Short Communication

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Analytical Studies on Biologically Active Compounds. *Part II.* Separation and quantitation of mixtures of isatin derivatives for application to metabolism studies

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The 2-oxo-3-indolyl nucleus is a fundamental structure in a number of biologically important compounds [1-4]. Isatin and its derivatives are well known for their biological properties [5-7]. Derivatives of isatin and N-methyl isatin have been found to act as central nervous system depressants and are used for prevention of small pox etc. [8]. It was found necessary to develop analytical system for the separation, identification and estimation of potential biologically active isatin derivatives and some of their possible metabolities [9-10].

The purpose of this paper is to describe the development of a simple TLC method for separation and quantitative determination of various mixtures of potential biologically active isatin derivatives.

Isatin and its derivatives were synthesized by known methods reported in the literature : isatin (I) [11], 1-methyl isatin (II) [12], isatin oxime (III) [13], isatin-3-hydrazone (IV) [14], isatin-3-phenyl hydrazone (V) [14], isatin-3-thiosemicarbazone (VI) [15], 1-methyl isatin-3-thiosemicarbazone (VII) [16], 1-acetyl isatin-3-thiosemicarbazone (VIII) [16] and 3-acetyl isatin-3-thiosemicarbazone (IX) [17]. The following solvent systems were used: (1) Methylene chloride - Ethyl acetate (75 : 25 v/v), (2) Chloroform - Methanol (96 : 4 v/v), (3) Pet. ether - Methylene chloride - Ethyl acetate (45 : 35 : 20 v/v), (4) Carbon disulphide - Ethyl acetate (70 : 30 v/v) and (5) Benzene - Ethyl acetate (55 : 45 v/v).

Thin layer chromatography. Generally, solutions of the compounds were prepared by dissolving 2 mg of pure compound in 1 ml of chloroform. Using an accurately calibrated micropipet, samples of the solutions were applied to silica gel G-60 plates (20 x 20 cm). All chromatograms were developed in chromatographic chambers saturated in solvent vapours. The solvent front was allowed to travel 15 cm, the plates were removed, air dried and sprayed with iodine solution, rechromatography was conducted in order to confirm R_f values.

* For correspondance.

Seven different mixtures of three, four and five compounds randomly selected were prepared for qualitative analysis. The same mixtures were prepared using accurately measured amounts for separation and quantitative determination of individual components.

Quantitative determination. The relationship between UV absorption and concentration of various compounds was established by preparing solution of compounds (0.5 - 2.5 mg/ml; at 0.5 mg intervals) and measuring their absorption at λ_{max} (Table 1), using a spectrophotometer Hitachi-113 with 1 cm cells. Standard curves of absorbance *vs* concentration of different compounds were then drawn.

Accurately measured solutions of different compounds were applied on thin layers, duplicates were run parallel. The chromatograms were developed and the parallel running spots were detected using iodine spray and marked. The areas corresponding to the marked spots were carefully scraped off and the layers were separately transferred to microbeakers with the aid of small funnel. All the compounds were eluted by employing the following procedure.

The compound was eluted from the coating material by adding 3 x 3 ml of anhydrous ethanol. The ethanolic extracts were centrifuged and collected by filteration, the combined extracts were finally diluted to 10 ml with ethanol and mixed. A similar elution procedure was adopted for a blank sample of the coating material. Spectrophotometric measurements were carried out, at λ_{max} using the same cells. The absorbance values obtained (after subtracting the absorbance due to the blank) were compared to the standard curves.

The elution and quantitative determination procedure described above was employed for all the compounds. All experiments were performed at least in triplicate and the mean values were recorded.

TABLE 1. R_F VALUES USING SOLVENT SYSTEM BENZENE ETHYL-ACETATE (55:45) AND THEIR λ_{max} USED FOR UV STUDIES.

Compound	R_f values	Variation in R_f values in different runs	λ_{max}
Isatin (I)	0.68	±0.03	243
1-Methyl isatin (II)	0.83	±0.01	242
Isatin-3-oxime (III)	0.44	±0.03	251
Isatin-3-hydrazone (IV)	0.72	±0.02	271
Isatin-3-phenyl hydrazone (V)	0.86	±0.01	258
Isatin-3-thiosemicarbazone (VI)	0.75	±0.04	271
1-Methyl isatin-3-thiosemicarbazone (VII)	0.78	±0.03	274
1-Acetylisatin-3-thiosemicarbazone (VIII)	0.60	±0.02	287
3-Hydroxy-3-acetonyl oxindoole (IX)	0.32	±0.01	245

Solvent system 5 (benzene - ethyl acetate; 55:45 v/v) was found to be the best for separation of all 9 compounds used. When a mixture of five compounds namely isatin, 1-methyl isatin, isatin-3-hydrazone, isatin-3-phenyl hydrazone and isatin-3-thiosemicarbazone was used, the chromatographic results indicated that the R_f values obtained agreed with the ones given by the individual standards (Table 1). Quantitation of the five spots was carried out by using UV absorption-concentration relationship (Fig. 1). The results obtained were compared to the standard curves absorbance *vs* concentration of the five compounds.

All experiments were performed in triplicate and the mean values were recorded. The recoveries of the compounds from the spots were 92-95%. The results are shown in Table 2.

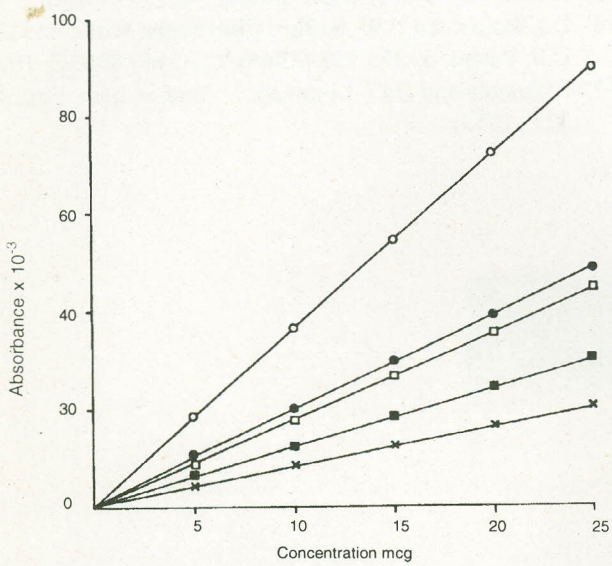
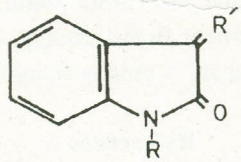
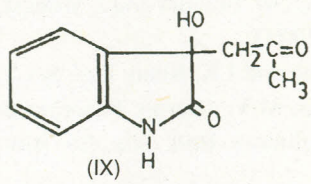


Fig. 1. UV absorption-concentration relationship of the following compounds.
Key: ○ = isatin. ● = 1-methyl isatin. □ = isatin -3-phenyl hydrazone. ■ = isatin -3-hydrazone. x = isatin -3-thiosemicarbazone.

The chromatographic results of seven different mixtures containing three, four and five compounds, randomly selected, were very similar to those already indicated. The method seems to be sufficiently accurate and could be employed for the routine analysis of isatin derivatives and their metabolities.



(I) R = H; R' = O (II) R = CH_3 : R' = O (III) R = H;R' = NOH (IV) R = H; R' = NNH_2 (V) R = H; R' = $NNHC_6H_5$ (VI) R = H; R' = $NNHCSNH_2$(VIII) R = CH_3; R' = $NNHCSNH_2$ (VIII) R = $COCH_3$; R' = $NNHCSNH_2$



(IX)

Structures I to IX

The R_f values are not absolute physical constant and may differ for many predictable reasons. Various parameters such as temperature, purity of the solvents and variation of the thickness of plates effect the R_f values. The development tank must be equilibrated with solvent prior to its use for at least 2 hr at room temperature. Freshly prepared solvents should be used, otherwise, the R_f values obtained are not completely reproducible. For quantitative determination, parallel spots were run simultaneously and detected first, separately, so that positions of the spots to be quantitated could be determined without spraying over their area. Using this procedure quantities as small as 1.5 mcg can be detected while quantities as small as 0.1 mg/ml can be quantitatively estimated using UV spectrophotometry.

TABLE 2. ABSORBANCE OF ISATIN, 1-METHYL ISATIN, ISATIN-3-HYDRAZONE, ISATIN-3-PHENYL HYDRAZONE AND ISATIN-3-THIOSEMICARBAZONE AT VARIOUS CONCENTRATIONS.

Concentrations mg/ml	Absorbance*									
	Isatin		1-methyl isatin		Isatin-3-hydrazone		Isatin-3-phenyl hydrazone		Isatin-3-thio-semicarbazone	
	A	B	A	B	A	B	A	B	A	B
0.5	0.006	0.005	0.017	0.011	0.005	0.003	0.038	0.036	0.013	0.009
1.0	0.009	0.008	0.020	0.019	0.016	0.016	0.052	0.051	0.019	0.016
1.5	0.013	0.012	0.036	0.034	0.024	0.022	0.064	0.063	0.025	0.023
2.0	0.024	0.023	0.055	0.053	0.029	0.030	0.071	0.070	0.036	0.032
Percent recovery	95		93		94.5		95		92	

*Pure compound = A and TLC recovery = B.

The method described above can be used for the detection and quantitation of these compounds and some of their possible metabolities expected to be excreted in urine from biological system.

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