Evaluation of Antispasmodic Activity in the Intact Dog II

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Results obtained with a method refined to determine more accurately the comparative effectiveness of the active components of an antispasmodic formula and their combinations are presented. Accompanying these results are data pertaining to the comparative antisialagogue activity of these components and their combination in the same kind of animal preparation. In both original and refined methods, a selected submaximal dose of methacholine chloride in mcg./Kg. (Me_{50}) is, in effect, used as a common reference point, so that a comparison may be made of the relative activities of the agents involved in terms of dose equivalents. These results indicate that the combination of components containing phenobarbital constitutes a potentiating union with respect to antispasmodic effectiveness but not with respect to inhibition of salivary secretion.

ATROPINE and many of its chemically related parasympatholytic agents have been known for well over 100 years. *In vitro* methods have been used for the most part in testing the cholinolytic activity of these and like substances. Methods of *in vitro* testing such as those reported by Luduena and Lands (1) provide the means which are in general acceptance today for the pharmacologic screening of parasympatholytic agents.

For testing the total quantitative response of the gastrointestinal tract to parasympatholytic agents, *in vivo* methods such as those described by Ingelfinger (2) in 1943 and Code *et al.* (3) in 1952 are superior to the *in vitro* methods. The tachyphylactic depression of gastrointestinal sensitivity to these blockers, as described by Seevers *et al.* (4) in 1954 and by Quigley *et al.* (5) in 1937, represents an obvious source of error in *in vivo* testing which must be recognized in the development of such bioassay methods. Even so, the studies of Turkanis and Jenkins (6) in 1960 demonstrated the feasibility of properly scheduled *in vivo* methods for quantitating parasympatholytic activity in the gastrointestinal tract (of the unoperated dog).

As originally conceived, the method emerging from these studies entailed initially the determination of the antispasmodic effectiveness of atropine, hyoscyamine and hyoscine individually and collectively, in the latter instance as two different combinations (CBA and CBB),[1] in terms of the dose equivalent with respect to the response of the canine gastrointestinal tract to an established standard dose of methacholine chloride (see Table V). The original method entailed further the statistical comparison of the sum of the individual dose equivalents (calculated), actually a weighted mean, with each collective equivalent (obtained) for the purpose of determining whether potentiation is inherent in the combinations. This method was implemented in the work of Turkanis and Jenkins (6), who determined the response durations which largely dictated the procedural approach to the problem.

It was discovered in the attempt to provide additional data for the original investigation that the ability to obtain a consistent dose-response relationship with this method was limited by some factor or factors then unknown. When a dose-response relationship involving the stimulus, methacholine chloride, and gastrointestinal activity was obtained at the outset of a determination, data consistent with those of the original investigation were obtained; but all too often this relationship was absent and a consequent low yield of valid data resulted from the determination.

In an effort to determine the underlying difficulty, the effect of the anesthetic agent on the response to the various antispasmodics under study was probed. It then became apparent, as other investigators have confirmed (7, 8), that the pentobarbital employed as the anesthetic agent is capable of ganglionic blocking activity, which increases proportionally to the depth of anesthesia and hence contributes varyingly, depending on anesthesia level, to the antispasmodic effectiveness of the compounds undergoing testing.

Although no attempt was made in the former investigation to determine the effect of the combination of the component antispasmodic compounds on a side effect, this was accomplished in the study herein reported. The parameter selected was salivary secretory activity and

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[1] Kindly supplied as Donna and Donnatal, respectively, by A. H. Robins Co., Inc., Richmond, Va.

though the attendant data were collected after the motility data, the procedures employed were identical in the two instances.

This phase of the study indicated that there is no potentiation of antisialagogue activity in the combination of alkaloids.

EXPERIMENTAL

The sensing device used in this investigation was a rubber sheath which could be inflated, after positioning, through the lateral openings of the blind end of a partially inserted Cantor tube (D 111, 12 French). The opposite end of the Cantor tube, leading from the rubber sheath, was attached at its opening to a glass T-tube, the latter being connected in turn with a rubber tube to a sensitive Sanborn transducer. The side-arm of the glass T-tube was provided with a rubber extension and clamp, and thus served as a valve for the inflation and deflation of the sheath when it was positioned in the gastrointestinal tract.

When the proper amount of initial pressure was contained in the unit-tubing-transducer system (determined by using the same strain gage dial setting each time), the recording was begun. To assess the influence of respiratory excursions on gastrointestinal pressure changes, respiratory activity was recorded along with gastrointestinal responses with a pneumograph, a second Sanborn transducer and strain gage amplifier.

The test animals employed were mongrel dogs of either sex ranging in weight from 12 to 22 Kg. During the course of the study, they were fed once daily and supplied with water *ad libitum*. Each dog was placed on a strict dietary schedule to promote regularity in bowel movements, so that a time might be arranged when there was little or no likelihood of the presence in the lower tract of amounts of fecal matter which would result in interference with the response to the drug. (There was almost always a great increase in the response to the spasmogen when considerable fecal matter was present.)

Tests were conducted to evaluate the possibility of the existence of tachyphylaxis in the response to the spasmogen and/or variable effects of the antispasmodics within the framework of the original procedure. No significant discrepancies were discovered at this point, however. The findings of Nash *et al.* (7) strongly implicate pentobarbital sodium as a blocker of parasympathetic ganglia. Such an action on the part of this substance, which was used as an anesthetic agent in the original procedure, would undoubtedly affect the results of the method if the anesthesia level were subject to variation during the course of the testing. The work of Koppanyi *et al.* (8) substantiates the (*para*-sympathetic) ganglionic blocking activity of pentobarbital sodium. These investigators demonstrated a dose-response relationship with respect to the antispasmodic activity of this barbiturate.

In view of this information, it seemed advisable to change the anesthetic agent from pentobarbital sodium to one which would have no appreciable effect upon autonomic ganglia and would be reasonably satisfactory in other respects. Several anesthetic agents, such as chloral hydrate and urethane, were tested but found unsatisfactory for one reason or another. Finally, chloralose (α-D-glucochloralose) was tried and determined to be the most suitable of the lot for this procedure.

All drugs were administered intravenously by injecting their solutions in the rubber tubing connecting an 18-gauge in-dwelling hypodermic needle with a 50-ml. saline-filled buret. A constant volume (3 ml.) of saline was released from the buret to flush each dose into the circulatory system of the animal. In this manner, the administration rate and interval of the compounds under study did not vary appreciably.

In accord with the data of the previous study, the average mean duration of the response to methacholine chloride was 8 minutes, with a range of 4 to 10 minutes. An interval of 15 minutes between doses of the spasmogen was adhered to throughout the entire series of tests. It had been determined in the early work with the original procedure that this interval is adequate for the avoidance of the cumulative effects of methacholine chloride.

In all cases, the onset of antispasmodic activity was exhibited by a fall in the base line of the record. The activity of atropine and hyoscyamine became apparent in the record within 5 minutes of the intravenous administration of these cholinergic blocking agents. And their entire effects were demonstrated in every instance within a 10-minute postinjection period. The establishment of the (partial) cholinergic block of hyoscine required at least 30 minutes following intravenous administration because of its more variable onset of activity. Based upon these findings, the first postantispasmodic dose of methacholine chloride was administered 10 minutes after atropine and hyoscyamine and 30 minutes after hyoscine, CBA, and CBB.

The antispasmodic activity of atropine and related belladonna alkaloids in doses somewhat less than that dose required to produce complete block of parasympathetic myoneural receptors persisted in this test method for periods up to 24 hours. To avoid cumulative effects, it was therefore necessary to administer a submaximal dose of these alkaloids no more frequently than once every 24 hours. Thus, only one antispasmodic dose was injected for each determination involving several methacholine chloride doses.

The latter agent, serving as the stimulus or spasmogen, was administered in varying doses following a given antispasmodic dose with the intent of obtaining a methacholine chloride$_{50}$ (Me_{50}), a dose of methacholine chloride the response to which is reduced by the antispasmodic to 50% of that to a preantispasmodic dose of this drug. The extent of the inhibition of each methacholine chloride dose by the antispasmodic was determined and expressed as a per cent of the preantispasmodic response (Table I).

A minimum of three postantispasmodic doses of methacholine chloride was employed to obtain the Me_{50} for a single antispasmodic dose: a high dose with an inhibition of less than 50%, a low dose with an inhibition of more than 50%, and a middle dose with an inhibition of approximately 50%. For every antispasmodic dose, each of the three or more doses of methacholine chloride, in mcg./Kg.,

was then plotted against the corresponding per cent inhibition of the preantispasmodic response (to methacholine chloride) on logarithmic probability paper. The line to fit these data points was drawn by inspection and the χ^2 test was applied to determine the goodness of fit. From this line of the plotted data, the Me_{50}, in mcg./Kg., for a given antispasmodic dose was read directly.

In a particular determination, two or three preantispasmodic doses of methacholine chloride were employed at 15-minute intervals, their responses averaged, and the per cent of this average represented by the response to each postantispasmodic dose of methacholine chloride evaluated.

Each antispasmodic was assigned a relative potency value which was based on the reciprocal of its Me_{50} 5.60 value (mg./Kg.) multiplied by the Me_{50} 5.60 value of atropine sulfate, 0.0300 mg./Kg. In this manner atropine was given a value of unity; the other agents, including CBA and CBB, were listed according to their relative potencies as multiples of this number.

The Me_{50} 5.60 value of each antispasmodic, atropine sulfate, hyoscyamine sulfate, and hyoscine hydrobromide was weighted by its relative concentration in the combination, CBB. The products so obtained were totaled. This total was then divided by the sum of the relative concentrations of the antispasmodic under study, and the resultant quotient represented the calculated Me_{50} 5.60 values of CBA and CBB.

Three Me_{50} dose determinations were made for each dose level of each of the two combinations of antispasmodics, CBA and CBB, to insure representative results at each level (Table I). Three such determinations per dosage level were not obtained in the case of each component alkaloid, since the ratios derived from the single values agreed with the concensus ratios for these alkaloids appearing in the literature.

The mean Me_{50} dose values for the various antispasmodic dosage levels were situated in separate distributions, as demonstrated by the fact that no mean value fell within the confidence limits of any other in either the CBA or CBB determinations.

To obtain a mean value with 95% confidence limits for the Me_{50} 5.60 values of each combination, CBA and CBB, three separate dose-response curves were drawn. Each curve represented one or the other of the following: the low responses, the medium responses, or the high responses to each of the three doses of the combinations. As opposed to this, the initial curve of each combination was drawn from the mean of the three Me_{50} doses (of methacholine chloride) per combination dose level. On the multicurve basis described previously, the antispasmodic values for the Me_{50} (5.60 mcg./Kg.) of the outside curves (low re-

TABLE I.—ANTISPASMODIC ACTIVITY OF BELLADONNA ALKALOIDS

Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]	Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]
dl-Hyoscyamine (atropine) sulfate	0.30	5.60	2.16	14.9	CBA	0.016	6.22	5.68	40.0
			5.54	60.4				11.36	80.0
			15.82	72.1				22.72	91.5
l-Hyoscyamine sulfate	0.009	2.87	1.16	20.5	CBA	0.016	6.40	3.03	33.4
			3.10	61.3				5.68	48.9
			5.54	69.8				9.47	56.0
l-Hyoscyamine sulfate	0.012	5.00	3.10	17.9				18.94	87.8
			5.54	64.6	CBA	0.016	6.50	5.55	45.9
			10.85	83.3				11.03	62.7
l-Hyoscyamine sulfate	0.015	7.75	5.29	46.5				22.06	76.0
			9.67	50.0	CBB	0.009	4.95	3.30	25.9
			20.90	79.1				5.57	55.6
l-Hyoscine (scopolamine) hydrobromide	0.005	5.15	2.86	26.7				13.30	96.3
			5.53	70.0	CBB	0.009	5.57	2.75	29.8
			9.93	86.7				5.49	37.8
l-Hyoscine hydrobromide	0.007	16.40	5.59	15.3				10.99	83.8
			11.20	38.9	CBB	0.009	3.99	2.98	28.2
			22.40	58.9				5.49	74.4
l-Hyoscine hydrobromide	0.009	33.70	5.54	8.7				11.19	97.4
			10.87	12.5	CBB	0.012	9.70	5.54	32.9
			21.70	42.5				10.93	51.2
CBA	0.009	2.31	1.10	13.1				19.50	75.7
			2.94	61.8	CBB	0.012	10.00	5.54	30.7
			5.55	94.6				10.93	50.0
CBA	0.009	2.20	1.07	25.0				19.50	75.0
			2.86	58.3	CBB	0.012	8.40	5.56	45.0
			5.54	87.5				11.20	53.4
CBA	0.009	2.30	1.10	20.6				18.65	66.7
			2.53	54.5	CBB	0.016	16.50	5.55	31.9
			5.54	83.4				22.06	36.2
CBA	0.012	3.86	2.38	26.7				29.40	89.4
			5.60	66.7	CBB	0.016	15.20	5.37	24.2
			9.52	93.4				10.75	44.0
CBA	0.012	3.45	2.36	28.1				26.88	60.0
			5.50	74.1				32.20	71.2
			11.02	88.9					
CBA	0.012	3.54	2.42	33.3	CBB	0.016	16.10	5.56	20.8
			5.52	69.5				16.00	46.0
			12.10	90.8				32.00	73.2

[a] A = Methacholine chloride mcg./Kg. [b] B = Per cent response.

TABLE II.—ANTISIALAGOGUE ACTIVITY OF BELLADONNA ALKALOIDS

Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]	Alkaloid	Dose, mg./Kg.	Me_{50}, mcg./Kg.	A[a]	B[b]
dl-Hyoscyamine (atropine) sulfate	0.030	18.2	11.29	14.8	CBA	0.009	11.6	5.55	10.9
			16.62	47.0				9.97	35.9
			24.92	73.1				16.62	75.2
l-Hyoscyamine sulfate	0.006	4.1	1.33	11.1	CBA	0.012	15.0	5.55	5.0
			2.99	32.0				13.29	40.0
			5.55	65.4				24.92	70.0
l-Hyoscyamine sulfate	0.009	9.9	5.55	16.4	CBA	0.012	18.0	5.55	7.0
			9.30	30.9				13.29	27.6
			3.29	81.0				24.92	69.1
			18.24	90.7	CBA	0.012	19.0	5.55	8.3
l-Hyoscyamine sulfate	0.012	15.0	5.15	5.6				13.29	30.9
			8.31	19.9				24.92	65.6
			16.62	54.8	CBB	0.006	6.6	5.55	38.1
			24.92	76.9				7.64	62.3
l-Hyoscine (scopolamine) hydrobromide	0.005	7.5	2.83	7.8				9.97	71.6
			5.55	31.8	CBB	0.006	8.6	5.55	27.2
			9.97	65.3				7.64	39.5
l-Hyoscine hydrobromide	0.007	8.6	5.55	29.8				9.97	60.5
			9.97	42.8	CBB	0.006	8.5	5.55	26.8
			16.62	87.0				7.64	38.3
l-Hyoscine hydrobromide	0.009	16.2	5.55	2.3				9.97	63.1
			9.97	18.1	CBB	0.009	13.2	5.55	2.3
			16.62	53.7				9.97	34.5
			26.58	81.4				22.60	85.5
CBA	0.006	8.15	5.55	22.6	CBB	0.009	11.8	5.55	11.7
			7.64	47.16				9.97	26.0
			9.97	64.15				22.60	89.6
CBA	0.006	9.0	5.55	25.57	CBB	0.009	11.5	5.55	10.2
			7.64	41.0				9.97	37.0
			9.97	55.17				22.60	89.8
CBA	0.006	10.5	5.55	22.1	CBB	0.012	14.2	5.55	6.0
			7.64	31.6				13.29	38.0
			9.97	49.0				24.92	87.0
CBA	0.009	10.8	5.55	8.5	CBB	0.012	17.8	5.55	1.5
			9.97	52.1				13.29	41.0
			16.62	74.4				24.92	68.0
CBA	0.009	12.2	5.55	10.7	CBB	0.012	13.9	5.55	7.0
			9.97	32.1				13.29	36.5
			16.62	73.2				24.92	88.0

[a] A = Methacholine chloride, mcg./Kg. [b] B = Per cent response.

sponse and high response) of each combination represented the broadest spread of such values possible. Consequently, the 95% confidence limits of the mean of the three values (low, medium, and high response curves) per combination represented the greatest possible range. Thus, greater significance attaches to the failure of the calculated antispasmodic value to fall within these confidence limits of the obtained value in the case of the CBB combination.

As a further check on the validity of the results, the *r* value (9) was calculated for each of the above-mentioned regression lines to determine whether the lines deviate significantly from parallel. Results from this work indicated no significant deviation from parallelism by these lines.

In the antisialagogue aspect of the study, Wharton's duct was cannulated *via* the oral cavity of the chloralose-anesthetized dog with polyethylene tubing (No. 10) which was connected to a saline-filled extension of glass tubing mounted on a millimeter stick. The data of this aspect of the study were treated in the same manner as those of the antispasmodic aspect. (Tables II–VI).

TABLE III.—COMPUTATION OF CALCULATED VALUE OF Me_{50} 5.60 mcg./Kg. FOR CBA (ANTISPASMODIC)

Alkaloid	Concn. in CBA			Actual Me_{50} 5.60, mcg./Kg.
l-Hyoscyamine sulfate	1037 × 0.01272	=		13.19064
dl-Hyoscyamine sulfate	194 × 0.00300	=		5.82000
l-Hyoscine hydrobromide	65 × 0.00543	=		0.35295
	1296			19.36359[a]

[a] 19.36359/1296 = 0.01493 mg./Kg. of CBA is the calculated value of the Me_{50} 5.60 mcg./Kg. Calculated value of CBB same as calculated value of CBA since phenobarbital in amount used has no activity.

TABLE IV.—COMPUTATION OF CALCULATED VALUE OF Me_{50} 18.2 mcg./Kg. FOR CBA (ANTISIALAGOGUE)

Alkaloid	Concn. in CBA			Actual Me_{50} 18.2, mcg./Kg.
l-Hyoscyamine sulfate	1037 × 0.01303	=		13.5121
dl-Hyoscyamine sulfate	194 × 0.0300	=		5.8200
l-Hyoscine hydrobromide	65 × 0.00882	=		0.5733
	1296			19.9054[a]

[a] 19.9054/1296 = 0.01536 mg./Kg. Calculated value of CBB same as calculated value of CBA since phenobarbital in amount used has no activity.

TABLE V.—ANTISPASMODIC ACTIVITY IN COMPARISON WITH ATROPINE SULFATE

Alkaloid	ED_{50} 5.60, mcg./Kg.	Potency Compared to Atropine Sulfate
dl-Hyoscyamine sulfate	0.0300	1.00
l-Hyoscyamine sulfate	0.01272	2.36
l-Hyoscine hydrobromide	0.00543	5.52
CBA[a]	0.01507	2.00
CBB[b]	0.00952	3.15

ED_{50} 5.60, mcg./Kg. calcd. for [a] CBA = 0.01493, for [b] CBB = 0.01493.

TABLE VI.—ANTISIALAGOGUE ACTIVITY IN COMPARISON WITH ATROPINE SULFATE

Alkaloid	ED_{50} 18.2, mcg./Kg.	Potency Compared to Atropine Sulfate
dl-Hyoscyamine sulfate	0.0300	1
l-Hyoscyamine sulfate	0.01303	2.30
l-Hyoscine hydrobromide	0.00882	3.40
CBA[a]	0.01350	2.22
CBB[b]	0.01443	2.08

ED_{50} 18.2, mcg./Kg. calcd. for [a] CBA = 0.01536, for [b] CBB = 0.01536.

DISCUSSION

The original procedure for the assay method under consideration was not entirely satisfactory because it yielded a low percentage of valid data. This procedure was operating properly when the results obtained were consistent with a previously established dose-response relationship. But on several occasions the collected data lacked coherence, at which time it was judged that the procedure was not functioning in the manner of which it was capable.

That the original procedure and the modification of this work constitute bioassay methods is attested by the fact that the potency ratios of atropine, hyoscine, and hyoscyamine obtained with them agree with the consensus values for these agents.

The task of the present investigation was involved then with increasing the yield of valid data from the original procedure through proper modification of it. Of those changes in the original procedure, the only productive one, as it turned out, was the substitution of α-chloralose for the ganglion-influencing pentobarbital as the anesthetic agent. This has served to bring about the desired end of increase in the percentage of valid tests by making considerably less critical the level of anesthesia required during testing.

The results of the work of Turkanis evidence a potentiation in the combination, CBA, which the results of this investigation did not show. This difference is not irreconcilable, however. Pentobarbital, as noted, was the anesthetic agent in the Turkanis procedure, while α-chloralose was used in that capacity in the present investigation. As has been mentioned previously, evidence is available to indicate that pentobarbital in proper dosage possesses ganglion-blocking activity, which activity is manifest in the gut by a decrease in motility. Thus, the effectiveness of any antispasmodic should be increased in the presence of the ganglion-influencing doses of pentobarbital employed in the original procedure; this is apparent when the methacholine chloride-antispasmodic ratio of the Turkanis work is compared with this ratio of the present study. The higher ratios of the calculated and the obtained values for the combination CBA in the Turkanis work, opposed to those of this study, reflect the assist provided the antispasmodic in every instance by the pentobarbital administered. That the obtained value ratio should be higher than the calculated value ratio in the Turkanis work reflects also the ability of the pentobarbital to potentiate when the combination of alkaloids is involved. In the study of this report, there was no evidence of increase in the antispasmodic effectiveness of CBA over the effectiveness of the sum of the components. In neither the CBA nor the individual alkaloid determination of this study was a barbiturate present.

The conclusion to be drawn from the results obtained with the modification of this investigation is that while the combination consisting of the alkaloids alone does not represent, with respect to the antimotility activity, a potentiating mixture in the absence of the phenobarbital of CBB, it does so in the presence of this phenobarbital. The further conclusion that neither combination amounts to enhancement of the total antisialagogue activity of the components is warranted by the results obtained.

SUMMARY

The relative antispasmodic effectiveness of atropine sulfate, hyoscyamine sulfate, and hyoscine hydrobromide both alone and in combination have been determined in a biological assay employing the surgically intact dog. The effect of the addition of phenobarbital to the combination has been evaluated.

In the procedure employed, the combination of antispasmodic alkaloids was observed to be no more effective than the sum of their individual activities when the dose of phenobarbital appearing in the combination, CBB, was absent. When this dose of phenobarbital was present, the combination of alkaloids demonstrated potentiation. Thus, while the combination of alkaloids alone does not appear to constitute a potentiating mixture, in so far as antimotility activity is concerned, the combination in the presence of phenobarbital does. At the same time, however, there does not appear to occur in either mixture an enhancement of the total antisialagogue activity of the component alkaloids.

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