SODIUM NOVOBIOCIN: STABILITY ASPECTS

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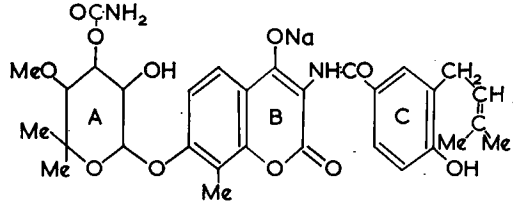
The stability of monosodium novobiocin has been examined. The solid antibiotic has been shown to be sensitive to light, but if properly protected, to be stable for a period of 2 years at 20°. In aqueous solution and suspension it is affected by increase of temperature, pH and certain heavy metals. Phosphate, and possibly sulphate, also cause decomposition. Concentrated solutions of novobiocin become coloured, particularly above 20° and in the presence of air. Light has little effect on potency but irradiation with ultra-violet light accelerates potency loss in solution. There appears to be no difference in the potency stability of three different batches of sodium novobiocin. Ultra-violet spectrophotometry cannot be used as a method of assay.

Novobiocin is a dibasic acidic antibiotic produced by the actinomycete *Streptomyces niveus*. In neutral to slightly alkaline solutions it exists as the monosodium salt (I), the disodium salt being formed in strongly alkaline solution.



(I)

The acidic form of novobiocin has been reported almost insoluble in water, but it is soluble at pH values between 7·5 and 9·5[1]. There are only few references in the literature to the stability of novobiocin. Stability in aqueous solution is said to be a function of pH and temperature; dilute solutions at pH 2 are stable at 24°, but a half-life of about 60 days is observed at pH values between 7 and 10. A 10 per cent solution of sodium novobiocin at pH 7·5 is said to have a half-life of 30 days at 24° and of several months at 4°. Mention is made of the light sensitivity of the dry antibiotic[2].

Methods

A large plate microbiological method was employed for determinations of potency. The unit of activity of novobiocin is defined as one microgram of pure novobiocin acid. The designs used were 8 × 8 Quasi-Latin squares capable of yielding results with a standard error of ±7 per cent ($P = 0{\cdot}95$) from the means of duplicate assays by separate operators. The test organism was *B. subtilis* (MB.32) seeded into neutral agar medium at pH 7·0 and incubated for 18 hours at 30°. The reference standard was sodium novobiocin of high potency and low moisture content, previously

standardised against a sample of novobiocin lodged with the Standards Department of the M.R.C., Mill Hill, for the purpose of creating a British National Standard. For assay, samples and standards were diluted in a phosphate buffer solution at pH 7·5 (0·98 per cent disodium hydrogen phosphate, 0·19 per cent potassium dihydrogen phosphate) to 10 and 5 u./ml., concentrations suitable for plating out. Colours were measured with the "Eel" Portable Colorimeter.

Experimental and Results

Stability of Solid Monosodium Novobiocin

Dry solid monosodium novobiocin (moisture content 3·0 per cent, by loss under reduced pressure at 60°) was stored at 4°, room temperature, in the dark or exposed to light, at 37° or at 50°. The results revealed no potency loss after 2 years storage at 4°, 20° or 37° in the dark. At 50°

TABLE I

EFFECTS OF CONCENTRATION AND TEMPERATURE ON POTENCY OF STORED MONOSODIUM NOVOBIOCIN SOLUTIONS

Temperature ° C.	Potency u./ml.					
	Initial	4 days	2 weeks	4 weeks	10 weeks	16 weeks
4	315,000				300,000*	
20	315,000		Solidified			
37	315,000	240,000	125,000			
4	68,000				61,000	
20	68,000		57,500	55,000		46,000
37	68,000	52,000	40,000	30,000		
4	13,800				13,000	
20	13,800		12,500	12,500		10,200
37	13,800	12,000	9,500	8,000		
4	2,500				2,450	
20	2,500		2,550	2,400		2,230
37	2,500	2,450	1,950	1,500		
4	530				505	
20	530		520	510		454
37	530	490	410	365		
4	110				100	
20	110		95	95		94·5
37	110	96	80	70		

* Solidified

approximately 10 per cent potency loss was recorded after 2 years. The samples exposed to light showed potency loss, regular mixing resulting in potency loss from 935 u./mg. to 680 u./mg.

A sample of solid sodium novobiocin, moisture content 2·7 per cent, was dried under reduced pressure at 50° for 3 hours to give material of moisture content 1·1 per cent. Another sample of the same batch was kept at 75 per cent relative humidity and 37° for 48 hours, after which the moisture content was 11 per cent. This material was blended with some of the sample containing 2·7 per cent moisture to give a product with moisture content of 5·8 per cent. The three samples of 1·1, 2·7 and 5·8 per cent moisture contents were heated in closed containers at 100° for 16 hours and assayed when the potency results obtained were 890, 740

and 685 u./mg. respectively. No change in the visible appearance of any sample was noticeable after heating.

Stability of Aqueous Solutions and Suspensions

Novobiocin is soluble in water at alkaline pH values, but is precipitated as the free acid in water at acid pH values. All solutions, unless otherwise stated, were prepared in distilled water and all samples, unless otherwise stated, were stored in the dark.

Stability at different concentrations. Solutions at the six concentrations levels were prepared: 312,500, 62,500, 12,500, 2,500, 500, and 100 u./ml. They were stored in 20 ml. ampoules at 4°, 20° or 37° and were assayed for potency, the colour development also being measured at intervals. The potency results are shown in Table I. A graph was drawn plotting the percentage potencies of the solutions stored at 37° against time. The 60 per cent, 70 per cent and 80 per cent lives for each concentration read from this graph were plotted against log concentration, as shown in Figure 1.

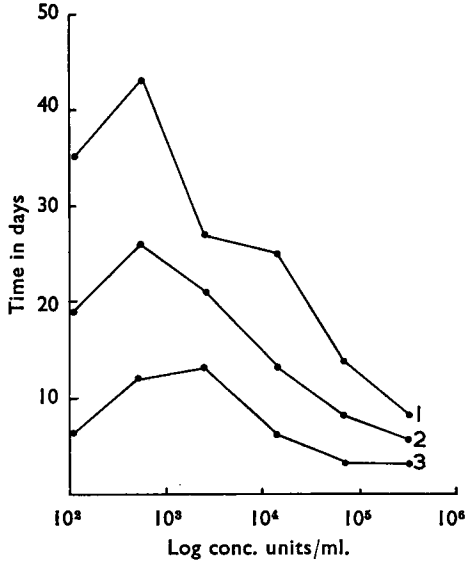


FIG. 1. Effect of concentration on the potency of sodium novobiocin solutions stored at 37°. 1. 60 per cent potency. 2. 70 per cent potency. 3. 80 per cent potency.

At a concentration of 2500 u./ml., or less there was no visually significant colour development at either 20° or 37°. A solution containing 12,500 u./ml., gave slight colour development when stored at 37° for six weeks but solutions of 62,500 and 312,500 u./ml. acquired appreciable colour after only 7 days storage. There was negligible colour development at 4° except in the 31·25 per cent solution, for which the Eel colorimeter reading (OB.2 filter) rose from 25 to 32 in 7 days.

Effect of pH value on stability. Sodium novobiocin as 5 per cent w/v solutions and suspensions in water at pH's 3·3, 5·0, 7·0 (suspensions) or 9·0 (solutions) was filled into 20 ml. ampoules, stored at 37° and assayed at intervals. The results are shown in Table II. In this series the pH values 3·3, 5·0 and 7·0 were obtained by means of phosphate-citrate buffers and pH 9·0 by means of a glycine-sodium hydroxide buffer. A similar series was prepared for room temperature (20°) storage, the only difference being that the solution at pH 9·0 was prepared with a sodium borate buffer. The results are shown in Table II.

Effect of air and antoxidants. We have already shown that sodium novobiocin solutions develop colour on storage; because of this the effect of air and the action of antoxidants on stability of potency and on colour development in sodium novobiocin solutions were investigated.

The antoxidants selected were sodium formaldehyde sulphoxylate (S.F.S.) and sodium metabisulphite. It was found that solutions of sodium novobiocin (50,000 u./ml.) containing 0·2 per cent S.F.S. formed a

TABLE II

EFFECTS OF pH AND TEMPERATURE ON POTENCY OF STORED NOVOBIOCIN SOLUTIONS AND SUSPENSIONS

pH	Temperature °C.	Potency u./ml.						
		Initial	6 days	21 days	6 weeks	12 weeks	24 weeks	16 months
3·3	20	46,900	47,000			55,000	47,000	49,700
	37	45,000	47,700	43,700				
5·0	20	43,800	36,000		53,500	51,200	48,000	47,000
	37	49,800	44,500	28,800				
7·0	20	47,200	48,300		43,200	34,800	25,000	
	37	45,700	30,400	9,500				
9·0	20	45,100	36,000		21,500	800		
	37	44,600	19,300	5,100				

precipitate and became cloudy within a week at room temperature or 37°. Nevertheless, despite the cloudiness, the solutions containing S.F.S. remained considerably lighter in colour, both at room temperature and at 37°, than the corresponding control (without S.F.S.) both solutions being stored in the presence of a large volume of air.

TABLE III

POTENCY OF SODIUM NOVOBIOCIN SOLUTIONS AND SODIUM NOVOBIOCIN SOLUTIONS CONTAINING 0·25 PER CENT SODIUM METABISULPHITE STORED IN AMPOULES SEALED UNDER NITROGEN AND IN 4-OZ. BOTTLES WITH A LARGE AIR SPACE

Solution	37° C.			20° C.
	Initial potency	14 days, u./ml.	4 weeks, u./ml.	4 weeks, u./ml.
Control (nitrogen)	53,000	35,000 (66 per cent)	28,900 (54·5 per cent)	40,300 (76 per cent)
Control (large air space)	53,000	33,900 (63 per cent)	19,900 (38 per cent)	45,300 (85 per cent)
Metabisulphite (nitrogen)	49,500	28,100 (56·5 per cent)	19,200 (39 per cent)	39,200 (79 per cent)
Metabisulphite (large air space) ..	49,500	23,900 (48 per cent)	<2,000	33,200 (67 per cent)

Two solutions of sodium novobiocin at a concentration of 50,000 u./ml. were prepared, and 0·25 per cent sodium metabisulphite was added to one of them. The latter solution was prepared by dissolving the sodium metabisulphite in a small quantity of water and adding NaOH solution to bring the pH to 7·0. This solution was then added to the sodium novobiocin solution, adjusted to the same pH value as the control (pH 8) with further NaOH and made up to volume. The

sodium metabisulphite was neutralised in this way to prevent precipitation of novobiocin free acid. Both these solutions were stored in 20 ml. ampoules sealed under nitrogen, and also in 4-oz. bottles with a 70: 30 air/solution ratio. The potency stability of these solutions at 20° and 37° is shown in Table III. The colours of the four solutions stored under nitrogen, or with a large air space, or containing 0·25 per cent sodium metabisulphite in the presence of either nitrogen or air, gave Eel colorimeter readings (OB.2 filter) of 16, 32, 7·5 and 41 respectively after 4 weeks' storage at 37°, the initial colour reading being 4·0.

Effect of buffer. The effect on the stability of sodium novobiocin solutions (50,000 u./ml.) of 2 per cent of disodium hydrogen phosphate,

TABLE IV

POTENCY OF SOLUTIONS OF THREE BATCHES OF SODIUM NOVOBIOCIN STORED AT ROOM TEMPERATURE OR 37°

Batch	Temperature °C.	Potency, u./ml.				
		Initial potency	4 days	1 week	2 weeks	4 weeks
A	R.T.	160,000			140,000	120,000
	37	160,000	110,000	95,000	75,000	
	37	185		155	125	115
B	R.T.	155,000			135,000	120,000
	37	155,000	115,000	85,000	65,000	
	37	175		105	90	95
C	R.T.	140,000			125,000	110,000
	37	140,000	110,000	85,000	70,000	
	37	150		100	95	90

sodium citrate or glycine was investigated and compared with that of a control. All the solutions, including the control, had pH values of 8·0, the one containing phosphate being adjusted to this pH value with 0·003 per cent v/v orthophosphoric acid. The potency results after 4 weeks' storage at 37° were 60, 25 and 61 per cent of the initial potencies for the control, sodium phosphate buffer and sodium citrate buffer solutions respectively. The solution containing glycine precipitated after 7 days' storage at 37°. The solutions developed colour and after 4 weeks' storage at 37° recorded Eel colorimeter readings (OB.2 filter) of 21, 27 and 22 for the control, phosphate and citrate buffer solutions respectively, the initial colour reading being 4·0.

Effect of visible and ultra-violet light on stability. Suspensions and solutions over a range of pH values were prepared, and, after storage in the dark or exposure to daylight, some being left undisturbed and others shaken daily, were assayed at intervals. A similar test was carried out on samples exposed to ultra-violet radiation from a 3·75 kw. lamp at 2 to 3 feet from the samples. Suspensions prepared at pH 3·6, 4·6, and 6·3 retained full potency for 6 months at 20° both when stored in the dark and when exposed to light and regularly shaken. Suspensions at pH 7·0 and solutions at pH 7·5 showed a loss in potency over the same period of approximately 40 per cent, there being no significant difference between samples stored in the dark and exposed to light. Suspensions at pH 3·8 and 5·4

exposed to ultra-violet light and regularly shaken showed no loss after 18 days whilst solutions at pH 7·8 showed approximately 25 per cent loss in activity over the same period, the control solution stored in the dark remaining stable.

Effect of heavy metals. The effect of five metals was investigated; copper, iron, nickel, zinc and lead. It was found necessary to add the solution of copper sulphate, ferric chloride, nickel sulphate, zinc sulphate or lead nitrate to suspensions of novobiocin (pH 6·3) as a precipitate formed when any of the metal salts were added to a sodium novobiocin solution at slightly alkaline pH. Each metal salt was used at a concentration of 20 p.p.m., calculated as metal. The concentration of sodium novobiocin was 2·5 per cent. None of the suspensions lost significant potency after 12 months' storage at 20°. The potency results after 3

TABLE V

COLOUR DEVELOPMENT OF SOLUTIONS OF THREE BATCHES OF SODIUM NOVOBIOCIN STORED AT ROOM TEMPERATURE OR 37° (EEL COLORIMETER—OB.2 FILTER)

Batch	Temperature, °C.	Initial colour	4 days	1 week	2 weeks	4 weeks
A	R.T.	10·5	22·0	27·5	15·0	18·0
	37	10·5			36·0	33·0
B	R.T.	9·5	18·0	22·0	13·0	15·0
	37	9·5			29·0	25·0
C	R.T.	20·0	34·0	70·0	26·0	30·0
	37	20·0			61·0	48·0

weeks' storage at 37° were 70, 64, 62, 32, 33 and 48 per cent of the initial potency for the control suspension, the suspension containing copper, iron, nickel, zinc and lead respectively.

Comparative stability of different batches of sodium novobiocin. The comparative stability of solutions of three different batches of monosodium novobiocin has been examined. The results are shown in Table IV. The colours of the stored solutions prepared from the three batches are recorded in Table V.

Solutions of sodium novobiocin that have lost most of their biological potency possess a distinct odour of ammonia. Such a solution, at a concentration of 50,000 u./ml., which had been stored for 4 weeks at 37°, was assayed both microbiologically, giving a figure of 29,000 u./ml., and spectrophotometrically, giving a figure of 49,000 u./ml.

DISCUSSION

Solid sodium novobiocin would appear to be reasonably stable for 2 years at temperatures up to 37° when protected from light. Exposure to light produced a dark yellow colour on the exposed surface, whereas the unexposed material below maintained the normal "off-white" colour. Mixing caused a significant drop in potency of exposed powder and the production of a uniform pale yellow colour throughout the bulk. It would appear that no special temperature precautions are required for the storage of solid sodium novobiocin, but it is important to protect from

light. An increase in moisture content renders solid sodium novobiocin less stable.

The potency results shown in Table I reveal the relatively lower stability of the concentrated solutions of sodium novobiocin. Figure 1 indicates that stability gradually increases as concentration decreases, the optimum concentration possibly being in the range 500 to 2500 u./ml., below which stability decreases. Further work is needed before this peak for optimum stability can be accepted.

The differing stabilities of the solutions at different concentrations might possibly be explained by the pH values of the solutions which were:

Concentration		pH
31·25 per cent	..	8·2
6·25 per cent	..	7·7
1·25 per cent	..	7·3
0·25 per cent	..	6·8
0·05 per cent	..	6·6
0·01 per cent	..	6·5

The lower stability at the higher concentrations might therefore be explained by the demonstration of the effects of hydrogen ion concentration on stability. From the results of colour development it is evident that solutions of concentration 6·25 per cent or less, stored under conditions that maintain potency also remain satisfactory in colour. Dilute solutions (0·25 per cent and less) show no visible colour development even though stored under conditions resulting in loss of potency.

The results in Table II indicate clearly that the stability of novobiocin in an aqueous system decreases with increase in pH value. This demonstration of lowered stability with increase in pH value made it useful to know how much of the antibiotic present in suspensions of free novobiocin acid is in true solution at various pH values. Spectrophotometry of filtrates of suspensions of free novobiocin acid of concentration 50,000 u./ml. at pH values 7·0, 6·0, 5·0, 4·0 and 3·0 revealed novobiocin contents of 14,500, 850, 350, less than 50 u./ml., and zero respectively.

At room temperature no apparent effect of metals on the stability of novobiocin has been demonstrated, but at 37° it appears that nickel, zinc or lead salts may have some action in accelerating potency loss.

No significant differences in the potency stability of solutions prepared from three different batches of sodium novobiocin has been detected however the extent to which colour develops on storage depends on the initial colour of the solution.

Although sodium novobiocin solutions are not sufficiently stable at pharmaceutical concentrations (say 0·1 per cent and above) to meet the requirements for shelf-life of a pharmaceutical product, suspensions of free novobiocin acid would meet these requirements (see Table II). However, the results in Table I indicate that solutions of concentration 6·25 per cent and less would be stable for several months if stored in a refrigerator and for about a month at room temperature.

Hoeksema and others[2] mention that a 10 per cent solution of novobiocin has a half-life of 30 days at 24°. Our results indicate much greater stability than this, for our solutions of concentration 6·25 per cent (see Table II) and 15 per cent (see Table IV) exhibited 80 per cent potency after 4 weeks' storage at room temperature (approximately 20°). We have also shown in preliminary work that a 10 per cent solution has a half-life of about 14 weeks at room temperature.

Novobiocin as a dry solid (sodium salt) is sensitive to visible light, but this sensitivity has not been clearly demonstrated for solutions and suspensions of novobiocin; on the other hand ultra-violet light causes potency losses in solutions, with apparently no effect on suspensions of free novobiocin acid under the particular conditions of the test.

Sodium metabisulphite appears to stabilise the colour of sodium novobiocin solutions in the absence of air, but in the presence of air it has the opposite effect; it causes loss of potency both in presence and absence of air. It is possible that the presence of sulphate arising from the oxidation of sodium metabisulphite might affect sodium novobiocin solutions in a manner similar to that which we have demonstrated for sodium phosphate.

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After Mr. Vergine presented the paper there was a DISCUSSION.