A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF β-GLYCYRRHETIC ACID (ENOXOLONE) AND ITS ESTERS IN BIOLOGICAL MATERIALS

BY T. J. COLEMAN AND D. V. PARKE

From the Department of Biochemistry, St. Mary's Hospital Medical School, University of London, London, W.2

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A method for the determination of β-glycyrrhetic acid (enoxolone) and its readily-hydrolysable esters in biological materials is described. The material containing the glycyrrhetic acid or its esters is hydrolysed with ethanolic sodium hydroxide, the glycyrrhetic acid is extracted from the acidified hydrolysate, submitted to two-dimensional, thin-layer chromatography on alumina, eluted with ethanol and estimated spectrophotometrically at 248 mμ. Mean recoveries of 100 μg. β-glycyrrhetic acid were 102 per cent (s.e.m. ± 4 per cent) from pure solution; 92 per cent (s.e.m. ± 4 per cent) from rat blood; 90 per cent (s.e.m. ± 4 per cent) from human urine; and recoveries of 20 μg. and 50 μg. from rat bile were 91 to 95 per cent. Mean recoveries of 100 μg. of β-glycyrrhetic acid hydrogen succinate were 95 per cent (s.e.m. ± 5 per cent) from pure solution; 100 and 110 per cent from rat blood; and 92 and 109 per cent from human urine.

β-GLYCYRRHETIC acid (enoxolone) is a triterpenoid obtained from liquorice root which has anti-inflammatory properties (Finney and Somers 1958). It has been used in the treatment of dermatitis (Colin-Jones, 1960) and more recently in the treatment of gastric ulcer (Doll, Hill, Hutton and Underwood, 1962). Recent metabolic studies with tritium-labelled glycyrrhetic acid in the rat have shown that metabolites are excreted mainly via the bile and less than 2 per cent of the activity is found in the urine (Parke, Pollock and Williams, 1963). It is therefore desirable to have a method for the determination of glycyrrhetic acid, which could be applied to bile and faeces as well as to blood and urine.

Several colorimetric methods for the determination of glycyrrhetic acid have been described, the colours being developed with vanillin and sulphuric acid (Weist, 1949), 2,6-di-t-butyl-*p*-cresol and sodium hydroxide (Brieskorn and Mahran, 1960) or by heating with a solution of antimony pentachloride in chloroform (Dr. S. Gottfried, personal communication). Because of lack of specificity or sensitivity, none of these methods is suitable for the determination of glycyrrhetic acid in biological materials.

Spectrophotometric methods have been described for the determination of glycyrrhetic acid in urine (Van Katwijk and Huis in't Veld, 1955) and in blood (Finney, Somers and Wilkinson, 1958), but both of these methods are unsatisfactory because of the lengthy chromatography procedure in the former and the absence of specificity in the latter.* Because of this lack of specificity, a rigorous separation of the β-glycyrrhetic acid is an essential preliminary to any estimation. Its separation from cholesterol

* Since the completion of this work a similar method for the determination of 18-β-glycyrrhetic acid in faeces has been published (Helbing, 1963).

and other steroids which in its chemical structure and solubilities it closely resembles, is particularly important.

A method has now been devised for the spectrophotometric determination of glycyrrhetic acid following a simple thin-layer chromatographic separation from sterols and other biological substances. This method is both sufficiently sensitive and specific for the determination of this triterpenoid in biological materials.

EXPERIMENTAL

Materials

β-Glycyrrhetic acid (3-hydroxy-11-oxo-18β-olean-12-en-30-oic acid), m.p. 285–7°, $[\alpha]_D^{19°} + 163°$ (Biorex Laboratories); β-glycyrrhetic acid hydrogen succinate (3-*O*-β-carboxypropionyl-11-oxo-18β-olean-12-en-30-oic acid), m.p. 315°, $[\alpha]_D^{20°} + 130°$, light absorption in ethanol, λ_{max} 248 mμ, ϵ_{max} 12,100 (Biorex Laboratories); cholesterol, m.p. 148°, and coprostanol (coprosterol), m.p. 101°, were used for the estimation procedures. Alumina (Aluminiumoxid G nach Stahl, Merck), silica gel (Kieselgel G nach Stahl, Merck) and Polyamide (Merck) were used as adsorbents in the thin layer chromatography.

Ultra-violet Absorption Spectra

The ultra-violet absorption spectrum of β-glycyrrhetic acid and subsequent quantitative estimations were determined with a Unicam SP 500 spectrophotometer. The light absorption of β-glycyrrhetic acid in ethanol (99·7 per cent, R.R. Grade for spectroscopy) showed maxima at 204 mμ (ϵ_{max}, 11600) and 248 mμ (ϵ_{max} 18700). The absorption at 248 mμ obeyed the Beer-Lambert Law over the range of 1 to 25 μg. of β-glycyrrhetic acid per ml. Previous spectrophotometric determinations have also been made at 248 mμ (Van Katwijk and Huis in't Veld, 1955; Finney, Somers and Wilkinson, 1958).

Chromatography

The principal problem was the separation of β-glycyrrhetic acid from cholesterol and other sterols which would interfere in the spectrophotometric determination of glycyrrhetic acid. β-Glycyrrhetic acid, β-glycyrrhetic acid hydrogen succinate, cholesterol, coprostanol, and extracts of biological materials containing β-glycyrrhetic acid were chromatographed on thin-layer plates (Desaga, Heidelberg) of alumina, silica gel and Polyamide. The chromatography plates were developed in various solvent systems and some R_F values are given in Table I.

The most efficient separation of β-glycyrrhetic acid from cholesterol, coprostanol and other biological substances was achieved by two-dimensional chromatography on thin-layer plates of alumina, developed first in acetone-chloroform (1 : 1 for 30 min.) and then at right angles in methanol-ammonia (s.g. 0·88) 4 : 1 for 90 min.). The α- and β-isomers of glycyrrhetic acid could be separated on alumina in methanol-ammonia (4 : 1), the R_F value for α-glycyrrhetic acid being 0·82.

The extracts of β-glycyrrhetic acid in solution in chloroform were applied to the adsorbent as a single spot of 2-5 mm. diameter. After development in the two solvent systems the chromatography plates were dried in air at room temperature to ensure the removal of any ammonia and the β-glycyrrhetic acid was located under ultra-violet light (254 mμ, Hanovia "Chromatolite") as a dark ultra-violet-absorbing spot at the correct R_F values (see Table I). This method of detection was sensitive to as little as 4 μg. of glycyrrhetic acid per cm.^{2} and in practice 2 μg. could be chromatographed and subsequently detected.

The β-glycyrrhetic acid was eluted from the alumina with ethanol, but the efficiency of the elution was found to be dependent on the pH of the eluent. At pH values of less than 2·0 the elution of β-glycyrrhetic acid was quantitative (100 ± 5 per cent), but became less at higher pH values (pH 4, 78 per cent; pH 5, 65 per cent; pH 6, 62 per cent).

TABLE I

APPROXIMATE R_F VALUES OF β-GLYCYRRHETIC ACID AND SOME STEROLS ON THIN-LAYER CHROMATOGRAPHY

Adsorbent	Solvent	R_F Values of			
		β-Glycyrrhetic acid	β-Glycyrrhetic acid hydrogen succinate	Cholesterol	Coprostanol
Alumina	n-Hexane(100)chloroform(1) ..	0	0	0·12	0·31
,,	Chloroform(1)acetone(1) ..	0	0	0·96	0·92
,,	Toluene(4)piperidine(1) ..	0·14	0	0·93	0·93
,,	n-Butanol(10)ammonia*(1) ..	0·96 (1) 0·36 (10)	0·96 (5) 0·36 (1) 0 (5)	0·89	0·89
	Methanol(20)ammonia*(1) ..	0·95 (1) 0·77 (10)	0·66 (1) 0·29 (5)	0–1·0†	0–1·0†
,,	Methanol(10)ammonia*(1) ..	0·92	0·92 (1) 0·66 (5)	0‡	0‡
,,	Methanol(4)ammonia*(1) ..	0·95	0·95 (5) 0·66 (1)	0	0
Silica gel	Toluene(2)piperidine(1) ..	0·50	—	0·92	0·92
,,	n-Butanol(10)ammonia*(1) ..	1·0	0·88	0·84	0·84
,,	Methanol(10)ammonia*(1) ..	0·98	0·98	0–1·0‡	0–1·0‡
,,	n-Hexane(8)ammonia*(1)-ethanol(1)	0	0	0·95	0·95
Polyamide	Chloroform(1)acetone(1) ..	0·55	—	0·91	—

* s.g. 0·88. † Continuous streaking. ‡ Streaking

Hydrolysis of Esters of Glycyrrhetic Acid

To simplify the chromatographic separation, extracts of glycyrrhetic acid were submitted to a preliminary hydrolysis procedure to convert esters of glycyrrhetic acid and sterols into the free compounds. The hydrolysis procedure, namely heating in 2N sodium hydroxide-ethanol (1 : 2 by vol.) for 5 min., quantitatively converts glycyrrhetic acid hydrogen succinate into free glycyrrhetic acid and has no destructive effect upon the glycyrrhetic acid (recoveries, 98 ± 5 per cent).

The removal of gross amounts of sterols before chromatography was necessary for efficient separation in the chromatography procedure and this was achieved by extraction of the saponified solutions with n-hexane. To prevent entrainment of glycyrrhetic acid into the hexane phase it is necessary to remove the ethanol from the hydrolysed solution. When

the ethanol is allowed to evaporate during the hydrolysis, extraction of the saponified solution (4 ml.) with n-hexane (5 ml.) removes most of the sterols but less than 3 per cent of the glycyrrhetic acid (recoveries, 97 per cent).

The glycyrrhetic acid may be quantitatively recovered from the hydrolysed solution (4 ml.) by acidification with 2 ml of 2N sulphuric acid, followed by a single extraction with 5 ml. of chloroform. By this procedure recoveries of β-glycyrrhetic acid were 97 ± 4 per cent of theoretical.

The Standard Procedure

Gently boil a solution of biological fluid (1 ml.) containing 20–100 μg. of β-glycyrrhetic acid or its esters with 2N sodium hydroxide (1 ml.) and ethanol (2 ml.) in a 10 ml. test-tube, allowing the ethanol to evaporate. Cool the solution, add water (4 ml.) and shake the mixture in the stoppered test-tube with n-hexane (5 ml.) for 5 min. Centrifuge the mixture to separate the two phases and remove and discard the hexane. Acidify the aqueous phase with 2N sulphuric acid (2 ml.) and extract the glycyrrhetic acid by shaking in the stoppered test tube with chloroform (5 ml.) for 5 min. Centrifuge the mixture again to separate the two phases and remove and discard the aqueous phase. Dry the chloroform by adding anhydrous sodium sulphate (0·25 g.), centrifuge, and remove the chloroform. Wash the sodium sulphate with chloroform (1 ml.) and add to the chloroform extract; concentrate to about 0·2 ml. Transfer the concentrated chloroform extract quantitatively to a thin-layer chromatography plate of alumina and develop first in one direction in acetone-chloroform (1 : 1) and then at right-angles in methanol-ammonia (4 : 1). Dry the plate for 10 min. in air at room temperature and detect the glycyrrhetic acid under ultra-violet light at the appropriate R_F value. Remove the area of adsorbent containing the triterpenoid and transfer to a 10 ml. stoppered test-tube containing 3 ml. of ethanol containing 1 per cent v/v of 2N hydrochloric acid. Shake the mixture for 5 min. and centrifuge to deposit the alumina. Measure the light-absorption of the supernatant ethanol at 248 mμ and evaluate the content of β-glycyrrhetic acid from a calibration curve. Obtain a blank by eluting an equivalent amount of chromatographed alumina. In our work the blank gave E (1 cm.) 248 mμ = 0·02, equivalent to 1·5 μg. β-glycyrrhetic acid, and this was subtracted from the experimental determinations.

Recoveries of β-Glycyrrhetic Acid

From pure solution. In a series of five estimations using the full standard procedure of hydrolysis, chromatography and spectrophotometric determination, the mean recovery of 100 μg. of β-glycyrrhetic acid from solution in ethanol was 102 per cent (s.e.m. ± 4 per cent). The mean recovery of 100 μg. of glycyrrhetic acid from ethanolic solutions containing also 100 μg. of cholesterol was 98 per cent (s.e.m. ± 4 per cent) and the recoveries from solutions containing 1·00 mg. and 10·0 mg. of cholesterol were 96 and 92 per cent respectively.

From blood. β-Glycyrrhetic acid in amounts of 100 μg. and 20 μg. were added to 1 ml. of heparinised rat blood. Estimation of the glycyrrhetic acid by the standard procedure gave mean recoveries for four

experiments of 92 per cent (s.e.m. ± 4 per cent) for 100 μg. and 104 per cent (s.e.m. ± 10 per cent) for 20 μg.

From urine. β-Glycyrrhetic acid in amounts of 100 μg. and 20 μg. were added to 5 ml. of human urine. The glycyrrhetic acid was estimated by the standard procedure except that the 4 ml. of water usually added after the alkaline hydrolysis was omitted. Mean recoveries for four experiments were 90 per cent (s.e.m. ± 4 per cent) for 100 μg. and 102 per cent (s.e.m. ± 8 per cent) for 20 μg.

From bile. β-Glycyrrhetic acid in amounts of 20 μg. and 50 μg. was added to 1 ml. of rat bile, collected by biliary cannulation, and the glycyrrhetic acid was estimated by the standard procedure. Recoveries were 91 and 93 per cent for 50 μg. and 94 and 95 per cent for 20 μg. Recoveries from concentrated rabbit bile were unsatisfactory due to the high mucin content and the material coagulated on addition of acid.

From faeces. Recoveries of β-glycyrrhetic acid from rat faeces were unsatisfactory (40–50 per cent) and location of the triterpenoid on the thin-layer chromatogram was difficult. Much of the added β-glycyrrhetic acid was not to be found at the expected R_F value. It is possible that the glycyrrhetic acid is changed chemically by the faecal bacteria into a substance with different chromatographic and light absorption characteristics.

Recoveries of β-Glycyrrhetic Acid Hydrogen Succinate

From pure solution. In a series of four estimations using the standard procedure the mean recovery of 100 μg. of β-glycyrrhetic acid hydrogen succinate from solution in ethanol was 95 per cent (s.e.m. ± 5 per cent).

From blood. β-Glycyrrhetic acid hydrogen succinate in amounts of 100 μg. and 20 μg. were added to 1 ml. of heparinized rat blood. Estimation of the glycyrrhetic acid by the standard procedure gave recoveries of 100 and 110 per cent for 100 μg., and 89 and 112 per cent for 20 μg.

From urine. Recoveries of 100 μg. of glycyrrhetic acid hydrogen succinate from 5 ml. of human urine were 92 and 109 per cent.

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References

Brieskorn, C. H. and Mahran, G. H. (1960). *Arch. Pharm. Berl.*, **293**, 1075–1078.
Colin-Jones, E. (1960). *Postgrad. med. J.*, **36**, 678–682.
Doll, R., Hill, I. D., Hutton, C. and Underwood, D. J. (1962). *Lancet*, **2**, 793–796.
Finney, R. S. H. and Somers, G. F. (1958). *J. Pharm. Pharmacol.*, **10**, 613–620.
Finney, R. S. H., Somers, G. F. and Wilkinson, J. H. (1958). *Ibid.*, **10**, 687–695.
Helbing, A. R. (1963). *Clin. chim. Acta.*, **8**, 756–762.
Parke, D. V., Pollock, S., and Williams, R. T. (1963). *J. Pharm. Pharmacol.*, **15**, 500–506.
Van Katwijk, V. M. and Huis in't Veld, L. G. (1955). *Rec. trav. chim. Pays-Bas*, **74**, 889–896.
Weist, F. (1949). *Kolorimetrische Bestimmung der Glycyrrhizinsaure als Aglukon*, Dissertation, Zurich.