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SHORT COMMUNICATIONS

A method of assay of CB 1414

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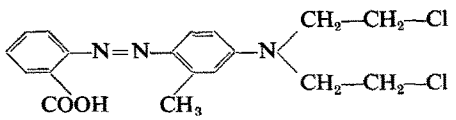
4-Di-2″-chloroethylamino-2-methylazo-benzene 2′-carboxylic acid (CB 1414),



was synthesized by Ross and Warwich in 1956,[1] and is a biological alkylating agent which becomes activated *in vivo* by reduction of the azo linkage.[2] Its hematological effects in dogs have been studied and it has been administered to patients with various malignant diseases.[3] A method for assay of microgram quantities is reported.

In solution of pH less than 5, CB 1414 absorbs in the visible region with a single maximum at 525 mμ, $E^{1\%}_{1cm} = 1200$. To prepare a stock solution of known concentration, 5·0 mg was dissolved in 1·0 ml of 5 N hydrochloric acid, which was then made up to 10·0 ml with ethanol. 0·20 ml of this solution was made up to 20 ml with a mixture of 5 N hydrochloric acid (1 vol) and absolute alchohol (9 vols). Alternatively, the sodium salt may first be formed by dissolving the CB 1414 in 1·0 ml of 1 N sodium hydroxide; an aliquot is made 5 N with respect to hydrochloric acid and diluted with 9 vols of ethanol; no color change occurred in 24 hr at room temperature. Under these conditions protonation of either the β-azo or the amino nitrogen atom had probably occurred because in neutral alcohol solution CB 1414 absorbed maximally at 410 mμ with less intensity. The relationships between the absorption bands and the ionic species present have been discussed in detail by Ross and Warwick.[4]

To assay CB 1414, 1·0 ml of the serum under test was pipetted into a test tube containing 20 mg of sodium bicarbonate, and well shaken. To the alkaline serum 10 ml of absolute alcohol was added and the protein was removed by centrifugation. The alcohol was evaporated from a flask under reduced pressure and the residue was dissolved in 5·0 ml. of 0·1 M phosphate buffer pH 3·4–3·6 which was decanted into a stoppered test tube. The evaporation flask was rinsed and the aqueous solution was extracted three times with 5·0 ml vols of benzene, which were then pooled and evaporated under reduced pressure. The residue was dissolved in a minimum of 0·30 ml of 5 N hydrochloric acid; nine times this volume of alcohol was then added. The resulting solution was further diluted with acid alcohol of the same proportions, if necessary, and the absorption was compared with the solution of known concentration. The recovery from the extraction steps was 90 per cent.

The use of the sodium bicarbonate in the above method permitted complete removal of the adsorbed CB 1414 from the protein surface by the alcohol. At low concentration all of the drug present in serum is adsorbed to serum protein.[5] The conditions under which the extraction was made by benzene from aqueous solution of pH 3·6 are the same as those chosen for the assay of the nitrogen mustard chlorambucil.[6] However no significant difference in the distribution between the organic and aqueous phases was detected in the pH range 2·5–5·5. Because the absorbance measurements were made in the visible spectral region, little background absorption was evident in the extracts from dog and human blood serum. Using a Hilger Uvispek spectrophotometer, with standard cells that require 3 ml of solution, concentrations in blood serum approaching 0·5 μg per ml have been detected.

Injected CB 1414 disappears rapidly from the circulation of dogs. To prepare the drug for injection into a 10-kg dog, 25 mg of the sodium salt of CB 1414 was dissolved in 3 ml of 0·9 per cent sodium chloride. This solution was mixed with 5 ml of whole blood, freshly drawn from a fore-leg vein. The

mixture was then reinjected into the leg vein. The quantity of drug was calculated to produce a maximum possible concentration in the plasma of about 65 μg/ml. In one experiment 125 mg of CB 1414 was injected into a 10 kg dog and this killed the dog in 60 min. When only the acid form of CB 1414 was available, a weighed amount was dissolved in 1 N sodium or potassium hydroxide. The pH was adjusted to 7·5 with strong dihydrogen phosphate solution prior to mixing with the dog's blood.

The concentration of CB 1414 in the circulating plasma was determined from 3-ml samples of heparinized blood which were withdrawn from the other fore-leg of the dog. Between the injection and the withdrawal of the first blood sample 3 minutes were allowed to elapse to permit mixing. The amounts recovered are listed in Table 1.

TABLE 1. RECOVERY OF CB 1414 FROM THE CIRCULATING PLASMA OF DOGS

Injected quantity 2·5 mg/kg (65 μg/ml of plasma)		Injected quantity 12·5 mg/kg (325 μg/ml of plasma)	
Time (min)	conc. in plasma (μg/ml)	Time (min)	conc. in plasma (μg/ml)
3	1·72	3	19
10	1·53	10	7·5
		20	6·5
30	0·5	30	5
60	0·3	60	died

Using a pump-oxygenator system, perfusion of an isolated dog liver *in vivo* with CB 1414 was carried out by Dr. W. Zingg of the Department of Surgery. 55 mg. of the drug in solution in 10 ml of whole blood was added to a reservoir directly connected to the hepatic artery. Half-a-dozen blood samples were withdrawn from the hepatic vein at 5 min intervals, in which no trace of CB 1414 was detected. The amount of drug added was calculated to produce a concentration of approximately 40 μg/ml of circulating plasma.

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Tetanus toxin activity and ganglioside content of rat brain

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THE ISOLATION of a receptor substance for the exotoxin of *Clostridium tetani* has been recently reported by van Heyningen and his associates.[1-5] This receptor substance consists of gangliosides.[2, 3] The criteria for the identification of gangliosides as the tetanus toxin receptor substance resides in the observation that gangliosides and tetanus toxin associate to form a complex. The complex formation