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Note

Rubeanic acid: a general spray reagent for the detection of steroids on thin-layer plates

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In the detection of steroids, aggressive reagents can be used with inorganic adsorbents, so that all classes of steroids can be visualized through colour or fluorescence reactions. Stahl[1] and Heftmann[2] compiled more than 60 reagents for specific and non-specific detection of steroids, while Heftmann *et al.*[3] made studies on the colour reaction of sulphuric acid with an impressive list of 142 steroid hormones, sterols, bile acids, cardenolides, sapogenins and alkaloids, giving various colours and fluorescence. Sulphuric acid was found to have a low specificity but high sensitivity. For biological materials, the Liebermann–Burchard reagent (acetic anhydride–sulphuric acid) is the most widely used spray reagent for quantitative analysis of cholesterol[4]. Recently, however, molybdophosphoric acid[5], which is non-specific and detects most reducing substances, together with chloramine-T[6] has been recommended for the detection of cholesterol.

In 1951 Lewis and Griffiths[7] reported that rubeanic acid, also known as dithiooxamide, a red crystalline powder, can form coloured complexes with various metal ions, lead, copper, manganese, nickel, mercury and bismuth, on thin-layer plates. Since then an ethanolic solution of rubeanic acid has been used to detect these inorganic ions on such plates. The formation of the coloured complex is completed by spraying with an aqueous solution of ammonia or developing the chromatogram in a chamber saturated with ammonia vapour. In this study, rubeanic acid has been found to detect up to 0.1 μg of various types of steroids.

EXPERIMENTAL

A 1% solution of rubeanic acid in concentrated sulphuric acid was sprayed on to pre-coated silica gel plastic sheets (0.2 MM, Merck) that had been spotted with various steroid standards dissolved in a suitable solvent, at different concentrations, and dispensed through a microlitre syringe. The plates were developed with chloroform–methanol (10:1). The spots were evaluated with a densitometer (Shimadzu Dual Wavelength TLC Scanner OS-930 equipped with a data recorder DR-2) at 254 nm. The colours produced after spraying were observed at room temperature and under short wavelength UV irradiation (Chromato-vue). The plates were then heated at 110°C and the colour responses again observed. The colour responses and the limits of detection are given in Table I.

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TABLE I

COLOUR REACTIONS OF STEROIDS WITH RUBEANIC ACID–SULPHURIC ACID SPRAY REAGENT

Abbreviations: BE = blue; BN = brown; BT = bright; GN = green; GY = grey; LT = light; OE = orange; PK = pink; PU = purple; RD = red; YW = yellow.

Steroid	*Colour after spraying at room temperature*	*Colour after heating at 110°C*				*Limit of detection (μg)*
		In daylight			*In short wavelength UV light*	
		*Time (min)**	*Initial*	*Final*		
Estradiol	–	2.25	YW	BT-PK	GN	0.1
Progesterone	–	5.00	YW-BN	LT-BN	GY	5.0
17-Hydroxyprogesterone		2.90	YW-BN	LT-BN	BN	0.25
Pregnenolone	OE		OE	PU	PU	1.0
Pregnenolone acetate	OE		OE	PU	PU	0.4
16-Dehydropregnenolone acetate	OE		OE	YW-BN	YW-BN	0.2
Testosterone	–	1.00	LT-BN	PU	BN	0.15
Methyltestosterone	BT-YW		YW	YW	YW	0.1
Testosterone acetate	–	1.50	LT-BN	PU	BN	0.1
Testosterone propionate	–	2.50	LT-BN	PU	BN	0.1
Δ^4-Androstene-3,17-dione	–	2.50	LT-BN	PU	BN	0.2
Dehydroepiandrosterone acetate	OE		OE	PU	DK-BN	0.2
Cholesterol	–	1.00	RD	PU	DK-PU	0.1
Cyclaudenol	–	1.80	OE-BN	OB-BN	BN	0.3
Stigmasterol	–	2.83	BE	PU	BN	0.4
Cycloartenol	–	2.00	BE	BN	DK-BN	0.5

* Time for the appearance of colour.

RESULTS AND DISCUSSION

Although only few of the steroids were detected at room temperature, all were detected when the thin-layer plates were heated at 110°C. Cholesterol and testosterone required the least time for the development of the coloured product upon heating at 110°C, while progesterone required the most. Rubeanic acid was most sensitive to estradiol, testosterone and its derivatives, as well as cholesterol. Estradiol gave an intense bright pink colour after heating which was found to be stable for quite a long period of time.

The Δ^5-3β-hydroxy steroids and their acetate derivatives, which contain either a conjugated or a non-conjugated carbonyl group at carbon-17, gave a characteristic orange spot, the intensity of which is proportional to its concentration. Because of the negative results given by the phytosterols, a carbonyl group at C-17 or C-20 together with a double bond at C-5 and C-6 seems to be important for the reaction at room temperature.

The negative reactions given by testosterone, testosterone acetate and testosterone propionate compared with the positive reaction given by methyltestosterone at room temperature indicated that the hindered hydroxyl group at C-17 is more important for the reaction than a hydroxyl group *per se*, or the Δ^4-3-keto group which is probably deactivated due to electron delocalization. It is possible that the

reaction is specific for a certain orientation of the hydroxyl group. The spray reagent described can therefore be used to differentiate methyl testosterone from testosterone or its derivatives.

Comparing detection limits, rubeanic acid gives similar results to those with the Komarowsky reagent (*p*-hydroxybenzaldehyde–sulphuric acid) for 3-keto steroids unsubstituted in the 2 position, and with antimony(III) chloride[1]. It is far superior to the Liebermann–Burchard spray reagent.

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