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GAS CHROMATOGRAPHIC DETERMINATION OF CHLORAL HYDRATE, TRICHLOROETHANOL AND TRICHLOROACETIC ACID IN BLOOD AND IN URINE EMPLOYING HEAD-SPACE ANALYSIS

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SUMMARY

A specific and sensitive gas chromatographic method for the quantitative determination of chloral hydrate and its metabolites trichloroethanol, trichloroethanol glucuronide and trichloroacetic acid in blood and urine is described. Use is made of the relative volatility of trichloroacetaldehyde, trichloroethanol and trichloroacetic acid methyl ester, which offers the possibility of head-space analysis. A known volume of the head-space gas, in equilibrium with its liquid phase (blood or urine), is directly injected into the gas chromatograph, which is equipped with an electron capture detector. Samples are divided in two portions: 1.0 ml for the determination of unconjugated trichloroethanol and 2.0 ml + 1.0 ml of concentrated sulphuric acid for the determination of chloral hydrate, trichloroethanol (conjugated + unconjugated) and trichloroacetic acid after esterification with dimethyl sulphate. Detection limits for chloral hydrate and trichloroethanol are 0.5 μg/ml in blood or urine and for trichloroacetic acid 0.1 μg/ml. Some preliminary pharmacokinetic studies on chloral hydrate and its metabolites in dogs and humans are reported.

INTRODUCTION

The principle metabolites of chloral hydrate (CH) in man are trichloroethanol (TCE), TCE glucuronide (TCE-Glu) and trichloroacetic acid (TCA), while the CNS depression that follows the ingestion of chloral hydrate is probably due entirely to TCE[1,2]. Recently, Garrett and Lambert[3] reported extensively on the pharmacokinetics of TCE and its metabolites in dogs. No detailed studies on the pharmacokinetics of CH and metabolites in humans have been reported so far.

A prerequisite for such studies is a sensitive assay for the simultaneous determination of unchanged drug and metabolites in the same blood or plasma sample. The spectrophotometric methods based on the Fujiwara reaction[4] are in general not specific enough for pharmacokinetic studies after the intake of therapeutic doses in man[5–7]. A specific gas chromatographic method with electron capture detection was described by Garrett and Lambert[8] in 1966 and a modification on this method was reported[3] by the same authors in 1973. However, time-consuming extractions of the rather polar substances into water-immiscible solvents are necessary in this

procedure. Direct GC injection of the biological sample has been described for the determination of CH and TCE[9], but the risk of contamination of the column and electron capture detector in this technique is substantial.

It was the purpose of the present study to develop a simple, sensitive and specific method for the assay of CH, TCE, TCE-Glu and TCA in blood and in urine. Trichloroacetaldehyde (dehydrated form of CH), TCE and TCA methyl ester are relatively volatile and this property offers the possibility of head-space analysis. When a dissolved substance is sufficiently volatile, the determination of its concentration in the vapour phase can be used as a measure of the concentration in the liquid phase, provided that an equilibrium between the vapour and liquid phases has been reached. The head-space is the vapour in equilibrium with its liquid phase. With a gas-tight injection syringe, part of the head-space vapour is injected directly into the gas chromatograph. In the literature, several volatile substances have been analysed in biological samples by applying this principle, *e.g.* inhalation anaesthetics[10] and ethanol[11]. This paper describes the head-space analysis of CH, TCE and TCA.

EXPERIMENTAL

Materials

Chloral hydrate (OPG, Utrecht, The Netherlands); trichloroethanol (Koch-Light, Colnbrook, Great Britain) was redistilled before use; trichloroacetic acid, p.a. (E. Merck, Darmstadt, G.F.R.); sulphuric acid, 95–97%, p.a. (Merck); dimethyl sulphate (BDH, Poole, Great Britain); lead acetate, p.a. (Merck) was used as a 20% solution in water. Sampling bottles were volumetric flasks (25.0 ml), cut at the calibration mark, with self-sealing silicone rubber caps (Beckman, Amsterdam, The Netherlands). Gas-tight Hamilton syringes varying in volume from 100 to 1000 μl were used for taking head-space gas and injection into the gas chromatograph.

Procedure

For the quantitative determination of CH and its metabolites in whole blood, a sample is divided in two portions immediately after it has been taken by vein puncture and a small drop of heparin solution has been added as anticoagulant:

(1) 1.0 ml for the analysis of unconjugated TCE (free TCE); and (2) 2.0 ml for the analysis of free TCE plus conjugated TCE (total TCE), of CH and TCA. These two portions are analysed as follows.

(1) A 1.0-ml volume of freshly taken whole blood is added to a vial containing 1.0 ml of lead acetate solution. The presence of lead acetate prevents the *in vitro* conversion of CH into TCE. The contents of the vial are gently homogenized and the vial is then placed in a 60° water-bath, such that the water level is just below the rubber cap, until equilibrium is reached between the liquid and vapour phase (3 h). With a gas-tight syringe, which is maintained at 60° so as to prevent condensation, a known amount of the head-space vapour is taken and analysed for free TCE by gas chromatography.

(2) A 2.0-ml volume of the same blood sample is added to another vial containing 1.0 ml of concentrated sulphuric acid. The function of this substance is outlined under Results and Discussion. After homogenizing the sample, the vial is equilibrated at 60° for 3 h. A known amount of the head-space vapour is taken and

analysed for CH and total TCE. The TCE-Glu concentration is calculated from the difference between the total TCE and free TCE. Then 0.10 ml of dimethyl sulphate is added by means of a syringe in order to convert the TCA into its methyl ester. After re-equilibration for 4 h, the head-space vapour is analysed for this compound.

The procedure for the analysis of urine samples is exactly the same as that for whole blood, except that the lead acetate solution is omitted. When relatively high concentrations were encountered, the urine samples were diluted with distilled water until proper measurement was possible.

The concentration of CH and metabolites in the samples was calculated with the aid of calibration graphs, prepared by adding known amounts dissolved in a maximum of 0.1 ml of distilled water to blank samples of blood or urine. These samples were run through the procedure as described. The peak height per 1000 μl of head-space vapour was plotted against known concentrations of the compounds. The detector response was calibrated daily by injection of a known amount of standard solution of TCE in ethanol (0.10 mg per 100 ml).

Gas chromatography

A Hewlett-Packard gas chromatograph, Model 402, with a Hewlett-Packard nickel-63 electron capture detector, Model 2-6195, and equipped with a Hewlett-Packard strip-chart recorder, Model 7127A, was used. Glass columns (1.8 m $\times$ 3 mm I.D.) were packed with 10% OV-17 on Gas-Chrom Q, 80–100 mesh (Applied Science Labs., State College, Pa., U.S.A.). The injector block was maintained at 150°, the column at 125° and the electron capture detector at 200°. Flow-rates were 20 ml/min for the carrier gas (nitrogen) and 60 ml/min for the purge gas (argon–methane, 95:5). The electron capture detector was operated at pulse 5 and the electrometer setting was kept continuously at range 10, attenuation 8.

RESULTS AND DISCUSSION

A major advantage of head-space analysis is that no time-consuming extraction procedure is required before analysis can be carried out. This advantage is particularly important for the relatively polar substances studied in this work, because they are difficult to extract into water-immiscible solvents. Garrett and Lambert[3,8] used diethyl ether, for which the following partition coefficients were determined: CH 0.191 and TCE 22. No partition coefficient is available for TCA, but it seems likely that it is very unfavourable with respect to the organic phase. Therefore, only TCE appears to have sufficient lipophilicity for a substantial yield on extraction. Nevertheless, Garrett and Lambert seemed to obtain satisfactory results for all compounds with extraction by diethyl ether. The other gas chromatographic method that has been described for the analysis of CH and its metabolites is the technique of direct injection of the biological sample[9]. However, it has been our experience that direct injection of aqueous solutions results in poor peak reproducibility with electron capture detection.

It is the relative volatility of trichloroacetaldehyde, TCE and TCA methyl ester that offers the possibility of head-space analysis, although a relatively high equilibration temperature and a long equilibration time are required. In Fig. 1, typical gas chromatograms are shown after direct injection of the head-space

gas in equilibrium with a blood sample containing CH, TCE and TCA. The times after which equilibrium was reached between the concentration in the liquid phase and in the head-space were verified by analysing five standard solutions of CH, TCE and TCA at several time intervals. In Fig. 2, the curves obtained for TCE and TCA are shown and it can be concluded that TCE reaches equilibrium after about 3 h and TCA after about 4 h. Also, a time of 3 h was found for CH. The relatively high temperature of 60° has been chosen arbitrarily; it results in an enrichment of the head-space vapour and it was observed that shorter equilibrium times were required than at lower temperatures.

When using the column packings described in the literature, CH and TCE exhibit relatively broad peaks. Therefore, several stationary phases were investigated, including OV-17, OV-17 plus orthophosphoric acid, Carbowax 20M and Carbowax 20M–terephthalic acid. A loading of 10% OV-17 on Gas-Chrom Q (80–100 mesh) proved to be the most satisfactory packing. A good separation was obtained and for all compounds symmetrical and well defined narrow peaks with short retention times were obtained (Fig. 1). The repeated injection of the same sample demonstrates the good reproducibility (standard deviation ±3%). The gas chromatogram of a

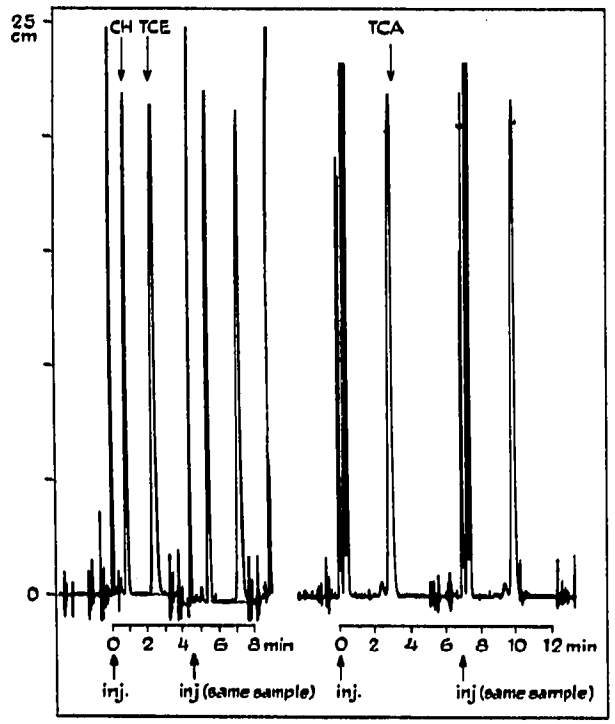

Fig. 1. Gas chromatograms of chloral hydrate (CH), trichloroethanol (TCE) and trichloroacetic acid methyl ester (TCA) after direct injection of head-space gas, in equilibrium with a 2.0-ml blood sample + 1.0 ml of concentrated sulphuric acid. Blood concentrations of the compounds were: CH, 10.2 μg/ml; TCE, 12.6 μg/ml (injection volume 1000 μl) and TCA, 10.5 μg/ml (injection volume 500 μl).

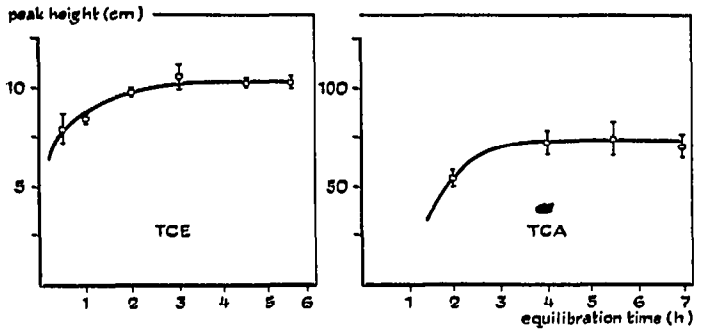

Fig. 2. Change of concentration (peak height per 1000 μl of head-space gas injected) of TCE and TCA methyl ester in the gas phase during equilibration at 60° (2.0 ml of blood + 1.0 ml of concentrated sulphuric acid). Concentration of TCE, 5.0 μg/ml in blood (injection volume 1000 μl) and concentration of TCA, 18.0 μg/ml in blood (injection volume 250 μl). Mean values ± standard deviation for five determinations.

blank blood or urine sample treated in the above manner is completely clean and there are no interfering peaks due to normal blood or urine constituents.

It seems obvious that for the quantitative determination of this type of compound, an electron capture detector should be used. The response of a normal flame ionization detector is determined by the number of C–H bonds per molecule and these are few in CH and its derivatives. However, the response of an electron capture detector depends on the electron affinity of the compound to be analyzed, and this is high for halogen-containing substances. Garrett and Lambert[3,8] found that the sensitivity of the flame ionization detector is only slightly greater than the Fujiwara UV method according to Friedman and Cooper[6], while their own method with electron capture detection was much more sensitive. In the present study, use was made of a nickel-63 electron capture detector with 0.75-μsec voltage pulses and intervals of 5, 15, 50 and 150 μsec. Although the sensitivity increases with increasing pulse interval, the sensitivity obtained with the lowest pulse interval is satisfactory for physiologically encountered drug concentrations. A practical disadvantage of the electron capture detector is its small linear dynamic range. However, this gave no problems when using a pulse interval of 5 and electrometer settings of range 10 and attenuation 8. Every response smaller than full-scale deflection (25 cm) proved to be out of the linear dynamic range (Fig. 3). If this was not the case, the injection volume was reduced. The quantitative parameter used in this study was the peak height per 1000 μl of head-space vapour. That there is no essential difference in choosing the peak height or peak area as a measure for the detector response can also be concluded from the results shown in Fig. 3.

Calibration curves for the different compounds obtained by the procedure described are shown in Fig. 4. There is a linear relationship between detector response and concentration, provided that the injection volume has been adapted to the linear dynamic range of the detector. The sensitivity, however, is dependent on the composition of the liquid phase, blood or urine and the compound in question. Also, sulphuric acid has a great influence on the concentration of the three substances in the head-space gas. It enhances the formation of trichloroacetaldehyde, which is the more volatile form of CH. The increase in response of TCA methyl ester is about

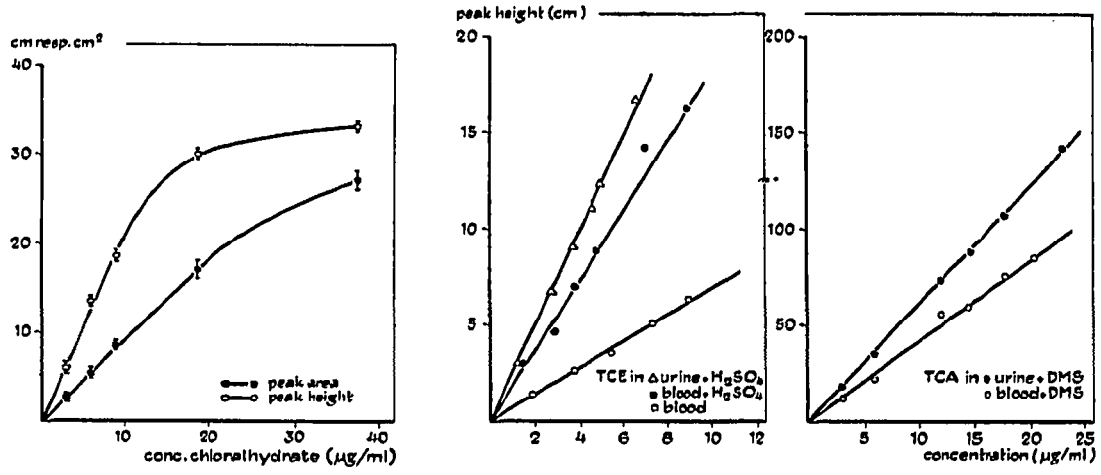

Fig. 3. Calibration curves for CH in blood. For each concentration, the injection volume was 1000 μl of head-space gas. Note the linearity over the first 25 cm, which corresponds to full-scale deflection of the recorder at range 10, attenuation 8 (electron capture pulse 5). Also, there is no essential difference between choosing peak height or peak area as a measure of the detector response. Mean values ± standard deviation for five determinations. ●, Peak area; ○, peak height.

Fig. 4. Calibration curves for TCE, TCE in the presence of sulphuric acid and TCA in blood and in urine. The detector response is measured as the peak height per 1000 μl of head-space gas. The volumes injected were such that the detector response was in the linear dynamic range. (a) TCE in urine + H_2SO_4 (△), blood + H_2SO_4 (●) and blood (○); (b) TCA in urine + dimethyl sulphate (●) and blood + dimethyl sulphate (○).

a factor 10 in the presence of sulphuric acid. The primary reason, however, for adding this strong acid to the samples is to achieve hydrolysis of TCE-Glu into TCE, so that total TCE can be determined and then conjugated TCE can be calculated by difference. Sulphuric acid also prevents the *in vitro* conversion of CH into TCE, as does lead acetate. This is necessary, for Butler[12] reported on the very rapid *in vitro* reduction of CH by erythrocytes. Therefore, it is important that immediately after they have been taken, the blood samples should be mixed with sulphuric acid or lead acetate. Analysis may then follow within 48 h.

There is also the possibility of determining plasma concentrations instead of blood concentrations, in which case the blood has to be centrifuged immediately after the sample has been taken.

For the esterification of TCA, dimethyl sulphate is used, and as this is an equilibrium reaction it could be expected that the amount of dimethyl sulphate added influences the amount of ester that is formed. It was observed that the addition of 0.30 ml instead of 0.10 ml of dimethyl sulphate results in an increase in the response by a factor of about 2.2. This effect means that the amount dimethyl sulphate must be carefully added by means of a calibrated syringe. At first, we tried to determine CH, TCE-total and TCA simultaneously, as there is a good gas chromatographic separation of the three compounds. However, dimethyl sulphate appeared to influence the response of TCE in an irreproducible manner and therefore TCE has

to be determined in the absence of dimethyl sulphate. This requires a separate equilibration period for the analysis of TCA.

The detection limits, under the conditions applied, are *ca.* 0.5 μg/ml for CH and TCE and 0.1 μg/ml for TCA in blood or urine. Lower detection limits can easily be obtained by increasing the pulse interval. The precision was determined by measuring the peak height after adding known concentrations of the compounds to a blank sample of blood or urine. Standard deviations did not exceed ±6% (five determinations), except for free TCE, for which the standard deviation was 6–8% (five determinations).

In conclusion, it can be stated that the method described for the determination of CH and its metabolites is simple and sensitive, and the precision is satisfactory.

Pharmacokinetic studies

The assay procedure described above has been used in the study of the pharmacokinetics and metabolism of chloral hydrate in man. It has also been used in the study of the relative bioavailability of different pharmaceutical formulations containing chloral hydrate[13].

In order to test the applicability of head-space analysis for the determination of CH and its metabolites, a preliminary experiment was undertaken with a dog. CH was administered i.v. (60 mg/kg) and blood samples were taken from the fore-leg or left hind-leg at several time intervals. The results are shown in Fig. 5 on a semi-

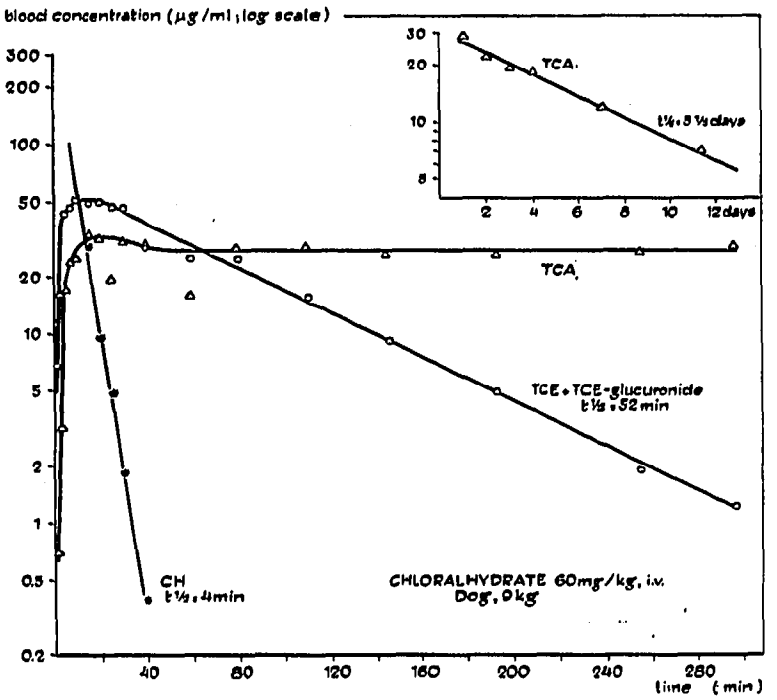

Fig. 5. Blood concentration *vs.* time curves for CH, TCE+TCE-Glu and TCA after i.v. injection of CH to a dog. CH is rapidly metabolized to TCE and TCA. Note the long half-life of TCA from the inset.

logarithmic scale. Chloral hydrate is very rapidly metabolized (biological half-life 4 min) and TCE and TCA are formed rapidly. In this experiment, no distinction was made between TCE and TCE-Glu. TCA has a very long half-life, which can be deduced from the inset in Fig. 5. These results for the dog are in good agreement with those obtained by Garrett and Lambert[3].

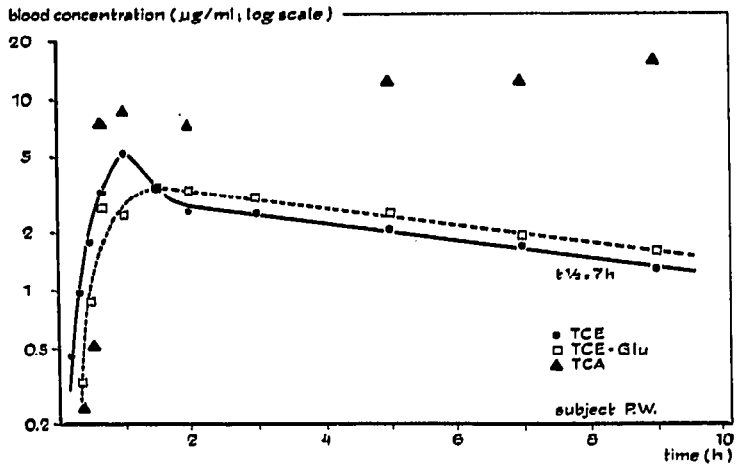

Fig. 6. Blood concentration *vs.* time curves for TCE (●), TCE-Glu (□) and TCA (▲) after oral administration of CH as an aqueous solution (15 mg/kg) to a human volunteer. No unchanged CH could be detected. Note the accumulation of TCA during TCE elimination. The half-life of TCA is 4–5 days.

In human experiments, CH was given orally as an aqueous solution (15 mg/kg) on an empty stomach. Blood samples were taken by vein puncture at several times after administration. Blood concentration–time curves for TCE, TCE-Glu and TCA are shown in Fig. 6. Although CH can be detected in concentrations of about 0.5 μg/ml in blood, no CH could be measured, even in the first sample 10 min after intake. However, there is a rapid increase in TCE and TCA concentrations and it seems likely that the conversion of CH into its metabolites takes place during the first passage through the liver. The half-life of TCE is about 7 h and the half-life of TCE-Glu is of the same order. From the kinetic point of view, this is understandable as the elimination of glucuronide is dependent on its formation from free TCE.

TCA has a very long half-life in man (4–5 days) and during daily administration of CH a considerable accumulation of this metabolite occurred. Because of its substantial protein binding, this might be of importance with respect to interactions of CH with other drugs[14]. Another important application of the present method could be the study of fate and metabolism of the organic solvent trichloroethylene in man, which is of great interest in occupational and industrial medicine[15].

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