Biochemical Pharmacology, Vol. 18, pp. 837–845. Pergamon Press. 1969. Printed in Great Britain

ESTIMATION OF CHOLINE ESTERS IN BRAIN BY A NEW GAS CHROMATOGRAPHIC PROCEDURE*

ISRAEL HANIN† and DONALD J. JENDEN

Department of Pharmacology, University of California, School of Medicine, Los Angeles, Calif. 90024, U.S.A.

(*Received* 27 *June* 1968; *accepted* 20 *September* 1968)

Abstract—A new gas chromatographic procedure for microestimation of choline esters has been successfully applied to extracts of rat brain. Brains are rapidly removed after decapitation and homogenized in 85% acetone, 15% 1 N formic acid containing hexyltrimethylammonium bromide as an internal standard. After centrifugation and extraction with ether, quaternary ammonium compounds are precipitated with ammonium reineckate and the chlorides are recovered in anhydrous methanol by treatment with Biorex 9 ion-exchange resin. The residue after evaporation is allowed to react with sodium benzenethiolate in anhydrous butanone. The resulting tertiary amines are analyzed by gas chromatography as previously described. ‡

Acetylcholine levels determined in this way agree with previous reports in the literature and with concurrent bioassays on frog rectus abdominis. Acetylcholine-like activity of rat brain assayed on this preparation can therefore be quantitatively accounted for by its acetylcholine content, and the results provide no support for the view that other pharmacologically active agents contribute significantly to it. Propionylcholine and butyrylcholine may be simultaneously determined by this procedure and could not be detected in fresh rat brain extracts, although both esters yielded the expected chromatographic peaks when added to the homogenate in a total quantity of 2 nmoles.

UNTIL 1964, bioassay was the only acceptable sensitive method for the microestimation of acetylcholine in tissue extracts. Bioassay preparations which are currently in most frequent use are the isolated frog rectus abdominis, guinea pig ileum, leech dorsal muscle and clam heart preparations, and the intact, anesthetized cat or rat.

These bioassay procedures are extremely sensitive. However, they are time-consuming and their reliability has been questioned in view of the fact that their response is not specific to one compound alone.[1–3] Esters related to choline will induce limited responses on these bioassay preparations.[4] Furthermore, pharmacological agents used in an experiment are likely to be present in the biological extract, and might sensitize or desensitize the bioassay preparation to acetylcholine.[5]

* This paper represents part of the research submitted in partial fulfilment of the requirements for the Ph.D. degree at the University of California, Los Angeles, and was conducted during the tenure of a USPHS predoctoral fellowship by Israel Hanin. The investigation was supported by research grants (NB 03007, GRSG 1 SOL FR 05354 and 1-FL-GM 22,905) from the National Institutes of Health and from the National Science Foundation (GB 7049).

† Present address: Laboratory of Preclinical Pharmacology, William A. White Building, St. Elizabeth's Hospital, Washington, D.C. 20032, U.S.A.

‡ D. J. JENDEN, I. HANIN and S. I. LAMB, *Analyt. Chem.* **40**, 125 (1968).

A considerable number of chemical procedures have been described, but none of these approaches the sensitivity and specificity obtainable by biological methods. A new procedure employing gas chromatography for the microestimation of acetylcholine and related esters has recently been developed in our laboratories.[6] The chemical principle involves *N*-demethylation of acetylcholine and related compounds by benzenethiolate to form the corresponding tertiary amines. Products of this reaction are volatile and are therefore amenable to estimation by gas chromatography. The reaction takes place in refluxing methyl ethyl ketone at 80° and is complete within 30 min.

This procedure has now been applied successfully to the estimation of levels of acetylcholine in rat brain extracts. A preliminary account has been presented elsewhere.[7, 8]

EXPERIMENTAL

Gas chromatography

An F & M 5750A dual column gas chromatograph equipped with flame ionization detectors was employed. Silanized glass columns (6 ft:1/4 in. o.d.) were packed with Polypak I (80/120 or 120/200 mesh) coated with 1% phenyldiethanolamine succinate (PDEAS) (Analabs). Column temperature was maintained at 170°. Injection port and flame detector temperatures were 204° and 200° respectively. Nitrogen was used as the carrier gas at a rate of 45 ml/min (80 psi). Air and hydrogen flow were maintained at 280 ml/min (30 psi) and 30 ml/min (26 psi) repectively. Each gas was separately passed through drying tubes containing molecular sieve (type 5A, Linde) before use. Injections were made with a Hamilton No. 701-N, 10 μl syringe. Detector response was recorded on a Varian Associates recorder, model G-2000.

Extraction of rat brains

Rat brains were extracted, with slight modifications, according to the procedure proposed by Toru and Aprison.[9] Brains were taken from male, albino, Sprague–Dawley rats weighing 300–350 g. After decapitation (Harvard guillotine), the brains were excised within 30 sec and dropped into a glass homogenizer containing 3 ml of an ice-cold solution composed of 85% acetone–15% 1 N aqueous formic acid. Precisely 20 nmoles of hexyltrimethylammonium bromide (HTA) was added at this stage and served as an internal standard which was carried through the entire procedure. The brain was thoroughly homogenized and the homogenate was diluted with the formic acid–acetone solution to make the final volume of the extract equivalent to 3 ml per g of wet brain. After standing in ice for 30 min, the homogenate was centrifuged for 15 min at 7000 rpm (5900 *g*) using a Sorvall RC-2 refrigerated centrifuge at 0°. The supernatant was saved and the pellet was resuspended in a solution containing 90% acetone, 10% aqueous 1 N formic acid, using 1·0 ml/g of original brain weight. After centrifuging at 7000 rpm (5900 *g*) for 15 min, the pellet was again extracted in the same manner, but the final centrifugation was at 35,000 *g* for 20 min. The supernatants were transferred to a 12-ml conical centrifuge tube, pooled and extracted with an equal volume of diethyl ether. Last traces of ether and acetone were removed with a stream of dry nitrogen.

Precipitation of quaternary ammonium compounds

The chemical estimation of acetylcholine in biological tissues has been complicated by the presence of interfering substances in these tissue extracts. Attempts have been made to circumvent the problem by the selective precipitation of acetylcholine in the form of an insoluble salt. Since its introduction by Kapfhammer and Bischoff in 1930,[10] precipitation with ammonium reineckate (AR) has been used as a common method for the isolation and separation of quaternary ammonium compounds, including acetylcholine. As little as 10 nmoles of acetylcholine has been successfully recovered by this method in the presence of a 300-fold molar excess of choline.[11] Choline served as a coprecipitant and incomplete recovery of acetylcholine was obtained unless an excess of choline was added. Reineckate precipitation was chosen as a suitable means of recovering choline esters in the anhydrous form necessary for the *N*-demethylation reaction. However, addition of excess choline to serve as a coprecipitant for trace amounts of choline esters is inappropriate because it precludes use of the method to estimate choline. Tetraethylammonium chloride (TEA) at a concentration of 1 mM was found to give complete coprecipitation of acetylcholine in the micromolar concentration range and did not interfere with the subsequent *N*-demethylation reaction.

TEA was added to the aqueous extract in an amount sufficient to make the final concentration 1 mM. An equal volume of filtered 2% aqueous ammonium reineckate solution was added and thoroughly mixed. Precipitation was complete after standing at 0° for 45 min. The precipitate was collected by centrifuging in an International clinical centrifuge for 3 min and dried in a vacuum desiccator over magnesium perchlorate after the supernatant was removed and discarded.

Reineckate was found to interfere with the demethylation reaction and to result in incomplete and variable yields. It was therefore necessary to reconvert the reineckate to some other salt. This has previously been accomplished by precipitation of silver reineckate[12, 13] and by an ion-exchange resin in an aqueous medium.[14, 15] In the present work, an anhydrous suspension of Biorex 9 (200–300 mesh), a quaternary pyridinium resin, was used to exchange chloride for reineckate.

The resin was exhaustively washed with several batches of anhydrous methanol and finally dried and stored over magnesium perchlorate under vacuum. A suspension of this material was made up in methanol at a concentration of 30 mg/ml. Of this, 0·5 ml was added to each tube containing the dried Reinecke precipitate and the mixture was triturated for a few minutes. After centrifuging, the supernatant was transferred to a clean centrifuge tube of the same type and evaporated to dryness with a stream of dry nitrogen. This procedure gave good recoveries which were identical for acetylcholine and hexyltrimethylammonium. The reineckate ion was completely removed.

Reaction with benzenethiolate

The preparation of reagents and the procedure for the *N*-demethylation reaction were identical with that described earlier,[6] with the following exception. Sequential treatment of sodium benzenethiolate with ethyl acetate and carbon dioxide was then reported to be essential for quantitative recovery of dimethylaminoethyl acetate at low concentration. It has now been found that these steps can be omitted provided that benzenethiol is added to the reaction mixture. In the work described here, the

demethylation reagent contained 50 mM sodium benzenethiolate and 25 mM benzenethiol in anhydrous butanone. It was made up freshly each day and stored under nitrogen until used.

RESULTS

Figure 1 shows a chromatogram obtained by applying the entire procedure to a dilute aqueous solution containing 25 nmoles hexyltrimethylammonium bromide

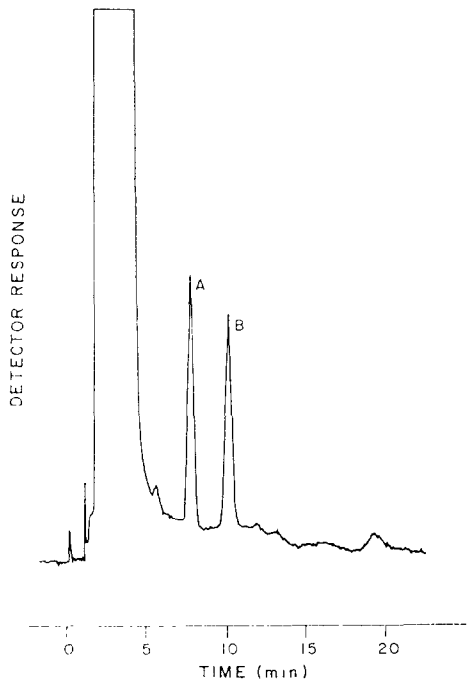

FIG. 1. Chromatogram obtained by subjecting to the entire procedure a 1-ml sample of a dilute aqueous solution containing authentic hexyltrimethylammonium bromide and acetylcholine chloride (25 μM and 50 μM respectively) in 0·1 M ammonium acetate buffer at pH 3·9. Full-scale detector response was $3{\cdot}2 \times 10^{-10}$ amp (attenuation 8×10).

and 50 nmoles acetylcholine chloride. Peaks A and B correspond in retention time to hexyldimethylamine and dimethylaminoethyl acetate, respectively, as can be seen by comparison with Fig. 2, in which a solution of the two authentic amines in chloroform was injected directly into the chromatograph. Minor variations in absolute retention time are seen as a result of small fluctuations in operating conditions, but the relative retention time (1·29:1) is very consistent and is identical for brain extracts and authentic standards. The ratio of the peak heights is constant for a given mole ratio and column; different chromatographic columns of the same type have given slopes for peak height ratio plotted against mole ratio varying from 0·44 to 0·54. A very slow change in this ratio as the column ages has also been observed and standardization checks are normally run at weekly intervals. A typical standardization curve is shown in Fig. 3. Each estimation was carried out on a 1-ml sample of 0·1 M ammonium acetate buffer at pH 3·9, containing 25 nmoles hexyltrimethylammonium bromide and amounts of acetylcholine chloride ranging from 0 to 100

nmoles. It is evident that a linear relationship exists between peak height ratio and starting mole ratio, that the results are reproducible and that the procedure is suitable for estimation of acetylcholine in high dilution. Figure 4 shows a typical chromatogram of an extract of rat brain processed in the manner described. From the ratio of heights of the peaks corresponding to hexyldimethylamine and dimethylaminoethyl acetate, the total quantity of acetylcholine in this brain was 24·1 nmoles, or 15·7 nmoles/g wet brain.

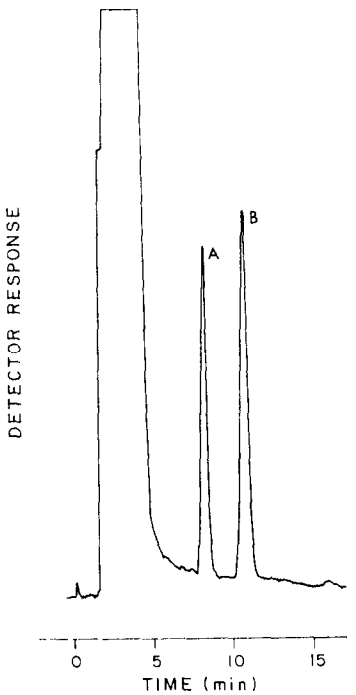

FIG. 2. Chromatogram of authentic hexyldimethylamine (A) and dimethylaminoethyl acetate (B). An injection was made at zero time of 3 μl of a chloroform solution of the two amines at concentrations of 0·5 mM and 1 mM respectively. Full-scale detector response was $3{\cdot}2 \times 10^{-10}$ amp (attenuation 8×10).

In order to establish rigorously that the indicated peak is attributable to dimethylaminoethyl acetate, it will be necessary to subject brain extracts to analysis with an integrated gas chromatograph/mass spectrometer system and demonstrate identical fragmentation patterns. It is hoped that the results of such a study will be reported soon.* However, voluminous circumstantial evidence has been published indicating the presence of acetylcholine in brain, and agreement in retention time and quantity expected strongly suggests that the peak is due to dimethylaminoethyl acetate.

There appear to be no reports in the literature regarding the occurrence of dimethylaminoethyl acetate in the brain. It was therefore necessary to demonstrate that the peak corresponding to this compound resulted from demethylation of acetylcholine rather than from spontaneous occurrence of the tertiary amine. This was achieved by processing a group of brains in a manner identical to that described except that benzenethiol and benzenethiolate were omitted from the reaction tube.

*See *Note added in Proof*.

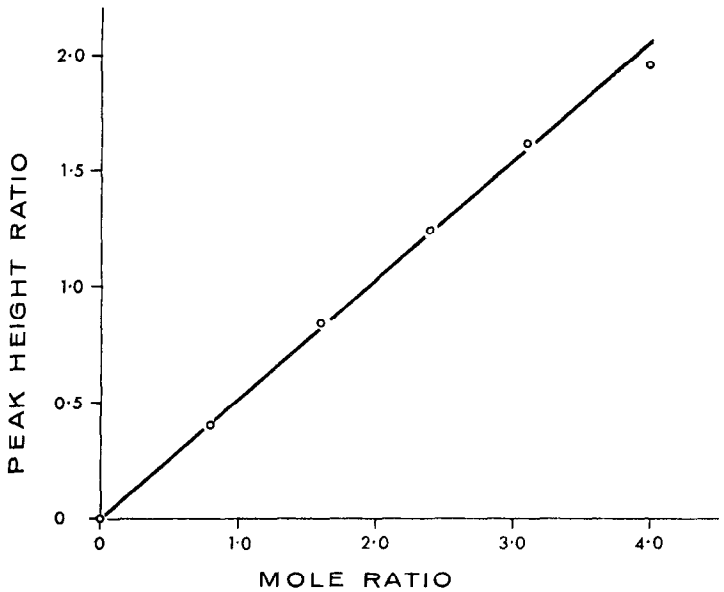

FIG. 3. Standardization data relating the ratio of peak heights for dimethylaminoethyl acetate and hexyldimethylamine to the initial mole ratio of acetylcholine and hexyltrimethylammonium (HTA) in dilute aqueous solution. The total quantity of HTA was constant at 25 nmoles. For further details see text.

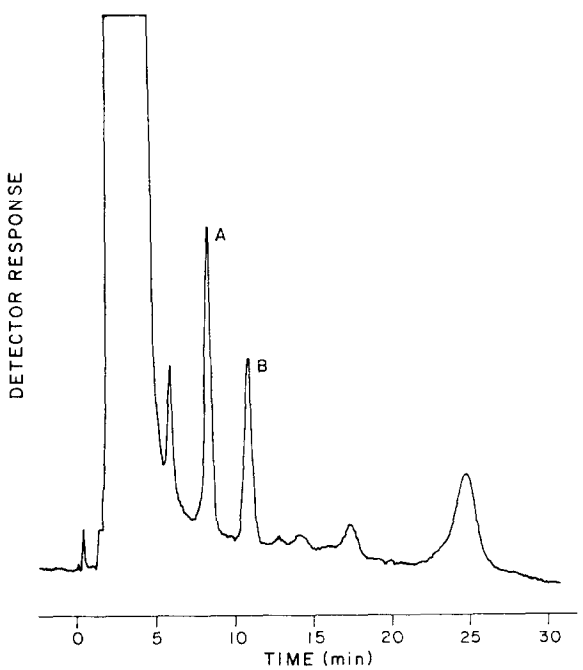

FIG. 4. Chromatogram of rat brain extract. Peaks A and B correspond to hexyldimethylamine and dimethylaminoethyl acetate respectively. The remaining components have not yet been identified. Six μl of the final chloroform extract was injected at zero time. Full-scale detector response was $1{\cdot}6 \times 10^{-10}$ amp (attenuation 4×10).

No dimethylaminoethyl acetate peak was observed in any of the resulting chromatograms.

Since choline is known to be present in brain in amounts far exceeding those at which acetylcholine is believed to occur,[16] it was of interest to determine whether a large excess of choline would interfere with the estimation of acetylcholine. A series of experiments was run in which choline was added, in up to 100-fold molar excess over acetylcholine, and no change in the recovery of acetylcholine was obtained. It may therefore be concluded that the choline content of brain is unlikely to interfere with the estimation of acetylcholine.

Another possible artifact which might arise during the procedure is transesterification catalyzed by the benzenethiolate ion through the formation of benzenethiol esters as intermediates. This possibility was examined by allowing sodium benzenethiolate to react, under the conditions used for demethylation, with a mixture of acetylhomocholine and propionylcholine chlorides at a concentration of 0·13 mM. Transesterification should yield acetylcholine and propionylhomocholine, which would presumably be demethylated to the corresponding tertiary amines. These products were sought chromatographically and could not be detected. Quantitative yields of dimethylaminopropyl acetate and dimethylaminoethyl propionate were obtained. Phenyl thiolacetate and acetoxyethylphenyl sulfide were also sought as possible products of side reactions, but could not be detected.

In order to verify the accuracy and quantitativeness of this method as applied to brain extracts, a series of experiments was run in which a known quantity of acetylcholine (10 or 20 nmoles) was added to part of a brain homogenate and the entire procedure was then applied to both parts. Two experiments of this type were run. In the first, four rat brains were pooled and the homogenate was divided into four equal parts, to two of which acetylcholine was added. In the second experiment, three brains were homogenized and from each homogenate two equal volumes were taken, to one of which acetylcholine was added. The mean difference between corresponding pairs of estimations represented a recovery of 95·4 $\pm$ 6·7 per cent of acetylcholine added.

It was of interest to compare results obtained by this method with those yielded by a conventional bioassay procedure. Twelve rat brains were extracted in the usual manner up to the point immediately before reineckate precipitation, when the aqueous extract was made up to 2 ml and divided into two equal parts. One of these was subjected to the gas chromatographic procedure described in this paper and the other was assayed on the frog rectus abdominis preparation as described by MacIntosh and Perry,[17] using the controls suggested by Feldberg[18] to allow for sensitization of the muscle to acetylcholine by extracts of brain. Since hexyltrimethylammonium (HTA) bromide was also present in the extract assayed, sensitization or desensitization of the frog rectus muscle to acetylcholine was sought. It was found that 4 μM HTA was the minimum concentration required to change the response to acetylcholine significantly (potentiation). The maximum concentration in the bioassay experiments was 1·25 μM and was equal for both standards and unknowns because of the controls referred to above. The results showed no significant difference between the acetylcholine content as estimated by bioassay and by gas chromatography (16·4 and 17·5 nmoles/g respectively, $t_{11} = 0{\cdot}88$).

It is evident from Fig. 4 that several peaks other than dimethylaminoethyl acetate

may be seen in chromatograms from brain extracts. The identity of these peaks has not yet been determined. Propionylcholine and butyrylcholine have been quantitatively recovered by using the identical procedure employed for acetylcholine, both from dilute aqueous solution and when added to brain homogenates. Chromatograms from brain extracts were compared with standards prepared from dimethylaminoethyl propionate and butyrate under the same conditions. The results indicate that if the corresponding esters are present in rat brain, their concentrations are very small (< 1 nmole/g) compared to that of acetylcholine.

DISCUSSION

The gas chromatographic procedure previously described by us for the micro-estimation of choline esters has been successfully applied to the identification and estimation of acetylcholine in rat brain. The results are in good agreement with estimates obtained previously[19] and in the present work by bioassay, and by Stavinoha and Ryan[20] using their gas chromatographic method based on reduction of the acetate moiety to ethanol. Although the identity of the chromatographic peak corresponding to dimethylaminoethyl acetate has not yet been rigorously established, there can be no reasonable doubt that it represents acetylcholine present in brain.*

Since parallel bioassay and gas chromatographic estimation on the same brain extracts yielded results in close agreement, it may be concluded that the 'acetylcholine-like activity' determined with the frog rectus abdominis preparation is in fact attributable to acetylcholine under the conditions of our experiments. Therefore these provide no support for the view that other choline esters or analogs contribute significantly to the pharmacological activity determined in this way. In particular, it has been shown that propionylcholine and butyrylcholine are not present in rat brain in significant amounts.

The procedure described is reproducible, accurate and quantitative. Although time-consuming, several estimations may be made simultaneously and the time required is no greater than that needed for an accurate bioassay. It has the additional advantages of specificity and allowing simultaneous estimation of several choline esters. Unlike bioassay, the results are not influenced by the presence of other drugs. The practical limit of detectability of the entire procedure as described here is about 25 ng. This limit can undoubtedly be extended considerably by scaling down the volumes, by use of more efficient columns and by more sensitive and specific detectors. The technique therefore shows promise of wide applicability in the study of cholinergic systems.

Note added in proof*—This study has now been published. See C.-G. HAMMER, I. HANIN, B. HOLMSTEDT, R. J. KITZ, D. J. JENDEN and B. KARLÉN, *Nature, Lond.* **220, 915 (1968).

REFERENCES

1. E. A. HOSEIN, P. PROULX and R. ARA, *Biochem. J.* **83,** 341 (1962).
2. E. A. HOSEIN and T. Y. KOH, *Can. J. Physiol. Pharmac.* **43,** 657 (1965).
3. E. A. HOSEIN and A. ORZECK, *Nature, Lond.* **210,** 731 (1966).
4. V. P. WHITTAKER, in *Handbuch der Experientellen Pharmakologie*, Vol. 15, p. 1. Springer-Verlag, Berlin (1963).
5. I. HANIN and D. J. JENDEN, *Experienta* **22,** 537 (1966).
6. D. J. JENDEN, I. HANIN and S. I. LAMB, *Analyt. Chem.* **40,** 125 (1968).
7. I. HANIN, D. J. JENDEN and S. I. LAMB, *Proc. west. Pharmac. Soc.*, **11,** 56 (1968).

8. I. HANIN, *A New Gas Chromatographic Procedure For the Microestimation of Acetylcholine in Tissue Extracts*, Ph.D. dissertation. UCLA, Department of Pharmacology (1968).
9. M. TORU and M. H. APRISON, *J. Neurochem.* **13**, 1533 (1966).
10. J. KAPFHAMMER and C. BISCHOFF, *Hoppe Seyler's Z. physiol. Chem.* **191**, 179 (1930).
11. F. H. SHAW, *Biochem. J.* **32**, 1002 (1938).
12. J. BANISTER, V. P. WHITTAKER and S. WIJESUNDERA, *J. Physiol. Lond.* **115**, 55P (1951).
13. J. BANISTER, V. P. WHITTAKER and S. WIJESUNDERA, *J. Physiol. Lond.* **121**, 55 (1953).
14. V. P. WHITTAKER, *Nature, Lond.* **181**, 856 (1958).
15. L. KUM-TATT, *J. Pharm. Pharmac.* **12**, 666 (1960).
16. J. C. SMITH and J. K. SAELENS, *Fedn. Proc.* **26**, 225 (1967).
17. F. C. MACINTOSH and W. L. M. PERRY, in *Methods in Medical Research*, Vol. 3, p. 78. Year Book Publishers, Chicago (1950).
18. W. FELDBERG, *J. Physiol. Lond.* **103**, 367 (1945).
19. B. HOLMSTEDT and G. LUNDGREN, in *Mechanisms of Release of Biogenic Amines*, p. 439. Pergamon Press, Oxford (1966).
20. W. B. STAVINOHA and L. C. RYAN, *J. Pharmac. exp. Ther.* **150**, 231 (1965).