A Specific Colorimetric Method for Estimation of Phlorrhizin in Biological Fluids

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The methods for estimation of phlorrhizin were reviewed by Lambrechts (1937), who pointed out that none of the methods discussed was specific. The most useful and sensitive, ultraviolet spectroscopy, does not distinguish between phlorrhizin and its aglucone phloretin. Furthermore, none of the methods was applicable to the estimation of phlorrhizin in urine. Braun, Whittaker & Lotspeich (1957) have described a method for estimation of phlorrhizin in urine, based on chromatographic separation combined with use of the Folin & Ciocalteu (1929) reagent. The method described here is based on the reaction used by Lehongre, Neumann & Lavollay (1950) for the estimation of hydrogen peroxide. Hydrogen peroxide in the presence of phlorrhizin gives a red colour, which is proportional to the amount of hydrogen peroxide present. We have found that if the amount of hydrogen peroxide is kept constant, the colour is proportional to the amount of phlorrhizin present. Neumann, Lehongre & Lavollay (1954, 1955, 1956*a, b*) claim that the preliminary step in the reaction is the formation of what they term 5-glucose-1:3:4:5-tetrahydroxybenzene by the Dakin (1909) reaction. This substance is then further oxidized in the presence of air to a red compound of unknown constitution.

METHODS

Phlorrhizin is first brought into solution in borate buffer (pH 9.1). The pH is important both because the rate of the reaction depends on pH and also because the final product acts as an indicator which is green at pH 12, red at pH 9 and yellow at pH 6.5. Since the rate of colour development also depends on electrolyte concentration the phlorrhizin is first extracted with butanol, and then re-extracted from the butanol with borate buffer. Hydrogen peroxide is then added to the borate extract and the colour compared with that of a standard phlorrhizin solution treated in a similar way.

Reagents. Hydrogen peroxide (100 vol.), 1 ml. diluted to 1 l., was used. Borate buffer (pH 9.1) was prepared by mixing 9 parts of 0.1 M-disodium tetraborate with 1 part of 0.4 M-boric acid. Barium hydroxide (0.3 N) and zinc sulphate (5%, w/v) solutions were prepared and standardized according to the directions of Somogyi (1945) for deproteinizing blood.

Preparation of phlorrhizin and its derivatives. Commercial phlorrhizin was purified by heating a solution (15 g./l.) to 80° for 10 min. with activated charcoal. The filtered solution was left overnight at 4°, and pure white crystals were obtained. Phloretin was prepared according to the method of Bach (1939), and recrystallized from acetic acid. Phlorin and phloretic acid were prepared by the method of Cremer & Seuffert (1912) and some phloretic acid was also prepared by the method of Neumann *et al.* (1956*a*). Phloroglucinol was obtained commercially.

Preparation of the standard. For estimation of phlorrhizin in clear solutions 0.2 mm-phlorrhizin in borate buffer (pH 9.1) can be used as standard. For biological fluids it is better to make up the standard in the appropriate fluid. By this means errors due to slight degrees of haemolysis of blood, presence of urinary pigments and losses in extraction are eliminated. These standards are prepared by addition of 0.4 ml. of aqueous 2 mm-phlorrhizin solution to 4 ml. of the fluid, e.g. bile or plasma. Preliminary trials showed that these standards gave results identical with those prepared by addition of solid phlorrhizin to the biological fluid. These standards were submitted to the same extraction procedure as the test solutions.

Extraction of phlorrhizin from biological fluids

Plasma. To 4 ml. of plasma were added 5 ml. of butanol and one drop of conc. HNO_3 . The tube was shaken for 3 min. The emulsion formed was separated by centrifuging at 3000 rev./min. for 10 min. The butanol layer was pipetted off, and 3 ml. was added to 5 ml. of borate buffer. The mixture was shaken for 3 min. and then the aqueous layer was collected.

Bile. To decolorize the bile 1 ml. was added to 14 ml. of water, 2 ml. of 0.3 N- $\text{Ba}(\text{OH})_2$ and 2 ml. of 5% ZnSO_4 . The solution was shaken and filtered. To 5 ml. of filtrate in a separating funnel were added 5 ml. of butanol and one drop of conc. HNO_3 . The butanol layer separated without centrifuging and the procedure was then continued as described for plasma.

Urine. To 5 ml. of urine in a separating funnel were added 5 ml. of butanol and one drop of conc. HNO_3 , and the procedure was continued as described for bile.

Estimation of phlorrhizin

Into each of two test tubes was measured 2 ml. of borate extract of the standard, and into two other test tubes 2 ml. of borate extract of the unknown solution containing 0.02–2.0 mg. of phlorrhizin. To one tube of standard and one of unknown was added 1 ml. of hydrogen peroxide solution and to the other two tubes 1 ml. of water.

The tubes were left at room temperature for 2 hr., and the extinctions read at 490 m μ . The concentration of phlorrhizin in the unknown solution was calculated from the extinctions corrected by subtraction of those of the water blanks.

By ultraviolet spectroscopy. Phlorrhizin, according to Lambrechts (1937), may exist in either enol or keto form, the former with maximum extinction at 325 m μ , and the latter with maximum extinction at 285 m μ . In aqueous solution the form depends on pH, and in the method of Lambrechts (1937) for plasma the extinction is read at 325 m μ . In ethanolic solution phlorrhizin is present in the keto form. For estimation of phlorrhizin in dog bile, the bile is diluted 250–1000 times with ethanol; this is filtered and the extinction value is read at 285 m μ .

By chromatography. For urine, a modification of the chromatographic method of Braun *et al.* (1957) was used in which the final estimation of the eluate was carried out by u.v. spectroscopy instead of by the Folin & Ciocalteu reaction. Ascending chromatograms were run for 5 hr. at 25° in the non-aqueous phase of butanol–acetic acid–water (4:1:5, by vol.). Urine (up to 0.5 ml.) was spotted on paper from an Agla micrometer syringe. The spot should contain 5–500 μ g. of phlorrhizin. If the spot contained more than 250 μ g., the final position of phlorrhizin could be located by u.v. fluorescence. For smaller amounts a control spot from 0.1 ml. of ethanolic phlorrhizin solution (5 mg./ml.) was run. Squares of paper 3 cm. $\times$ 3 cm. containing the final spots were cut out and eluted with absolute ethanol. Elution by putting a filter paper into a solution of ethanol is unsatisfactory and is accompanied by considerable loss of phlorrhizin. We have found the most effective method is to suspend the square of paper and to run 5 ml. of absolute ethanol slowly over it from a dropping funnel. The extinctions of ethanol eluates were read at 285 m μ .

Spectroscopy. A Uvispek spectrophotometer with 1 cm. silica cuvettes was used.

RESULTS

Relation between phlorrhizin concentration and extinction. A series of solutions of phlorrhizin, from 0.02 to 2.0 mM, in borate buffer was made up. To 2 ml. of each solution without any preliminary extraction was added 1 ml. of hydrogen peroxide, and the extinction was measured after 2 hr. Over the range of concentrations tested the extinction varied in a linear manner with the original phlorrhizin concentration, an extinction of 1.0 corresponding with a concentration of 0.8 mM.

Effect of electrolyte concentration on colour development. In preliminary experiments it was observed that the use of two borate-buffer solutions of the same pH but different molar concentration leads to different rates of colour development. That electrolyte concentration can affect the rate of colour development is shown in Fig. 1. This source of error, which might be appreciable if solutions are concentrated before estimation, is prevented by preliminary extraction with butanol.

Estimation of phlorrhizin in plasma. Table 1 shows the results of estimating phlorrhizin in dog

plasma. In these experiments phlorrhizin was added to plasma. The phlorrhizin was also estimated by the method of Lambrechts (1937). Plasma containing no phlorrhizin, when estimated by this method, always gives a blank value which varies in different plasma specimens, and if slight haemolysis has occurred the high blank values may interfere seriously with the estimation. With the hydrogen peroxide method even moderate amounts of haemolysis do not interfere with the estimation.

Fig. 1. Relation between the extinction value at 490 m μ and the time after adding the hydrogen peroxide. Hydrogen peroxide (1 ml.) was added to 2 ml. of 0.5 mM-phlorrhizin made up in borate buffer (■), borate buffer containing 0.5% of NaCl (Δ), borate buffer containing 10% (w/v) of NaCl (●) and borate buffer containing 20% (w/v) of NaCl (○).

Table 1. *Estimations of phlorrhizin in dog plasma to which different amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Lambrechts' method
0	0	0.04
0.06	0.04	0.09
0.12	0.13	0.16
0.23	0.23	0.26
0.47	0.48	0.51
0.70	0.68	0.72
0.94	0.95	0.97
1.17	1.19	1.19
1.41	1.41	1.44
1.64	1.66	1.66
1.88	1.84	1.89

Estimation of phlorrhizin in bile. Jenner & Smyth (1957) have shown that when phlorrhizin is administered to animals, large amounts appear in the bile. For this reason, relatively high concentrations of phlorrhizin were made up in dog bile. The bile was obtained by cannulation of the common bile duct after ligation of the cystic duct. Although bile contains substances which have a high extinction value at 285 m μ , it is possible to use u.v. spectroscopy for estimation of phlorrhizin in bile since because of the large amounts of phlorrhizin expected the bile can be greatly diluted. Table 2 gives figures for the amounts of phlorrhizin in bile as estimated by u.v. spectroscopy and by the hydrogen peroxide reaction.

Estimation of phlorrhizin in urine. It is not possible to use u.v. spectroscopy for urine unless this is combined with a chromatographic separation of phlorrhizin from other substances which give high extinction values in the u.v. range. Solutions of phlorrhizin in dog urine were made up and estimated both by the hydrogen peroxide method and the chromatographic separation com-

bined with u.v. spectroscopy. The results are given in Table 3.

Specificity of the reaction. The reaction has been tested with solutions in borate buffer of phloretin, phlorin, phloretic acid and phloroglucinol. No colour is obtained with any of these substances. The absence of reaction with phloretin is of particular interest, since considerable doubt exists as to whether phlorrhizin or its aglucone is responsible for the biological activity, and hitherto no simple method has been available for distinguishing between the two substances.

Interfering substances. Any substance which reacts with hydrogen peroxide will interfere with the reaction. Such substances, e.g. glucose and ascorbic acid, in the amounts in which they are likely to be present in biological fluids, are not extracted in sufficient quantities to interfere with the reaction. All the phlorrhizin derivatives are extracted with butanol, but of these phloroglucinol is the only one which interferes with the reaction, possibly because of its strong reducing properties. It can, however, readily be distinguished by u.v. spectroscopy, and if necessary can be separated by the chromatographic procedure described.

SUMMARY

A simple colorimetric method is described for the estimation of phlorrhizin in biological fluids. The method appears to be highly specific for phlorrhizin, and is not given by any phlorrhizin derivative which has been tested.

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Table 2. *Estimations of phlorrhizin in dog bile to which various amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Spectroscopic method
0	0	0.02
2.60	2.52	2.54
3.86	4.10	3.78
5.05	4.87	4.89
6.54	6.54	6.65
7.71	7.92	7.67
8.94	8.88	9.03

Table 3. *Estimations of phlorrhizin in dog urine to which different amounts of phlorrhizin had been added*

Concn. of added phlorrhizin (mM)	Concn. found (mM)	
	Hydrogen peroxide method	Chromatographic method
0	0	0.02
0.04	0.02	0.07
0.09	0.08	0.09
0.18	0.18	0.20
0.27	0.27	0.29
0.54	0.53	0.57
0.81	0.81	0.82
1.08	1.06	1.08
1.35	1.37	1.32
1.89	1.85	1.84