compounds listed in Table II were screened *versus* the G46 strain of *S. typhimurium*, except for I*a*, I*b*, Ie, I*f*, V*b*, Vc, VII*a*–VII*c*, and VIII*c*, which were assessed against the ATCC 13311 microorganism.

Evaluation of II*b* against thymidylate synthetase and dihydrofolate reductase was done according to a literature method (34).

REFERENCES

(1) J. R. Dimmock and M. L. C. Wong, *Can. J. Pharm. Sci.*, **11**, 35 (1976).

(2) S. M. Kupchan, *Pure Appl. Chem.*, **21**, 227 (1970).

(3) O. K. Kabiev and S. M. Vermenichev, *Izv. Akad. Nauk Kaz. SSR Ser. Biol.*, **9**, 72 (1971); through *Chem. Abstr.*, **75**, 47091u (1971).

(4) M. Yamaguchi, M. Taniguchi, I. Kubo, and T. Kubola, *Agr. Biol. Chem.*, **41**, 2475 (1977).

(5) S. M. Kupchan and D. R. Streelman, *J. Org. Chem.*, **41**, 3481 (1976).

(6) P. W. Le Quesne, M. P. Pastore, and R. F. Raffauf, *Lloydia*, **39**, 391 (1976).

(7) S. M. Kupchan, J. A. Lacadie, G. A. Howie, and B. R. Sickles, *J. Med. Chem.*, **19**, 1130 (1976).

(8) S. M. Kupchan and G. Tsou, *J. Org. Chem.*, **38**, 1055 (1973).

(9) R. T. Williams, "Detoxication Mechanisms," 2nd ed., Wiley, New York, N.Y., 1959, p. 278.

(10) N. J. Harper, in "Progress in Drug Research," vol. 4, E. Jucker, Ed., Birkhauser Verlag, Basel, Switzerland, 1962, p. 221.

(11) H. J. E. Lowenthal, in "Protective Groups in Organic Chemistry," J. F. W. McOmie, Ed., Plenum, London, England, 1973, p. 330.

(12) P. M. Gullino, F. H. Crantham, S. H. Smith, and A. C. Haggerty, *J. Natl. Cancer Inst.*, **34**, 857 (1965).

(13) A. Burger, *J. Med. Chem.*, **21**, 2 (1978).

(14) A. F. Casy, N. J. Harper, and J. R. Dimmock, *J. Chem. Soc.*, **1964**, 3635.

(15) N. J. Harper, A. F. Casy, and J. R. Dimmock, *ibid.*, **1964**, 4280.

(16) J. R. Dimmock and W. G. Taylor, *J. Pharm. Sci.*, **64**, 241 (1975).

(17) J. R. Dimmock, A. M. Qureshi, L. M. Noble, P. J. Smith and H. A. Baker, *ibid.*, **65**, 38 (1976).

(18) H. Gershon and L. Shanks, *J. Med. Chem.*, **20**, 606 (1977).

(19) H. Zimmer and J. Rothe, *J. Org. Chem.*, **24**, 28 (1959).

(20) E. J. Forbes and M. J. Gregory, *J. Chem. Soc. B*, **1968**, 207.

(21) M. I. Vinnik and I. S. Kislina, *Kinet. Catal.*, **15**, 271 (1974).

(22) A. K. Barbour, M. W. Buxton, P. L. Coe, R. Stephens, and J. C. Tatlow, *J. Chem. Soc.*, **1961**, 808.

(23) G. S. Hammond, *J. Am. Chem. Soc.*, **77**, 334 (1955).

(24) J. W. Baker, I. Schumacher, and D. P. Roman, in "Medicinal Chemistry," 3rd ed., A. Burger, Ed., Wiley–Interscience, New York, N.Y., 1970, p. 631.

(25) J. R. Dimmock, C. B. Nyathi, and P. J. Smith, *J. Pharm. Sci.*, **67**, 1543 (1978).

(26) P. J. Nelson and A. F. A. Wallis, *Tappi*, **56**, 132 (1973).

(27) "Handbook of Chemistry and Physics," 49th ed., R. C. Weast, Ed., Chemical Rubber Co., Cleveland, Ohio, 1968, p. C-182.

(28) M. Hayashi, *J. Prakt. Chem.*, **123**, 289 (1929).

(29) "Dictionary of Organic Compounds," 4th ed., J. R. A. Pollock and R. Stevens, Eds., Eyre and Spottiswoode Publishers, London, England, 1965, p. 2477.

(30) *Ibid.*, p. 751.

(31) L. P. Hammett, "Physical Organic Chemistry," 2nd ed., McGraw-Hill, Toronto, Canada, 1970, p. 356.

(32) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep. (Part 3)*, **3**(2), (Sept. 1972).

(33) J. R. Dimmock, P. J. Smith, and S. K. Tsui, *J. Pharm. Sci.*, in press.

(34) S. K. Srinivasan, V. S. Gupta, and R. E. Howarth, *Biochem. Pharmacol.*, **26**, 1027 (1977).

(35) T. Koppanyi and A. G. Karczmar, "Experimental Pharmacodynamics," 3rd ed., Burgess, Indianapolis, Ind., 1964, p. 67.

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New and Simple Methylene Blue Colorimetric Assay for Glycyrrhizin in Pharmaceuticals

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Abstract □ A new colorimetric method for glycyrrhizin in licorice and drug preparations is given. The method is based on coupling the acidic genin, glycyrrhetinic acid, with methylene blue and measuring the extinction of the coupled compound solution in chloroform–alcohol.

Keyphrases □ Glycyrrhizin—analysis, methylene blue colorimetry □ Antiulcerogenic agents—glycyrrhizin, methylene blue colorimetric analysis □ Colorimetry—analysis, glycyrrhizin, methylene blue □ Methylene blue—analysis, glycyrrhizin

Licorice, the root and subterranean stem of different varieties of *Glycyrrhiza glabra*, has long been used in medicine. The drug and some of its preparations are official in many pharmacopoeias. Besides being a valuable flavoring and sweetening agent, the drug has demulcent, expectorant, and antispasmodic action. Recently, it was shown to be effective in gastric ulcer treatment and to have a cortisone-like action in rheumatic arthritis and other inflammatory diseases (1–3). These activities are due to the active constituent glycyrrhizin, which is the calcium and potassium salt of glycyrrhizic acid; the latter is the diglucopyranosiduronic acid of the pentacyclic triterpenoid sapogenin, β-glycyrrhetinic acid. Simple derivatives of β-glycyrrhetinic acid such as the disodium salt of carbenoxolone have been used extensively in gastric ulcer treatment (4).

BACKGROUND

Glycyrrhizin has been estimated by gravimetric assays of variable weighing forms (5–7); volumetric assays such as direct titration of glycyrrhizic acid, glycyrrhetinic acid, or their salts (6, 8); colorimetric assays (6, 9–12); and colorimetric and spectrophotometric determination after chromatographic separation (13–18).

The reported glycyrrhizin content of licorice has varied greatly. Some investigators attributed this variation to the different analytical methods

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utilized; they considered the figure given in the BPC 1963 (2%) to be low and favored the higher figure given in the later edition (7%) on the basis of good agreement with the 6–13% found by Houseman (5). However, recent reports criticized these high figures and showed most early gravimetric and colorimetric methods to be extremely nonspecific (4, 6), producing results that are appreciably higher than the true value.

Killacky *et al.* (4) criticized earlier colorimetric methods that rely on the reaction between β-glycyrrhetinic acid and vanillin–sulfuric acid as well as spectrophotometric methods that rely on UV absorption at 248 nm on the basis of the nonspecificity of the aromatic aldehyde–sulfuric acid color reaction. The numerous flavonoids, coumarins, and polyphenolic derivatives present in licorice extracts would absorb at 248 nm and are difficult to eliminate.

Some methods for the determination of glycyrrhizin and β-glycyrrhetinic acid were reported recently. These methods attempted chromatographic separation of the active principle by reversed-phase high-pressure liquid chromatography (HPLC) (4), ion-exchange HPLC (19), rod TLC (20), or GLC (21, 22).

A simple, specific method for glycyrrhizin estimation was developed through direct coupling of the acidic genin, β-glycyrrhetinic acid, with a basic dye, methylene blue.

EXPERIMENTAL

Instrument—A spectrocolorimeter[1] was used.

Material—One batch of powdered licorice[2], two batches of licorice[3] liquid extract, and one batch of glycyrrhizin flakes[4] were used.

Reagents—Aqueous methylene blue[5] (0.1%), chloroformic glycyrrhetinic acid reference[6] solution (5 mg/100 ml), and citrate–phosphate–borate–hydrochloride buffer[7] (Teorell and Stenhagen buffer) were prepared.

Determination of Optimum Coupling Conditions—Aliquots (20 ml) of reference glycyrrhetinic acid solution were shaken separately with 1 ml of dye solution and 20 ml of pH 6–12 buffer solution (Fig. 1). Solutions were shaken continuously for 1 min and set aside for 5 min. The chloroform layer was decanted into a 50-ml measuring flask.

The coupled compound was completely extracted by reshaking with an additional 10 and 5 ml of chloroform. The mixed chloroform extract was diluted to volume with ethanol, and the extinction of the chloroform–ethanol solution was measured at 640 nm (Fig. 1). The extractibility of the free dye was determined in a similar manner from blank experiments at the same pH values (Fig. 1).

Obedience to Beer's Law—Aliquots of glycyrrhetinic acid reference solution, corresponding to 0.05–1 mg of glycyrrhetinic acid, were coupled with methylene blue as described. Two series of experiments were attempted at pH 9.2 and 8.4. However, the extinction was measured in chloroform–ethanol (4:6). A linear relationship was maintained at both pH values (slopes of 0.21 and 0.14, respectively).

Molar Ratio of Coupled Compound—Eleven different coupling experiments were attempted with glycyrrhetinic acid aliquots ranging from 1 to 9 g-mole $\times 10^{-4}$. They were coupled, at pH 9.2, with aliquots ranging from 9 to 1 g-mole $\times 10^{-4}$ of methylene blue, respectively (Fig. 2).

Assay—For powdered licorice, 1.0 g was refluxed four times for 15 min with four successive 15-ml portions of 70% ethanol. Each portion was decanted and filtered through the same filter paper. The alcohol was evaporated, 20 ml of warm water was added, and the procedure was completed as described for the liquid extract, starting with: "After cooling, 5 ml of ethanol . . . . "

For the liquid extract and flakes, 1.0 g, accurately weighed, of the liquid extract (or 0.25 g of flakes) was dissolved with 20 ml of boiling water. After complete dissolution, the mixture was transferred into a separator, the flask was washed with two 10-ml portions of boiling water, and the washings were added to the original mixture in the separator. After cooling, 5 ml of ethanol and 1 ml of 6 N H_2SO_4 were added; then the mixture was shaken very gently with successive portions of 20, 20, and 10 ml of chloroform. Each chloroform fraction was separated as completely as possible, taking care not to pass down any precipitated matter

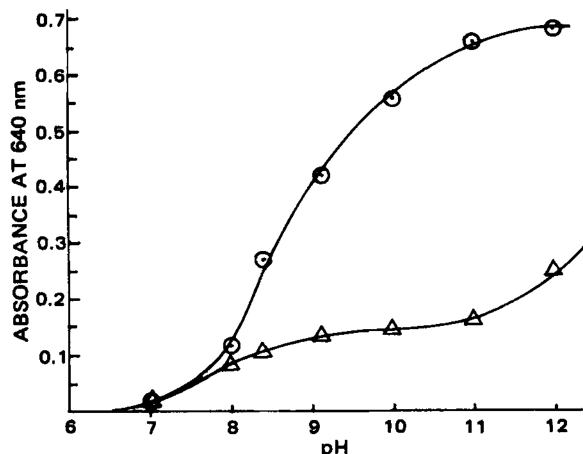

Figure 1—*Extractibility of methylene blue (△) and glycyrrhetinic acid–methylene blue coupled compound (○) at different pH values.*

or emulsion at the interphase. The chloroform extracts were discarded.

The aqueous phase with any suspended insoluble matter was quantitatively transferred into a boiling flask, using 5 ml of distilled water to wash off any clinging matter on the separator walls. Ten milliliters of 6 N H_2SO_4 was added, and the mixture was heated on a boiling water bath for 75 min.

The hydrolyzed mixture was transferred quantitatively, while hot, into a separator, and the flask was washed with 5 ml of distilled water. The aqueous acid solution was extracted with four 20-ml and one 10-ml portions of chloroform. Each chloroform fraction was filtered through a small piece of cotton surmounted by 2 g of anhydrous sodium sulfate. The chloroform filtrate was collected into a 100-ml measuring flask, and the volume was completed with chloroform.

Coupling Experiments—A 10-ml aliquot was transferred into a separator containing 20 ml of pH 9.2 buffer solution, 2 ml of dye solution, and 10 ml of chloroform. The mixture was shaken continuously for 1 min. (After separation of the two phases, the aqueous phase must be blue in color. If it has a greenish or yellow color, additional 1-ml aliquots of the dye solution must be added and the mixture must be reshaken.) The chloroform layer was separated into another separator containing 5 ml of pH 9.2 buffer. Then the mixture was shaken and, after complete separation, the chloroform layer was separated into a 100-ml measuring flask (without filtration or dehydration).

The contents of the first and second separators were reextracted with 10-, 5-, and 5-ml portions of chloroform in the same manner, and the combined chloroform extract in the measuring flask was completed to volume with ethanol.

A standard solution was prepared according to the same coupling procedure but with 10 ml of reference glycyrrhetinic acid solution instead of the 10-ml sample aliquot.

The extinctions of the test and standard solutions were measured at

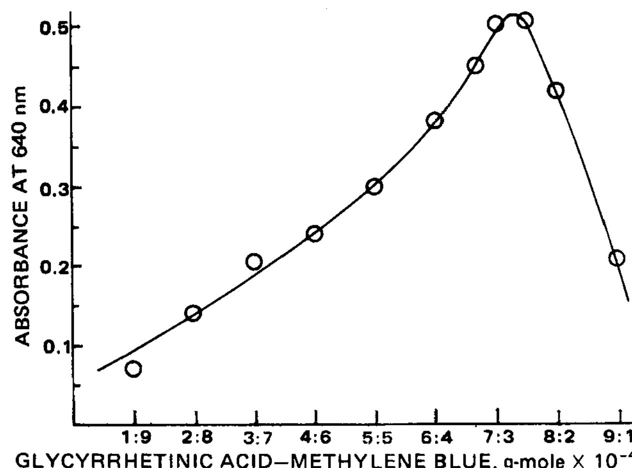

Figure 2—*Molar ratio curve of glycyrrhetinic acid–methylene blue coupled compound.*

[1] Spekol, Carl Zeiss, Jena, East Germany.
[2] Caesar and Loretz-Hilden.
[3] Lot 934, CID Laboratories, Cairo, Egypt, and batch 33, Memphis Chemical Co., Cairo, Egypt.
[4] CID Laboratories, Cairo, Egypt.
[5] Reidel-De Haen AG, Seelze-Hannover.
[6] Merck.
[7] According to "Scientific Tables," 7th ed., Ciba-Geigy, Basel, Switzerland, 1971, p. 280.

Table I—Glycyrrhizin Content of Certain Preparations

Sample	Original Preparation		Enriched Preparation		
	Combined Glycyrrhetinic Acid, %	Glycyrrhizin, %	Added Glycyrrhetinic Acid, mg	Found Glycyrrhetinic Acid, mg	Error, %
Liquid extract[a]	1.534	2.7	5.626	5.753	+2.26
Liquid extract[b]	0.649	1.14	5.138	5.227	+1.73
Powdered licorice	0.835	1.46	1.285	1.348	+4.9
Glycyrrhizin flakes[a]	8.21	14.37	6.779	6.901	+1.8

[a] CID Laboratories. [b] Memphis Chemical Co.

640 nm against a blank prepared at the same time by the same coupling procedure but with 10 ml of chloroform instead of the sample aliquot.

The glycyrrhizin percentage in the assayed preparation was calculated using:

$$\%\ \text{glycyrrhizin} = \frac{E_t \times 0.5 \times 1.75}{E_s \times a} \quad \text{(Eq. 1)}$$

where E_t is the extinction of the test, E_s is the extinction of the reference, a is the weight taken of the preparation, and 1.75 is the transformation factor of glycyrrhetinic acid to glycyrrhizin.

The reliability of the method was checked by assaying some pharmaceutical preparations before and after enrichment with authentic glycyrrhetinic acid (Table I).

DISCUSSION

Preliminary studies with methylene blue, safranin, brilliant cresyl blue, and basic fuchsin indicated that methylene blue was the best for coupling with glycyrrhetinic acid. The coupled compound was of comparably high extinction. The serious λ_{max} inconsistency of the methylene blue coupled compound in chloroform solutions was overcome by measuring the extinction in a chloroform–alcohol mixture in which the coupled compound showed a consistent λ_{max} at 640 nm.

The coupled compound was highly extractable with chloroform at all alkaline pH values; however, obedience to Beer's law was studied only at pH 9.2 and 8.4 rather than at higher pH values at which the free dye showed higher extractibility.

The specificity of the method was proved by resolution of the coupled compound (extraction with chloroform from acidic solution). Examination of the chloroform solution by TLC (23, 24) revealed one principal spot corresponding to glycyrrhetinic acid.

Molar ratio studies proved the coupled compound to be formed from three molecules of the acid and one molecule of the basic dye.

The proposed assay ensures complete glycyrrhizin extraction and complete elimination of free acidic constituents such as simple organic acids, sugar acids, free glycyrrhetinic acid, and other triterpene acids. The procedure also ensures complete glycyrrhizic acid hydrolysis and complete extraction of the liberated glycyrrhetinic acid.

The aliquot technique was followed in the coupling experiment to allow for possible repetition of that step.

The glycyrrhetinic acid recovery (Table I) was calculated to be 101.7–104.9%. This consistent positive error may be explained by a probable slight increase in solubility of the uncoupled dye in higher concentrations of the coupled compound (in the case of enriched samples).

The method is simple, rapid, and sensitive. The assay procedure, by ensuring complete initial elimination of free acids, renders the method specific for the acidic genins in the hydrolysate, which was proved chromatographically to consist principally of β-glycyrrhetinic acid.

REFERENCES

(1) F. E. Revers, *Ned. Tijdschr. Geneesh.*, **90**, 135 (1946). *Ibid.*, **92**, 2968, 3567 (1948); through Ref. 4.

(2) P. B. Hudson, A. M. Mittelman, and M. Podberezec, *N. Engl. J. Med.*, **251**, 641 (1954); through *Chem. Abstr.*, **49**, 3482 C (1955).

(3) National Research Development Corp., British pat. 713,651 (Aug. 18, 1954); through *Chem. Abstr.*, **49**, 3482 (1955).

(4) J. Killacky, M. S. F. Ross, and T. D. Turner, *Planta Med.*, **30**, 310 (1976).

(5) P. A. Houseman, *Am. J. Pharm.*, **84**, 351 (1912); through *Chem. Abstr.*, **84**, 558 (1913). P. A. Houseman, *J. Assoc. Offic. Org. Chem.*, **6**, 191 (1922).

(6) A. F. Selim, thesis, Cairo University, Cairo, Egypt, 1971, p. 199.

(7) I. A. Murawjew and N. A. Buska, *Aptechn. Delo*, **8**, 23 (1959); through *Chem. Abstr.*, **54**, 6033 (1960). *Ibid.*, **10**, 24 (1960); through *Chem. Abstr.*, **55**, 27778 (1961).

(8) I. A. Murawjew and W. D. Ponomarew, *Pharm. Zentralhalle*, **105**, 153 (1966); through *Chem. Abstr.*, **68**, 107868 (1968). I. A. Murawjew and W. D. Ponomarew, *Acta Pharm. Jugoslav.*, **16**, 3 (1966); through *Chem. Abstr.*, **69**, 44029 (1968). I. A. Murawjew and W. D. Ponomarew, *Med. Ind. USSR*, **16**, 43 (1962); through *Chem. Abstr.*, **57**, 3565 (1962).

(9) F. Wiest, dissertation, University of Zurich, Zurich, Switzerland, 1949.

(10) R. H. Cundiff, *Anal. Chem.*, **36**, 1871 (1964).

(11) C. H. Brieskorn and G. H. Mahran, *Arch. Pharm.*, **293**, 1075 (1960).

(12) C. H. Brieskorn and W. Wallenstaeter, *Arch. Pharm. (Weinheim)*, **300**, 8, 717 (1967).

(13) J. Gootjes and W. T. Manta, *Rec. Trav. Chim. Pays Bas*, **73**, 886 (1954); through *Chem. Abstr.*, **49**, 9223c (1954).

(14) H. Hada and M. Inagaki, *J. Pharm. Soc. Jpn.*, **78**, 795 (1958); through *Chem. Abstr.*, **52**, 17357 (1958).

(15) G. Kurono and S. Sasaki, *Yakugaku Zasshi*, **90**, 497 (1970); through *Chem. Abstr.*, **73**, 28969d (1970).

(16) T. J. Coleman and D. V. Parke, *J. Pharm. Pharmacol.*, **15**, 84 (1963).

(17) K. S. H. Finny, G. F. Somers, and J. H. Wilkuison, *ibid.*, **10**, 687 (1958).

(18) B. Pasich, B. Adamezewski, and J. Wisniewski, *Farm. Pol.*, **22**, 35 (1966); through *Chem. Abstr.*, **61**, 15018 (1966).

(19) S. Ogawa, A. Yoshida, and Y. Mitani, *Yakugaku Zasshi*, **96**, 122 (1976); through Ref. 4.

(20) T. Namba, M. Yoshizaki, T. Tomimori, M. Tsuboi, and K. Kato, *ibid.*, **95**, 809 (1975); through *Chem. Abstr.*, **83**, 120949b (1975).

(21) D. Larry, J. M. Fuller, and P. G. Harrill, *J. Assoc. Off. Anal. Chem.*, **53**, 698 (1970). *Ibid.*, **55**, 570 (1972).

(22) T. Vondenhof, K. W. Glombitza, and M. Steiner, *Sci. Pharm.*, **41**, 155 (1973).

(23) "British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973.

(24) E. Stahl, "Thin-Layer Chromatography," 2nd ed., Springer-Verlag, Berlin, Germany, 1969.