THE USE OF OXIDISED CELLULOSE FOR THE DETERMINATION OF STRYCHNINE IN PHARMACEUTICAL PREPARATIONS

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With oxidised cellulose as a carboxylic cation exchange medium strychnine can be separated from extraneous interfering materials in a pure form for spectrophotometric assay. When brucine is also present a 2-point spectrophotometric procedure is adopted. Results compare well with chemical assays with a coefficient of variation of about 1 per cent. The oxidation procedure used in the official assay of nux vomica completely destroys brucine with no loss of strychnine.

IN 1955 Brealey and Proctor[1] reported spectrophotometric methods for the determination of the therapeutically active ingredient in injection solutions containing chlorocresol. It was realised that these methods had only a limited application and investigations were therefore continued with the aim of finding a general method by which any injection solution capable of spectrophotometric assay could be so determined in the presence of any interfering bacteriostatic agent. Early in 1956 Freeman[2] described the use of oxidised cellulose for alkaloidal analysis and we have applied it[3] to the separation and determination of the active principle and bacteriostatic agent in seventeen different injection solutions, almost all official preparations. In these the active ingredient is assayed spectrophotometrically. Phenol, chlorocresol and benzyl alcohol all interfere with the direct spectrophotometric determination of injections but may be separated by oxidised cellulose.

Many of the preparations examined could not be purified sufficiently for spectrophotometric determination by oxidised cellulose alone, because of the very high proportion of interfering materials which also occasionally prevented the repeated use of the column. Some preliminary purification, usually by treatment with alumina, was therefore necessary. Where the sample contained either coloured materials which were not retained on alumina, or salts which had been shown by Freeman[2] to hinder the retention of alkaloids on oxidised cellulose, it was necessary to extract with solvent.

The methods are divided into those involving (*a*) strychnine and (*b*) nux vomica. The preliminary purification and chromatographic stages were identical for both.

PREPARATIONS CONTAINING STRYCHNINE ALONE

Tablets and pills, and a liquid containing nicotinamide, caffeine and riboflavine were examined.

Preliminary Purification

(*a*) Treatment with Brockman alumina.

Apparatus, Pyrex tube (2·5 × 30 cm.) with tap, plugged with glass wool.

Preparation of column. Slurry 20 g. of alumina with 70 per cent ethanol, transfer to the tube and allow to settle under gentle air pressure.

Preparation of sample. Weigh a suitable amount of the powdered sample into a 50 ml. conical flask, heat on a steam bath with 25 ml. of 70 per cent ethanol and filter through No. 30 paper onto the alumina column. Extract the residue in the flask with two further portions of 15 ml. of 70 per cent ethanol and transfer to the column. After these extracts have passed through the column wash with small quantities of 70 per cent ethanol and collect the total eluate in a 100 ml. graduated flask. After dilution to volume with the same solvent transfer a suitable aliquot to an oxidised cellulose column.

(*b*) Solvent extraction with chloroform B.P.

Preparation of sample. Pipette a suitable aliquot into a 100 ml. separating funnel, make alkaline with a few drops of dilute ammonia and extract with 20 ml. of chloroform for two minutes. Transfer the chloroform layer into a second separating funnel and wash with 10 ml. of water. Run off the washed extract into a flask, and extract the original sample in a similar manner with three further portions of 20 ml. of chloroform. Wash these further extracts with the same 10 ml. of water and add to the original extract. Transfer the bulked chloroform extracts to an oxidised cellulose column.

Chromatography

Prepare a 1 g. column of oxidised cellulose, Eastman Kodak (16 to 22 per cent carboxylic content) as described in our previous communication[3]. If preliminary purification is unnecessary make an extract or dilution of the sample with 70 per cent ethanol and transfer a suitable aliquot to the column. When preliminary purification has been made by solvent extraction wash the oxidised cellulose column with absolute ethanol before adding the chloroform solution. A suitable amount of strychnine for chromatography is about 0·5 mg.

After the alkaloidal extract has passed through the column under gentle air pressure, adjusted to give a flow rate of about 3 ml. per minute, wash the column with successive amounts of water until the eluate shows an extinction of less than 0·005 at 250 mμ. Where purification by solvent extraction has been used pass 10 ml. of absolute ethanol through the column before washing with water. After washing, elute the alkaloid from the column with N sulphuric acid and collect 50 ml. of eluate in a graduated flask.

Spectrophotometric Determination

Measure the extinction of the eluate from the oxidised cellulose column in a suitable spectrophotometer at 254 mμ in a 1 cm. cuvette, with N

sulphuric acid in the compensation cuvette. Calculate the amount of strychnine from the formula,

$$\text{per cent strychnine} = \frac{E\,(1\text{ per cent, }1\text{ cm.}) \times 100.}{375}$$

Table I gives the results with tablets, pills and a tonic. Replicates were within 1 per cent of the mean. These results were not compared with chemical assays the accuracy of which is poor in comparison. The quantitative nature of the method may, however, be seen from the following recovery experiments.

TABLE I

SAMPLES CONTAINING STRYCHNINE ONLY

Sample	Strychnine content	
	Expected	Found
Compound tablets of ferrous carbonate	0·033 grains/tab.	0·033 grains/tab.
Tablets of ferrous carbonate with arsenic and strychnine	0·0100 ,,	0·0098 ,,
		0·0108 ,,
Tablets of reduced iron with arsenic and strychnine	0·0166 ,,	0·0161 ,,
		0·0167 ,,
Compound Pills of Phenolphthalein B.P.C.	0·0125 ,,	0·0125 ,,
Compound tablets of glycerophosphates	0·0109 ,,	0·0119 ,,
Tonic	0·084 mg./ml.	0·087 mg./ml.

1. A sample of strychnine pills was dissolved in 70 per cent ethanol and made up to a suitable volume. One aliquot was examined directly after dilution in N sulphuric acid; a further aliquot was passed through an oxidised cellulose column, washed and eluted with N sulphuric acid. Comparison of the two solutions showed that 98·6 per cent of the strychnine was recovered from the column.

2. To a sample of compound tablets of ferrous carbonate, containing an expected amount of 0·033 grains of strychnine per tablet, a weight of strychnine equivalent to 0·0300 grains/tablet was added and the whole subjected to alumina treatment followed by chromatography.

The recovered amount of strychnine was 0·0297 grains/tablet, or 99·0 per cent recovery.

3. To a sample of tablets of ferrous carbonate with arsenic and strychnine, containing an expected amount of 0·0100 grains strychnine per tablet, a weight of strychnine equivalent to 0·00982 grains/tablet was added and the whole subjected to treatment with alumina followed by chromatography.

The recovered amount of strychnine was 0·0100 grains/tablet, or 102 per cent recovery.

The purity of the strychnine obtained may be judged by the absorption spectrum of a final N sulphuric acid eluate from the tonic, which coincides with the absorption spectrum of pure strychnine between 240 mμ and 295 mμ.

PREPARATIONS CONTAINING NUX VOMICA

A number of pharmaceutical preparations contain nux vomica. The official chemical method of assaying strychnine in nux vomica also

extracts brucine, which is removed by chemical destruction under controlled conditions. This is followed by re-extraction and titration of the strychnine.

Various workers[4–6] have shown that strychnine can be determined spectrophotometrically in the presence of brucine. We have found these two alkaloids to be isolated in a pure state from preparations containing nux vomica by the use of oxidised cellulose and that as a result it is possible to estimate strychnine with an accuracy of about ± 1 per cent. Furthermore, these determinations can generally be carried out within two hours with only 500 μg. of strychnine. A chemical assay requires at least 20 mg.

Details of the 2-point spectrophotometric procedure are given below. In N sulphuric acid strychnine exhibits a characteristic spectrum with a maximum at 254 mμ, whilst brucine has two maxima at 264 mμ and 300 mμ respectively. In a mixture of approximately equal quantities of the two alkaloids, as in nux vomica, a maximum is observed at about 262 mμ with an inflexion at 300 mμ, so that these two wavelengths are most suitable for the application of a 2-point method.

The E (1 per cent 1 cm.) values of strychnine and brucine at 262 mμ and 300 mμ are as follows:—

	Strychnine	*Brucine*
262 mμ ..	322	312
300 mμ ..	5·16	216

It can be shown that, for a solution containing only strychnine and brucine the observed extinctions are A and B at 262 mμ and 300 mμ respectively, it follows that $x = 0{\cdot}318A - 0{\cdot}460B$ where x is the concentration of strychnine per cent. Nine different preparations containing nux vomica were examined.

Tincture of Nux Vomica B.P.

(*a*) 5 ml. of sample was diluted to 100 ml. in ethanol, 10 ml. was placed on a 1 g. column of oxidised cellulose, washed with 20 ml. of alcohol, then with 50 ml. of water, and the alkaloids finally eluted with N sulphuric acid and diluted to 50 ml.

(*b*) To 5 ml. of sample was added 5 ml. of a 0·125 per cent solution of strychnine in ethanol, the mixture diluted to 100 ml. and 5 ml. chromatographed as above.

The tincture was found to contain 0·118 and 0·120 per cent of strychnine, and the tincture to which 0·125 per cent of strychnine was added contained 0·244 and 0·244 per cent, so that 0·125 per cent strychnine was recovered from each of the latter two samples.

These results agree with the result of the official chemical assay (0·119 per cent of strychnine).

Mixture of Potassium Bromide and Nux Vomica B.P.C.

This preparation contains 4·17 per cent of tincture of nux vomica and 4·57 per cent of potassium bromide in addition to a small amount of amaranth. The presence of potassium bromide and the dye-stuff

prevented the direct application of the mixture to an oxidised cellulose column, and preliminary purification by solvent extraction was necessary.

Some initial experiments were done on two mixtures. The first was prepared by dissolving 0·0520 g. of strychnine in 20 ml. of ethanol, adding 0·104 g. of amaranth, 4·57 g. of potassium bromide and 2·5 ml. of chloroform and diluting to 1 litre with water. The second solution was prepared with the same quantities of ingredients, together with 0·0508 g. of brucine.

10 ml. of each solution was made alkaline with dilute ammonia and extracted with chloroform as described in the preliminary purification method.

The first mixture gave duplicate assays of 0·00524 and 0·00520 per cent of strychnine (theory = 0·00520 per cent). The second mixture gave an average result of 0·00514 per cent (theory = 0·00520 per cent), with a coefficient of variation of $\pm 1{\cdot}22$ per cent (four determinations).

A further mixture containing potassium bromide, amaranth, strychnine, 0·0052 per cent, and brucine was prepared and assayed in duplicate by three methods:—

(i) Solvent extraction and chromatography as described above. (ii) Extraction as above, destruction of the brucine by the official method, and re-extraction of the strychnine followed by spectrophotometric determination. (iii) The official method. Found by methods (i) 0·00525 and 0·00525, (ii) 0·00506 and 0·00517, and (iii) 0·0052 and 0·0053 per cent of strychnine.

The agreement is good and confirms the accuracy of the spectrophotometric method which also saves time.

Prepared Nux Vomica B.P.

The official method requires a continuous extraction procedure with boiling chloroform for at least four hours. Preliminary experiments showed that the alkaloids were extracted easily by boiling with 70 per cent ethanol. When an aliquot of this extract was chromatographed on oxidised cellulose and washed with water only, high results were obtained and the ultra-violet spectrum suggested that this was due to extraneous absorption. By washing with chloroform the interfering material was eliminated and close agreement with chemical assays was obtained. From the calculated strychnine (and brucine) concentrations a composite absorption curve was obtained using spectrophotometric data for the pure alkaloids and corresponded closely with that of the extract, indicating the high state of purity obtained by the chromatographic procedure.

An improvement was made by extracting the sample in a Soxhlet apparatus with 70 per cent ethanol for two hours. Longer periods of extraction of four hours yielded no increase of strychnine.

To test the reproducibility of the method a total of six assays in duplicate were carried out on one sample. The mean was 1·22 per cent strychnine with a coefficient of variation of $\pm 1{\cdot}4$ per cent. Duplicate chemical assay gave results of 1·22, 1·23 per cent of strychnine.

The method finally adopted therefore for this material is as follows:—

Weigh 1 g. of finely powdered sample into a Soxhlet thimble, add 80 ml. of 70 per cent ethanol and reflux for two hours. After cooling, transfer the extract to a 100 ml. graduated flask and make up to volume with 70 per cent ethanol. Place 5 ml. of this solution on an oxidised cellulose column, prepared as previously described. Wash the column with 10 ml. of absolute ethanol, 50 ml. of chloroform, 10 ml. of ethanol and 50 ml. of water in succession. Elute the alkaloids with 50 ml. of N sulphuric acid, collect the eluate in a graduated flask and measure the extinctions at 262 mμ and 300 mμ. Calculate the strychine content from the equation.

Miscellaneous Preparations

Six further preparations examined gave no difficulties and need not be discussed individually. The details of method of purification, quantities, etc., are given in Table II, and the results in Table III. Determinations were carried out in duplicate, and replicates were within 1 per cent of the mean.

TABLE II

METHODS FOR SAMPLES CONTAINING NUX VOMICA

Sample	Amount taken	Purification method	Dilution	Aliquot taken for chromatography
Dry Extract of Nux Vomica B.P. ..	0·10 g.	None	→100	10 ml.
Liquid Extract of Nux Vomica B.P. ..	5 ml.	,,	→100 : 10 → 100	10 ,,
Compound Pills of Aloin and Podophyllin B.P.C. 1934	3 pills	(*a*)	None	Whole
Compound Pills of Aloin B.P.C. 1934 ..	3 ,,	,,	→200	30 ml.
Pills of Aloes and Nux Vomica B.P.C. ..	5 ,,	,,	→200	20 ,,
Compound Bismuth Mixture with Pepsin B.P.C. 1934	10 ml.	(*b*)	None	Whole

TABLE III

RESULTS ON SAMPLES CONTAINING NUX VOMICA

Sample	Strychnine content		
	Expected	Found	Chemical assay
Dry Extract of Nux Vomica B.P.	5·0 per cent	5·00 per cent	5·14 per cent
Liquid Extract of Nux Vomica B.P. ..	1·50 ,, ,,	1·52 ,, ,,	1·54 ,, ,,
Compound Pills of Aloin and Podophyllin B.P.C. 1934	0·0025 grains/pill	0·00209 grains/pill	
Compound Pills of Aloin B.P.C. 1934 ..	0·025 ,,	0·0217 ,,	
Pills of Aloes and Nux Vomica B.P.C. ..	0·0125 ,,	0·0120 ,,	
Compound Bismuth Mixture with Pepsin B.P.C. 1934	0·0105 per cent	0·0117 per cent	0·0114 per cent

THE EFFECT OF OXIDATION ON STRYCHNINE-BRUCINE MIXTURES

The described method was suitable for examining one aspect of the validity of the correction factor (1·02) applied to compensate for loss of strychnine in the official method of assay of preparations containing nux vomica.

Preliminary experiments were done by mixing suitable aliquots of strychnine sulphate solution with an aqueous solution of potassium nitrate and potassium sulphate of a concentration equivalent to that

obtained by neutralisation of the official oxidation mixture. The recovery of strychnine with the recommended 1 g. of oxidised cellulose was low and variable, but quantitative recoveries were obtained with a 2·5 g. column. For six determinations the average recovery was 100·3 per cent with standard deviation ±0·2 per cent.

An aqueous solution of strychnine sulphate equivalent to 1·00 per cent of strychnine was prepared and the acidity adjusted by mixing 10 ml. of this solution with 5 ml. of 9 per cent sulphuric acid immediately before adding 2 ml. of nitric acid and a few crystals of sodium nitrite. In this way possible errors due to incomplete dissolution of the strychnine were avoided. After exactly 30 minutes at 20° the solution was adjusted to about pH 7 with N potassium hydroxide, transferred to a 250 ml. graduated flask with water and diluted to volume. Immediately 5 ml. was pipetted on a column of 2·5 g. of oxidised cellulose, washed with 100 ml. of water and the alkaloid eluted with N sulphuric acid. The strychnine was determined by spectrophotometric measurement at 254 mμ and the results of five assays gave an average recovery of 100·0 per cent, with standard deviation of ±0·3 per cent. There is thus no evidence for any destruction of strychnine.

To complete the investigation the effect of the official oxidation mixture on brucine was examined. After oxidation of 0·1 g. quantities and chromatographic treatment it was impossible to measure spectrophotometrically any residual brucine. There was a small amount of general extraneous absorption which, if calculated as brucine, would give a maximum of 0·05 per cent of brucine, implying a minimum of 99·95 per cent destruction. Thus, there are no grounds for the application of the correction factor for loss of strychnine by oxidation.

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DISCUSSION

The paper was presented by MR. D. A. ELVIDGE.

The CHAIRMAN. The reason for the factor of 1·02 in the B.P. assay of nux vomica became less obvious as the result of this work.

DR. D. C. GARRATT (Nottingham). It was Corfield who introduced the factor of 1·02, after showing that the weight of strychnine was less than the figure obtained by titration. Foster's work may now have solved the question.

MR. H. B. HEATH (Sudbury) had made a number of spectrophotometric determinations of strychnine and brucine mixtures and found that the

method was inaccurate and he asked how it followed that $x = 0{\cdot}318A - 0{\cdot}460B$. He had found that sodium nitrite had a very marked absorption at 300 mμ. What was the number of the Brockman grade of alumina used?

DR. G. E. FOSTER (Dartford). How was the theoretical amount of strychnine reported in Table III determined?

DR. W. MITCHELL (London). Could coarsely crushed nux vomica be readily extracted by boiling with ethanol? The liquid reaching the extraction thimble would be stronger than 70 per cent.

MR. S. G. E. STEVENS (London). Did all batches of oxidised cellulose give the same type of absorption characteristics and was there any experience when other permitted dyes were present?

DR. L. SAUNDERS (London). It was not clear that oxidised cellulose was comparatively unstable at room temperature and had to be kept refrigerated. Had it been found necessary to apply blanks for absorbing material derived from the oxidised cellulose and had the exchange capacities been determined in order to standardise the material?

MR. H. B. HEATH (Sudbury). Was it necessary to wash the column after eluting with sulphuric acid?

DR. G. E. FOSTER said it was not clear whether the authors had carried out assays of nux vomica using the B.P. chloroform extraction method as well as the ethanol method recommended. His experience of spectrophotometric methods had been that although one worker with his own spectrophotometer might obtain results accurate within 0·1 per cent, when assays were carried out in independent laboratories the agreement was not so good.

DR. G. BROWNLEE (London) raised the question of the use of the term "error" by chemists to record the observed deviations of replicates and by other scientists to indicate the error of the estimate. Had not the time arrived for analysts to record the latter? The analyst would lose nothing by stating the intrinsic error of his estimation, and he would strengthen his own hand by recording the variations from day to day or from laboratory to laboratory.

DR. GARRATT agreed with Dr. Brownlee. The precision of many determinations was known, but not the accuracy.

MR. H. D. RAPSON (Dorking). Spectrophotometers might be subject to error owing to diurnal temperature variations, although the more recent instruments did not show such an error. He would use a set of standard solutions.

MR. C. A. JOHNSON (Nottingham). Despite the instability of the oxidised cellulose, could it not be used over a long period for a number of determinations?

In reply MR. ELVIDGE said that there were two simultaneous equations for the additive absorption spectrum, but these were not included in the paper. For pure strychnine the standard deviation was calculated as $\pm 0{\cdot}3$

per cent, but with strychnine and brucine there would be a slightly larger error. They had found that all the salts came through the column, only strychnine being left and, therefore, there was no interference from sodium nitrite. The alumina was Brockman Grade I. He had had no experience with coarsely crushed nux vomica. The temperature of the alcohol vapour recorded by a thermometer in the Soxhlet thimble indicated a strength of 70 to 72 per cent. After saying that the figure of 0·033 grains of strychnine was determined on one sample of tablets, and it was assumed that others had the same amount. There were different grades of oxidised cellulose. For strychnine assay a content of 16 to 22 per cent of free carboxyl was satisfactory, but for some other alkaloids the 10 to 12 per cent grade must be used. Amaranth and dyes of a similar type were used in the preparations. Oxidised cellulose was unstable, particularly in the dry state, but appeared more stable under water, and the powdered material was always kept in a refrigerator. In all the batches examined there was virtually no blank, and even at 200 mμ it was found to be only 0·002. Provided simple solutions practically free from suspension were used, up to 10 ot 12 consecutive determinations could be carried out successfully. It was advisable after, say, a month to discard the column. The variation of wavelength in one of his calibrated spectrophotometers had been no greater than $\frac{1}{2}$ mμ during one year. It was doubtful whether that shift would cause any appreciable error.