(6) Sodium laurate, of the soaps examined, is the most toxic to earthworm segments. The toxicity of the others are in the order named: sodium myristate, sodium oleate, sodium ricinoleate, sodium palmitate and sodium stearate.

REFERENCE.

(1) U. S. P. Revision Committee, JOUR. A. PH. A., 24, 891 (1935).

A STUDY OF THE ASSAYS OF THE POWDERED EXTRACTS OF BELLADONNA AND HYOSCYAMUS.*,[1]

BY HOWARD H. FRICKE[2] AND K. L. KAUFMAN.[3]

In past years, trouble has been encountered in the determination of the alkaloidal content of the powdered extracts of belladonna and hyoscyamus. The potency of these drugs makes it desirable to remedy the difficulties underlying the assays of their extracts. Our work has been carried on in an effort to determine the exact origin of these difficulties, and thus point the way to more satisfactory analytical methods. Most of the experimental studies were made on the pure alkaloids and the powdered extract of belladonna.

The presence of volatile basic substances which were being determined as atropine, was noted by various workers including Goris and Larsonneau (1), Markwell and Walker (2), DeKay and Jordan (3) and the A. D. M. A. Contact Committee's Sub-Committee (4). A few of the volatile bases have been analyzed, but the chemical natures of most of them have not been determined. The A. D. M. A. Contact Committee's Sub-Committee (4) found that the physiological activity of the volatile bases was weak and inconstant.

Various methods of assay have been suggested by different workers in an attempt to eliminate these volatile bases so that the product may be more easily and accurately standardized. At present there is no process which accomplishes this satisfactorily.

Tsakalotos (5) and several other workers found that the mydriatic alkaloids were not decomposed during the assay process as long as they were not exposed to excessively alkaline solutions or were not left in contact with alkaline solutions for long periods. Schousen (6) and several other authors, however, have found that atropine and hyoscyamine are sensitive to water-bath temperature and may be partially hydrolyzed when subjected to water-bath heat. Durrett (7) stated that the hydrolysis of the mydriatic alkaloids could be prevented by the addition of a little dehydrated alcohol near the end of the evaporation of the final chloroform extract. The A. D. M. A. Contact Committee's Sub-Committee (4) has reported that the final heat treatment of the U. S. P. XI assay causes little or no destruction of the alkaloids.

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[1] Based on a thesis submitted to the Committee on Graduate Studies of the State College of Washington by Howard H. Fricke in partial fulfilment of the requirements for the degree of Master of Science.

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The assay methods for the powdered extract of belladonna were very similar in the various revisions of the pharmacopœia until 1930. The discovery of the volatile bases obtained in the assay procedure brought forth several difficult problems. As a result of much study, the U. S. P. XI assay process was made official.

The chief differences between the U. S. P. X and U. S. P. XI methods are as follows:

1. The U. S. P. X directed that the alkaloids be purified by extracting a suitable portion of the sample with chloroform, then removing the alkaloids from this solvent with 2 per cent sulfuric acid. The acid solution is then extracted with chloroform. The U. S. P. XI method purifies the alkaloids by filtering out the diluent and then extracting the filtrate with chloroform after first making it alkaline with ammonia water.

2. The U. S. P. X method directed that the chloroform be removed by evaporating to about five cc. on a water-bath, adding an equal volume of alcohol and removing the remaining chloroform by evaporation. The U. S. P. XI method directs that the chloroform be removed by evaporating to dryness on a water-bath, and then heating at dryness about fifteen minutes. The residue is dissolved in about five cc. of chloroform and again heated on a water-bath for fifteen minutes. This treatment is repeated a second time.

EXPERIMENTAL.

The materials used for the experimental studies were obtained from various sources. Certain special tests were performed to determine their purity.

A. STABILITY OF ATROPINE AND HYOSCYAMINE IN SOLUTION.

In a previous communication, we have summarized a large number of experiments on the stabilities of atropine and hyoscyamine in solution (8). This work has been checked and extended.

TABLE I.—THE EFFECT OF ALCOHOL ON THE STABILITIES OF ATROPINE AND HYOSCYAMINE IN CHLOROFORM.*

Alkaloid.	Treatment with Alcohol When Evaporated to 5 Cc.	Water-Bath Treatment.	Average Gm. of Alkaloid Returned.	Average Per Cent Alkaloid Returned.
Atropine		Evaporated to dryness	0.03006	100.00
Atropine	Added 5 cc. alcohol	Evap. remaining chloroform	0.03063	100.00
Atropine	Added 5 cc. alcohol	Evap. remaining chloroform	0.03052	100.00
Atropine	Added 5 cc. alcohol	Heated 30 min. after evap'n.	0.03006	100.00
Atropine	Added 5 cc. alcohol	Heated 60 min. after evap'n.	0.02988	99.61
Atropine	Added 5 cc. alcohol	Heated 60 min. after evap'n.	0.02977	99.22
Atropine		Heated 60 min. after evap'n.	0.02936	97.88
Atropine	Sufficient alc. to avoid dryness**	Heated 60 minutes	0.03034	100.00
Hyoscyamine		Evaporated to dryness	0.03000	100.00
Hyoscyamine	Added 5 cc. alcohol	Evaporated remaining $CHCl_3$	0.03046	100.00
Hyoscyamine	Added 5 cc. alcohol	Heated 30 min. after evap'n.	0.02988	99.61
Hyoscyamine	Added 5 cc. alcohol	Heated 60 min. after evap'n.	0.02948	98.26
Hyoscyamine	Sufficient alc. to avoid dryness**	Heated 60 minutes	0.03034	100.00

NOTE: All averages are based on the results of at least four determinations.

* The analytical grade of chloroform was used.

** Several additions of alcohol were made at suitable intervals.

TABLE II.—THE RELATIVE EFFICIENCIES OF THE U. S. P. X AND U. S. P. XI ASSAY PROCESSES AND THE EFFECT OF THE U. S. P. XI FINAL HEAT TREATMENT UPON THE RESULTS OF THE ASSAY.

U. S. P. X Assay.		U. S. P. XI Assay.		U. S. P. X Assay.* Fortified Extract.		U. S. P. XI Assay.* Fortified Extract.		U. S. P. X Shake-Out. U. S. P. XI Complete.		U. S. P. XI Shake-Out. U. S. P. X Complete.	
Alkaloid Returned.**				Atropine Returned.**				Alkaloid Returned.**			
Grams.	Per Cent.	Grams.	Per Cent.	Grams.	Per Cent.	Grams.	Per Cent.	Grams.	Per Cent.	Grams.	Per Cent.
0.9872	79.00	0.8208	65.70	0.0480	96.00	0.0459	91.90	0.8208	65.70	0.9964	79.71
0.9768	78.14	0.8184	65.44	0.0487	97.32	0.0466	93.18	0.8138	65.12	0.9906	79.17
0.9896	79.17	0.8024	64.20	0.0477	95.54	0.0472	94.44	0.8092	64.74	1.0058	80.47
0.9792	78.34	0.8116	64.93	0.0485	97.04	0.0466	93.18	0.8184	65.49	0.9988	79.90
1.0126	81.00	0.8266	66.13	0.0481	96.24			0.8266	66.13		
Average	Average	Average	Average	Average	Average	Average	Average	Average	Average	Average	Average
0.9891	79.13	0.8160	65.28	0.0482	96.43	0.0466	93.18	0.8178	65.44	0.9979	79.81
±0.0096	±0.76	±0.0072	±0.57	±0.0003	±0.60	±0.0003	±0.63	±0.0050	±0.40	±0.0044	±0.37

* Atropine returned calculated only on the basis of 50 mg. added.

** Per cent of alkaloid returned based on 1.2500 Gm. of alkaloid per 100 Gm. of extract.

However, it should be made clear that the experimental results, when chloroform is the solvent, will vary with the grade of reagent used. It was found that technical quality chloroform gave much lower results than analytical grade.

The effect of alcohol (95 per cent) on the stabilities of atropine and hyoscyamine was determined by evaporating to about five cc. a solution of the alkaloid in a volatile solvent, and then adding 5 cc. of neutral alcohol. The mixture was then subjected to various heat treatments. The results are summarized in Table I.

B. THE RELATIVE EFFICIENCIES OF THE U. S. P. X AND U. S. P. XI ASSAY PROCESSES AND THE EFFECT OF THE FINAL HEAT TREATMENT.

During the last two and one-half years, much criticism has arisen regarding the U. S. P. XI assay methods for belladonna and hyoscyamus preparations. Accordingly, the processes of the Tenth and Eleventh Revisions of the Pharmacopœia were compared by analyzing the same sample of powdered extract by each method. Extreme care was exercised to follow the exact assay procedure as given in the respective Pharmacopœias. The results obtained are recorded in Table II.

The efficiency of the extraction methods and the inherent loss of each was determined by adding 50.0 mg. atropine to the extract. This fortified product was assayed by each method. The efficiency of each process was indicated by the per cent atropine returned. The results of the experiments are given in Table II.

The effect of the final heat treatment required by the U. S. P. XI was determined on a number of 5.0 Gm. samples as follows: The method of the Tenth Revision was used for shaking out and purifying the alkaloidal solutions. A comparison was made by using the Eleventh Revision shake-out method and the Tenth Revision completion process. The effect of the heat treatment is shown by comparing these results with those obtained by the U. S. P. X and U. S. P. XI assay methods. The results of these analyses are shown in Table II.

C. THE EFFECTS OF VARIOUS DILUENTS ON THE ASSAY PROCESS.

Various diluents may be employed to adjust powdered extracts to their proper strength. Most manufacturers employ some type of starch for this purpose. Many experiments were performed in an effort to determine the effects of some common starches on the assay procedure.

The starches selected were corn, wheat, rice, arrow-root and potato. These were chosen because they are inexpensive, easy to obtain and furnish a variety of characteristics.

Mixtures of starch and atropine were allowed to stand five months before analysis in order to determine if any alkaloid was being adsorbed. The results obtained were then compared with the analyses of freshly prepared mixtures of starch and atropine.

The effect of chlorophyll on the assay process was determined by adding three drops of chlorophyll to mixtures of 30.00 Gm. of starch and 375.0 mg. of atropine. These mixtures were also allowed to stand five months before analysis.

The procedure for these experiments follows: the starch was triturated thoroughly with the atropine. Two Gm. of this mixture was accurately weighed and digested in three parts of alcohol (95 per cent) and one part of water. The solution was made alkaline with ammonia T.S. and extracted with chloroform. The combined chloroform extractions were evaporated on a water-bath to 5 cc., then an equal volume of alcohol was added. The remaining chloroform was removed and the alkaloids determined by titration with $N/50$ sulfuric acid and $N/50$ sodium hydroxide solutions. Part of the combined chloroform solutions were determined by adding 15.00 cc. of $N/50$ sulfuric acid to the solution when it had evaporated to a volume of 5.0 cc. The remaining chloroform was then removed and the alkaloids titrated with $N/50$ sodium hydroxide solution. Methyl red was used as the indicator in all cases. The results of the experiments are summarized in Tables III, IV, V, VI and VII.

TABLE III.—THE EFFECT OF CORN STARCH AND CHLOROPHYLL ON THE ASSAY OF ATROPINE.

Treatment of Combined Chloroform Extractions before Removing the Last 5 Cc. of Chloroform.	Newly Made Mixture of Corn Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Corn Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Corn Starch, Atropine and Chlorophyll. Atropine Returned.	
	Grams.	Per Cent.*	Grams.	Per Cent.*	Grams.	Per Cent.*
Added 5 cc. C_2H_5OH			0.02127	86.12	0.02000	80.96
Added 5 cc. C_2H_5OH			0.02191	88.70	0.01595	64.58
Added 5 cc. C_2H_5OH			0.02225	90.10	0.01815	73.48

Added 5 cc. C_2H_5OH			0.02225	90.10	0.01803	73.00
			Average	Average	Average	Average
			0.02127	88.75	0.01803	73.00
			±0.00033	±1.44	±0.00104	±4.21
Added 15 cc. H_2SO_4**	0.02283	92.42	0.02312	93.60	0.02225	90.10
Added 15 cc. H_2SO_4**	0.02219	89.86	0.02254	91.26	0.02208	89.39
Added 15 cc. H_2SO_4**	0.02191	88.70	0.02254	91.26	0.01971	79.80
Added 15 cc. H_2SO_4**	0.02282	92.42	0.02346	95.01	0.02312	93.60
	Average	Average	Average	Average	Average	Average
	0.02244	90.85	0.02266	92.78	0.02179	88.22
	±0.00039	±1.57	±0.00037	±1.52	±0.00104	±4.21

* Analyses calculated on the basis of 24.70 mg. per sample taken as 100 per cent.

** Fiftieth-normal sulfuric acid solution was used.

TABLE IV.—THE EFFECT OF RICE STARCH AND CHLOROPHYLL ON THE ASSAY OF ATROPINE.

Treatment of Combined Chloroform Extraction before Removing the Last 5 Cc. of Chloroform.	Newly Made Mixture of Rice Starch and Atropine.		Five-Month Old Mixture of Rice Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Rice Starch, Atropine and Chlorophyll.	
	Grams.	Per Cent.*	Grams.	Per Cent.*	Grams.	Per Cent.*
Added 5 cc. C_2H_5OH	0.02237	90.56	0.01734	70.20	0.02063	83.54
Added 5 cc. C_2H_5OH	0.02092	84.71	0.01919	77.69	0.02000	80.96
Added 5 cc. C_2H_5OH	0.02202	89.16	0.02046	82.98	0.01901	77.00
Added 5 cc. C_2H_5OH	0.02081	84.24	0.02237	90.56	0.01907	77.22
	Average	Average	Average	Average	Average	Average
	0.02153	87.17	0.01984	80.33	0.01968	79.68
	±0.00066	±2.69	±0.00158	±6.41	±0.00064	±2.57
Added 15 cc. H_2SO_4**	0.02364	95.71	0.02069	83.77	0.02034	82.37
Added 15 cc. H_2SO_4**	0.02173	87.98	0.01595	64.58	0.02063	83.54
Added 15 cc. H_2SO_4**	0.02428	98.28	0.02173	87.98	0.01971	79.80
Added 15 cc. H_2SO_4**	0.02046	82.98	0.02104	85.18	0.02063	83.54
	Average	Average	Average	Average	Average	Average
	0.02253	91.24	0.01985	80.38	0.02033	82.31
	±0.00143	±5.76	±0.00195	±7.90	±0.00031	±1.26

* Analyses calculated on the basis of 24.70 mg. per sample taken as 100 per cent.

** Fiftieth-normal sulfuric acid solution was used.

TABLE V.—THE EFFECT OF WHEAT STARCH AND CHLOROPHYLL ON THE ASSAY OF ATROPINE.

Treatment of Combined Chloroform Extractions before Removing the Last 5 Cc. of Chloroform.	Newly Made Mixture of Wheat Starch and Atropine.		Five-Month Old Mixture of Wheat Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Wheat Starch, Atropine and Chlorophyll.	
	Grams.	Per Cent.*	Grams.	Per Cent.*	Grams.	Per Cent.*
Added 5 cc. C_2H_5OH	0.02433	98.55	0.02439	98.83	0.02329	94.30
Added 5 cc. C_2H_5OH	0.02376	96.18	0.02346	95.01	0.02439	98.83
Added 5 cc. C_2H_5OH	0.02439	98.83	0.02410	97.58	0.02300	93.13
Added 5 cc. C_2H_5OH	0.02410	97.58	0.02469	99.92	0.02387	96.64
	Average	Average	Average	Average	Average	Average
	0.02414	97.78	0.02416	97.83	0.02364	95.72
	±0.00021	±0.90	±0.00038	±1.54	±0.00049	±2.01
Added 15 cc. H_2SO_4**	0.05954	96.81	0.02410	97.58	0.02300	93.13
Added 15 cc. H_2SO_4**	0.05918	96.23	0.02439	98.83	0.02428	98.28
Added 15 cc. H_2SO_4**	0.05896	95.88	0.02439	98.83	0.02376	96.18
Added 15 cc. H_2SO_4**			0.02346	95.01	0.02364	95.71
	Average	Average	Average	Average	Average	Average
	0.05923	96.31	0.02408	97.56	0.02376	95.82
	±0.00021	±0.34	±0.00031	±1.28	±0.00035	±1.40

* Analyses calculated on the basis of 24.70 mg. per sample taken as 100 per cent.

** Fiftieth-normal sulfuric acid solution was used.

TABLE VI.—THE EFFECT OF POTATO STARCH AND CHLOROPHYLL ON THE ASSAY OF ATROPINE.

Treatment of Combined Chloroform Extractions before Removing the Last 5 Cc. of Chloroform.	Newly Made Mixture of Potato Starch and Atropine.		Five-Month Old Mixture of Potato Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Potato Starch, Atropine and Chlorophyll.	
	Grams.	Per Cent.*	Grams.	Per Cent.*	Grams.	Per Cent.*
Added 5 cc. C_2H_5OH	0.02410	97.58	0.02469	99.92	0.02410	97.58
Added 5 cc. C_2H_5OH	0.02347	95.01	0.02428	98.28	0.02439	98.83
Added 5 cc. C_2H_5OH	0.02312	93.60	0.02439	98.83	0.02376	96.18
Added 5 cc. C_2H_5OH	0.02469	99.92	0.02312	93.60	0.02357	95.44
	Average	Average	Average	Average	Average	Average
	0.02384	96.53	0.02412	97.66	0.02396	97.01
	±0.00055	±2.21	±0.00050	±2.03	±0.00029	±1.20
Added 15 cc. H_2SO_4**	0.02469	99.92	0.02399	97.11	0.02399	97.11
Added 15 cc. H_2SO_4**	0.02376	96.18	0.02376	96.18	0.02410	97.58
Added 15 cc. H_2SO_4**	0.02410	97.58	0.02364	95.71	0.02469	99.92
Added 15 cc. H_2SO_4**	0.02410	97.58	0.02410	97.58	0.02469	99.92
	Average	Average	Average	Average	Average	Average
	0.02416	97.81	0.02387	96.64	0.02437	98.63
	±0.00026	±1.04	±0.00017	±0.70	±0.00032	±1.29

* Analyses calculated on the basis of 24.70 mg. per sample taken as 100 per cent.

** Fiftieth-normal sulfuric acid solution was used.

TABLE VII. THE EFFECT OF ARROW-ROOT STARCH AND CHLOROPHYLL ON THE ASSAY OF ATROPINE.

Treatment of Combined Chloroform Extractions before Removing the Last 5 Cc. of Chloroform.	Newly Made Mixture of Arrow-Root Starch and Atropine.		Five-Month Old Mixture of Arrow-Root Starch and Atropine. Atropine Returned.		Five-Month Old Mixture of Arrow-root Starch, Atropine and Chlorophyll.	
	Grams.	Per Cent.*	Grams.	Per Cent.*	Grams.	Per Cent.*
Added 5 cc. C_2H_5OH	0.02347	95.01	0.02410	97.58	0.02376	96.18
Added 5 cc. C_2H_5OH	0.02329	94.30	0.02439	98.83	0.02312	93.60
Added 5 cc. C_2H_5OH	0.02364	95.71	0.02312	93.60	0.02376	96.18
Added 5 cc. C_2H_5OH	0.02439	98.83	0.02376	96.18	0.02347	95.01
	Average	Average	Average	Average	Average	Average
	0.02370	95.96	0.02384	96.55	0.02353	95.24
	±0.00035	±1.43	±0.00040	±1.66	±0.00023	±0.94
Added 15 cc. H_2SO_4**	0.02439	98.83	0.02502	100.00	0.02474	100.00
Added 15 cc. H_2SO_4**	0.02428	98.28	0.02376	96.18	0.02387	96.64
Added 15 cc. H_2SO_4**	0.02474	100.00	0.02410	97.58	0.02439	98.83
Added 15 cc. H_2SO_4**	0.02474	100.00	0.02439	98.83	0.02427	98.28
	Average	Average	Average	Average	Average	Average
	0.02454	99.28	0.02432	98.15	0.02432	98.44
	±0.00020	±0.75	±0.00039	±1.27	±0.00025	±0.98

* Analyses calculated on the basis of 24.70 mg. per sample taken as 100 per cent.

** Fiftieth-normal sulfuric acid solution was used.

DISCUSSION AND SUMMARY.

1. The quality of the chloroform used (technical, dried technical or dried analytical grades) definitely influences the amount of alkaloid recovered from solutions of atropine and hyoscyamine in this solvent. It is suggested that this fact may clarify some of the contradictory statements in the literature.

2. Solutions of atropine and hyoscyamine in 95 per cent neutral alcohol are stable when heated on a water-bath for one hour. If 5 cc. of alcohol is added before the final few cc. of volatile solvent is evaporated from a chloroform solution of atropine or hyoscyamine, the decomposition of the alkaloidal residue when heated at

dryness on the water-bath is retarded. This confirms the work of Durrett (7) and others. However, the slight advantage of alcohol does not justify its use if analytical grade chloroform is employed for the assay process.

3. The decreased yield of the U. S. P. XI method is chiefly due to the removal of volatile basic substances, but is partly due to loss of alkaloids.

4. The alkaloids are more thoroughly extracted by the U. S. P. X process, as shown by the greater amounts of atropine returned from a fortified sample of powdered extract.

5. The U. S. P. X shake-out method removes more of the chlorophyll and resins which interfere in the U. S. P. XI extraction process. Concordant results are also easier to obtain. This confirms the work of the A. D. M. A. Contact Committee's Sub-Committee (4) and Hayden (9).

6. The most satisfactory results were obtained by using the U. S. P. X extraction method and then completing the assay by the U. S. P. XI process because: *First*, the alkaloids can be extracted with little difficulty; *second*, the assay can be completed in one day; *third*, the volatile bases, which would be calculated as alkaloid, are removed; *fourth*, concordant results can be obtained by using a good grade of chloroform; and *fifth*, the destruction of the alkaloids is less than the approximate error of the assay if analytical grade chloroform is used.

7. Experiments with "synthetic powdered extracts" indicate that atropine is not adsorbed to any appreciable extent by chlorophyll, with the possible exception of a mixture containing corn starch, atropine and chlorophyll. It should be noted, however, that this combination occurs in many powdered extracts.

8. Of the starches tested, only that from rice showed any evidence of adsorption of atropine during the five month test period.

9. Corn and rice starch are diluents from which atropine is difficult to extract. When wheat starch is used as the diluent, this difficulty is partly overcome.

10. Atropine may be readily extracted from mixtures containing potato or arrow-root starches. Neither of these form troublesome emulsions. They were the most satisfactory diluents examined.

The results summarized in 7, 8, 9 and 10 indicate that the type of starch used as a diluent affects the analyses, a marked relationship existing between the size of the starch granules and the efficiency of extraction.

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