β-D-glucopyranosyl, $R_2 = R_3 = R_4 = R_5 = H$
XIX: $R_1 = R_2 = R_3 = R_4 = R_5 = CH_3$

[3] British Drug Houses (60–120 mesh).
[4] E. Merck AG, Darmstadt, Germany.

Table I—Xanthonic Patterns (Relative Percent Yield) of *S. angustifolia* during Growth[a,b]

Xanthones	4–6-Week-Old Plant		Plant with Fruits	
	Roots	Aerial Parts	Roots	Aerial Parts
Tetraoxygenated free xanthones	60	48	22	10
Pentaoxygenated free xanthones	12	17	2	6
Tetraoxygenated xanthone *O*-glucosides	2	10	50	64
Pentaoxygenated xanthone *O*-glucosides	—	3	5	6
Unidentified phenolic constituents	26	22	21	14
Yield, %	0.038	0.22	1.8	5.5

[a] The mean of three experiments is recorded. [b] One- to two-week-old seedlings, grown on a potting soil, gave only traces of 1,3,5,8- and 1,3,7,8-tetraoxygenated xanthones but relatively larger amounts of polyphenols which could not be identified.

eluates afforded 1,3,5,8-tetraacetoxyxanthone (5.5 mg). This compound, on hydrolysis with methanolic potassium hydroxide (5%) and the usual workup of the hydrolyzed product, gave 1,3,5,8-tetrahydroxyxanthone as a brown solid; the melting point and mixed melting point were 295–300° (2).

Xanthone IV (1,3,7,8-Tetrahydroxyxanthone)—The later benzene and benzene–chloroform eluates, in a similar way, furnished 1,3,7,8-tetrahydroxyxanthone (11 mg); the melting point and mixed melting point were 330–333° (3).

Separation of Xanthones from Fraction B—The total phenolic constituents from this fraction showed five iodine-positive spots on analytical TLC. Preparative TLC, using benzene–acetic acid (100:2) as the developer, afforded three xanthones as pure entities.

Xanthone V (1-Hydroxy-2,3,4,7-tetramethoxyxanthone)—The upper bright-yellow zone, R_f 0.75, was eluted with chloroform. The solution was evaporated to give a yellow solid (3 mg), mp 114–115°. Its melting point, mixed melting point, R_f value, and UV and mass spectra (M^+, 332) were identical with those of 1-hydroxy-2,3,4,7-tetramethoxyxanthone (4).

Xanthone VI (1,8-Dihydroxy-3,5-dimethoxyxanthone)—The middle yellow zone, R_f 0.62, was eluted with chloroform, and the chloroform concentrate showed an elongated spot on analytical TLC. It was subjected again to preparative TLC and, after the usual workup, the residue (about 1 mg) was obtained as a yellow microcrystalline solid, mp 184–186°. Its melting point, mixed melting point, R_f values, and UV absorption spectrum suggested its identity as 1,8-dihydroxy-3,5-dimethoxyxanthone. The diacetate derivative, prepared by treating it with acetic anhydride and pyridine on a steam bath (4 hr), was found to be identical to 1,8-diacetoxy-3,5-dimethoxyxanthone by co-TLC.

Xanthone VII (1,8-Dihydroxy-3,7-dimethoxyxanthone)—After the usual workup, the lowest yellow zone, R_f 0.45, gave yellow crystals (7 mg), mp 177–178°. The melting point, mixed melting point, R_f value, and UV and PMR spectra of the compound were identical with those of 1,8-dihydroxy-3,7-dimethoxyxanthone (2).

Isolation of Xanthones from Alcoholic Extract—The alcoholic extract was concentrated (to about 200 ml) and kept at 0° for 7 days, and a brown gum (5.8 g, Fraction C) separated. The alcoholic supernate was further concentrated to a syrupy liquid. It was poured into water (100 ml), and the mixture was kept at ambient temperature overnight. The precipitated yellow solid (0.72 g, Fraction D) was collected by filtration. The aqueous mother liquor was successively extracted with ether, ethyl acetate, and *n*-butyl alcohol (5 × 100 ml each).

The solvent was evaporated from the combined ether extracts, and a yellow solid (0.18 g, Fraction E) was obtained. The combined ethyl acetate extracts, on concentration, gave a yellow solid (0.42 g, Fraction F). On evaporation, the ethyl acetate mother liquor gave a second yellow solid (0.08 g, Fraction G). Likewise, the *n*-butyl alcohol extract gave a brown gum (2.1 g, Fraction H).

Separation of Xanthone *O*-Glucosides from Fraction C—Analytical TLC (*n*-butyl alcohol–acetic acid–water, 4:1:2) and paper chromatography[5] (aqueous acetic acid, 15%) indicated the presence of four xanthone *O*-glucosides in this fraction. A small portion (40 mg) of this mixture was hydrolyzed with emulsin according to a previously described procedure (4). In the aqueous hydrolysate, the presence of glucose as the only glucone moiety was detected by cochromatography (4). The aglucone moieties were identified as I, II, 1,3,4,5,8-pentahydroxyxanthone, and 1,3,4,7,8-pentahydroxyxanthone by their melting points, mixed melting points, co-TLC with reference samples, and comparison of UV absorption spectra of the individual compounds and their methyl ethers with those of authentic markers (4, 7).

A larger portion (0.5 g) of this fraction was permethylated with methyl iodide and sodium hydride in tetrahydrofuran at ordinary temperature (4). The product was dissolved in chloroform and chromatographed over a 40 × 1.8-cm silica gel column. The elution was carried out with chloroform (500 ml) and chloroform–ethyl acetate (2:1 and 1:1, 500 ml each). Fractions (50 ml) were collected and monitored by TLC.

Permethyl Ether of Xanthone *O*-Glucoside XIII (2′,3′,4′,6′-Tetra-*O*-methyl-1-*O*-β-D-glucopyranosyl -3,5,8-trimethoxyxanthone)—Fractions 3–6, on evaporation, yielded a straw-colored amorphous powder (22 mg). The 60-MHz PMR spectrum of the compound in deuterochloroform was characteristic of the heptamethyl ether of 3,5,8-trioxygenated xanthone 1-*O*-glucoside. Thus, the glucosyl six protons and 21 methoxyl protons appeared in the 3.2–4.0-ppm region; the glucosyl anomeric proton showed a broad one-proton signal around 5 ppm; and the aromatic protons appeared at 6.40 (1H, d, J = 2 Hz), 6.64 (1H, d, J = 2 Hz), 6.76 (1H, d, J = 9 Hz), and 7.34 (1H, d, J = 9 Hz) ppm. It did not give any molecular ion peak in its mass spectrum, but characteristic fragment ion peaks appeared at *m/e* 302 and 219 due to 1-hydroxy-3,5,8-trimethoxyxanthone and 2,3,4,6-tetra-*O*-methylglucosyl moieties, respectively.

The glucoside methyl ether was hydrolyzed with 2 *N* HCl, and the aglucone was extracted with chloroform. The residue from the chloroform solution crystallized from ethyl alcohol as pale-yellow needles, mp 204–205°. The melting point, mixed melting point, R_f value, and UV absorption spectrum of the compound established its identity as 1-hydroxy-3,5,8-trimethoxyxanthone (2). The glucone fraction from the aqueous hydrolysate was obtained as a syrup, $[\alpha]_D^{30}$ +74° (c 0.52, water), *m/e* 236 (M^+). The R_f value and spectral (PMR and mass) and optical properties of the compound were indistinguishable from those of 2,3,4,6-tetra-*O*-methylglucose.

Permethyl Ether of Xanthone *O*-Glucoside XIV (2′,3′,4′,6′-Tetra-*O*-methyl-1-*O*-β-D-glucopyranosyl -3,7,8-trimethoxyxanthone)—Fractions 8–10, on evaporation, gave a yellow solid (172 mg); *m/e* 302 and 219.

Anal.—Calc. for $C_{26}H_{32}O_{11}$: C, 60.00; H, 6.15. Found: C, 59.61; H, 6.42.

Hydrolysis of the glucoside with 2 *N* HCl and the usual workup of the aglucone afforded a yellow solid, mp 148–149°. The compound was identical with decussatin in all respects (melting point, mixed melting point, co-TLC, and UV absorption spectrum) (2). The glucone part was identified as 2,3,4,6-tetra-*O*-methylglucose as shown previously.

The chloroform–ethyl acetate (1:1) eluates, on evaporation, gave an ivory-colored solid (14 mg); it showed two major spots, R_f 0.38 and 0.28, on analytical TLC. These compounds were separated by preparative TLC using chloroform–acetic acid (100:2).

Permethyl Ether of Xanthone *O*-Glucoside XV (2′,3′,4′,6′-Tetra-*O*-methyl-1-*O*-β-D-glucopyranosyl -3,4,5,8-tetramethoxyxanthone)—The less polar component, R_f 0.38, from preparative TLC was obtained as a glassy solid (8 mg); *m/e* 332 and 219.

Anal.—Calc. for $C_{27}H_{36}O_{12}$: C, 61.13; H, 6.79. Found: C, 60.62; H, 6.88.

Hydrolysis of the glucoside with 2 *N* HCl and workup of the product in the usual manner furnished 1-hydroxy-3,4,5,8-tetramethoxyxanthone (melting point, mixed melting point, co-TLC, and UV absorption spectrum) (7) and 2,3,4,6-tetra-*O*-methylglucose (co-TLC and PMR spectrum).

Permethyl Ether of Xanthone *O*-Glucoside XVI (2′,3′,4′,6′-Tetra-*O*-methyl-1-*O*-β-D-glucopyranosyl -3,4,7,8-tetramethoxyxanthone)—The more polar component, R_f 0.28, from the preparative TLC was obtained as a pale-yellow gum (17 mg); *m/e* 332 and 219.

Anal.—Calc. for $C_{27}H_{36}O_{12}$: C, 61.13; H, 6.79. Found: C, 60.82; H, 6.94.

The compound, on hydrolysis with 2 *N* HCl, gave 1-hydroxy-3,4,7,8-tetramethoxyxanthone (melting point, mixed melting point, co-TLC, and PMR spectrum) (7) and 2,3,4,6-tetra-*O*-methylglucose (co-TLC and PMR spectrum).

Separation of Xanthones from Fraction D—The yellow solid showed two major and four minor spots on analytical TLC. Co-TLC behavior of the minor components suggested that these compounds were xanthones I, II, V, and VI (2, 7). The two major components were sepa-

[5] Whatman No. 1.

rated by repeated preparative TLC using benzene–acetic acid (100:2).

Xanthone VIII (1-Hydroxy-3,7,8-trimethoxyxanthone)—The upper zone, R_f 0.35, was eluted with chloroform, and the solvent was evaporated. The residue crystallized from ethyl alcohol as bright-yellow needles (18 mg), mp 149–150°. The melting point, mixed melting point, R_f value, and UV absorption spectrum of the compound were in excellent agreement with those of decussatin (2).

Xanthone IX (1,3,8-Trihydroxy-7-methoxyxanthone)—The lower zone, R_f 0.18, was eluted with chloroform. The residue from chloroform crystallized from acetone as yellow needles (5 mg), mp 295–298°; λ_{max} (ethyl alcohol) (log ϵ): 238 (4.44), 262 (4.54), and 336 (4.29) nm; *m/e* 274 (M^+, relative intensity, 100%), 259 (18), 245 (22), 231 (24), 203 (5), and 202 (4). The melting point and UV absorption spectrum of the compound were indistinguishable from those reported for 1,3,8-trihydroxy-7-methoxyxanthone (8). The compound, on methylation with ethereal diazomethane, gave decussatin (2).

Separation of Xanthones from Fraction E—Analytical TLC indicated the presence of seven xanthones, I, II, and V–IX, which were separated as described previously.

Separation of Xanthone *O*-Glucosides from Fraction F—This fraction showed one major and two minor spots on analytical TLC, R_f 0.52, 0.48, and 0.33 (chlorofrom–methanol, 2:1).

Xanthone *O*-Glucoside XII (3-Hydroxy-1-*O*-β-D-glucopyranosyl-4,5-dimethoxyxanthone)—The major component, R_f 0.52, crystallized from ethyl alcohol as pale-yellow crystals (24 mg), mp 268–270°: $[\alpha]_D^{30}$ −112.8° (c 0.32, methyl alcohol).

Anal.—Calc. for $C_{21}H_{22}O_{11}\cdot H_2O$: C, 53.84; H, 5.12. Found: C, 54.27; H, 5.06.

Hydrolysis of the compound with emulsin gave 1,3-dihydroxy-4,5-dimethoxyxanthone (4) and glucose. Permethylation of the glucoside followed by acid hydrolysis yielded 1-hydroxy-3,4,5-trimethoxyxanthone (melting point, mixed melting point, co-TLC, and PMR spectrum) (4) and 2,3,4,6-tetra-*O*-methylglucose (co-TLC and PMR spectrum). These properties were identical with those reported for 3-hydroxy-1-*O*-β-D-glucopyranosyl-4,5-dimethoxyxanthone (9).

From the alcoholic mother liquor, after the separation of the glucoside (XII), further quantities of xanthone *O*-glucosides XIII (7 mg) and XIV (12 mg) were obtained as their permethyl ethers following the procedure described for Fraction C.

Separation of Xanthone *O*-Glucosides from Fraction G—This fraction showed one major and one minor spot, R_f 0.46 and 0.37, respectively, on analytical TLC (chloroform–methanol, 2:1).

Xanthone *O*-Glucoside XVII (3-Methoxy-1-*O*-β-D-glucopyranosyl-7,8-dihydroxyxanthone)—The major component crystallized from ethyl alcohol as straw-colored crystals (26 mg), mp 198–203°; $[\alpha]_D^{30}$ −103° (c 0.28, methyl alcohol).

Anal.—Calc. for $C_{20}H_{20}O_{11}\cdot H_2O$: C, 52.86; H, 4.84. Found: C, 52.51; H, 4.98.

Hydrolysis of the glucoside with emulsin gave 1,7,8-trihydroxy-3-methoxyxanthone and glucose. Permethylation followed by acid hydrolysis gave decussatin and 2,3,4,6-tetra-*O*-methylglucose.

Separation of Xanthone *O*-Glucosides from Fraction H—This fraction, when processed for the isolation of heterosides (7), gave swertiamarin (melting point, mixed melting point, and PMR spectrum of the tetraacetate) (1.8 g) (10) as the major constituent plus small quantities of xanthone *O*-glucosides X and XII–XIV. The xanthone *O*-glucosides were identified as before.

RESULTS AND DISCUSSION

Preliminary examination of the petroleum ether and alcoholic extracts of the roots of *S. angustifolia* by analytical TLC suggested the presence of over a dozen xanthones of varying polarity. The TLC patterns of these constituents and those obtained from the aerial portions (stems, leaves, and flowers) did not show any significant qualitative difference. Therefore, the whole plant was used for batch extractions with larger samples. The individual parts were also extracted in small batches to determine the quantities of free xanthones and xanthone *O*-glucosides during plant growth. The results are recorded in Table I.

From the whole plant of *S. angustifolia*, 14 tetraoxygenated and five pentaoxygenated xanthonic constituents were isolated. Chemical transformation, spectroscopic analyses (UV, IR, PMR, and mass spectra), and comparison with reference samples (2–7) established their structures. Xanthones XVIII and XIX were isolated from a permethylated sample of the natural mixture of xanthones. The xanthone *O*-glucosides XI and XIV–XVII were previously unreported in nature.

The occurrence of V and XII in this species is taxonomically significant. Xanthones containing these two oxygenation patterns were encountered only once before in *Swertia*—*viz.*, *S. bimaculata* (4). These two types of xanthones, however, constitute major characters in a number of *Frasera* species (11). Until recently, the genus *Frasera* was considered chemotaxonomically different from *Swertia* on the basis of differences in the oxygenation patterns of their contained xanthones. Work in this laboratory has shown (4) for the first time that xanthones of *S. bimaculata* bear oxygenation patterns characteristic of both *Swertia* and *Frasera* species. The isolation of xanthone V and xanthone *O*-glucoside XII from *S. angustifolia* provides additional support in favor of the proposed phylogentic similarity of the two genera.

Another aspect of this investigation that has taxonomic significance was the relative abundance of the differently oxygenated xanthones occurring in *S. angustifolia*. The relative abundance of the complementary pair of 1,3,5,8- and 1,3,7,8-tetraoxygenated xanthones was inversely proportional in all members of the genera *Swertia* and *Gentiana* investigated so far (3). In *S. angustifolia* also, 1,3,7,8-tetraoxygenated xanthones constituted the major oxygenation pattern. The glucosylation in the xanthone *O*-glucosides of this species was exclusively at the 1-position.

The formation of xanthone *O*-glucosides in *S. angustifolia* was discernible only from the onset of maturity (4–6-week-old plant). This phenomenon is rather surprising in view of the fact that glycosides are less toxic to plants than their phenolic aglucones (12); therefore, glycosidation of xanthones is expected at an early stage of growing but was not observed in *S. angustifolia*.

Phenolic substances are known to be responsible for the general resistance showed by higher plants toward parasitic bacteria and fungi (13). The question of whether an unaltered phenol or the corresponding *O*-glycoside is a true protective agent of a higher plant was answered recently (14) for the case of the wilting disease of safflower (*Carthamus tinctorius* L.) caused by *Fusarium oxysporum* f. sp. *carthami*. Carthamidin (4′,5,7,8-tetrahydroxyflavanone), a constituent of healthy safflower, was strongly effective as a protective agent against the fusarial wilt while the corresponding 5-*O*-glucoside was practically inactive. Further work is clearly necessary to establish whether or not the observed difference between the free phenolic compound and the corresponding *O*-glucoside as a protective agent is a general phenomenon.

The content of the xanthone *O*-glucosides was greater in the aerial portions than in the roots (Table I). This result is particularly germane to the use of the plant extract for therapeutic purposes (1). Recent investigations in this laboratory have shown that free xanthones and their *O*-glucosides have different biological properties (2, 7, 15–18).

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Quantitative Determination of Amitriptyline in Blood

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Abstract □ An assay was developed and standardized for amitriptyline and its hydroxylated tertiary amine metabolites in blood and other biological tissues. This method is capable of determining 5–15 ng of these compounds/ml and is based on reacting the drug as base with 9-bromomethylacridine to form a quaternary product which, on photolysis, yields fluorescence in a stoichiometric fashion. The precision of the method is usually around ±5%.

Keyphrases □ Amitriptyline—fluorometric analysis, blood and tissues □ Fluorometry—analysis, amitriptyline, blood and tissues □ Antidepressants—amitriptyline, fluorometric analysis, blood and tissues

Blood amitriptyline levels in humans following a single oral dose are generally 20 ng/ml or less (1). These low levels were difficult to determine until recently, primarily because of a lack of appropriate methods with adequate sensitivity and precision.

Several methods were reported (1–6), but only three were capable of assaying blood levels of less than 20 ng/ml. A GLC method (3) has not yet been used to study amitriptyline pharmacokinetics. A method based on UV reflectance photometry was used for various psychoactive drugs including amitriptyline (5), but its ability to determine blood levels of this tricyclic compound following a single oral dose has not been demonstrated. One GLC method (6) was reported to exhibit reasonably good sensitivity and precision.

A method based on a fluorometric approach (7–9) could determine the absorption and elimination profiles of amitriptyline in humans following a single oral dose of 1 mg/kg (4). In this method, amitriptyline extracted from blood is reacted with 9-bromomethylacridine to form a quaternary ammonium product which, on photolysis, yields fluorescence. Both quaternization and photolysis are stoichiometric with a high degree of precision.

This paper describes the development of a fluorometric assay capable of determining levels as low as 5 ng of amitriptyline/ml in biological fluids. The method has been applied to assaying patient blood samples and is being used to generate basic pharmacokinetic data on amitriptyline in animals and humans.

EXPERIMENTAL

Materials and Equipment—The acridine reagent was synthesized as described earlier (8). Various chemicals and solvents, the laboratory equipment, and other instrumentation were essentially the same as described previously (9). Amitriptyline[1] was used as a hydrochloride salt.

Table I—Percent Maximum Extraction of Amitriptyline from Blood (n = 4)

Solvent	Relative Fluorescence	Blood Blank	Maximum Extraction, %
Hexane	2762 ± 345[a,b]	100 ± 21[a,c]	100
Hexane–1.5% isoamyl alcohol	2707 ± 242	86 ± 5	98
Toluene–1.5% isoamyl alcohol	2020 ± 106	180 ± 0	73
Benzene–1.5% isoamyl alcohol	2435 ± 78	202 ± 53	88
Toluene	2030 ± 259	138 ± 30	73
Benzene	2148 ± 43	108 ± 11	78

[a] Mean ± *SD*. [b] Formed emulsions. [c] n = 2.

General Procedure—*Amitriptyline, as the base, was reacted with* excess 9-bromomethylacridine in 200 μl of acetonitrile in the presence of glass beads as the catalyst. The reaction mixture was incubated at 50° for 18 hr, evaporated to dryness, and finally reconstituted in 100 μl of acetonitrile. An aliquot was subjected to TLC to separate the quaternary salt. The separated quaternary salt was photolyzed under UV light, and the fluorescent material was eluted with methanolic sulfuric acid for fluorometric determination[2].

Standardization of Assay—Various steps, starting from the isolation of amitriptyline from blood, its reaction with the acridine reagent, and finally fluorometric determination, were standardized to derive the optimum conditions. Since the assay primarily would be applied to blood, all standardization steps were carried out in the presence of a blank blood extract, *i.e.*, amitriptyline was added either to the blank whole blood and extracted out or to the evaporated extract of 3-ml blank blood, prior to reaction with the acridine reagent.

Extraction from Blood—Of the organic solvents examined, n-hexane containing 1.5% isoamyl alcohol was optimum for single extraction (Table I). Other optimum conditions included a blood-to-solvent ratio of 3:20, an extraction pH of 13 ± 0.5, and a shake time of 30 min at 150 cpm (Table II).

Quaternization Reaction—A range of 9-bromomethylacridine concentrations was reacted with amitriptyline and assayed. The optimum acridine concentration for the quaternization of 0.1–1 μg of amitriptyline was 0.47×10^{-3} M and higher (Table III).

TLC and Photolysis—A 20-μl aliquot of the reconstituted mixture was spotted on the synthetic-backed 100-μm silica gel plate. The plate was dried and developed in many trial solvents, including ethyl acetate–methanol–water–acetic acid–cyclohexane (100:20:12:5:5). This solvent system caused a good separation of the amitriptyline quaternary product (R_f 0.42) from the quaternary products of 10-hydroxy and

[1] Supplied by Merck Sharp & Dohme Research Laboratories, West Point, Pa.
[2] Aminco-Bowman spectrophotofluorometer.