A COLORIMETRIC METHOD FOR THE ESTIMATION OF 2-METHYL-3—(3′-METHYL-2′ - PYRIDYL)-4(3H) QUINAZOLINONE (SRC-820)*

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Summary : A colorimetric method for the estimation of 2-methyl-3-(3' methyl-2'—pyridyl)-4(3H) quinazolinone (SRC-820 R) is described. The method involves preilminary acid hydrolysis of the substance in sulphuric acid, followed by diazotization and coupling with 2-naphthol. 10 to 100 μg of the substance could be estimated using this method.

Key words : 2-methyl-3(3'-methyl-2'-pyridyl)-4 (3H) quinazolinone acid hydrolysis diazotization colorimetric estimation

INTRODUCTION

2-methyl-3-(3′ -methyl-2′ - pyridyl)-4 (3H) quinazolinone (SRC-820 R) has recently been prepared and found to exert an effect, ** in experimental animals, similar to that of methaqualone (3, 6). Besides, the derivative acts also as a minor tranquilizer like chlordiazepoxide and meprobamate. The biochemical and pharmacological properties of this compound are under study at present. As a first step, it was considered desirable to devise a simpler and yet reliable fluorometric method (2) which involves the use of costly lithium-borohydride and a sensitive spectro-fluorometer.

The method to be described is based on diazotization of the amine(s) formed on acid hydrolysis (4) and coupling the diazo derivative(s) with 2-naphthol (5) to yield an orange-yellow dye which is estimated colorimetrically.

METERIALS AND METHODS

Colorimetric and ultraviolet absorption studies were carried out with "Uvispek" spectrophotometer (Model H, Hilger and Watts). Reduction of SRC-820 or methaqualone was achieved with nominal amounts of lithium borohydride in tetrahydrofuran or anhydrous ether (2). For hydrolysis of the compound, the following acids were used : hydrochloric (6*N*),

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sulphuric (4.5 *N* or 3.5 *N*) and acetic (8.7 *N*) acids. In preparative experiments, each *mg* of the substance was hydrolysed with 5.0 *ml* of 4.5 *N* sulphuric acid, for 60 min in a boiling water bath.

For thin-layer chromatography (TLC), acid hydrolysates were first extracted 5 times with amyl alcohol, each time using a volume 0.8 times that of the hydrolysate. The separated amyl alcohol layer was concentrated under reduced pressure and used for spotting. The residual aqueous layer was neutralised with solid sodium carbonate and extracted with ether. The ether layer was separated and used for charging the TLC plates. TLC was carried out on glass plates (19 x 20 cm) coated with "silica-gel-G (according to Stahl)" R (E. Merck), at room temperature. The following solvent systems were used : (a) dioxane : benzene : amonia, 20:75:5, (b) dioxane:benzene:ethylacetate, 20:75:5 and (c) n-butanol:glacial acetic acid : water, 4:1:5. Detection of the components was accomplished by (a) scanning the TLC plates under ultraviolet light, (b) spraying with sulphuric acid (18 *N*) and heating at 110° C for 10 min, (c) spraying with Dragendorff reagent (1) and (d) spraying with $HgNO_3$ reagent (8).

Diazotization was carried out with freshly prepared sodium nitrite (0.1 *M*) in ice-bath (0-4°C) for 1 min. The diazo derivative formed was coupled with 2-naphthol (7.2 *mg*%) in sodium hydroxide (2 *N*). The details as finally followed in the colorimetric estimation are described later. The colour of the dye was estimated at 380 $m\mu$ in the "Uvispek" spectrophotometer.

The influence of time, the concentration of acid and the amount of substance on hydrolysis was studied with reference to the intensity of colour obtained after diazotization and coupling.

Known quantities of SRC-820 (10-40 μg) were added to normal human serum separately, and the serum was extracted twice with chloroform (5 *ml* and 3 *ml*). The separated chloroform layer was then treated with 2 *ml* of 0.05 *N* NaOH and the chloroform layer siphoned off and dried over anhydrous sodium sulphate. A known aliquot of the chloroform layer was evaporated and subjected to acid hydrolysis, diazotization and coupling. Suitable controls without added SRC-820, blanks and standards were run under identical conditions. Recoveries were then calculated. Diazotization with and without preliminary acid hydrolysis was carried out in the control experiment.

Serum pH, after addition of known amounts of SRC-820, was adjusted to a range of values between 6.0 to 8.0 and extracted as before. The influence of pH on the recovery of added SRC-820 was then determined.

RESULTS

When alkaline hydrolysis (5) was tried for the colorimetric estimation of μg quantities of SRC-820, the results were found to be non-linear and the reaction mixture turned turbid on

standing. Reproducibility was also found to be poor. In subsequent trials, recourse to acid hydrolysis was made, which yielded consistent results.

After acid hydrolysis in 4.5 *N* H_2SO_4, its absorption spectrum was found to have changed significantly (Fig. 1). About 60% reduction in optical density (O.D.) at 230 *mμ* and three fold increase at 300 *mμ* could be observed. A similar pattern of changes was observed when SRC-820 was first reduced with $LiBH_4$ and then hydrolysed (Fig. 1). No such increase could

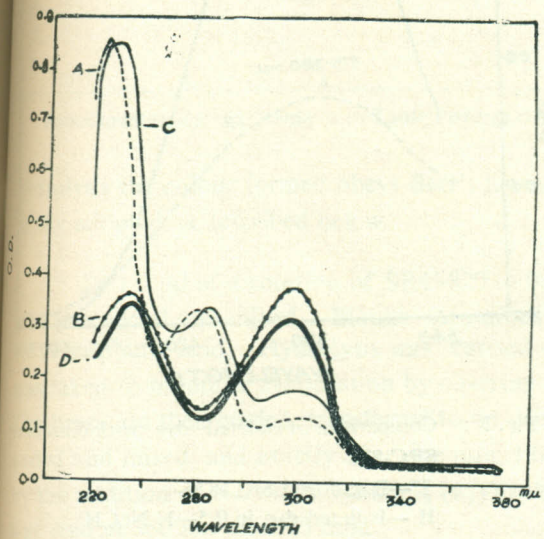

Fig 1 : Ultraviolet Spectra of SRC-820 before and after acid treatment
A—Pure SRC-820 in 0.01N H_2SO_4
B—Pure SRC-820 after H_2SO_4 treatment
C—Reduced SRC-820 in 0.01 N H_2SO_4
D—Reduced SRC-820 after H_2SO_4 treatment

be seen in the case of methaqualone under these conditions. In the case of SRC-820, the increase in O.D. at 300 *mμ* was found to be linear with the amount taken and corresponds well with the colour obtained after diazotization and coupling (Fig. 2).

Of the three solvent systems tried for TLC, good resolution was achieved in n-butanol ; acetic acid:water system. Two major components having R_f values, 0.857 (component I) and 0.80 (component II) could be detected. Component I gave positive test with Dragendorff reagent. Anthranilic acid, under identical conditions has an R_f value of 0.86.

Ultraviolet absorption spectra of component I and II were studied; component I in 95% ethanol showed a major peak at 240 *mμ* and a minor one at 260 *mμ*. The same substance in HCl or H_2SO_4 had an absorption maximum at 290 - 295 *mμ*. Component II in 95% ethanol showed a single peak at 270 *mμ*. Component I on diazotization and coupling yielded a dye with an absorption maximum at 380 *mμ*, identical to that of the isolated dye from 1 *g* of the material (Fig. 3). Ccmponent II, on the other hand did not undergo diazotization.

The isolated diazo derivative after hydrolysis gave positive tests for phenol (ferric chloride and Millon-Nasse tests).

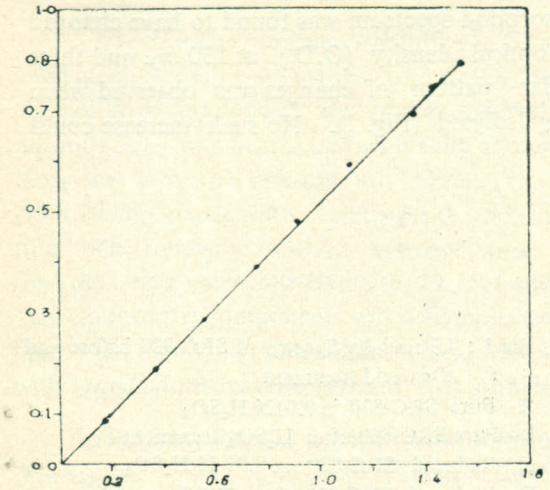

Fig. 2 : Relationship between increase in O.D. at 300 $m\mu$ and diazotization reaction
Abscissa : Increase in O.D. at 300 $m\mu$
Ordinate : Increase in O.D. after diazotization and coupling (380 $m\mu$)

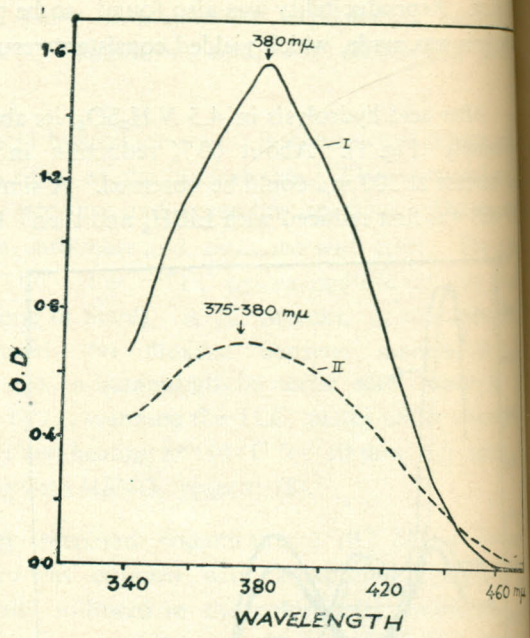

Fig. 3 : Comparison of isolated dye with diazotized SRC-820
I —Diazotized SRC-820
II —Isolated dye in 0.5—N NaOH

Consistent results could not be obtained when hydrolysis of SRC-820 was carried out with hydrochloric or acetic acids. Sulphuric acid gave reproducible results and hence chosen for hydrolysis. With sulphuric acid, the optimum time of hydrolysis was 30 min (Table I) and the optimum concentration of the acid was 3.5 *N* (Table II). Increasing the time of hydrolysis or the strength of acid resulted in reduction of the colour. Under the optimum conditions of

TABLE I : Effect of time on acid hydrolysis of SRC-820

Experiement	*Time in min*	*O.D. at* 380 $m\mu$
1.	10	0.488
2.	20	0.581
3.	30	0.596
4.	40	0.588
5.	60	0.582
6.	80	0.556

50 μg of SRC-820 was hydrolysed with 4.5 N H_2O_4 for varying periods of time in a boiling water bath. The hydrolysate was diazotized and coupled with 2-naphthol. The colour of the dye was estimated at 380 $m\mu$

TABLE II : Effect of concentration of sulphuric acid on hydrolysis of SRC-820

Experiment	*Strength of H_2SO_4 (in normality)*	*O.D. at 380 mμ*
1.	0.5	0.197
2.	1.0	0.339
3.	2.0	0.466
4.	2.5	0.487
5.	3.5	0.512
6.	4.5	0.454

Experimental details same as in Table I except the time of hydrolysis was 30 min.

hydrolysis the colour formed obeys Beer's law and is linear even upto 200 μg. The method, as finally adopted, is described below.

To 1.0 *ml* of a solution of SRC-820 in 0.01 *N* sulphuric acid, 1.0 *ml* of 7.0 *N* sulphuric acid was added and mixed. Blanks contained 1.0 *ml* of 0.01 *N* sulphuric acid and 1.0 *ml* of 7.0 *N* sulphuric acid. Hydrolysis was carried out in a boiling water bath for 30 min. Care was taken to minimise evaporation by covering the mouths of the test tubes with glass bulbs. The tubes are then cooled, transferred to an ice-bath, 0.2 *ml* of freshly prepared 0.1 *M* $NaNO_2$ added and mixed, and exactly after one min, 1.0 *ml* of 8.0 *N* NaOH added. This was followed by the addition of 4.0 *ml* of 7.2 *mg%* (w/v) solution of 2-naphthol in 2 *N* NaOH. The colour was read at 380 *mμ* after 15 min.

Reproducibility of the method was tested on four different days (Table III). The mean O.D. for 10 μg was 0.0964 $\pm$ 0.003.

TABLE III : Estimation of SRC-820

Experiment	*Concentration of SRC-820 in μg*	*Range of O.D. at 380 mμ*	*Mean O.D. ± S.D.*
1.	10	0.088—0.098	0.093 ± 0.004
2.	20	0.181—0.192	0.189 ± 0.005
3.	30	0.282—0.293	0.286 ± 0.004
4.	40	0.382—0.391	0.387 ± 0.003
5.	50	0.471—0.480	0.477 ± 0.004
6.	60	0.585—0.606	0.596 ± 0.008
7.	70	0.684—0.698	0.690 ± 0.005
8.	80	0.780—0.801	0.792 ± 0.006
9.	100	0.977—0.988	0.983 ± 0.004

Time of hydrolysis, 30 min; acid, H_2SO_4, 3.5 N; other details are the same as in Table I.

Recovery of added SRC-820 (from serum) ranged between 100-114.4% (Table IV). Prior adjustment of the serum pH to values ranging from 6.0 to 8.0 before extraction resulted in 88.8 to 108.6% recovery.

TABLE IV : Recovery of added SRC—820 from serum

Experiment	*μg of SRC-820 added to serum*	*O.D. at 380 mμ*	*Test— control*	*%Recovery*
Reagent blank	—	0.000	—	—
Control	—	0.027	—	—
1.	10	0.096	0.069	100.0
2.	15	0.169	0.142	101.4
3.	20	0.242	0.215	114.4
4.	30	0.337	0.310	106.9
5.	40	0.458	0.430	113.4

Extraction from serum with chloroform. For other experimental details, see text.

DISCUSSION

Substitution of quinazoline at the 3-position with methylpyridyl radical appears to render the molecule more labile than substitution with toluyl radical, since methaqualone does not undergo diazotization as easily as SRC-820 under these conditions of hydrolysis. Methaqualone, however, undergoes hydrolysis when concentrated acid was employed (4). In the case of SRC-820, on the other hand, the use of acid higher than 3.5 *N* results in reduction of the colour obtained on diazotization and coupling with 2-naphthol (Table III).

The formation of aromatic amine(s) after acid hydrolysis is inferred from the diazotization reaction and subsequent hydrolysis of the isolated diazo derivative to phenol. By analogy with the hydrolysis of methaqualone, this compound also may, on hydrolysis, give rise to anthranilic acid, 2-amino-3-methyl pyridine and acetic acid.

Separation of the hydrolytic products by TLC showed that only one of the products (component I) undergoes the diazotization reaction. Component II, on elution from silica gel gave very faint colour. It is well known that 2-aminopyridines are difficult to diazotize (7), and the failure of component II to undergo diazotization reaction could be explained if it were a 2-aminopyridine. However, no chemical characterization of the reaction product has been made in the present investigation.

Recoveries of the added SRC-820 from serum indicate that the added substance could be extracted without difficulty and variations in the pH of the serum from 6.0 to 8.0 do not affect the recovery.

The colorimetric method described in this paper makes use of ordinary laboratory chemicals and equipment and is well within the competence of small laboratories.

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