J Forensic Sci, Oct. 1972, Vol. 17, No. 4

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Methods for the Detection and Determination of Ethchlorvynol in Biological Tissue

Ethchlorvynol (Placidyl^{R}) is a moderately popular hypnotic drug often used in place of barbiturates. The drug was first marketed in 1955 and thought to be a safe drug, free from dependence properties. However, subsequent reports have shown that ethchlorvynol can cause dependence to develop [*1,2*] with severe withdrawal problems resulting from chronic high dosage [*3,4*].

Approximately seven years after ethchlorvynol was marketed, a method of analysis in biological tissues was first reported by Algeri et al [*5*]. Although this method was somewhat limited in sensitivity and specificity, it did provide a means of determining tissue concentrations. Other wet chemical methods of analysis were developed by Wallace et al [*6*] and Andryauskis et al [*7*]. The application of gas chromatography for analysis has been described by numerous investigators [*8–10*]. The use of thin layer chromatography for ethchlorvynol analysis has been comparatively neglected.

The reported wet chemical methods of analysis are somewhat lengthy, lacking in the high degree of specificity required in medico-legal work, and do not describe effects of interfering substances or analysis of putrid samples. For these reasons investigations were carried out to develop a screening method, and a wet chemical method having a high degree of specificity.

Screening Method

Ethchlorvynol when reacted with diphenylamine in sulfuric acid/acetic acid solution yields a red color (λ max. 510 nm) [7].

Materials

Ethchlorvynol (obtained from the Abbott Co., North Chicago, Ill.) was purified by vacuum distillation in the dark (70–78 C at 7 mm Hg uncorrected).

Diphenylamine reagent—0.7 percent weight/volume diphenylamine in sulfuric acid/glacial acetic acid/water (1:1:1).

Conway microdiffusion units.

Chloroform (distilled).

From the thesis submitted by H. W. Peel in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Maryland. Received for publication 27 March 1972; accepted for publication 20 May 1972.

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Method

Two milliliters of blood, 2 g of homogenized tissue, or 3 ml of urine, is introduced into the outer well of a microdiffusion cell, which has been pregreased with silicone grease. The fluid or tissue homogenate is diluted with 1 ml of water. One milliliter of diphenylamine reagent is added to the center well, and the diffusion cell is sealed with a glass cover. The unit is warmed at 40 C for 2 h. Development of a red color in the center well indicates more than 50 μg of ethchlorvynol in the outer well (equivalent to a blood or tissue concentration larger than 2.5 mg/100 ml). If no red color develops, the unit is allowed to stand overnight at room temperature; a pink color in the center well at this time indicates 10 to 50 μg of drug present (0.5 to 2.5 mg/100 ml in blood or tissue). If the reagent appears colorless at this point, the solution of the center well is transferred to a small centrifuge tube, 2 drops of chloroform added, shaken well and allowed to settle. A pink color in the chloroform layer indicates 2 to 10 μg ethchlorvynol (0.1–0.5 mg/100 ml in blood or tissue).

Discussion

Numerous substances were investigated that would give a sensitive test for ethchlorvynol. These included diphenylamine, *p*-anilinophenol, and aniline. The diphenylamine reagent similar to that prepared by Andryauskis et al, was found to provide the most sensitive test for the drug. A brief study was carried out to investigate the usefulness of this procedure as a quantitative method, but it was discarded for the following reasons. The reagent was unstable above 50 C; maximum color development required 2 h; the diffusion of ethchlorvynol was slow at lower temperatures.

The microdiffusion procedure was examined with respect to possible interfering substances. The following compounds cause no color development with diphenylamine reagent: methanol, ethanol, isopropanol, *n*-propanol, *n*-butanol, *t*-butanol, acetone, ethylacetate, benzene, toluene, ether, methylpentynol, ethinamate, and chloral hydrate. A yellow color developed with acetaldehyde and paraldehyde which cause no real interference. Formaldehyde produced an interfering black color. Samples containing greater than 4 percent formaldehyde cannot be used for this test.

Qualitative and Quantitative Identification of Ethchlorvynol Using TLC

Ethchlorvynol when subjected to steam distillation from acid solution forms 3-ethyl-2-penten-4-ynol which can be easily reacted to form the semicarbazone derivative which exhibits a maximum at 289 nm [*6,11*].

Materials

Ethchlorvynol standard—as above

Semicarbazide reagent: semicarbazide hydrochloride (5.6 g) and potassium hydroxide (2.8 g) are dissolved in 40.7 ml M/15 KH_2PO_4 and brought to a volume of 50 ml with M/15 Na_2HPO_4, and filtered. This solution is stable at room temperature for about 4 weeks, after which a precipitate may form.

Chloroform (distilled if necessary)

Sulfuric Acid (5% volume/volume)

Thin layer chromatography equipment: Silica Gel G plates, 250 μm in thickness, were prepared and activated at 115 C for 20 min. The glass tanks (23 by 12 by 23 cm) were lined with Whatman 3M filter paper, and the tops sealed with starch/glycerine paste. The total ascending distance of solvent flow was 15 cm.

Methods

Two milliliters of blood or 2 g of homogenized tissue or 2 ml of urine is transferred to an 800-ml Kjeldahl flask to which 15 ml of 5% sulfuric acid is added, and the sides are rinsed down with water. An adapter which is attached to the condenser remains immersed in the reagent throughout the distillation. The mixture is slowly steam distilled (10–15 min), so that the distillate bubbles directly into a 25-ml graduated cylinder containing 1 ml of semicarbazide reagent. Twenty five milliliters of total distillate solution is collected. The distillate is mixed and filtered, and the absorbance measured at 289 nm against a water blank.

Further examination by thin layer chromatography may be carried out by extracting the distillate, or a portion thereof, with three 10 ml portions of chloroform. The combined extracts are evaporated to a small volume on a steam bath, before taking to dryness in a current of air at room temperature. The semicarbazone residue is dissolved in a minimum of solvent (ethanol/chloroform, 9:1), and quantitatively spotted on a silica gel plate. The plate is developed in a solvent system of benzene-dioxane-ethanol-ammonia (150, 120, 15, 15) and allowed to dry, Ethchlorvynol, or its semicarbazone derivative, are made visible as red spots by spraying with the diphenylamine reagent described previously. The Rf values for ethchlorvynol and the semicarbazone derivative are 0.75 and 0.39, respectively (relative to 1.82 and 0.95, respectively for codeine).

If the thin layer chromatography step is carried out in order to purify the initial semicarbazone distillate, the semicarbazone spot may be extracted from the plate and examined further. The semicarbazone derivative is visible under ultraviolet light as a blue fluorescence. The spot area is carefully transferred to a 15 ml centrifuge tube, shaken thoroughly with 5 ml of water, and the aqueous extract is filtered. The absorbance of the resulting solution is determined at 289 nm, against a water blank.

Calibration Data (see Fig. 1)

Distillation from aqueous solutions of ethchlorvynol.[3]

Concentration (μg/ml)	Absorbance	Standard Error
50	0.258	0.007
100	0.523	0.003
150	0.791	0.009
200	1.082	0.050

Distillation of blood containing ethchlorvynol.[3]

The blood mixtures were equilibrated at least 2 h before distillation.

Concentration (μg/ml)	Absorbance	Standard Error
0	0.003	0.002
10	0.096	0.001
20	0.141	0.002
50	0.307	0.006
100	0.526	0.009
150	0.775	0.030
200	1.042	0.070

[3]The average absorbance was obtained from at least 8 determinations, using at least 3 different standards.

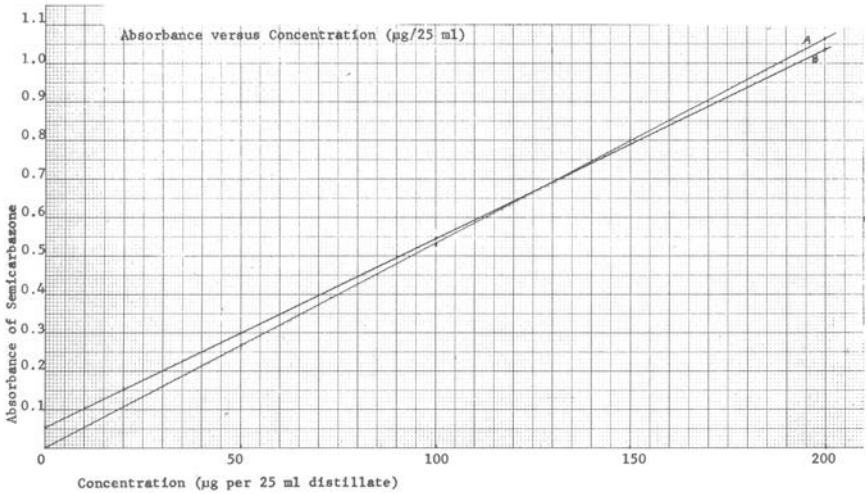

FIG.1—*Calibration curve of ethchlorvynol.*

The absorbance of blood blanks was found to be fairly constant. Thirty-five different blood and liver samples containing no ethchlorvynol were steam distilled in the normal manner, and read against water. Fresh and slightly putrid blood samples were included in the examination. Markedly putrid blood and tissue were found to cause high blank values. Putrid samples from ethchlorvynol cases can be purified by thin-layer chromatography before final quantitation.

Discussion

The general manner in which the samples are steam distilled, reacted with semicarbazide and analyzed is somewhat different than the original method by Wallace et al. The total analysis time was reduced somewhat. Also, it was found that the range of concentrations of drug occurring in cases could be better accommodated by this method of always distilling the same volume. In cases where low therapeutic levels of ethchlorvynol occurred, the distillate may be extracted twice with 10-ml portions of chloroform, carefully evaporated to dryness, and reconstituted in 5 ml of water before determining the absorbance.

Evaporation of ethchlorvynol or the semicarbazone derivative to dryness on a steam bath was found to result in serious loss of the former, and significant loss of the latter. For this reason, the semicarbazone solutions were finally evaporated to dryness at room temperature.

The TLC procedure was found to be useful for qualitative determinations important in medico-legal work, and for purifying contaminated or putrid samples. The recovery of the semicarbazone derivative after TLC was found to be 75 to 82 percent.

A number of volatile substances were examined to see if their presence would interfere with the semicarbazone method. No interference was shown by methanol, ethanol, *n*-propanol, isopropanol, acetone, benzene, chloroform, or ether. One-milliliter solutions containing ethchlorvynol (100 µg), and the following substances were distilled in the usual manner with no significant problems: formaldehyde 2 percent vol/vol; "Mylo Fix" embalming preservative 2 percent vol/vol; "perma-Glo" embalming preservative 2 percent vol/vol; acetaldehyde 1 percent vol/vol; and paraldehyde 1 percent vol/vol. Solutions

containing larger than 10 percent formaldehyde interfere with the analysis. In these cases purification can be effected by thin layer chromatography. Chloral hydrate was found to cause an inhibiting background spectrum when present in concentrations larger than 50 mg percent. The two acetylenic type drugs tested, ethinamate and methylpentynol, cause no interference.

Acknowledgments

H. W. Peel wishes to express his appreciation to the Royal Canadian Mounted Police for their assistance.

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