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Determination of chloral hydrate and its metabolites in blood plasma by capillary gas chromatography with electron capture detection

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Abstract

A sensitive, accurate, and reliable method is described for the quantitative determination of chloral hydrate (CH) and its metabolites in blood plasma of mice and rats. Metabolites of CH include trichloroacetic acid (TCA), trichloroethanol (TCE), and trichloroethanol glucuronide (TCE-Glu). This new method uses capillary gas chromatography with electron-capture detection (GC/ECD). Procedures for improving sample stability and quality assurance are also described that were not mentioned in previous literature. Rat or mouse plasma (50 μl) is acidified (or treated enzymatically for TCE-Glu determination) and extracted with peroxide free methyl *t*-butyl ether. Distilled diazomethane (CH_2N_2) is added to derivatize TCA to its methyl ester. Detection limits were estimated at 0.2 μg/ml for CH and TCE, and 0.1 μg/ml for TCA. Detector response to TCA and TCE were shown to be linear in the range of 3.125–200 μg/ml ($r \geq 0.9996$). For CH, the response fits a second-order equation in this same range ($r = 0.99994$)

Keywords: Chloral hydrate

1. Introduction

Chloral hydrate (2,2,2-trichloro-1,1-ethandiol) is a rapidly effective sedative and hypnotic drug that is often prescribed to infants, young children, and elderly patients prior to surgical procedures to relieve anxiety or produce sleep [1]. CH is also used in veterinary medicine as a central nervous system depressant and anesthetic [2]. The general public is exposed to CH in small amounts through drinking water since it is formed as a disinfection by-product when water is treated with chlorine [3–5]. A number of assays have shown CH to be genotoxic [6–8] and hepato-carcinogenic in male mice [9–11]. Due to its current pediatric use and widespread potential for human exposure, the U.S. Food and Drug Administration nominated chloral hydrate as a priority compound for in-depth toxicological evaluation by the National Toxicology Program (NTP) in 1992.

As part of this evaluation, a sensitive, accurate, and reliable analytical procedure was needed to measure CH and its metabolites simultaneously in the blood plasma of mice and rats following single or

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multiple dose administration of the drug. Previously reported methods were found to be unreliable for our use under the strict GLP guidelines required for the support of this NTP study. Significant modifications were made to the method described by Gorecki et al. [15]. Instead of using a packed column, split injections (30:1) were made into a capillary column which produced sharper peaks, better resolution, increased sensitivity, and more accurate quantitation. Problems concerning sample stability are also addressed. A novel approach was taken to stabilize samples by removing reactive peroxides from the extraction solvent. TCE-Glu controls, essential for quality assurance, were prepared in our laboratory. These modifications along with advancements in instrumentation and data processing software produced more accurate and dependable results.

The method was applied to investigate the pharmacokinetics of CH metabolism in mice and rats following oral administration as described by Beland et al. [16]. In vivo CH is metabolized to trichloroacetic acid (TCA), trichloroethanol (TCE), and its conjugate trichlorethanol-glucuronide (TCE-Glu) [12]. Each of these compounds, including CH, were detected in the plasma of dosed animals by this method. Monochloroacetic acid (MCA) and dichloroacetic acid (DCA) have also been reported to be metabolites [13,14] but neither MCA nor DCA were observed in mice or rats during our investigation. This unique analytical procedure requires very small sample volumes (50 μl) and can measure very low levels of CH, TCA, and free TCE metabolites simultaneously in plasma. Total TCE (free TCE+ TCE-Glu) is analyzed by pre-treating a separate sample of the plasma with β-glucuronidase. TCE-Glu is determined by subtracting the free TCE concentration from the total TCE concentration in the plasma.

2. Experimental

2.1. Chemicals and reagents

CH, TCA, and β-glucuronidase [EC 3.2.1.31, bacterial, optimum activity at pH 6.8] were purchased from Sigma (St Louis, MO, USA). TCE, methyl trichloroacetate (MTCA), *N*-methyl-*N*′nitro-*N*-nitroso guanidine (MNNG), lithium aluminum hydride (LAH), and 1,1,1,2-tetrachloroethane were obtained from Aldrich (Milwaukee, WI, USA). Methyl *t*-butyl ether (MTBE) was purchased from Baxter Healthcare (Muskegon, MI, USA) and further purified to remove peroxides as described in Section 2.5. Ethereal diazomethane was prepared from MNNG as specified in Section 2.7. TCE-Glu controls were prepared as follows using modifications of a procedure described by Kadlubar et al. [18].

To 2 ml of 100 m*M* Tris buffer (pH 7.8) containing 5 m*M* $MgCl_2$ was added 12 μmol UDPGA (Sigma), 2.4 mg rat liver microsomes from a rat induced with 3-methylchol-anthrene as described by Fu et al. [19], and 680 nmol (0.1 mg) trichloroethanol. The mixture (Control A) was incubated for 30 min at 37 °C. Incubations were also conducted by omitting UDPGA (Control B), by omitting trichloroethanol (Control C), or using heat-denatured microsomes (Control D). Analysis of Control A indicated a total TCE concentration of 50 μg/ml with approximately 80% conjugated as TCE-Glu.

The argon had a minimum purity of 99.999%. Deionized/glass distilled water (DDH_2O) was used for preparing reagents. All other chemicals were analytical grade.

2.2. Gas chromatography system

The analysis was carried out using a Hewlett-Packard Model 5890 Series II gas chromatograph (GC) equipped with a model 7673 auto-injector, a split-splitless injection port, and a ^{63}Ni electron-capture detection system (Agilent Technologies, Palo Alto, CA, USA). HP ChemStation software (version A.03.21) was used to program and operate the system.

2.3. Gas chromatography conditions

The column was a DB 1701, 30 m×0.25 mm× 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Helium with a flow-rate of 0.72 ml/min was used as the carrier gas. Nitrogen was the make-up gas for the ECD and had a flow-rate of 60 ml/min. Injection port and detector temperatures were 220 and 250 °C, respectively. The initial column temperature was 80 °C. Following injection, the

oven temperature was held at 80 °C for 2 min, heated at 10 °C/min to 110 °C, heated at 40 °C/min to 160 °C and held at 160 °C for 2 min. The column was then re-conditioned at the end of each run by continuing to heat at 40 °C/min to 230 °C, and holding at 230 °C for 2 min before cooling the oven back down to the original conditions. Injections of 1 μl were made into the GC using a split ratio of 30:1.

2.4. Solvent purification system

The solvent purification system consisted of a 3-l two-neck round bottom flask with a removable plug, a 1-l solvent still head with drainage arm, a condenser, a heating mantle with power controller, Teflon™ (PTFE) sleeves for all connecting joints, adapters for an argon purge line and for dispensing the distillate (Lab Glass, Inc., Vineland, NJ, USA). An argon line was connected to the still to remove oxygen from the system. The flow of argon was controlled using a fine flow control valve and a simple bubble meter/indicator containing mineral oil. A chiller (Lauda RM6, Brinkmann Instruments, Westbury, NY, USA) was used to circulate antifreeze (50% aqueous ethylene glycol) through the condenser. All parts were thoroughly cleaned and oven dried prior to assembly.

2.5. Purification of the extraction solvent

The extraction solvent was purified by reflux with LAH (~1.5 g/l) under argon for 3 h. The distillate was collected and maintained under argon. All receiving vessels and vials from this point were pre-filled and capped with a headspace of argon to avoid exposure of the solvent to oxygen.

2.6. Diazomethane generating apparatus

Diazomethane (CH_2N_2) was prepared by distillation using an apparatus constructed of all smooth glass. (It should be noted that CH_2N_2 has been reported to explode on occasion when distilled in a ground glass apparatus [17].) The apparatus (Fig. 1) consisted of a glass reaction tube (12 cm×2 cm I.D.) fitted with a 19/22 Wheaton two-arm adapter. One arm of the adapter was connected to argon with a fine flow control. The opposite end extends close to the bottom of the reaction tube. The other arm is joined to a Pasteur pipette with PTFE tubing to allow transfer of the CH_2N_2 to a glass collection tube. The collection tube was supported in a beaker of crushed ice. The entire apparatus was assembled over a containment tray in a well-ventilated fume hood.

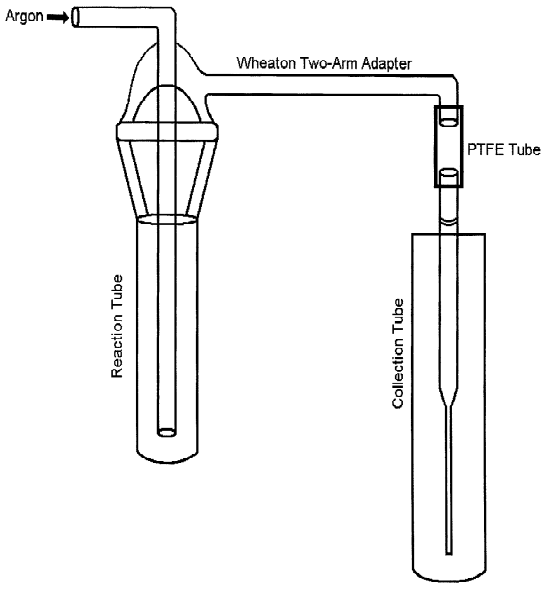

Fig. 1. Diazomethane generating apparatus.

2.7. Preparation of ethereal diazomethane

The CH_2N_2 collection tube was purged with argon and approximately 15 ml of purified MTBE added to it. (The MTBE was chilled before placing it in the beaker of crushed ice.) Three milliliters of DDH_2O were added to the reaction tube followed by a small scoop (~0.25 g) of MNNG. One milliliter of 10 *M* KOH was added to the reaction tube and the apparatus connected. Yellow CH_2N_2 gas is generated which passes through the cold MTBE. Argon was bubbled through the mixture to gently mix the reactants and help transfer the CH_2N_2 to the MTBE in the collection tube. The process was repeated with additional MNNG and/or KOH as necessary until the MTBE turned bright yellow. The solution was

wrapped in foil to protect from light and stored at −5 °C. Solutions were used for about 1 week and discarded once the color faded to pale yellow.

2.8. Preparation of plasma standards

Aqueous standard solutions of CH, TCA, and TCE were prepared, each at a concentration of 80 mg/ml in DDH_2O. A stock plasma standard containing 800 μg/ml of each analyte was then prepared by adding 20 μl of each of the three 80 mg/ml standard solutions into 1940 μl of control rat plasma. The stock plasma standard was mixed thoroughly by vortex, divided into 250-μl aliquots, and placed immediately in a freezer at ≤−60 °C.

Working plasma standards were prepared the day of analysis at concentrations of 0 (control), 2.5, 5, 10, 40, and 160 μg/ml. (These concentrations covered the ranges found in mice and rats dosed at up to 200 mg CH/kg body wt.). An aliquot of the stock plasma standard prepared above was thawed in a cold water bath and appropriate serial dilutions made with control plasma. The standards were kept chilled in an ice water bath or frozen if not used immediately to prevent degradation.

2.9. Preparation of extraction solvent containing internal standard

A stock internal standard was prepared at a concentration of 0.4 mg/ml by weighing an appropriate amount of 1,1,1,2-tetrachloroethane and diluting it with purified MTBE. The solution was capped under a headspace of argon, protected from light, and stored refrigerated.

The extraction solvent containing a working concentration (0.2 μg/ml) of the internal standard was prepared by making a 1 to 2000 dilution of the stock internal standard into purified MTBE. The solution was capped under a headspace of argon, mixed, and kept at room temperature in an amber glass bottle.

2.10. Preparation of β-glucuronidase solution

The β-glucuronidase solution was prepared at 2.5 mg/ml in 0.1 *M* phosphate buffer (pH 6.8) and stored refrigerated.

3. Sample extraction

3.1. Sample preparation for determining CH, TCA, and free-TCE

Plasma samples were thawed in a rack containing cold water and vortexed to ensure homogeneity. Crushed ice was added to the water to keep the samples chilled. A 50-μl aliquot of each standard or sample was transferred to a corresponding 4 ml PTFE screw-capped glass tube followed by 100 μl of 3 *M* H_2SO_4. The contents were mixed by vortex. A fine stream of argon was blown into each tube to displace air. Two milliliters of the extraction solvent containing internal standard were added to each tube. The tubes were capped with argon, vortexed, and centrifuged at 1500 *g* for 10 min at 5 °C. One milliliter of the MTBE layer was transferred to the corresponding screw-capped GC vial and 50 μl of diazomethane added. The vials were capped under argon, inverted to mix, and placed on the preprogrammed auto-sampler carousel for analysis by GC/ECD.

3.2. Sample preparation for determining total-TCE and TCE-glucuronide

Plasma samples were thawed as described in Section 3.1 above. A 50-μl aliquot of each sample or control was transferred to the appropriate 4-ml tube followed by 100 μl of β-glucuronidase solution. The tubes were capped, vortexed, and incubated in a 37 °C water bath for 1 h. The tubes were chilled to reduce vapor pressure prior to opening. Two milliliters of extraction solvent containing internal standard was added to each tube. The contents were mixed by vortex and the tubes centrifuged at 1500 *g* for 10 min at 5 °C. One milliliter of the MTBE layer was transferred to the corresponding GC vial and 50 μl of diazomethane added. (The diazomethane is added for dilution and background correction.) The vials were capped tightly, inverted to mix the contents, and analyzed by GC/ECD.

3.3. TCE-Glu controls

TCE-Glu controls were included in each assay to ensure complete hydrolysis of the conjugate by the

β-glucuronidase incubation. Samples of Control A and Control B were treated identical to plasma samples as described in Section 3.2. An additional sample of Control A was treated without β-glucuronidase using 100 μl of 0.1 *M* phosphate buffer.

4. GC analysis

Plasma standards, samples, and controls were extracted as described in Sections 3.1 and 3.2. Standards were injected at the beginning of the assay for calibration. Additional standards (5 and 40 μg/ml) were included in the middle and end of the injection sequence as controls.

Injection volumes of 1 μl were made into the GC using the conditions described in Section 2.3. A standard curve was plotted for each analyte by careful integration of the peaks and using the internal standard method of quantitation. Plasma CH, TCA, and Free-TCE concentrations were determined by direct comparison to the corresponding standard curve. The enzymatically-treated samples were used for determining the Total-TCE. Plasma TCE-glucuronide concentrations were obtained by subtracting the Free-TCE from the Total-TCE.

5. Results and discussion

The method described employs significant modifications to the procedure used by Gorecki et al. [15]. Revisions include using a capillary column with split injections instead of a packed column. These conditions produce sharper peaks, better resolution, and enhanced sensitivity. Detection limits for CH, TCA, and TCE in plasma samples were estimated to be 0.2, 0.1, and 0.2 μg/ml, respectively. Maximum extractability of TCA is achievable by the addition of only 100 μl of 3 *M* H_2SO_4. The enzyme β-glucuronidase from *Escherichia coli* was selected since its optimum activity (pH 6.8) closely matches the pH of plasma. Incubation times for the hydrolysis of TCE-Glu were reduced from 18 to 1 h and completeness of the reaction confirmed by use of controls. TCE results for Control A (enzyme-treated) were 50 μg/ml while results for Control A (phosphate buffer alone) were less than 10 μg/ml. These results verify that Control A contained a significant amount of TCE bound as the glucuronide. Control A has the same total TCE concentration as Control B which contains only free TCE. Complete hydrolysis is therefore indicated when the TCE results for Control A (enzyme-treated) and Control B are equivalent.

MTBE was selected as the extraction solvent since it exhibits extraction efficiencies for CH and its metabolites similar to diethyl ether but is somewhat less volatile and contains no added alcohol or BHT. An internal standard was added to the MTBE to enhance the quantitative accuracy of the analysis. The compound, 1,1,1,2-tetrachloroethane was found to produce an excellent reference peak with ideal retention in the middle of the chromatogram. The internal standard concentration will be the same in each sample and for any given sample the ratio of analyte concentration (CH, TCA, and TCE) relative to that of the internal standard is constant regardless of the exact amount injected. Using the internal standard method of quantitation, variations in the amount of sample introduced on-column can be compensated for. By this method, peak areas of the analytes for each sample are normalized to a constant value for the internal standard.

Sample stability is crucial to conducting accurate pharmacokinetic investigations. To prevent degradation, plasma samples were stored frozen at ≤ -60 °C and thawed promptly just prior to analysis using cold water. Evaluation of the stability of extracted samples indicated that CH containing plasma extracted with un-distilled MTBE showed a loss of CH with a corresponding increase in TCA over time (approx. 0.3–5% per h). This instability is thought to be caused by traces of peroxides present in the solvent. Refluxing the MTBE with lithium aluminum hydride followed by distillation was used to remove peroxides. The process was carried out under argon to avoid exposure of the solvent to oxygen. CH containing plasma extracted with distilled MTBE showed negligible conversion of CH to TCA (0.1–0.2% per h) following repeated injections. When distilled MTBE was used, samples showed little degradation during assays lasting nearly 7 h.

The figures show typical chromatograms obtained by this method. A chromatogram of control rat

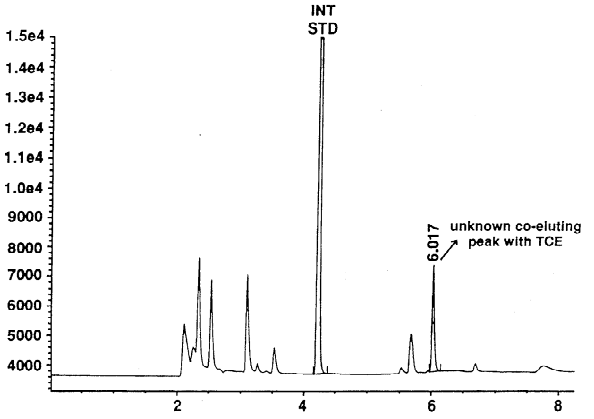

Fig. 2. Sample chromatogram of a control blank rat plasma extracted and derivatized as described.

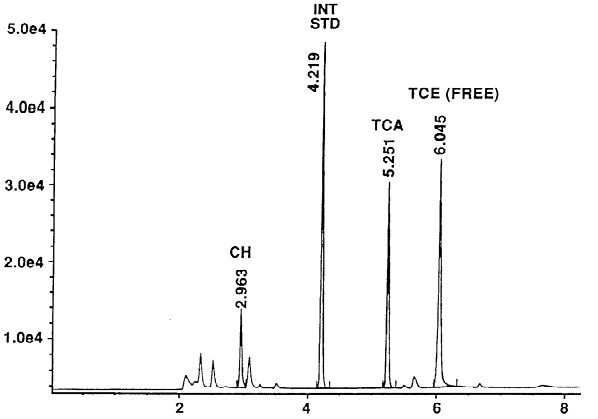

Fig. 4. Mouse plasma sample 15 min after receiving a 200 mg/kg dose (acid treatment).

plasma is shown in Fig. 2. Fig. 3 is a chromatogram of rat plasma spiked with CH, TCA, and TCE at 10 μg/ml each. Fig. 4 is that of a mouse plasma collected 15 min after the animal received a 200 mg/kg dose of chloral hydrate. Fig. 5 is the same mouse plasma treated enzymatically. Figs. 6–8 are typical calibration curves for CH, TCA, and TCE, respectively, using spiked plasma as standards. Excellent linear fits were obtained for TCA and TCE. For CH, curve fits were best when a second-order equation was used. An unidentified peak with a retention time of approximately 6.0 min and consistent area was observed in samples from both mice and rats that co-elutes with TCE. Calibration curves for TCE were calculated by including the peak area of the unknown with each calibration point. The other peaks of interest are well separated with retention times of approximately 3.0, 4.2, and 5.3 min for CH, internal standard, and TCA-methyl ester, respectively. DCA-methyl ester elutes at 4.5 min producing a signal about 1/3 that of TCA on an equivalent molar basis.

The chromatography has been observed to degrade

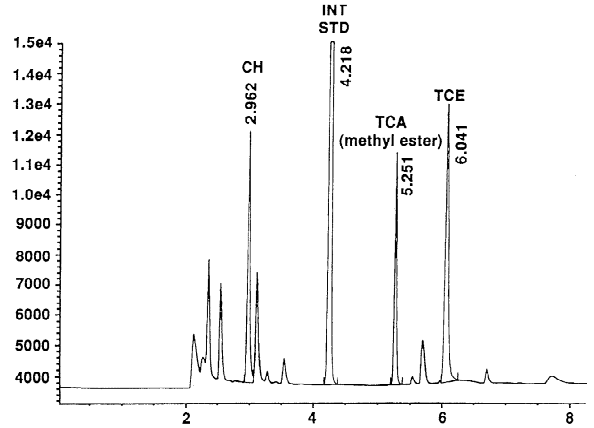

Fig. 3. Sample chromatograph of spiked plasma (10 μg/ml each of CH, TCA, and TCE).

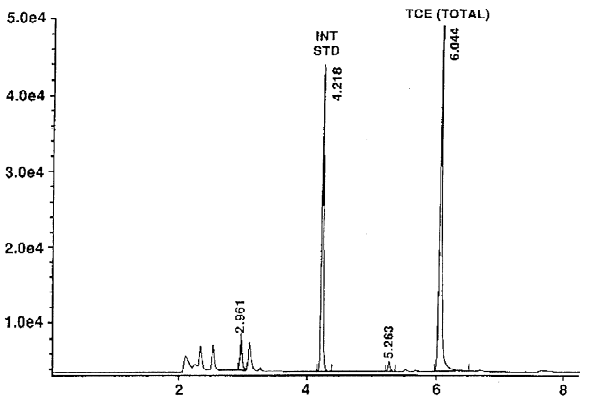

Fig. 5. Mouse plasma sample 15 min after receiving a 200 mg/kg dose (enzyme treatment).

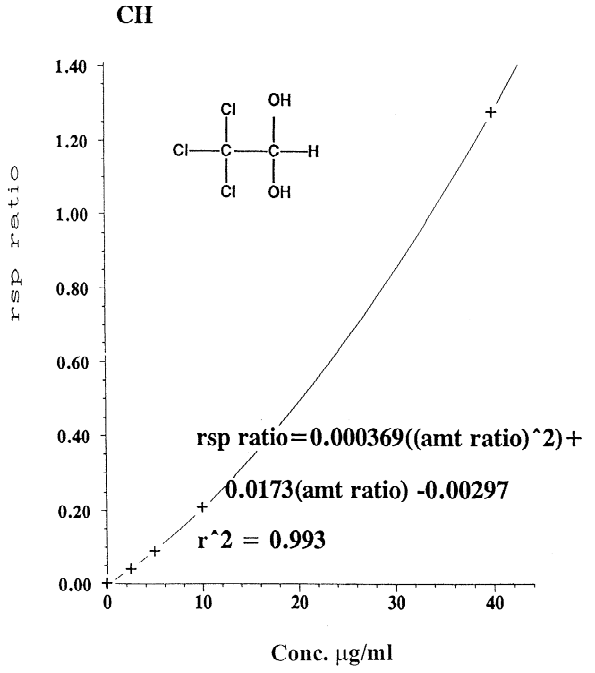

Fig. 6. Calibration curve for chloral hydrate.

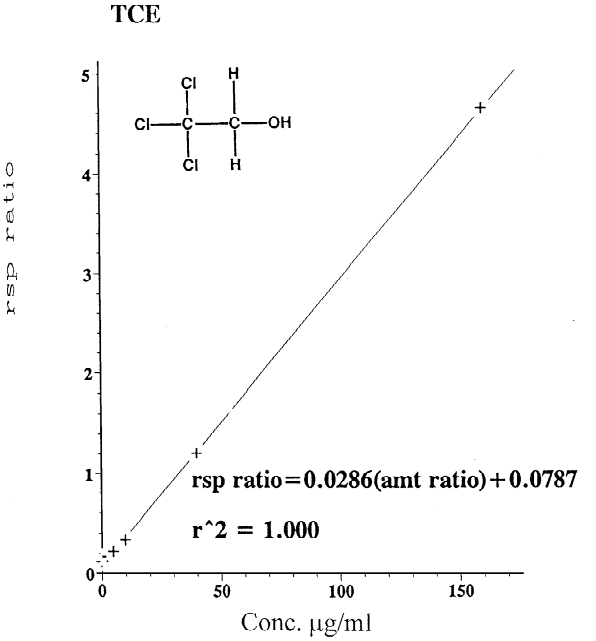

Fig. 8. Calibration curve for trichloroethanol.

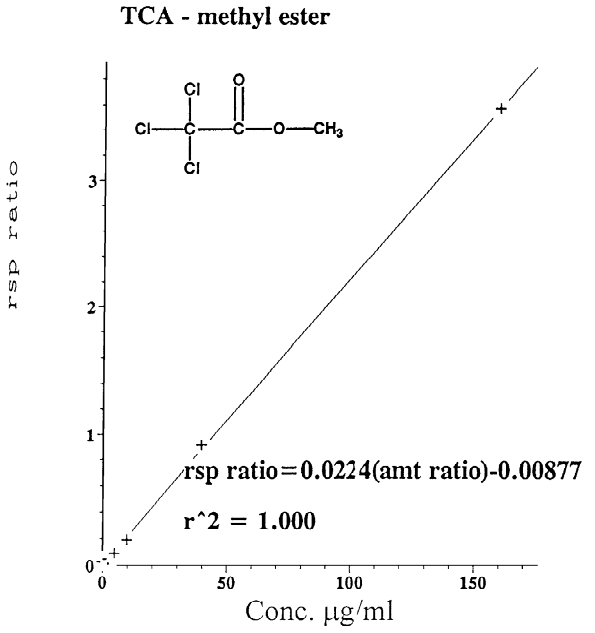

Fig. 7. Calibration curve for trichloroacetic acid methyl ester.

after a large number of samples have been injected. Co-extractants from the plasma matrix can accumulate in the injection sleeve, eventually causing peak tailing and diminished response for TCE. This problem is prevented by periodic cleaning or replacement of the silanized glass sleeve insert in the injection port and reconditioning the column.

Statistical data regarding accuracy, precision, repeatability, and recoveries by this method are shown in Tables 1 and 2. Accuracy and precision data were determined from repeat injections of 5 and 40 μg/ml plasma standards. Recoveries of CH and metabolites from plasma were determined by comparison to direct spikes into MTBE. Methyl-trichloroacetate was used for determining recovery of TCA.

6. Conclusion

This method was selected to perform pharmacokinetic investigations of chloral hydrate in mice and rats because of its sensitivity and reliability.

Table 1
Accuracy/precision

Drug/ metabolite	Concentration added (μg/ml)	Concentration recovered mean±SD (n=9)	Coefficient of variation (%)
CH	5.0	4.98±0.20	4.0
	40.0	40.4±1.0	2.5
TCA	5.0	4.95±0.46	9.3
	40.0	40.3±0.5	1.3
TCE	5.0	4.64±0.19	4.0
	40.0	39.5±1.5	3.8

Table 2
Recoveries of CH and metabolites from plasma

Drug/ metabolite	Recovery mean±SD (%)	Coefficient of variation (%)
CH	84±9 (n=6)	10.3
TCA	99±3 (n=4)	3.3
TCE	94±2 (n=4)	2.0

Analyses were conducted under GLP guidelines of the US Food and Drug Administration.

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