pyridine was extracted with ether and dried (sodium sulfate). After evaporation of the ether, the product was recrystallized from benzene to give 0.3 g (40%) of V as pale-yellow crystals, mp 128–130°.

Anal.—Calc. for $C_{17}H_{13}N_3O_7$: C, 54.98; H, 3.50; N, 11.32. Found: C, 54.80; H, 3.60; N, 11.40.

2-Methoxy-6-methyl-1-nitro[1,4]benzodioxino[2,3-*c*]quinoline (VI)—A stirred mixture of 0.37 g (0.001 mole) of V and 10 ml of an aqueous solution of sodium hydroxide (10%) was gently warmed on the steam bath for 2 hr. It was then cooled and neutralized with hydrochloric acid (pH 7). The precipitated product was recrystallized from ethanol to give 0.17 g (52%) of VI as yellowish-green needles, mp 208–210°. The IR spectrum showed the absence of an OH group. The NMR spectrum showed: *(a)* τ 7.41 (protons of the 6-CH_3 group, s), *(b)* τ 5.98 (protons of the 2-OCH_3 group, s), *(c)* τ 1.9 and 2.6 (three and four aromatic protons, two doublets showing an *AB* system), and *(d)* τ 3.02 (five aromatic protons, s).

Anal.—Calc. for $C_{17}H_{12}N_2O_5$: C, 62.96; H, 3.70; N, 8.64. Found: C, 63.06; H, 4.09; N, 8.46.

REFERENCES

(1) G. S. Turpin, *J. Chem. Soc.*, **59**, 714(1891).

(2) O. L. Brady and C. Waller, *ibid.*, **1930**, 1218.

(3) E. Misslin and A. Bau, *Helv. Chim. Acta*, **2**, 295(1919).

(4) V. A. Petrow and E. L. Rewald, *J. Chem. Soc.*, **1945**, 313.

(5) H. Musso, *Chem. Ber.*, **96**, 1927(1963).

(6) M. L. Crossley, P. F. Dreisbach, C. M. Hofmann, and R. P. Parker, *J. Amer. Chem. Soc.*, **74**, 573(1952).

(7) R. C. Clapp, J. H. Clark, J. P. English, C. E. Fellows, R. E. Gritz, and R. G. Shepherd, *ibid.* **74**, 1989(1952).

(8) M. P. Olmsted (Smith Kline and French Laboratories), U.S. pat. 2,947,746 (1960); through *Chem. Abstr.*, **55**, 11443(1961).

(9) A. Sekera, *J. Mond. Pharm.*, **5**, 5(1962); through *Chem. Abstr.*, **58**, 4421(1963).

(10) French pat. 1,173,149 (1959); through *Chem. Abstr.*, **56**, 3488(1962).

(11) J. M. Venditti, A. Goldin, I. Kline, and D. R. Shelden, *Cancer Res.*, **23**, 650(1963).

(12) R. U. Schock, *J. Amer. Chem. Soc.*, **79**, 1672(1957).

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Separation of Anthraquinone Glycosides and Aglycones Using Electropaperography

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Abstract □ A method was developed using an electropaperography apparatus for the separation of hydroxyanthraquinone derivatives, both as glycosides and as aglycones. By using this technique, a mixture of emodin, alizarin, and chrysophanic acid was separated and identified by paper chromatography. Similarly, emodin maltoside and chrysophanic acid maltoside were separated and identified, and the glycosides of senna leaflets were separated and their corresponding aglycones were identified.

Keyphrases □ Anthraquinone aglycones and corresponding glycosides—extraction and separation from plant material by electropaperography □ Senna leaflets—extraction, separation, and identification of glycosides and aglycones by electropaperography □ Electropaperography—separation and identification of anthraquinone glycosides and aglycones

For a number of years, considerable work has been done on the identification of naturally occurring hydroxyanthraquinone derivatives. Numerous methods can be found for the separation of anthraquinone derivatives in several vegetable laxative drugs. Most methods involve the use of either TLC (1) or column (2) or paper (3) chromatography. Characterizations of the anthraquinone derivatives by paper electrophoresis were first described by Core and Kirch (4) and Siesto and Bartoli (5). These workers used single strip paper electrophoresis to accomplish the separation of some hydroxyanthraquinones. The R_f value of each individual compound was determined after each run.

The principle of applying, to a flowing liquid inside a porous medium, an electric field at right angles to the central stream of the mixture to be separated was initially proposed by several investigators (6–9). This method, usually referred to as electropaperography or continuous electrophoresis, is particularly useful for the preparative separations of multicomponent mixtures. The electrolyte solution flows in a direction normal to the lines of force of the electrical field, and the mixture to be separated is added continuously at a small spot in the flowing medium. Components of the mixture are deflected in different directions according to their electrophoretic mobilities and, after passage through the entire plane of the flowing medium, can be collected continuously at various positions. Electropaperography differs from other methods in one important and fundamental respect, *i.e.*, the more slowly migrating components are not required to move over a path that has been previously traversed by the more rapidly migrating components; as a result, absolute separations can be effected. The main purpose of this research was to use this method in the separation of certain plant anthraquinone derivatives, a method that has not been reported previously for these compounds.

EXPERIMENTAL

Separation of Emodin, Alizarin, and Chrysophanic Acid Aglycones—After the components of the electropaperography apparatus[1] (10) were thoroughly cleaned and the apparatus was assembled, the two electrode vessels were filled with 0.01 *M* sodium borate buffer, having an ionic strength of approximately 0.025 (pH 9.6), to approximately 2.5 cm above the horizontal portion of the platinum electrodes. The three liquid compartments or solvent vessels on top of the fractionation unit were then filled with the borate buffer to a given level, depending on the desired speed of fluid flow down the curtain. The buffer levels of the three liquid compartments were kept constant by adding fresh buffer solution. The fractionation surface was then placed on the position that makes an angle of 18° with the fractionation unit. The precut paper curtain[2] was positioned with the fractionation area centered over the supporting fractionation surface plate; the electrode flaps as well as the three solvent flaps were then inserted in their respective containers. The curtain was permitted to be wetted by capillarity for 2 hr, and the effluent was removed before applying the sample to paper.

A 1% solution of equal parts of pure samples of emodin[3], alizarin[4], and chrysophanic acid[3] (11) were prepared using 0.1 *M* aqueous potassium hydroxide as the solvent. The sample solution was then fed by a 1-mm wide filter paper wick to the prewetted paper curtain at point 1 marked on the paper curtain. A potential of 700 v was then applied across the platinum electrodes. After a steady state was established, a current of 14 mamp was observed. To avoid pH gradients that are formed during the experiment, the electrodes were washed with fresh buffer every 24 hr.

After the potential was applied for 96 hr, the sheet was removed and dried in a stream of warm air directed perpendicularly to the fractionation area of the paper. When the sheet was inspected under visible light, three distinct bands could be seen; the red band led to drip points 5–7, the purple band led to drip points 9–12, and the yellow band led to drip points 16 and 17. The various fractions in the collecting tubes were then acidified with hydrochloric acid and extracted with chloroform. The combined chloroform fractions of each tube were evaporated in air to about 0.5 ml.

The chloroform fractions were then spotted on paper[5] and chromatographed by the ascending method using toluene alone as the running solvent (3). The paper was allowed to remain in the sealed jar for about 2 hr. Following removal from the jar, the paper was dried and sprayed with a 0.5% solution of magnesium acetate in methanol and heated in the 90° oven for a few minutes. The developed chromatogram showed purple to pink coloration in daylight and in UV light. The fractions from tubes 5–7 resulted in a spot having an R_f value of 0.55, which was found to represent emodin when compared on the same chromatograph with a reference sample of pure emodin[3]. Similarly, fractions from tubes 9–12 were shown to contain alizarin (R_f 0.99) when chromatographed with pure alizarin[4].

The final proof of identity was established through a comparison of IR spectra of the separated aglycones with those of the pure compounds.

Separation of Emodin Maltoside and Chrysophanic Acid Maltoside—The sample was prepared by dissolving equal parts of emodin maltoside[3] and chrysophanic acid maltoside[3] (11) in approximately 0.1 *M* cold aqueous potassium hydroxide to result in a concentration of 1%. The background electrolyte used was 0.01 *M* sodium borate buffer solution. The precut paper curtain[2] was wetted for 2 hr, and the sample solution was then fed by a 1-mm wide filter paper wick to point 3 on the paper. A potential of 600 v was then applied; after a steady state was established, a current of 9 mamp was observed. At the end of 144 hr, the curtain was removed and dried in the oven. On inspection under visible light, two distinct bands were seen; the red band led to drip points 6–10 and the yellow band led to drip points 20–22. The contents in tubes 6–10 were then combined, as were also the contents in tubes 20–22. Each fraction from the resulting collecting tubes was acidified and allowed to evaporate in air to about 0.5 ml. Samples of each fraction as well as samples of the pure glycosides were chromatographed on paper[5] using a mixture of water–acetone–benzene (2:1:4). After 2 hr, the paper was dried and developed with magnesium acetate solution. Fractions from tubes 6–10 showed a single spot (R_f 0.10) corresponding to emodin maltoside when compared with the reference sample of emodin maltoside[3]. Fractions from tubes 20–22 showed a single spot (R_f 0.32) corresponding to chrysophanic acid maltoside when compared in like manner with pure chrysophanic acid maltoside[3].

As a further proof of the completeness of separation of the glycosides, samples from each set of tubes were hydrolyzed with 10% hydrochloric acid by refluxing in a boiling water bath followed by extraction with chloroform. The chloroform fractions were evaporated in air and chromatographed using toluene as the running solvent. Tubes 6–10 yielded a single spot (R_f 0.55) corresponding to emodin when chromatographed with the pure emodin[3]. Similarly, tubes 20–22 yielded chrysophanic acid (R_f 0.98) when chromatographed with pure chrysophanic acid[3].

Table I—Fractionation of Senna Leaflet Glycosides and Their Aglycones

	Fraction					
	14	15	16	17	18	19
	R_f Value					
Glycosides, sodium borate complexed	0.78	0.70	0.64	0.46	0.45	0.41
Glycosides, in acid form	0.64	0.68	0.75	0.10	0.51	0.72
Aglycones, potassium hydroxide hydrolyzed	0.98	0.96	0.94	0.92	0.93	0.91
Aglycones, hydrochloric acid–ferric chloride hydrolyzed	0.98	0.21	0.98 0.20	0.83	0.99 0.82	0.98

Separation and Identification of Senna Leaflet Hydroxyanthraquinone Glycosides and Their Aglycones by Electropaperography—The extraction procedure of the crude drug was based on the method described by Su and Ferguson (12) using powdered senna[6].

Separation of Aglycones—The chloroform layer was completely air dried, and a 1% solution of the residue was prepared using 0.1 *M* aqueous potassium hydroxide solution. The background electrolyte solution (0.01 *M* sodium borate buffer, pH 9.6) was fed continuously at point 3 by a 1-mm wide filter paper wick to the prewetted[2] paper curtain. A voltage of 400 and a current of 12 mamp were maintained for 144 hr after which fractions 1–22 were removed, air evaporated to about 5 ml, spotted on paper[5], and chromatographed using toluene as the running solvent. Pure samples of rhein[3], aloe-emodin[3], and chrysophanic acid[3] were spotted on the same paper for comparison. Following removal from the jar, the paper was developed using the magnesium acetate solution previously described.

Fractions 12 and 13 yielded single spots with R_f values of 0.02 and 0.03, respectively, corresponding to rhein. Similarly, fractions 15 and 16, with R_f values of 0.98 and 0.99, respectively, corresponded to chrysophanic acid. Fractions 17 and 18, with R_f values of 0.83 and 0.82, respectively, corresponded to aloe-emodin.

Separation of Glycosides from Senna—The alcoholic extract was air dried, and a 1% solution was prepared in 0.1 *M* aqueous potassium hydroxide. This solution was fed continuously by a 1-mm wide wick to the borate buffer prewetted paper curtain[2] at position 3. A voltage of 400 and a current of 10 mamp were maintained during 96 hr, after which the contents of fractions 1–20 were transferred to beakers and air evaporated to about 5 ml. These fractions containing the sodium borate-complexed glycosides were then spotted on paper[5] and chromatographed using water–acetone–benzene (2:1:4). The chromatogram was developed using magnesium acetate solution. The R_f values of those fractions showing spots are

[1] Misco electropaperography apparatus, Microchemical Specialties Co., Berkeley, Calif.
[2] Schleicher and Schuell 598.
[3] Synthesized by N. M. Ferguson.
[4] Fisher Scientific Co., New York, N.Y.
[5] Whatman No. 1.
[6] Senna leaves, *Cassia augustifolia*, Delile, Tinnevelly No. 1, NF X, 40 mesh, Lot No. WEB-414, E-10317, S. B. Penick & Co., New York, N.Y.

shown in Table I. The remaining solution from each fraction was then acidified to release the glycosides from the sodium borate complex, dissolved in alcohol, spotted, chromatographed, and developed as before. The R_f values obtained are shown in Table I.

To determine the exact composition of the glycosides of senna leaves as separated by electropaperography, samples of each fraction were treated with potassium hydroxide and refluxed for 2 hr in a boiling water bath. The solutions, after being cooled, were acidified with hydrochloric acid followed by extraction with chloroform. The latter solutions were in turn evaporated in air to 20 ml, and samples were chromatographed on paper[5] using toluene as the running solvent. Development of the spots with magnesium acetate solution resulted in the R_f values shown in Table I.

The remaining chloroform fractions were then evaporated in air, dissolved in dilute potassium hydroxide solution, and transferred to the reflux flasks. They were hydrolyzed with a combination of hydrochloric acid and ferric chloride by refluxing in a boiling water bath for 3 hr. The content of each flask was then evaporated in air to one-half the volume and extracted with chloroform. The chloroform solutions were evaporated in air to 3 ml and chromatographed on paper[5] using toluene as the running solvent. Development of the spots with magnesium acetate solution resulted in the R_f values shown in Table I.

RESULTS AND DISCUSSION

Although the technique of electropaperography was applied in the last decade to the separation of proteinaceous substances, it has not yet been developed to a state where it can be routinely applied to the separation and purification of many plant constituents.

In this study, this method was applied to the separation of some hydroxyanthraquinone glycosides and their aglycones, using 0.01 *M* sodium borate buffer solution as the background electrolyte. The borate ions complex with the free hydroxy groups and, since the position of the free hydroxy groups varies with each isomer, the degree of borate complexing also varies and, in turn, changes the molecular charge on the compound. The results were reproducible when constant conditions were maintained with respect to the concentration of the buffer and the sample, the pH, the applied potential, the rate of fluid flow, and the kind of paper curtain employed.

Electropaperography is not always the best method of separation even for the substances that it can separate. Older methods such as fractional precipitation by organic solvents, salting out, dialysis, ion exchange, paper chromatography, and TLC are often considered to be faster or more efficient. Electropaperography should be used only for those separations for which it is more efficient. It is an ideal tool for the separation of substances that have high electrophoretic potentials and large differences in mobility.

Good separations were obtained when using a mixture of the pure aglycones chrysophanic acid, alizarin, and emodin. A mixture of pure glycosides consisting of chrysophanic acid maltoside and emodin maltoside was satisfactorily separated, and the identity of the fractions was proved by determination of their R_f values as well as the R_f values of their aglycones. Further proof was obtained from IR spectral studies. In applying this method to a crude drug, senna leaflets were extracted and the free aglycones were separated and identified. The glycosides present were separated, hydrolyzed, and identified through the R_f values of their aglycones. Since some senna leaflet glycosides occur in the dimeric form, more than one aglycone may be produced through hydrolysis.

From Table I it is seen that the senna leaflet glycosides were separated into six fractions. Fraction 14, on hydrolysis with potassium hydroxide, produced chrysophanic acid (R_f 0.98). No further change in R_f value occurred on hydrolysis with hydrochloric acid–ferric chloride solution, showing fraction 14 to consist of the monomeric glycoside chrysophanic acid glycoside. Fraction 15 yielded a single spot (R_f 0.96) on hydrolysis with potassium hydroxide. On further hydrolysis in hydrochloric acid–ferric chloride solution, only one spot (R_f 0.21) was seen. Therefore, this fraction consisted principally of di-rhein glycoside. Through a similar line of reasoning, fraction 16 was found to contain the aglycones rhein and chrysophanic acid, resulting in the dimeric compound rhein–chrysophanic acid glycoside. Similarly, fraction 17 consisted of di-aloe-emodin glycoside, fraction 18 of aloe-emodin–chrysophanic acid glycoside, and fraction 19 of di-chrysophanic acid glycoside.

SUMMARY AND CONCLUSIONS

Methods were developed for the isolation and identification of some hydroxyanthraquinone compounds from plant materials and for the isolation of some hydroxyanthraquinone glycosides from plant material together with a means of identifying them through their corresponding aglycones. By using these techniques, senna leaflets were shown to contain rhein, chrysophanic acid, and aloe-emodin as free aglycones together with chrysophanic acid glycoside, di-rhein glycoside, rhein–chrysophanic acid glycoside, di-aloe-emodin glycoside, aloe-emodin–chrysophanic acid glycoside, and di-chrysophanic acid glycoside. The methods described here should find wide application in the isolation of other types of plant constituents from drug plants, because electropaperography provides larger amounts of individual constituents than the conventional paper chromatography or TLC systems.

REFERENCES

(1) E. Stahl, "Thin Layer Chromatography, A Laboratory Handbook," 2nd ed., Springer Verlag, New York, N.Y., 1969, pp. 706–709.

(2) L. H. Briggs, G. A. Nicholls, and R. Patterson, *J. Chem. Soc.*, **1952**, 1718.

(3) T. J. Betts, J. W. Fairbairn, and V. K. Mital, *J. Pharm. Pharmacol.*, **10**, 436(1958).

(4) A. C. Core and E. R. Kirch, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 513(1958).

(5) A. J. Siesto and A. Bartoli, *Farmaco, Ed. Prat.*, **12**, 517(1951).

(6) H. Svensson and I. Brattsten, *Arkiv Kemi*, **I**, 401(1949).

(7) W. Grassmann and K. Hannig, *Naturwissenschaften*, **37**, 397(1950).

(8) M. Vier, "Electrophoresis Theory, Methods and Applications," vol. II, Academic, New York, N.Y., 1967, pp. 449–451.

(9) R. J. Block, E. L. Durrum, and G. Zweig, "A Manual of Paper Chromatography and Electrophoresis," Academic, New York, N.Y., 1958, pp. 537–551.

(10) W. T. Gordon and T. K. Ling, "Spectrolation, and Continuous Flow Electrophoresis with the Misco and Kird-Misco Variable Angle Plate Spectrolators," Microchemical Specialties Co., Berkeley, Calif., pp. 1–20.

(11) N. M. Ferguson and J. H. Gardner, *J. Amer. Chem. Soc.*, **72**, 2887(1950).

(12) C. Y. Su and N. M. Ferguson, *J. Pharm. Sci.*, **62**, 899(1973).

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