Plant Anticancer Agents XXX: Cucurbitacins from *Ipomopsis aggregata* (Polemoniaceae)

MUNEHISA ARISAWA *, JOHN M. PEZZUTO,
A. DOUGLAS KINGHORN ˣ, GEOFFREY A. CORDELL, and
NORMAN R. FARNSWORTH

Received January 11, 1983, from the *Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612.* Accepted for publication February 25, 1983. *Present address: Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan.

Abstract □ Isocucurbitacin B (I), 3-epi-isocucurbitacin B (II), and cucurbitacin B (III) were identified as the principal cytotoxic constituents of *Ipomopsis aggregata* (Pursh) V. Grant (Polemoniaceae). The structure of the new compound, II, was determined through analysis of its spectrometric characteristics.

Keyphrases □ *Ipomopsis aggregata* (Polemoniaceae)—isolation and identification of cytotoxic constituents □ Cucurbitacins—cytotoxic principles from *Ipomopsis aggregata* □ 3-Epi-isocucurbitacin B—novel cytotoxic constituent of *Ipomopsis aggregata*

Ipomopsis aggregata represents a small New World genus of herbs in the family Polemoniaceae (1). This species is the most wide-ranging member of the genus, extending in distribution from British Columbia to northwest Mexico (2). In previous phytochemical work, an uncharacterized saponin was isolated from an extract of the entire plant of *I. aggregata* (3), but there appear to have been no prior studies to determine the biological activity of extracts and isolates derived from this taxon.

In a continuing search for tumor inhibitors of plant origin[1], we found that a chloroform extract of the combined roots, stems, leaves, flowers, and fruits of *I. aggregata* ex-

I R_1 = α-OH; R_2 = H

II R_1 = β-OH; R_2 = H

IV R_1 = α-OCOCH₃; R_2 = COCH₃

V R_1 = β-OCOCH₃; R_2 = COCH₃

III R_1 = H; R_2 = H

VI R_1 = COCH₃; R_2 = COCH₃

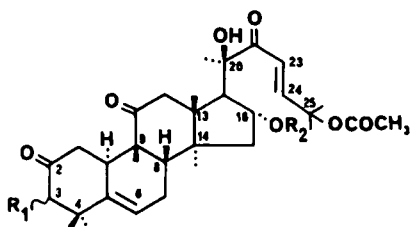
[1] For the previous article in this series, see Ref. 4.

hibited significant cytotoxic activity against Eagle's carcinoma of the nasopharynx in cell culture (KB), but no activity when tested at a dose range of 1.87–15.0 mg/kg (ip) against the P388 lymphocytic leukemia system in mice[2]. In the present contribution we wish to report the isolation of three cytotoxic cucurbitacins from this plant, namely, isocucurbitacin B (I), a new compound 3-epi-isocucurbitacin B (II), and cucurbitacin B (III).
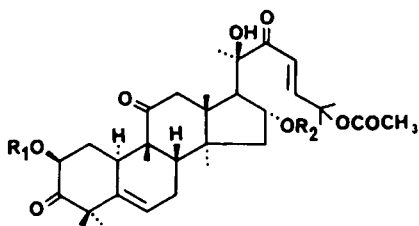
EXPERIMENTAL[3]

Plant Material—The combined roots–stems–leaves–flowers–fruits of *Ipomopsis aggregata* (Pursh) V. Grant (Polemoniaceae) were collected in Idaho during July 1980[4].

KB Cell Culture Assays—Substances were evaluated for cytotoxic activity essentially by the procedure of Geran *et al.* (5), as described previously (6). All assays were performed in duplicate, and the results were expressed as the effective dose to inhibit 50% of the growth observed in control tubes which were treated with solvent only. Mithramycin was used as a positive cytotoxic control substance (ED_{50} = 0.1 µg/mL).

Extraction and Fractionation—The air-dried, milled plant material (10 kg) was extracted sequentially with petroleum ether (bp 60–80°C) and methanol. The methanol extract was concentrated *in vacuo* to yield a residue (1.57 kg), which was partitioned between 3-L portions of chloroform and water. Cytotoxic activity was found to be concentrated in the chloroform fraction (ED_{50} = 0.64 µg/mL), which was further partitioned between petroleum ether (2 L) and methanol–water (9:1, 2 L). After removal of the solvent, the cytotoxic aqueous methanol extract (600 g, ED_{50} = 0.30 µg/mL) was chromatographed over silica gel[5] (5 kg), by elution with chloroform and mixtures of chloroform–methanol of increasing polarity. A total of 22 fractions (2-L each) were collected. Fractions 9–11, eluted from this column with chloroform–methanol (99:1), were cytotoxic (ED_{50} = 0.27, 0.01, and 0.10 µg/mL, respectively). These combined fractions were chromatographed over a Florisil[6] column that was eluted with chloroform. Fractions eluted with this solvent were combined to afford a cytotoxic residue (1.32 g, ED_{50} = 0.05 µg/mL). Final purification of the cytotoxic constituents was achieved by preparative TLC on silica

gel GHLF[7] plates, using cyclohexane–ether–ethyl acetate (1:1:1) and chloroform–acetone–methanol (14:1:2) as solvents. Compounds I (33 mg), II (31 mg), and III (120 mg) were obtained from preparative TLC zones found to be cytotoxic, by crystallization from methanol.

Characterization of Biologically Active Isolates—Isocucurbitacin B (I, 33 mg, 0.00033%) crystallized in the form of colorless needles, mp 220–222°C, $[\alpha]_D^{25}$ +38° (c 0.2, CHCl$_3$) [lit. (7) mp 223–223.5°C, $[\alpha]_D$ +43° (c 1.61, CHCl$_3$)]; IR ν_{max} (KBr): 3550, 3450, 2950, 1720, 1680, 1620, 1440, 1360, 1230, 1170, 1120, 1090, 1030, and 980 cm^{-1}; UV λ_{max} (MeOH) (log ϵ): 232 (4.32) nm; ^{1}H-NMR (60 MHz): δ 0.81 and 0.96 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.18 and 1.26 [two s, 6, 4-(CH$_3$)$_2$], 1.32 (s, 3, 14-CH$_3$), 1.41 (s, 3, 20-CH$_3$), 1.54 [s, 6, 25-(CH$_3$)$_2$], 2.00 (s, 3, 25-OCOCH$_3$), 3.89 (br s, 1, 3-H), 4.25 (br s, 1, 20-OH exchangeable with D$_2$O), 4.33 (m, 1, 16-H), 5.92 (m, 1, 6-H), 6.42 (d, 1, J = 15.6 Hz, 23-H), and 7.07 ppm (d, 1, J = 15.6 Hz, 24-H); MS m/z (70 eV) (relative intensity): 498 (M$^+$ −60, 2%), 386 (1), 385 (1), 369 (1), 219 (1), 203 (2), 164 (4), 137 (3), 135 (3), 113 (6), 112 (9), 111 (8), 109 (8), 97 (10), and 96 (100). These data (IR, UV, and ^{1}H-NMR) are in agreement with those previously reported for isocucurbitacin B (I) (7, 8), although no mass spectral data for this compound appear to have been published. Identity was established as isocucurbitacin B by direct comparison with an authentic sample[8] (MS, co-TLC).

Isocucurbitacin B (I, 10 mg) was treated with acetic anhydride–pyridine (1:1, 1 mL) at room temperature overnight. Workup in the usual manner afforded a triacetate (IV, 8 mg) as colorless needles, mp 120–123°C; ^{1}H-NMR (60 MHz): δ 0.97 and 1.01 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.18 (s, 3, 4-CH$_3$), 1.22 (s, 6, 4-CH$_3$ and 14-CH$_3$), 1.39 (s, 3, 20-CH$_3$), 1.57 [s, 6, 25-(CH$_3$)$_2$], 1.85 (s, 3, 16-OCOCH$_3$), 2.01 (s, 3, 25-OCOCH$_3$), 2.17 (s, 3, 3-OCOCH$_3$), 4.25 (br s, 1, 20-OH exchangeable with D$_2$O), 4.93 (s, 1, 3-H), 5.17 (m, 1, 16-H), 5.90 (m, 1, 6-H), 6.37 (d, 1, J = 15.6 Hz, 23-H), and 7.16 ppm (d, 1, J = 15.6 Hz, 24-H); MS m/z (70 eV) (relative intensity): 582 (M$^+$ −60, 1%), 522 (1), 507 (1), 487 (2), 427 (1), 411 (2), 385 (1), 383 (1), 368 (2), 367 (4), 279 (3), 219 (3), 177 (3), 166 (8), 150 (3), 135 (4), 113 (8), 112 (6), 111 (6), 109 (6), 97 (11), and 96 (100).

3-Epi-isocucurbitacin B (II, 31 mg, 0.00031%) was obtained in the form of a white powder, mp 92–98°C, $[\alpha]_D^{25}$ −28.5° (c 0.2, CHCl$_3$); IR ν_{max} (CHCl$_3$): 3450, 2960, 2920, 1720, 1690, 1630, 1450, 1360, 1250, 1130, 1090, 980, and 940 cm^{-1}; UV λ_{max} (MeOH) (log ϵ): 229 (4.47) nm; ^{1}H-NMR (60 MHz): δ 0.85 and 0.96 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.07 and 1.25 [two s, 6, 4-(CH$_3$)$_2$], 1.31 (s, 3, 14-CH$_3$), 1.41 (s, 3, 20-CH$_3$), 1.55 [s, 6, 25-(CH$_3$)$_2$], 2.00 (s, 3, 25-OCOCH$_3$), 4.11 (s, 1, 3-H), 4.24 (br s, 1, 20-OH exchangeable with D$_2$O), 4.36 (t, 1, J = 7.4 Hz, 16-H), 5.90 (m, 1, 6-H), 6.44 (d, 1, J = 15.6 Hz, 23-H), and 7.06 ppm (d, 1, J = 15.6 Hz, 24-H); MS m/z (70 eV) (relative intensity): 498 (M$^+$ −60, 2%), 385 (1), 327 (2), 287 (2), 286 (7), 285 (3), 271 (4), 266 (4), 253 (9), 187 (4), 185 (7), 175 (11), 173 (25), 159 (6), 147 (5), 131 (5), 129 (16), 115 (22), 113 (7), 112 (11), 111 (8), 101 (13), 97 (16), and 96 (100); mass measurement: calc. for C$_{30}$H$_{42}$O$_6$, 498.2981; observed, 498.2986.

3-Epi-isocucurbitacin B (II, 10 mg) was acetylated as described for I, and the triacetate V (7 mg) obtained after workup was found to exhibit the following data: colorless needles, mp 103–107°C; ^{1}H-NMR (60 MHz): δ 1.05 and 1.10 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.17 and 1.26 [two s, 6, 4-(CH$_3$)$_2$], 1.30 (s, 3, 14-CH$_3$), 1.41 (s, 3, 20-CH$_3$), 1.58 [s, 6, 25-(CH$_3$)$_2$], 1.86 (s, 3, 16-OCOCH$_3$), 2.03 (s, 3, 25-OCOCH$_3$), 2.18 (s, 3, 3-OCOCH$_3$), 4.26 (br s, 1, 20-OH exchangeable with D$_2$O), 5.13 (s, 1, 3-H), 5.19 (m, 1, 16-H), 5.85 (m, 1, 6-H), 6.38 (d, 1, J = 15.5 Hz, 23-H), and 7.16 ppm (d, 1, J = 15.5 Hz, 24-H); MS m/z (70 eV) (relative intensity): 582 (M$^+$ −60, 1%), 522 (1), 487 (4), 427 (4), 411 (9), 385 (3), 367 (6), 325 (4), 219 (4), 189 (5), 187 (7), 177 (6), 171 (5), 161 (4), 159 (7), 137 (5), 135 (9), 133 (6), 121 (5), 113 (14), 112 (41), 111 (30), 109 (9), 105 (9), 97 (19), and 96 (100).

Cucurbitacin B (III, 120 mg, 0.0012%) was obtained in the form of a white powder, mp 160–162°C, $[\alpha]_D^{25}$ +44° (c 0.1, CHCl$_3$) [lit. (7) mp 184–186°C, crystallized from EtOH; $[\alpha]_D^{25}$ +87° (EtOH)]; IR ν_{max} (KBr): 3550, 3400, 2950, 1720, 1680, 1620, 1450, 1360, 1230, 1120, 1050, 1020, and 980 cm^{-1}; UV λ_{max} (MeOH) (log ϵ): 229 (4.47) nm; ^{1}H-NMR (60 MHz): δ 0.97 and 1.07 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.25 and 1.29 [two s, 6, 4-(CH$_3$)$_2$], 1.34 (s, 3, 14-CH$_3$), 1.43 (s, 3, 20-CH$_3$), 1.56 [s, 6, 25-(CH$_3$)$_2$], 2.01 (s, 3, 25-OCOCH$_3$), 4.25 (t, 1, J = 7.4 Hz, 16-H), 4.28 (br s, 1, 20-OH exchangeable with D$_2$O), 4.43 (dd, 1, J = 12.8 and 5.7 Hz, 2-H), 5.76 (m, 1, 6-H), 6.49 (d, 1, J = 15.6 Hz, 23-H), and 7.08 ppm (d, 1, J = 15.6 Hz, 24-H); MS m/z (70 eV) (relative intensity): 498 (M$^+$ −60, 4%), 480 (1), 385 (1), 299 (1), 285 (1), 219 (1), 203 (1), 187 (2), 175 (3), 173 (3), 133 (3), 121 (3), 115 (3), 113 (4), 112 (8), 111 (7), 109 (5), 105 (4), 97 (11), and 96 (100). The above spectral data are consistent with those published pre-

viously for cucurbitacin B (7–15). Identity as cucurbitacin B was established by direct comparison with an authentic sample[8] (MS, co-TLC).

Cucurbitacin B (III, 15 mg) was acetylated as described for I, and the triacetate VI (9 mg) obtained after workup was found to exhibit the following data: colorless needles, mp 114–117°C; ^{1}H-NMR (60 MHz): δ 1.03 and 1.10 (two s, 6, 9-CH$_3$ and 13-CH$_3$), 1.32 [s, 9, 4-(CH$_3$)$_2$ and 14-CH$_3$], 1.42 (s, 3, 20-CH$_3$), 1.58 [s, 6, 25-(CH$_3$)$_2$], 1.87 (s, 3, 16-OCOCH$_3$), 2.03 (s, 3, 25-OCOCH$_3$), 2.13 (s, 3, 2-OCOCH$_3$), 4.27 (br s, 1, 20-OH exchangeable with D$_2$O), 5.20 (t, 1, J = 7.3 Hz, 16-H), 5.48 (dd, 1, J = 13.4 and 5.7 Hz, 2-H), 5.77 (m, 1, 6-H), 6.42 (d, 1, J = 15.6 Hz, 23-H), and 7.18 ppm (d, 1, J = 15.6 Hz, 24-H); MS m/z (70 eV) (relative intensity): 582 (M$^+$ −60, 1%), 522 (1), 487 (4), 427 (5), 411 (4), 385 (1), 367 (5), 325 (4), 219 (3), 189 (6), 187 (6), 177 (6), 159 (5), 137 (5), 111 (12), 109 (9), 107 (6), 105 (7), 97 (19), and 96 (100).

Biological Activity of the Isolates—Isocucurbitacin B (I, NSC-106400), 3-epi-isocucurbitacin B (II, NSC-359240), and cucurbitacin B (III, NSC-049451) exhibited ED$_{50}$ values of 0.016, 0.050, and 0.032 µg/mL, respectively, in the KB cell culture system.

DISCUSSION

The known tetracyclic triterpenes isocucurbitacin B (I) and cucurbitacin B (III) were isolated and identified in the present study from a cytotoxic fraction of *I. aggregata* whole flowering plants. The molecular ions of both compounds were characteristically absent from their electron-impact mass spectra (10), so that the highest fragment peaks observed occurred at m/z 498, representing in each case a loss of one acetate unit from the parent ion. In its ^{1}H-NMR spectrum, I was typified by the presence of a singlet assignable for the C-3 methine proton at δ 3.89, which, since it was shifted downfield in isocucurbitacin B diacetate (IV) to δ 4.93, suggested the presence of a 2-keto-3-hydroxy ring A moiety in I (8, 15). Isocucurbitacin B (I) is a relatively rare compound, previously being reported only from three species of two Cucurbitaceae genera (9). Cucurbitacin B (III), on the other hand, appears to be the most widely distributed cucurbitacin known, since it occurs in more than 15 genera of the Cucurbitaceae, and in species of the Begoniaceae, Cruciferae, and Euphorbiaceae (9, 12, 16, 17). Cucurbitacins have not previously been reported from the family Polemoniaceae.

The spectrometric parameters (IR, UV, ^{1}H-NMR, and MS) of II were very similar to those observed for isocucurbitacin B (I). Thus, comparable with I, no molecular ion was evident in the mass spectrum of II, with the highest observable fragment peak occurring at m/z 498, of elemental composition C$_{30}$H$_{42}$O$_6$, as determined by high-resolution MS. In its ^{1}H-NMR spectrum, signals indicating the presence of eight quaternary attached C-methyl groups, one acetate methyl group, two methine protons on carbons bearing hydroxyl groups, and three olefinic protons were observed. Following mild acetylation, triacetate V was produced from II, for which paramagnetic C-3 and C-16 methine proton shifts occurred from δ 4.11 and 4.36, respectively, in II, to δ 5.13 and 5.19 in V. By analogy to conclusions reached on the basis of almost identical C-3 ^{1}H-NMR assignments made for isocucurbitacin D (the C-25 deacetyl derivative of I) and 3-epi-isocucurbitacin D (7), II could therefore be assigned a 2-keto-3-hydroxy ring A moiety, and is the C-3 epimer of I.

The role of cucurbitacin B (III) in nature has been partially characterized. For example, III has been shown to stimulate feeding by the spotted cucumber (18) and diabroctic beetles (19). Further, it acts as an antigibberellin (20). In regard to antitumor activity, both isocucurbitacin B (I) and cucurbitacin B (III) have been shown in earlier work to demonstrate significant inhibitory activity against cultured KB cells (7, 12, 21, 22). We presently extend these results through the demonstration of the cytotoxic activity of II. Previous studies have also shown III to be active *in vivo* against the Walker 256 (im) carcinosarcoma and Lewis lung carcinoma in rats (7). The therapeutic index, however, appears too low for use as a clinical agent (7), and it is likely that the same would be found for I and II. An additional biological activity demonstrated by III, however, which may be worthy of additional investigation, is the prevention of experimentally induced hepatitis and cirrhosis (23).

Interestingly, it has been observed previously that cucurbitacin D, a constituent of *Phormium tenax* Forst. (Liliaceae) is degraded to isocucurbitacin D and 3-epi-isocucurbitacin D as a result of lengthy passage over silica gel during column chromatography (8). Therefore, since large-scale gravity column chromatography was employed in this study to obtain I–III from *I. aggregata*, it is possible that I and II are progeny of III and are not indigenous to this plant. For studies concerned primarily with chemotaxonomic relationships, it may be suggested that rapid techniques of isolation, preferably avoiding the use of silica gel, should be used to obtain cucurbitacins from plants.

REFERENCES

(1) J. C. Willis in "A Dictionary of Flowering Plants and Ferns," 8th ed., H. K. Airy-Shaw, Ed., University Press, Cambridge, England, 1973, p. 594.

(2) C. L. Lundell and collaborators, "Flora of Texas," vol. 1, part III, Texas Research Foundation, Renner, Tex., 1966, p. 285.

(3) M. E. Wall and K. H. Davis, Jr., in "Toxins of Animal and Plant Origin," A. de Vries and E. Kochra, Eds., Grodon and Breach, New York, N.Y., 1972, p. 597.

(4) M. Arisawa, S. S. Handa, D. D. McPherson, G. A. Cordell, H. H. S. Fong, and N. R. Farnsworth, *J. Nat. Prod.*, in press.

(5) R. I. Geran, N. H. Greenberg, M. M. McDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep.*, 3(3), 1 (1972).

(6) J. M. Pezzuto, S. K. Antosiak, W. M. Messmer, M. B. Slaytor, and G. R. Honig, *Chem.-Biol. Interactions*, 43, 323 (1983).

(7) S. M. Kupchan, A. H. Gray, and M. D. Grove, *J. Med. Chem.*, 10, 337 (1967).

(8) S. M. Kupchan, H. Meshulam, and A. T. Sneden, *Phytochemistry*, 17, 767 (1978).

(9) W. O. Eisenhut and C. R. Noller, *J. Org. Chem.*, 23, 1984 (1958).

(10) H. E. Audier and B. C. Das, *Tetrahedron Lett.*, 1966, 2205.

(11) R. W. Doskotch, M. V. Malik, and J. L. Beal, *Lloydia*, 32, 115 (1969).

(12) D. Lavie and E. Glotter, *Prog. Chem. Org. Nat. Prod.*, 29, 307 (1971).

(13) P. J. Curtis and P. M. Meade, *Phytochemistry*, 10, 3081 (1971).

(14) J. Konopa, J. Zieliński, and A. Matuszkiewicz, *Arzneim.-Forsch.*, 24, 1554 (1974).

(15) C. A. Rice, K. S. Rymal, O. L. Chambliss, and F. A. Johnson, *J. Agr. Food. Chem.*, 29, 194 (1981).

(16) P. R. Enselin, C. W. Holzapfel, K. B. Norton, and S. Rehm, *J. Chem. Soc. (C)*, 1967, 964.

(17) A. M. Tessier, A. Bouquet, and R. R. Paris, *Plant. Méd. Phytothér.*, 9, 328 (1975).

(18) G. C. Sharma and C. V. Hall, *J. Am. Soc. Hort. Sci.*, 96, 750 (1971).

(19) R. L. Metcalf, R. A. Metcalf, and A. M. Rhodes, *Proc. Natl. Acad. Sci. USA*, 77, 3769 (1980).

(20) J. Guha and S. P. Sen, *Nature (New Biol.)*, 244, 223 (1973).

(21) J. Konopa, J. Zieliński, A. Matuszkiewicz, and M. Hrabowska, *Adv. Antimicrob. Antineoplast. Chemother., Proc. Int. Congr. Chemother., 7th 1971*, (Pub. 1972), 2, 163; through *Chem. Abstr.*, 79, 73776y, 1973.

(22) J. Konopa, A. Matuszkiewicz, M. Hrabowska, and K. Onoszka, *Arzeim.-Forsch.*, 24, 1741 (1974).

(23) T.-W. Han, H.-H. Ma, Y.-C. Chao, and L.-M. Chou, *Chung-hua I Hsueh Tsa Chih (Peking)*, 59, 206 (1979); through *Chem. Abstr.* 92, 209055r, 1980.

Assay of Benzenoid Drugs in Tablet and Capsule Formulations by Second-Derivative Ultraviolet Spectrophotometry

A. G. DAVIDSON [x] and S. M. HASSAN [*]

Received December 8, 1982, from the *Department of Pharmacy, University of Strathclyde, Glasgow, G1 1XW, United Kingdom.* Accepted for publication February 2, 1983. *Present address: Analytical Chemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

Abstract □ Rapid second-derivative assay procedures are described for tablet and capsule formulations of benzenoid drugs, which eliminate the interference of the formulation excipients in simple UV spectrophotometric methods. Accuracy, precision, and selectivity of the technique are discussed. The general application of the procedures for the batch assay of tablet and capsule dosage forms containing ≥2 mg of a benzenoid drug is indicated by the results obtained for 18 such formulations, in good agreement with pharmacopeial assay results and/or declared amounts. The procedures are sufficiently sensitive to permit unit dose assays of these formulations.

Keyphrases □ Benzenoid drugs—tablet and capsule formulations, second-derivative UV spectrometry □ Formulations—benzenoid drugs, second-derivative UV spectrometry, tablets and capsules □ UV spectrometry, second-derivative—benzenoid drugs, tablet and capsule formulations

The interference of formulation excipients in the conventional ultraviolet (UV) spectrophotometric procedures for certain formulated drugs is well recognized. It is a particular problem in the assay of tablets and capsules of benzenoid drugs, which, in general, are both weakly absorbing and also formulated at a relatively low dosage level (typically 1–50 mg/unit dose). The high excipient–drug ratio and high sample weight required for these formulations result in background-irrelevant absorption of a sufficiently high intensity to possibly prohibit the application of simple spectrophotometric methods.

Techniques that reduce or eliminate matrix interference in the assay of formulated drugs include the Morton–Stubbs (1) correction procedure, which requires that the irrelevant absorption is linear over the wavelength range of the absorption band of the drug; orthogonal polynomials, which can cope with nonlinear irrelevant absorption if the correct choice of the polynomial order, wavelength range, and interval is made (2, 3); compensation spectrophotometry, in which the reference solution contains the matrix or sample at the same concentration present in the sample solution (4, 5); and difference spectrophotometry, which may be applied if an absorbance difference can be