Biochemical Pharmacology, Vol. 17, pp. 2503–2505. Pergamon Press. 1968. Printed in Great Britain

PRELIMINARY COMMUNICATION

Extraction of acetylcholine from aqueous solutions

(*Received* 24 *April* 1968; *accepted* 24 *July* 1968)

RADIOACTIVELY labelled acetylcholine (ACh) is an important tool in studies of the uptake of ACh into synaptosomes[1] or brain slices,[2] in radiochemical assays of cholinesterase[3] and choline acetyltransferase[4] and in the estimation of choline by acetylation.[5] Optimal use of this compound is only achieved when small amounts may be isolated by a rapid and specific method. The methods generally used today include precipitation with reineckate,[6] dipicrylamine,[7] aurichloride,[8] and tetraphenylboron (Kalignost)[9] or column chromatography[10] (Review, see Whittaker, 1963).[11] These methods are time-consuming and not very sensitive and some of them are less suitable for scintillation counting due to the presence of coloured precipitates or to the large volumes of aqueous solution obtained.

Kalignost was previously found to be a suitable precipitating reagent for radioactively labelled ACh since the precipitate readily dissolved in acetonitrile to give a colourless solution.[4] The radioactivity could therefore be determined by scintillation counting with a minimum of quenching.

The present method is based on the observation that ACh forms a complex with Kalignost and that this complex is insoluble in water, but readily soluble in nitriles, benzylalcohol and ketones. It was therefore possible to extract acetylcholine in the presence of Kalignost from an aqueous into a non-aqueous phase.

Ethyl butyl ketone is readily available,has a low density (0·81), is only slightly miscible with water, and was therefore selected in the present investigation. Other ketones above hexanones and some nitriles like vinylacetonitrile may also be used.

Table 1 shows that ACh (0·1 mM of chloride 150,000 cpm) dissolved in 5 ml of sodium phosphate buffer could be completely precipitated by 5 mg of Kalignost and that the extent of precipitation was hardly affected by the buffer concentration. When the aqueous suspension of ACh-Kalignost was shaken with 1 ml of ethyl butyl ketone, the precipitate disappeared and the ACh was recovered in the ketone phase. Under these conditions only a part of the ACh was extracted in the case of the high buffer concentration. If the aqueous solution of ACh was shaken with ethyl butyl ketone containing Kalignost, avoiding prior precipitation, the ACh was similarly extracted into the ketone phase. The latter procedure has been adopted in the present work and is recommended because it is the easiest and most rapid way of isolating ACh from an aqueous solution. In the absence of Kalignost, ACh was not extracted into the ketone. The percentage ACh extracted was independent of the ratio between the volumes of the aqueous and ketone layers when this varied from 2:1 to 20:1.

TABLE 1. PRECIPITATION AND EXTRACTION OF ACETYLCHOLINE WITH KALIGNOST AND ETHYL BUTYL KETONE*

Aqueous solution	Precipitation with Kalignost	Precipitation with Kalignost and extraction with ketone	Extraction with ketone-Kalignost	Extraction with ketone-only
0·1 mM acetylcholine + 1 mM sodium phosphate	98	94	95	< 2
0·1 mM acetylcholine + 100 mM sodium phosphate	92	58	59	< 2

* Values are expressed as per cent acetylcholine isolated.

As evident from Table 1 the proportion of ACh extracted was dependent upon the amount of ions in the aqueous phase. Separate experiments demonstrated that the extraction is not dependent upon the molar concentration of an ion, but on the total amounts of the ion present. The interference by lithium, sodium, calcium and magnesium could easily be overcome by increasing the amounts of Kalignost in the ketone (Table 2). The interference by potassium ion was much more serious, since Kalignost is a precipitating reagent for this ion and is used in its quantitative estimation.[12] At low potassium concentrations, the ACh could be extracted by using a mixture of ketone and acetonitrile or vinylacetonitrile. The results from Table 2 also showed that ACh was extracted preferentially to potassium since 1 mg Kalignost (3 μmole) could theoretically have extracted at most 15 per cent of the potassium (20 μmole) while it did extract 57 and 92 per cent respectively of the ACh mixed with the potassium. The percentage extracted ACh was almost independent of the type of anions in the aqueous phase since the presence of sodium fluoride, chloride, nitrate, bromide, sulphate and phosphate at similar sodium ion concentration gave only small differences in extracted amounts of ACh. Sucrose, in the high molarities used in density gradient centrifuging, has insignificant effect on the extraction of ACh.

TABLE 2. PERCENTAGE OF ACETYLCHOLINE EXTRACTED FROM AN AQUEOUS SOLUTION AT VARYING AMOUNTS OF KALIGNOST*

Composition of aqueous phase		Kalignost (mg) in ketone phase				
Salt	Concn (mM)					
		1	2	4	10	20
LiCl	200	13	25	43	69	87
NaCl	200	13	25	45	70	86
CaCl$_2$	50	75	79	83	86	92
MgCl$_2$	50	76	91	85	93	93
KCl†	4	66	78	84	88	93
KCl‡	4	89	94	95	96	98
Ringer–Locke§		16	28	44	78	86

* 5 ml water containing 0·2 μM acetylcholine (20,000 cpm) and cations was shaken with 1 ml ketone containing Kalignost. The results are expressed as percentage acetylcholine extracted into the ketone phase.

† 0·2 ml acetonitrile + 0·8 ml ketone.

‡ 1·0 ml vinylacetonitrile.

§ Ringer–Locke: 150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 10 mM NaHCO$_3$, 5 mM Glucose.

When comparing 5 ml of 40 mM sodium chloride solutions containing 100, 1, 0·01, 0·0002 μM ACh chloride respectively, the percentage ACh extracted by 2 mg Kalignost in 1 ml of ketone was: 57, 64, 64 and 56 respectively; by 4 mg: 78, 82, 81, 70 and by 10 mg: 92, 93, 94 and 81. Thus the percentage ACh extracted by the present procedure was found to be almost independent of the ACh concentration. This makes the method very suitable for comparisons of samples with widely varying ACh concentrations. Lower concentrations than 0·2 mμ M ACh could not be determined accurately with the radioactive labelling intensity used, so the lowest concentration given is by no means the limit of extraction. The solubilities of ACh complexes with Kalignost or reineckate in water are around 50 μM.[11] The extraction procedure represents therefore considerable improvement over the precipitation methods. Besides, the extraction of such small amounts is technically a much easier procedure than the handling of minute precipitates.

The extraction of ACh was independent of pH between 3–7. The extracted ACh could be removed into the aqueous phase by shaking the ketone layer with 1 ml of 0·2 N HCl, leaving nearly all the Kalignost in the ketone layer. Traces of ketones may be extracted with cyclohexane which is easily removed by passing a fine stream of air over the solution.

The present method may be used whenever ACh should be isolated from an aqueous solution. The extraction is completed after shaking for a few minutes and the two layers may be separated by brief centrifugation. The ketone phase may be washed several times with 5 ml water containing 2 mg of Kalignost without apparent loss of ACh. The method is particularly useful for scintillation counting of the radioactive ACh. The colourless ketone layer may be transferred to a counting vial containing

1 ml of acetonitrile and 10 ml toluene scintillation mixture (0·4% of w/v 2,5-diphenyloxazole and 0·02% w/v of 1,4-*bis*-(-4-methyl-5-phenyloxazol-2-yl) benzene). It may also be used to extract and concentrate a solution of ACh before paper and TLC or electrophoresis.

In our laboratory it has been used in the assay of choline acetyltransferase based on 1-^{14}C-acetylCoA. Less than 0·1% of the acetate and less than 0·3% of the acetylCoA were extracted into the ketone phase. By washing the ketone phase once with water containing Kalignost, the blanks were further reduced.

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