A SOURCE OF ERROR IN THE ASSAY OF STRYCHNINE SALTS AND PREPARATIONS CONTAINING STRYCHNINE

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Strychnine, the most important alkaloid obtained from *Strychnos nux vomica* L., was discovered in 1817 by Pelletier and Caventou[1] and since that time has been widely used in medicine. On this account the assay of nux vomica and of strychnine preparations has been investigated many times and such assays are now carried out by a few well established procedures. Owing to the sparing solubility of the alkaloid in most common solvents chloroform, of which six parts dissolve one of strychnine, has been almost universally used as solvent for extraction of the alkaloid from preparations being assayed for strychnine. It has been usual for the solvent to be removed from the chloroform extract by distillation, after which the residual base may be either weighed, after drying to constant weight, or titrated with standard acid. Two colorimetric methods for completing the assay have found considerable application, particularly when small amounts of strychnine are to be estimated. Denigès[2] modification of Malaquin's colour test has been critically studied by Allport and his collaborators[3,4] with the object of using it for colorimetric determination of strychnine. In this procedure the alkaloid is reduced in acid medium with zinc amalgam and the product treated with sodium nitrite solution when the red colour produced is a measure of the strychnine present. The colour developed with ammonium vanadate in the presence of sulphuric acid[5] also affords a satisfactory means of colorimetric assay. The extent to which these procedures have found favour is indicated in Table I, which gives a summary of the methods employed by some official and semi-official books of standards.

In our laboratories, where strychnine salts and preparations are examined, the analytical methods used cover all of those included in Table I. We have repeatedly found, however, that there was a small discrepancy between the results obtained when the residue from the chloroform extract was weighed as strychnine and when it was titrated with standard acid, the former always giving the higher result. A series of assays carried out by independent workers convinced us that the observed difference was not due to experimental error and it was therefore decided to examine critically the assay procedures. The present paper describes the results of this investigation.

Experimental and Results

The Assay of Strychnine Salts

The B.P. method for the assay of strychnine hydrochloride consists of extraction with chloroform of the alkaloid from a solution of the salt, rendered alkaline by addition of dilute solution of ammonia, evaporation

TABLE I

SUMMARY OF OFFICIAL AND SEMI-OFFICIAL ASSAY PROCEDURES

Publication	Preparation	Solvent used	Method for estimating strychnine in residue from extract
B.P. 1953	Nux vomica, its dry extract, liquid extract and tincture	Chloroform	Titration with standard acid
B.P. 1953	Strychnine hydrochloride and solution of strychnine hydrochloride	Chloroform	Titration with standard acid
B.P.C. 1954	Strychnine sulphate	Chloroform	Titration with standard acid
B.P.C. 1954	Injection of strychnine and mixture of strychnine	Chloroform	Colorimetric estimation using solution of ammonium vanadate as reagent
B.P.C. 1954	Easton's syrup and tablets of Easton's syrup	Chloroform	Weigh residue after drying to constant weight at 105° C.
National Formulary IX	Strychnine phosphate, tablets of strychnine nitrate and tablets of strychnine sulphate	Chloroform	Titration with standard acid

of the solvent from the extract and titration of the residue with standard acid using solution of methyl red as indicator. We have used this official procedure for the assay of strychnine salts but the analytical figures obtained have always been lower than those recorded when the final residues were weighed as strychnine instead of being titrated. In some experiments the residues were titrated potentiometrically but the results were identical with those obtained using methyl red as indicator. Some typical results are shown in Table II.

TABLE II

COMPARISON OF RESULTS BY WEIGHING AND TITRATION

Salt	Sample	$C_{21}H_{22}O_2N_2$ content, expressed as per cent. in undried sample	
		From weight of base	By titration of base
Strychnine sulphate	1	78·8	76·6
,, ,, ...	2	78·3	76·4
,, ,, ...	3	79·6	76·2
Strychnine hydrochloride ..	1	84·1	80·8
,, ,, ..	2	84·0	81·4
,, ,, ..	3	84·6	80·9

Chemical Reaction between Chloroform and Strychnine

Our work on strychnine salts suggested that some reaction between the alkaloid and chloroform may occur during the assay and in order to investigate the possibility weighed portions of pure strychnine base were transferred to tared flasks, 100 ml. portions of chloroform added to each and the resulting solutions evaporated to dryness. The residues were weighed after drying at 105° C, and then titrated with standard acid. Particular attention was paid to the removal of the chloroform from the solutions and, in selected experiments, the following three procedures were adopted: (1) Evaporation without the addition of ethanol. (2) Evaporation to small volume, addition of 5 ml. of ethanol

and evaporation to dryness (B.P. method). (3) Evaporation to about 20 ml., evaporation continued to dryness while two portions (10 ml.) and three portions (5 ml.) of ethanol were added.

Table III summarises the results of these experiments in which strychnine, containing 98·9 per cent. $C_{21}H_{22}O_2N_2$ estimated by titration, was used.

TABLE III

ESTIMATION OF STRYCHNINE USING THREE PROCEDURES OF SOLVENT REMOVAL

Weight of strychnine	Procedure for removing solvent	Weight of base recovered	Percentage of strychnine recovered	
			By weight	By titration
0·5039 g.	1	0·5123 g.	101·7	96·1
0·5018 g.	2	0·5085 g.	101·3	97·7
0·4895 g.	3	0·4935 g.	100·8	96·5

The final titration liquors from these experiments were acidified with nitric acid and tested for ionisable chlorine by addition of silver nitrate solution; in all cases chloride was found to be present. In order to investigate the formation of ionisable chlorine quantitatively a further series of experiments was performed in which the final titration liquors, obtained during the strychnine determination, were titrated electrometrically with 0·02 N silver nitrate solution. All three methods of solvent evaporation were employed and, in some cases, chloride formation was increased by boiling the alkaloidal solution under a reflux condenser for periods up to 5 hours before the chloroform was removed by evaporation. In extension of this work, the amount of alkaloid used was increased to allow the base recovered from the chloroform solution to be divided into two portions, one of which was titrated in the usual manner and the other being dissolved in glacial acetic acid and titrated with 0·1 N perchloric acid solution after the addition of mercuric acetate, solution of crystal violet in glacial acetic acid being used as an indicator. A summary of the results obtained is given in Table IV.

It was at first concluded that our experimental results could be explained by the formation of a small amount of strychnine hydrochloride, due to the interaction of the alkaloid and chloroform, but this conclusion proved to be erroneous as the increase in the weight of the base on evaporation of its solution was far greater than that due to the ionisable chlorine found.

Isolation of Chloro-compound

During experiments in which solutions of strychnine in chloroform were boiled for some hours it was noticed that an insoluble product gradually separated from the solution and first appeared after one or two hours boiling. The yield of material could be increased by continuing the boiling for several days and in this way sufficient was collected for examination. By recrystallisation from water, it was obtained as colourless needles, melting with decomposition above 300° C. The following analytical figures were obtained using a sample dried at 105° C. Found: C, 57·2; H, 5·3; N, 6·63; Cl, 17·5; ionisable Cl 7·65 per cent.

TABLE IV

SUMMARY OF ANALYTICAL RESULTS

Expt. No.	A Assay by titration on original strychnine	Weight of strychnine taken g.	Method of evaporation	Weight of strychnine recovered g.	Strychnine recovered by weight per cent.	B Strychnine recovered by 0·1 N H_2SO_4 titration per cent.	Ml. 0·02 N $AgNO_3$ required for Cl^-	Difference per cent. between A and B		Strychnine recovered by 0·1 N $HClO_4$ titration per cent.
								Calculated from $AgNO_3$ titration	Found	
1	98·3	0·5180	1	0·5309	102·5	96·25	1·90	2·5	2·05	—
2	98·5	0·4464	1	0·4566	102·3	97·4	0·97	1·45	1·1	—
3	99·2	1·3044	1	1·3230	101·5	97·6	3·77	1·8	1·6	99·8
4	98·3	0·5496	2	0·5569	101·3	96·2	2·15	2·6	2·1	—
5	98·5	0·4490	2	0·4555	101·5	97·5	1·05	1·6	1·0	—
6	99·2	1·2348	2	1·2557	101·8	98·0	3·38	1·7	1·2	99·7
7	98·3	0·5741	3	0·5768	100·5	96·2	1·90	2·25	2·1	—
8	98·5	0·4579	3	0·4620	100·9	97·5	1·00	1·5	1·0	—
9	99·2	1·2180	3	1·2295	100·9	98·3	2·49	1·2	0·9	100·0
10*	98·5	0·4474	1	0·4616	103·2	95·3	2·65	4·0	3·2	—
11*	99·2	1·3020	1	1·3329	102·4	94·4	10·00	5·0	4·8	99·9
12*	99·2	1·0347	1	1·0677	103·2	92·8	10·3	6·5	6·4	99·6
13*	99·2	1·0095	1	1·0599	105·1	90·2	14·34	8·7	9·0	100·0

* In Expts. 10 and 11—strychnine boiled under reflux with chloroform for 2–3 hours; in Expt. 12 for 3 hours and in Expt. 13 for 5 hours; before evaporation by method 1.

Equivalent weight by (*a*) titration with 0·1 N silver nitrate = 463·4, (*b*) non-aqueous titration with 0·1 N perchloric acid = 464·4. $[\alpha]_D^{20} + 17°$ (*c.*, 2 in water). Examination by paper chromatography indicated that

the material consisted of a single substance. For this purpose 0·01 ml. of a 1 per cent. w/v solution in ethanol was placed on a strip of Whatman No. 1 paper and the chromatogram developed using a chloroform–ethanol–glacial acetic acid 80:15:5 mixture as solvent. After drying the paper the alkaloid was located by immersion of the strip in Dragendorff's reagent[6]. Figure 1 shows a chromatogram comparing the chloro-compound with strychnine, strychnine hydrochloride and strychnine recovered by evaporation of the solvent from a chloroform solution, previously boiled for two hours. These data provided conclusive evidence that the chloro-compound, which may be formed during the assay, is distinct from strychnine and its hydrochloride.

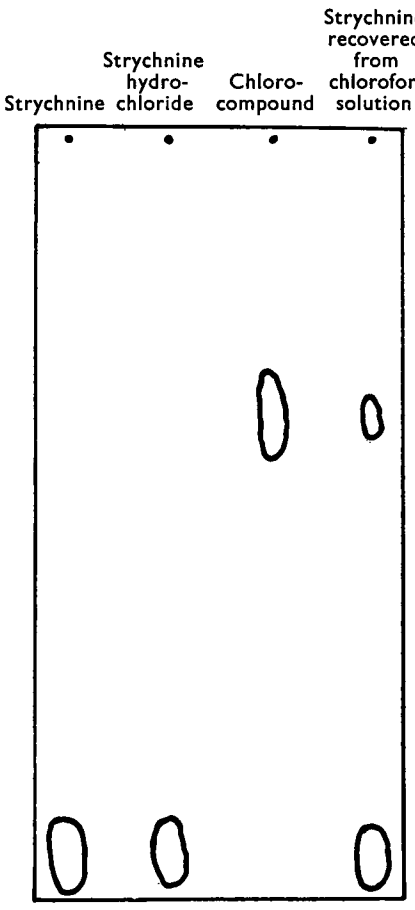

FIG. 1. Chromatogram using Whatman No. 1 paper and chloroform-ethanol-glacial acetic acid 80:15:5 mixture as solvent.

Proposed Amendment to B.P. Assay of Strychnine Hydrochloride

As a result of the work already described some modification of the B.P. assay procedure is necessary if the error due to the formation of the chloro-compound is to be eliminated. To avoid heating the chloroform solution we decided to explore the possibility of removing the alkaloid from the chloroform extract, obtained during the assay, by shaking with a known volume of standard acid, separating the acid layer and titrating the excess of acid with standard alkali. For this purpose the following experiment was repeated six times. Strychnine, weighing approximately 0·5 g. was dissolved in 25 ml. of 0·1 N sulphuric acid solution and the excess of acid titrated with 0·1 N sodium hydroxide solution using methyl red solution as indicator, the resulting assay figure being noted. The solution was then made alkaline by the addition of 5 ml. of sodium hydroxide solution and extracted with four portions (20 ml.) of chloroform, each extract being washed with two portions (10 ml.) of water.

The mixed chloroform extracts were shaken with 25 ml. of 0·1 N sulphuric acid solution and the mixture allowed to separate. The chloroform layer was removed and washed with three portions (15 ml.) of water, the washings being added to the acid extract. The mixed acid extract and washings were then titrated with 0·1 N sodium hydroxide solution using solution of methyl red as indicator. The results of these experiments are summarised in Table V.

TABLE V

SUMMARY OF RESULTS OBTAINED FOR THE AMENDED B.P. ASSAY

Weight of strychnine taken g.	Assay expressed as per cent. $C_{21}H_{22}O_2N_2$ before extraction	Assay expressed as per cent. $C_{21}H_{22}O_2N_2$ after extraction	Per cent. recovery
0·5368	99·4	99·0	99·6
0·5070	99·2	98·9	99·7
0·4581	99·2	99·1	99·9
0·4616	99·3	99·0	99·7
0·4222	99·1	98·9	99·8
0·4644	99·4	99·2	99·8

It is clear from these results that satisfactory assay figures can be obtained by the above procedure which eliminates the need to evaporate the chloroform extract containing the alkaloid. The main objection to the suggested modification is that chloroform being heavier than water causes the acid extraction and subsequent washing of the chloroform layer to be tedious and time consuming.

An alternative procedure, which affords satisfactory results, is to perform the B.P. assay but to titrate the final alkaloidal residue in glacial acetic acid solution with 0·1 N perchloric acid as shown in Table IV.

Colorimetric Estimation

For the determination of small quantities of strychnine, colorimetric methods, or more usually absorptiometric methods, are often employed after the alkaloid has been isolated by suitable means, which may involve extraction with chloroform. It therefore became of interest to ascertain whether the presence of the chloro-compound had any influence on the two colour reactions most widely used for quantitative work.

Colorimetric readings were obtained in the following manner for (1) strychnine, (2) a weighed amount of strychnine dissolved in chloroform and recovered from the solution, after standing overnight, by evaporation of the solvent and (3) the chloro-compound.

Malaquin's reaction. 0·2 g. of zinc amalgam activated before use by momentarily immersing in mercuric chloride solution was added to 5 ml. portions of the solutions containing 0·02 to 0·1 mg. of the substance, under examination, in 10 per cent. hydrochloric acid. The solutions were heated on a boiling water bath for nine minutes, cooled in running water for fifteen minutes and each treated with 1 drop of 0·1 per cent. sodium nitrite solution. The light absorption of each reaction mixture was then measured within 5 minutes with a Spekker absorptiometer using No. 547 Kodak filters and a blank, consisting of a reaction mixture

containing no alkaloid. The readings obtained are shown graphically in Figure 2.

Ammonium vanadate reaction. To 1 ml. portions of solution containing 0·04 to 0·2 mg. of substance, under examination, 5 ml. of reagent, containing 0·5 g. of ammonium vanadate in 100 ml. of 60 per cent. v/v sulphuric acid was added. After standing for two minutes 4 ml. of water was added and the light absorption of each reaction mixture

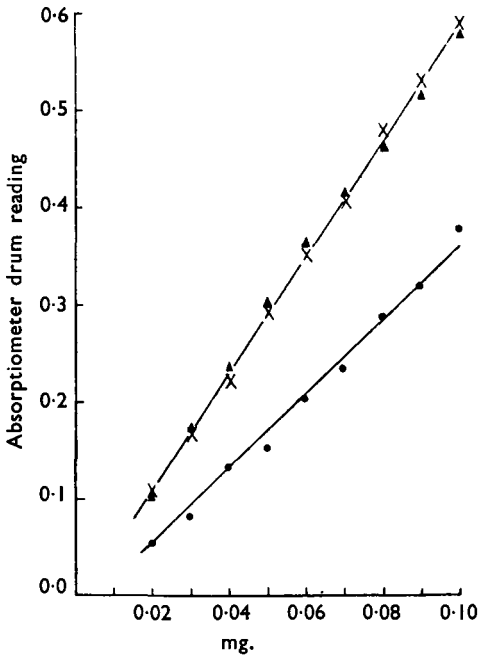

FIG. 2. Data for Malaquin colour reaction. ▲, strychnine; ×, strychnine recovered from chloroform solution; ●, chloro-compound.

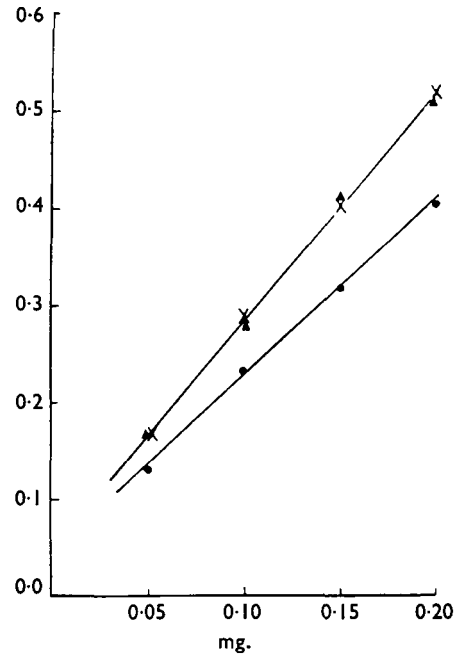

FIG. 3. Data for ammonium vanadate reaction. ▲, strychnine; ×, strychnine recovered from chloroform solution; ●, chloro-compound.

measured after 15 minutes with a Spekker absorptiometer using No. 547 Kodak filters and water as blank.

The readings obtained are shown graphically in Figure 3.

DISCUSSION

The work described in this paper leaves no doubt that a reaction takes place between chloroform and strychnine when a solution of the alkaloid in this solvent is heated, even for the short period needed to remove the chloroform by evaporation during an assay. By prolonged heating of such solutions a chlorine containing reaction product separates from the solution and may be recrystallised from water. Paper chromatography has been used to establish that this chloro-compound may be formed in small amounts during the assay of strychnine salts by the normal procedure.

The reaction product was at first thought to be an addition compound containing one molecule of alkaloid and one of chloroform but analytical evidence was not in harmony with this view. Several samples on ultimate analysis gave analytical figures in close agreement but, so far, we have been unable to derive a satisfactory formula for the compound on the basis of the accepted structure of strychnine. It is noteworthy that part of the chlorine in the molecule is ionisable and part non-ionisable, while the product is dextro-rotatory in contrast with strychnine which has a laevo-rotation. Klemperer and Warren[7] have recently reported the isolation of strychnine dichloromethochloride, $C_{22}H_{23}O_2N_2Cl_3\frac{1}{4}H_2O$, from chloroform solutions of the alkaloid. It is probable that we have obtained the same substance, but the analytical figures reported by us are not in good agreement with the proposed formula and we prefer to postpone any statement regarding its structure until we have carried out further work.

Evidence has been provided that the formation of the chloro-compound causes a small but significant error in the assay of strychnine salts and preparations by assay procedures depending on weighing or titrating the alkaloid recovered from the chloroform extract by evaporation of the solvent. This error may be eliminated or reduced to negligible proportions by titrating the recovered alkaloid in glacial acetic acid with standard perchloric acid or by performing an extraction titration by shaking the chloroform extract with excess of standard sulphuric acid and titrating the excess of acid with standard sodium hydroxide.

The magnitude of the error will doubtless depend upon the conditions under which assays are performed in different laboratories and we are prepared to believe that some analysts will observe smaller errors than those reported in this paper. Nevertheless, we are convinced that the reaction between chloroform and the alkaloid constitutes a real source of error in the standard assay procedure and one which it is desirable to eliminate. In this connection the official assay of nux vomica, involving continuous extraction of the drug with ethanol-chloroform mixture, would seem to call for investigation.

Small quantities of strychnine are sometimes estimated colorimetrically using either the Malaquin reaction or the colour developed with ammonium vanadate in the presence of sulphuric acid. It has been shown that the presence of the chloro-compound does not affect the determination of strychnine by these methods, within the normal limits of experimental error.

Summary

1. In the assay of strychnine salts by the standard procedure a discrepancy has been observed between the results obtained when the recovered alkaloid is weighed as strychnine and when it is titrated with standard acid.

2. A chlorine containing reaction product, for which analytical data are given, has been isolated from chloroform solutions of strychnine.

3. The formation of the chloro-compound interferes with the standard assay of strychnine salts.

4. Amendments to the standard assay procedure have been proposed with the object of eliminating the source of error.

5. The effect of the chloro-compound on the two most widely used colorimetric methods for the determination of strychnine has been investigated.

We wish to thank Mr. F. J. McMurray for the micro analyses reported in this paper.

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DISCUSSION

The paper was presented by DR. G. E. FOSTER.

DR. D. C. GARRATT (Nottingham) asked whether the authors could have given attention to nux vomica and its preparations, because he felt sure that in their assay an empirical factor must have been worked out on a weight basis and not on the titration figure, and it would have been helpful to know if that correction factor was correct. In the assay of small quantities of strychnine it was always assumed that the weight obtained was that of pure strychnine, but he wondered whether there was not a balance of errors.

DR. F. HARTLEY (London) said that the preparation of the chloro-compound by refluxing the alkaloid with chloroform for some hours was not equivalent to the condition encountered in the assay procedure and asked whether the authors had assessed the rate at which the chloro-compound was formed.

DR. W. MITCHELL (London) suggested that the point could be emphasised with some advantage that the volumetric method gave low results and the gravimetric method high results, but neither gave the correct result. The authors had shown that non-aqueous titration gave an accurate result but suggested another method which was tedious. He suggested that it would be advantageous to adopt the convenient non-aqueous titration. In addition to the existing errors in the assay of nux vomica it was now necessary to take into account the loss due to the use of chloroform and he suggested that for initial extraction, benzene or some other solvent might be used instead of chloroform.

DR. A. H. BECKETT (London) said that the South African workers had based their conclusions on evidence which was completely unsatisfactory, but Dr. Foster had provided some rather interesting figures. The

equivalent weight given of 464·4 fitted exactly for one ionised chlorine atom and he hoped that the nitrogen figure 6·63 was an error and that it should have been 6·03.

DR. G. BROWNLEE (London) pointed out that should the chloro-compound prove to be quaternary in nature it would be expected to have very different pharmacological effects from those of strychnine.

DR. G. E. FOSTER, in reply, said that Mr. Caws was engaged in an investigation into the assay of nux vomica itself, and he hoped to publish a further paper. It was not necessary to reflux strychnine with chloroform to obtain the chloro-compound as they had recently found that this could be obtained by allowing a chloroform solution of strychnine to stand in the cold. The authors had not investigated the rate of reaction. It was true that non-aqueous titration gave the right result but that was because the factor used was that for strychnine. He had also queried the analytical figure for nitrogen, but the data had been checked and the printed figure was that actually found.