THE ISOLATION AND IDENTIFICATION OF 1-ETHINYLCYCLOHEXYL CARBAMATE AND ITS METABOLITE FROM TOXICOLOGICAL SPECIMENS

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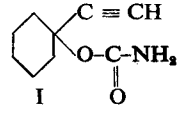
Sensitive tests involving the formation of the mercuric and silver salts of ethinamate (1-ethinylcyclohexyl carbamate) are reported for the detection and identification of μg. quantities of the drug. Conditions for the isolation of this compound and its metabolite from toxicological specimens are described. The infra-red spectra of both the carbamate and its metabolite isolated from a human brain are also given.

ETHINAMATE (Valmid,1-ethinylcyclohexyl carbamate; I) is a central nervous depressant, hypnotic and sedative which has found popular use in Singapore and is a problem in forensic chemistry. Although several papers have been published on its pharmacological actions (Langecker, Schumann and Junkmann, 1953; Gruber, Kohlstaedt, Moore and Peck, 1954; Franke, 1954; Foltz, Dracos and Gruber, 1955; and Swanson, Anderson and Gibson, 1956) there is a scarcity of chemical methods for its isolation and identification from toxicological specimens.



I

Langecker, Schumann and Junkmann (1953) and Nakamura (1958) have recommended the extraction of ethinamate at pH 8. The disadvantages of extraction at this pH are that many other substances which react with the mercuric chloride test of Langecker and which also interfere with the colour reactions of Nakamura are extracted as well. The precipitation method with ammoniacal silver nitrate of Langecker and others lacks specifiity in that halide, aldehydes and methylpentynol, another commonly used sedative, interfere with the test. Recently Moss and Jackson (1961) have reported a paper chromatographic method for the detection of this compound and other carbamates using furfural and concentrated hydrochloric acid as the colour developing reagents.

This paper describes some sensitive and specific tests for the detection and identification of ethinamate. A study was also made on the metabolite isolated from a human brain.

EXPERIMENTAL

Reagents

Aqueous ethinamate solution: 50 μg./ml. *Ammoniacal potassium mercuric iodide solution:* dissolve 2·5 g. potassium iodide and 5 g. mercuric iodide in 100 ml. of water, add 50 ml. 5 per cent ammonia, boil for 10 min., allow the solution to stand overnight and filter through a filter paper.

Alkaline potassium mercuric iodide solution: dissolve 2·5 g. potassium iodide and 5 g. mercuric iodide in 100 ml. of water; add 50 ml. of 5 per cent potassium hydroxide and filter. 10 per cent *aqueous solution potassium cyanide. 2,4-Dinitrophenylhydrazine solution:* dissolve 3 g. of 2,4-dinitrophenylhydrazine in 20 ml. concentrated sulphuric acid, 60 ml. water and 20 ml. 95 per cent ethanol; allow the solution to stand overnight and filter. 5 per cent *aqueous mercuric chloride solution. Tollen's reagent:* prepare by addition of 125 ml. 0·1 N silver nitrate, 15 ml. 6 N sodium hydroxide, 20 ml. 25 per cent ammonium hydroxide and 90 ml. of water. *Isobutanol:* A.R. grade.

Preparation of Mercuric Salt of Ethinamate

Procedure 1: To a small test tube containing an aqueous solution of ethinamate is added 5 drops of ammoniacal potassium mercuric iodide reagent; this is warmed over a micro burner for 2 min. The mercuric salt of ethinamate is obtained as a white precipitate or gives a turbidity to the solution depending on the amounts of carbamate present. The sensitivity is 10 μg. of carbamate (in 1 ml.). Addition of a drop of 10 per cent potassium cyanide solution dissolves the precipitate. If obtained in mg. quantities, the precipitate can be recrystallised from ethanol: benzene (1 : 1) solution yielding a crystalline product, m.p. 209°. Found N, 5·19. Calc. for $C_{18}H_{24}HgN_2O_4$, N, 5·27.

Procedure 2: Alkaline potassium mercuric iodide solution is used instead of ammoniacal potassium mercuric iodide solution. If ethinamate is present an immediate precipitate is obtained. On warming a brown precipitate is obtained because of the hydrolysis of the carbamate to an ammonium salt which then reacts with the alkaline potassium mercuric iodide to give the brown precipitate.

Bromural, carbromal, formaldehyde, acetaldehyde, formic acid and methylpentynol do not interfere in procedure 1 but do so in procedure 2.

Preparation of 2,4-*Dinitrophenylhydrazone of Cyclohexene Methyl Ketone from the Mercuric Salt of Ethinamate*

The mercuric salt obtained in procedure 1 is centrifuged and the supernatant liquid is pipetted off. To the residue is added 0·5 ml. concentrated hydrochloric acid. The solution is heated over a small flame until the residue dissolves. 0·5 ml. of 2,4-dinitrophenylhydrazine solution is added and the resulting solution is heated to boiling. The 2,4-dinitrophenylhydrazone of cyclohexene methyl ketone is obtained as a red fluffy precipitate. When recrystallised from 95 per cent ethanol it has a m.p. 206°. The limit of detection is 200 μg. of carbamate (in 1 ml.). Found: C, 55·17; H, 5·54; N, 45·81. Calc. for $C_{14}H_{16}N_4O_2$, C, 55·24; H, 5·26; N, 45·70.

Addition Product of Ethinamate and Mercuric Chloride

To an aqueous solution containing the carbamate is added 5 drops of mercuric chloride solution. The addition product is formed as a white precipitate. The sensitivity is 20 μg. of the carbamate (in 1 ml.). The compound can be recrystallised from 95 per cent ethanol, m.p. 195°.

Modified Test for Ethinamate with Tollen's Reagent

One ml. of Tollen's reagent is added to a solution containing the carbamate and allowed to stand for 10 min. A few drops of isobutanol is added and shaken. If ethinamate is present the organic layer attains a reddish brown colour. The sensitivity of this test is 15 μg. of the carbamate (in 1 ml.).

Isolation of Ethinamate from Gastric Lavage

In most cases of poisoning by sedatives or hypnotics stomach washouts are available for toxicological analysis. The carbamate can be isolated by the following procedure.

A suitable portion of the stomach washout is acidified with 10 ml. of 10 per cent hydrochloric acid and the acidic solution is extracted with ether. The ethereal solution is evaporated and the residue tested for the presence of the carbamate. If the residue contains oil the latter can be removed by boiling with water, cooled and extracted with light petroleum (b.p. 40–60°). The light petroleum fractions are rejected and the aqueous layer is extracted with ether. The residue obtained after the evaporation of the ethereal solution is dissolved in the minimum amount of water and tested for ethinamate by the procedures already described.

Isolation of Ethinamate and its Metabolite from Viscera

A death from this drug took place early in 1959 in Singapore. At the time of the investigations there were no indications of the type of poisons taken and hence all the organs were submitted for toxicological analysis. Because only 15 ml. of urine and the same volume of blood were available for examination, no significant conclusions could be obtained from the analyses. The extraction of the drug from the other organs are given below.

Extraction of the stomach. Half of the stomach was macerated, treated with 400 ml. 95 per cent ethanol and 20 ml. 10 per cent tartaric acid and boiled for 1 hr. The alcoholic solution was filtered, evaporated and the residue treated with absolute ethanol. The ethanolic solution was again filtered and evaporated. The residue, an oily substance, was treated with light petroleum which was decanted off. The residue on trituration with 50 per cent ethanol and chilling deposited crystals which were filtered and sublimed under reduced pressure. The melting point of the white sublimate (3·1 g.) was 96–98° alone or mixed with an authenticated sample of ethinamate. The infra-red absorption spectra of the compound in chloroform and potassium bromide were taken and found to be similar to those of the carbamate itself. The substance also gave positive results for all the tests for the carbamate already described.

Extraction of the brain. The whole brain (475 g.) was macerated and digested with 300ml. of 95 per cent ethanol and 20 ml. 10 per cent tartaric acid. The alcoholic solution was filtered, evaporated and the residue was treated with absolute ethanol. The solution was again filtered and evaporated. The residue was taken up in ether and filtered.

The ethereal solution was shaken with 30 ml. 0·1 N hydrochloric acid to remove any basic material that might be present. The ether layer was washed with water, 30 ml. of 0·5 per cent sodium hydroxide solution and finally with water again. The ethereal solution was evaporated and the residue on tituration with 50 per cent ethanol yielded crystals (84 mg.) m.p. 112–116°. On repeated recrystallisation from the same solvent the m.p. was raised to 142°. The crystals can be sublimed under reduced pressure (0·1 mm. Hg) without decomposition. Found C, 83·52; H, 11·74; O, 5·26.

TABLE I

COLOUR REACTIONS OF ETHINAMATE, ITS DERIVATIVES AND METABOLITE

Reagents	Substances			
	Ethinamate (I)	Mercuric salt of (I)	Mercuric chloride addition product of (I)	Metabolite
Sulphuric acid	Bright red	Red to orange	Light yellow	Red
Frodhë	Red	Red to orange	No colour	Orange
Marquis	Orange	Orange	No colour	Red
Erdman	Red to orange	Brown to yellow	No colour	Brown
Mecké	Brown	Brown to yellow	No colour	Violet
Mandelin	Brown to green	Red to yellow	No colour	Orange
Sulphuric acid-vanillin	Red	Red to orange	No colour	Orange
Sulphuric acid-dimethylamino-benzaldehyde	Brown	Red to orange	No colour	Red

This metabolite forms a mercuric salt, mercuric chloride addition product, and a silver salt under the conditions already described. However, its silver salt when treated with isobutanol does not give any coloration to the organic solvent. Like the parent substance this metabolite also gave colour reactions when treated with concentrated sulphuric acid or sulphuric acid-containing reagents (Table I).

The hydrochloric acid and sodium hydroxide washings obtained earlier were basified and acidified respectively and extracted with ether. The residues obtained on evaporation of the ether solutions were treated with the Tollens reagent and no precipitate was obtained from both fractions.

Extraction of the liver and kidney. A portion of the liver (285 g.) and 1 kidney were extracted separately by the same procedure as described earlier for the brain. Only traces of oil were obtained in both cases and attempts to crystallise the oils were unsuccessful. However, the oils gave precipitates with mercuric chloride, Tollens reagent and ammoniacal potassium mercuric iodide, indicating the presence of a terminal acetylenic group in the molecule. The silver salts from kidney and liver extracts gave no colour in isobutanol.

DISCUSSION

The reaction between compounds which possess the grouping $-C{\equiv}CH$ and potassium mercuric iodide in potassium hydroxide solution proceeds according to the following equation (Shriner and Fuson, 1948)—

$$2\,RC{\equiv}CH + K_2HgI_4 + 2\,KOH \rightarrow (RC{\equiv}C-)_2Hg + 4\,KI + 2\,H_2O$$

The disadvantages of using potassium hydroxide are mainly due to the interference of compounds which are easily hydrolysed to ammonia. Ethinamate itself on prolonged standing or on heating in the presence of potassium hydroxide and potassium mercuric iodide gives a brown precipitate. Traces of formaldehyde, formic acid, acetone, acetaldehyde and paraldehyde, which are subtsances commonly encountered in toxicological analysis, also interfere with the test if potassium hydroxide is used together with potassium mercuric iodide. However, when ammonia is

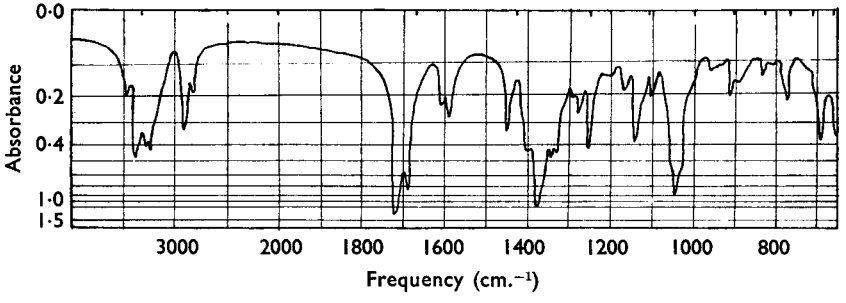


FIG. 1. Infra-red spectrum of ethinamate in potassium bromide.

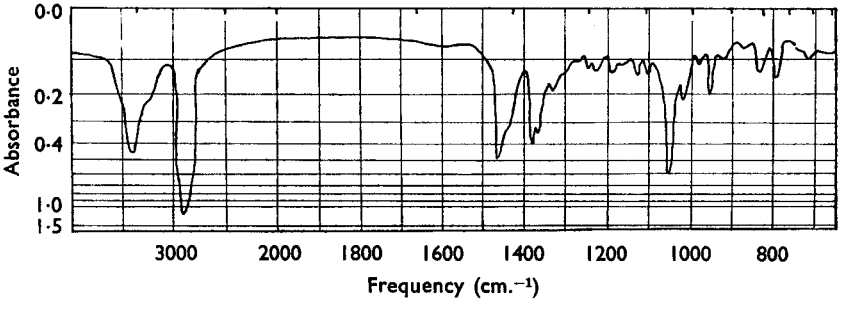


FIG. 2. Infra-red spectrum of the brain metabolite of ethinamate in potassium bromide.

used in place of potassium hydroxide the reagent is specific for the detection of ethinamate under the conditions described. The substances mentioned above do not interfere.

When the mercuric salt is treated with an aqueous solution of potassium cyanide the free carbamate is regenerated and can be extracted with ether and subjected to other confirmatory tests.

Reaction between ethinamate and Tollen's reagent. The disadvantages of using Tollen's reagent for the detection of ethinamate in toxicological specimens are mainly due to interference of halides and aldehydes in the samples. However, when the modified procedure is used it was found that only ethinamate gives a brown precipitate which is soluble in isobutanol. The precipitates formed when Tollen's reagent reacts with halides and aldehydes are not soluble in isobutanol. The modified procedure is therefore specific.

Infra-red Absorption Spectra

The infra-red spectra of ethinamate and its metabolite isolated from the human brain are shown in Figs. 1 and 2. The spectrum of the carbamate shows two intense bands at 1712 and 1685^{-1}. They reflect the vibrational characteristics of the C=O and NH_2 groups of the molecule respectively. In the –CO–NH– region two absorption bands are observed at 1610 and 1590 cm.$^{-1}$. Characteristics C–H and N–H stretching vibrations are observed in the 3500 and 2900 cm.$^{-1}$ regions.

In contrast to the marked absorption of the C=O and NH_2 groups seen in the spectrum of ethinamate these bands are absent in the spectra of the metabolite. This observation clearly indicates the absence of the carbamate group in the molecule of the metabolite. Two strong bands

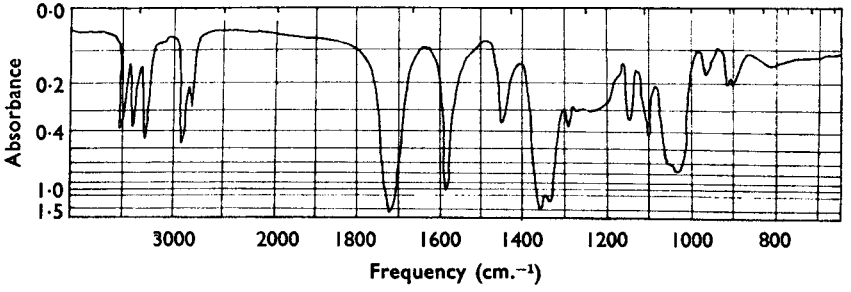


FIG. 3. Infra-red spectrum of ethinamate in chloroform.

are observed in the 3400 and 2900 cm.$^{-1}$ regions which indicate the presence of OH and $-CH_2-$ groups in the molecule. The absence of the carbamate group from the infra-red spectra is in agreement with the elemental analysis which shows the metabolite has an empirical formula of $C_{21}H_{16}O$.

The chemical reactions of the metabolite indicate the presence of a terminal acetylenic group similar to that of ethinamate in the molecule. However both the spectra of ethinamate and the metabolite do not exhibit any characteristic absorption in the 2100–2140 cm.$^{-1}$ region which is caused by the stretching of the carbon-carbon triple bond linkage (Wotiz and Miller, 1949; Wotiz, Miller and Palchak, 1950). In the ethinamate spectra the small band at 3280 cm.$^{-1}$ may be considered as indicative of the stretching of the C–H bond in the –C≡C–H grouping (Bellamy, 1958). The spectrum of a chloroform solution of the carbamate (Fig. 3) gives a better defined band at 3300 cm.$^{-1}$ which can be attributed to the terminal acetylenic group. However with the spectrum of the metabolite the detection of a similar band is made complicated by the fact that this band may be hidden in the broad OH band at 3400 cm.$^{-1}$ region. Attempts were made to acetylate the hydroxyl group of the metabolite to bring out the hidden band at 3300 cm.$^{-1}$ However the acetylated compound could not be obtained in a pure state and hence no conclusion could be drawn from its infra-red spectrum.

Although no less than 6 g. of ethinamate was isolated from the stomach only 84 mg. of a metabolite containing an ethinyl group was obtained from 475 g. of the brain and insignificant amounts from the liver and the kidney. It seems that the ethinyl group is easily metabolised. Experiments with rat tissue slices and on dogs blood bear out the metabolic liability of this structural group (Perlman and Johnson, 1952).

McMahon (1958) and Murata (1960) have recently made extensive studies on the metabolism of ethinamate in man and they have both established that one of the metabolites isolated from the urine is a monohydroxy derivative. The metabolite obtained from the human brain during this study is different from the one obtained by these workers although the structure of the former has not yet been established.

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