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Determination of chloral hydrate metabolites in human plasma by gas chromatography-mass spectrometry

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Abstract

Chloral hydrate (CH) is a widely used sedative. Its pharmacological and toxicological effects are directly related to its metabolism. Prior investigations of CH metabolism have been limited by the lack of analytical techniques sufficiently sensitive to identify and quantify metabolites of CH in biological fluids. In this study a gas chromatography/mass spectrometry (GC/MS) method was developed and validated for determining CH and its metabolites, monochloroacetate (MCA), dichloroacetate (DCA), trichloroacetate (TCA) and total trichloroethanol (free and glucuronidated form, TCE and TCE-Glu) in human plasma. Of these, DCA and MCA are newly identified metabolites in humans. The drug, its plasma metabolites and an internal standard, 4-chlorobutyric acid (CBA), were derivatized to their methyl esters by reacting with 12% boron trifluoride-methanol complex (12% BF_3-MeOH). The reaction mixture was extracted with methylene chloride and analyzed by GC/MS, using a selected ion monitoring (SIM) mode. The quantitation limits of MCA, DCA, TCA, and TCE were between 0.12 and 7.83 μM. The coefficients of variation were between 0.58 and 14.58% and the bias values ranged between −10.03 and 14.37%. The coefficients of linear regression were between 0.9970 and 0.9996.

Keywords: Chloral hydrate; Monochloroacetate; Dichloroacetate; Trichloroacetate; Trichloroethanol; Gas chromatography-mass spectrometry

1. Introduction

Chloral hydrate (CH) is a widely used sedative for children and adults [1] and is also a metabolite of some chlorohydrocarbons, such as tri- and tetra-chloroethylene [2–4]. Since the pharmacology and toxicology of CH [5–7] and these chloroethylenes [2–4] are directly related to their metabolism, identifying and quantifying CH metabolites are important in interpreting the pharmacological and toxicological effects of these

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compounds. It has been reported that CH is metabolized to trichloroacetate (TCA), trichloroethanol (TCE) and its glucuronide (TCE-Glu) in human [8–10]. However, prior methods for analyzing CH metabolites either lacked specificity or were unable to determine simultaneously all the metabolites. Since CH and metabolites lack strong UV absorption and radioisotopes are impractical for human investigation, high performance liquid chromatography is not an appropriate analytical tool to employ. An early study indicated that CH and its metabolites could not be analyzed by GC using a flame ionization detector [11]. Although CH and its chlorinated metabolites were sensitive to an electron capture detector (ECD), the published GC/ECD methods are still not able to determine CH and all of its known metabolites simultaneously [9,10]. Despite the fact that GC/MS is more selective and sensitive than these other techniques, previous GC/MS methods have been utilized only for structure identification of CH metabolites [12] and for quantitation of TCE [13,14].

In this study, we developed and validated a sensitive and selective GC/MS method that is applicable to the study of CH kinetics and metabolism in humans. The technique enables the simultaneous identification of all the known metabolites of CH from a small plasma sample.

2. Experimental

2.1. Materials

Chloral hydrate (10% in syrup) used in clinical investigation was obtained from Geneva Pharmaceuticals (Broomfield, CO). Other chemicals used in the study were, DCA sodium salt (TCI America, Portland, OR), MCA, CBA, dichloroaldehyde diethyl acetal and methyl esters of DCA, MCA, TCA and 12% BF_3-MeOH (Aldrich, Milwaukee, WI), TCA and pure CH (Sigma, St. Louis, MO) and methylene chloride (Fisher Scientific, Pittsburgh, PA). All chemicals used were reagent grade, except methylene chloride, which was pesticide grade. They were used without further purification. Deionized water was prepared in our laboratory using a Milli-Q Water System (Millipor, Bedford, MA). Fresh frozen human plasma was purchased from Civitan Regional Blood Center (Gainesville, FL).

2.2. Drug administration and plasma sample collection

This study was approved by the Institutional Review Board of the University of Florida and informed consent was obtained from each subject. Eleven children with congenital lactic acidosis were hospitalized in the General Clinical Research Center of Shands Hospital, University of Florida. CH was administered orally (50 mg kg^{-1} in syrup) and venous blood samples (0.4 ml) were collected in heparinized tubes at 0, 1/4, 1/2, 1, 2, 4, 6, 8, 12 and 24 h after the dose. Blank control samples were collected at the same time spots a day before CH administration. Samples were placed on ice and then centrifuged at 1200 g for 15 min at 4°C in a Sorvall RT6000B refrigerated centrifuge (DuPont, Delaware, NJ). The supernatants were stored at -20°C before derivatization.

2.3. Derivatization and extraction

Following oral administration of CH to children, the drug and metabolites in plasma samples plus the internal standard CBA were derivatized to their methyl esters by reacting with 12% boron trifluoride-methanol complex. The derivatization procedures followed published methods [15] with minor modification. A plasma sample (200 μl) and an internal standard (CBA, 500 μM in water, 50 μl) were mixed with 500 μl of 12% BF_3-MeOH in a sealed glass cell culture tube. The mixture was heated at 110°C for 15 min. After cooling, 1 ml of methylene chloride and 1 ml of water were added to the reaction solution. The mixture was vortexed using a Vortex Genie 2 mixer (Fisher Scientific, Pittsburgh, PA) for 2 min, allowed to stand for 10 min, and then centrifuged at $2500 \times g$ in a Beckman J-6B centrifuge (Beckman Instruments, Palo Alto, CA) at 10°C for 8 min. The methylene chloride layer was transferred to a sample vial for GC/MS analysis.

Table 1
Mass fragments for selected ion monitoring in GC/MS analysis

Compound	CH	CH-$(Me)_2$	MCA-Me	TCA-Me	DCA-Me	CBA-Me	TCE
RT (min)	4.71	6.02	8.47	10.15	10.76	12.42	15.95
Fragments (m/z)	47	48	49	59[a]	59[a]	49	49[a]
	82[a]	49[a]	51	63	63	59	77
	84	50	59[a]	66	77	74[a]	79
	111	51	64	82	79	82	82
	113	76	66	84	83		84
		84	77	117	85		86
	117	86	79	119			112
		112	108	141			114
	119	114	110	143			
	146						
	148						

[a] Target ion fragment used for quantitation.

To evaluate the conversion of TCE-Glu to free TCE during the derivatization, some samples (0.2 ml each) were directly mixed with 0.5 ml of methanol (in comparison with 0.5 ml 12% BF_3-MeOH used for derivatization) and 1 ml water, and then extracted with 1 ml of methylene chloride, following the same procedures described above. The amount of TCE determined from direct derivatization of plasma included both free TCE and that from degradation of TCE-Glu. The amount of TCE-Glu was calculated as the difference between samples that were derivatized first and then extracted and those that were extracted without derivatization.

2.4. GC/MS conditions

The GC/MS system consisted of a Hewlett-Packard (Palo Alto, CA) 5890 series II plus gas chromatograph, a 5972A series mass selective detector, a 6890 series auto-injector, a G1512A autosampler controller and a Gateway P5-133 computer using ChemStation software (version C.03.02). The column was a 30 m long HP-Wax (crosslinked polyethylene glycol) with 0.25 mm ID and 0.15 μm film thickness. The carrier gas was 99.995% pure helium (zero grade, Jacksonville Welding Supply, Jacksonville, FL) and it was used at a flow rate of 1.21 ml min^{-1} (40.8 cm s^{-1}) with a head pressure of 9.4 psi at 40°C. The sample in methylene chloride solution was injected using a splitless mode and the injection volume was 1 μl. Temperatures of the injection port and mass selective detector interface were set at 150 and 280°C, respectively. The temperature gradient of the GC oven was programmed to be initiated at 40°C for 2 min, raised to 100°C at 5°C min^{-1}, then raised to 240°C at 50°C min^{-1} and held at the final temperature for 2 min. An electron impact (EI) ionization mode with the ionization energy of 70 eV was used for the MS detector. Perfluorotributylamine was the calibration compound.

Employing a full mass spectra scan mode in comparison with the authentic compounds identified chemical structures of CH and metabolites. The MS detector was calibrated by standard spectra autotune for structural identification. The MS detector was calibrated by maximum sensitivity autotune and a selected ion monitoring (SIM) mode was used for the quantitation of CH and its metabolites. As shown in Table 1, specific mass fragments were selected for characterizing TCE, methylated CH and methyl esters of MCA, TCA, DCA and CBA, while only the target ions were applied for quantitation using the real time executive integrator in the ChemStation software.

Table 2
Precision and accuracy of the assay in human plasma

Compound	Spiked (μM)	Intra-day ($n = 5$) Mean ± SD (μM)	RSD (%)	Bias (%)	Inter-day ($n = 5$) Mean ± SD (μM)	RSD (%)	Bias (%)
		$r^2 = 0.9976$			$r^2 = 0.9970$		
CH	146.01	151.08 ± 4.50	2.98	3.48	144.17 ± 10.42	7.23	−1.26
	73.01	69.97 ± 7.80	11.15	−4.15	78.98 ± 1.81	2.29	8.18
	36.5	34.17 ± 3.31	9.7	−6.38	37.79 ± 1.30	3.43	3.54
	18.25	18.88 ± 1.27	6.75	3.43	17.00 ± 0.57	3.37	−6.85
	9.13	10.25 ± 1.29	12.58	12.27	10.44 ± 0.54	5.19	14.37
		$r^2 = 0.9996$			$r^2 = 0.9992$		
MCA	5.66	5.68 ± 0.16	2.82	0.37	5.74 ± 0.36	6.27	1.41
	2.83	2.92 ± 0.13	4.45	3.21	2.79 ± 0.26	9.31	−1.36
	1.42	1.47 ± 0.09	6.21	3.68	1.52 ± 0.11	7.34	6.8
	0.71	0.72 ± 0.07	9.77	0.72	0.73 ± 0.06	8.59	3.25
	0.36	0.33 ± 0.03	10.22	−8.33	0.32 ± 0.04	11.07	−10.03
		$r^2 = 0.9989$			$r^2 = 0.9971$		
DCA	79.84	79.61 ± 1.28	1.61	−0.29	77.88 ± 3.16	4.06	−2.45
	39.92	38.91 ± 0.43	1.1	−2.54	42.27 ± 1.35	3.2	5.88
	19.96	21.87 ± 0.64	2.93	9.56	22.1 ± 0.85	3.83	10.73
	9.98	10.7 ± 0.31	2.92	7.19	10.64 ± 0.31	2.96	6.57
	4.99	4.93 ± 0.20	4.02	−1.27	4.63 ± 0.16	3.48	−7.27
		$r^2 = 0.9971$			$r^2 = 0.9974$		
TCA	243.77	253.8 ± 19.87	7.83	4.11	238.58 ± 22.62	9.48	−2.13
	121.89	115.47 ± 6.65	5.76	−5.27	128.57 ± 5.72	4.45	5.48
	60.94	58.43 ± 3.04	5.21	−4.11	56.24 ± 3.13	5.57	−7.71
	30.47	30.92 ± 0.81	2.62	1.48	29.81 ± 2.22	7.44	−2.18
	15.27	17.37 ± 1.34	7.74	13.77	17.05 ± 2.49	14.58	11.64
		$r^2 = 0.9991$			$r^2 = 0.9988$		
TCE	227.58	229.42 ± 6.86	2.99	0.81	230.83 ± 3.22	1.39	1.43
	113.79	110.23 ± 0.64	0.58	−3.13	109.28 ± 12.76	11.68	−3.96
	56.89	59.59 ± 2.29	3.84	4.75	57.36 ± 2.37	4.14	0.82
	28.45	30.50 ± 1.42	4.64	7.21	31.20 ± 1.27	4.08	9.67
	14.22	13.93 ± 0.42	3.04	−2.03	13.62 ± 0.60	4.42	−4.19

2.5. Calibration curves

Calibration of CH and its metabolites was performed by adding the compounds to human plasma (Civitan Regional Blood Center, Gainesville, FL). The concentration of the internal standard, CBA, was selected because its peak area is comparable to that of the highest levels of CH metabolites. Peak plasma concentrations of CH and its metabolites following oral administration of a 50 mg kg^{-1} dose of CH were usually < 250 μM. Therefore, the concentration ranges of calibration for TCA, MCA, DCA and TCE were set between 0.1–250 μM. Specific concentrations of CH and metabolites tested in plasma are listed in Table 2. The calibration curves were plotted as concentration versus the ratio of peak area of the compound/peak area of the internal standard.

2.6. Recovery of derivatization and extraction

TCE and methyl esters of DCA, MCA and TCA were dissolved in methylene chloride to make a stock solution containing 500 μM of each component. This solution was diluted to 250, 125,...0.1 μM with methylene chloride and three dilutions at each concentration were made. To evaluate the efficiency of extraction, the solutions

were treated in a manner similar to that described in Section 2.3, i.e. a 1 ml aliquot of each of the methylene chloride solution was mixed with the derivatization reagent (0.5 ml of 12% BF_3-MeOH) and washed with 1 ml water, vortexed for 2 min and centrifuged at 2500 g at 10°C for 8 min. The methylene chloride layer was separated from the extraction mixture, and this extract and the original unextracted methylene chloride solutions were assayed by GC/MS.

The results from the solutions with or without extraction were analyzed by linear regression. The efficiency of extraction was calculated as the ratio of slope of the calibration curve of each compound through extraction vs. that of the calibration curve of the original solution without extraction, and multiplied by a factor of volume change during extraction (factor of extraction efficiency = $\text{slope}_{(\text{extract})}/\text{slope}_{(\text{non-extract})}$ X factor of volume change). The calibration curves were obtained by using the same method for calibration of the compounds spiked in water and plasma through derivatization. On the other hand, the recovery of derivatization was calculated as the ratio of the slope of the calibration curve of each compound obtained from derivatization versus that of each reference methyl ester directly spiked in methylene chloride, and divided by the factor of extraction efficiency (recovery of derivatization reaction = $\text{slope}_{(\text{deriv})}/\text{slope}_{(\text{non extract})}$/factor of extraction efficiency = $\text{slope}_{(\text{deriv})}$ X $\text{slope}_{(\text{extract})}$.

2.7. Method validation

Precision was determined by calculating the intra-day and inter-day coefficients of variance or relative standard deviations (RSD %). Intra-day and inter-day variances were measured using the same plasma or water stock solutions prepared for calibration curves. To measure the intra-day variance, five sets of derivatized and extracted samples were made at each concentration level, using the plasma or water stock solutions. One set of derivatization and extraction was carried out on each of the following four successive days for measuring the inter-day variance. The intra-day variance was calculated based on the five trial measurements accomplished in the first day, and the inter-day variance was calculated based on the results of five trial analyses performed on five consecutive days. Accuracy was determined by comparing concentrations measured from extracted samples with those determined from solutions to which compounds were directly added, and was expressed as bias (percentage difference between the measured and added concentrations). The intra-day calibration curves were used to quantitate CH and its metabolites in human plasma.

2.8. Limit of quantitation

To determine the limit of quantitation for each compound, besides the calibration solutions used above, different sets of solutions of each compound in human plasma over a range 0.01–20.00 μM were prepared and assayed. The limit of quantitation for each compound was determined as the lowest concentration (mean of the results of five assays) at which the standard deviation was $< 15\%$ of the mean.

3. Results

3.1. Typical total ion chromatograms (TIC)

Presented in Fig. 1 are the TIC of the authentic compounds spiked in water (panel A), the TIC of a plasma sample collected from a child before CH administration (B) and the TIC of a plasma sample of the child collected 1 h after an oral administration of 50 mg kg^{-1} CH in syrup (panel C). The concentrations of CH, MCA, TCA, DCA and TCE were 8.93, 1.84, 28.42, 119.82 and 84.41 μM, respectively (panel A). The concentrations of CH, MCA, TCA, DCA and TCE were 7.48, 1.08, 112.37, 34.81 and 320.77 μM, respectively (panel C). CBA was the internal standard, at a concentration of 125 μM. The compounds detected are methyl esters of MCA, TCA, DCA, CBA and methylated CH as trichloroacetaldehyde dimethyl acetal (CH-Me_2) and free TCE (including free TCE and the part of TCE formed from degradation of TCE-Glu during derivatization).

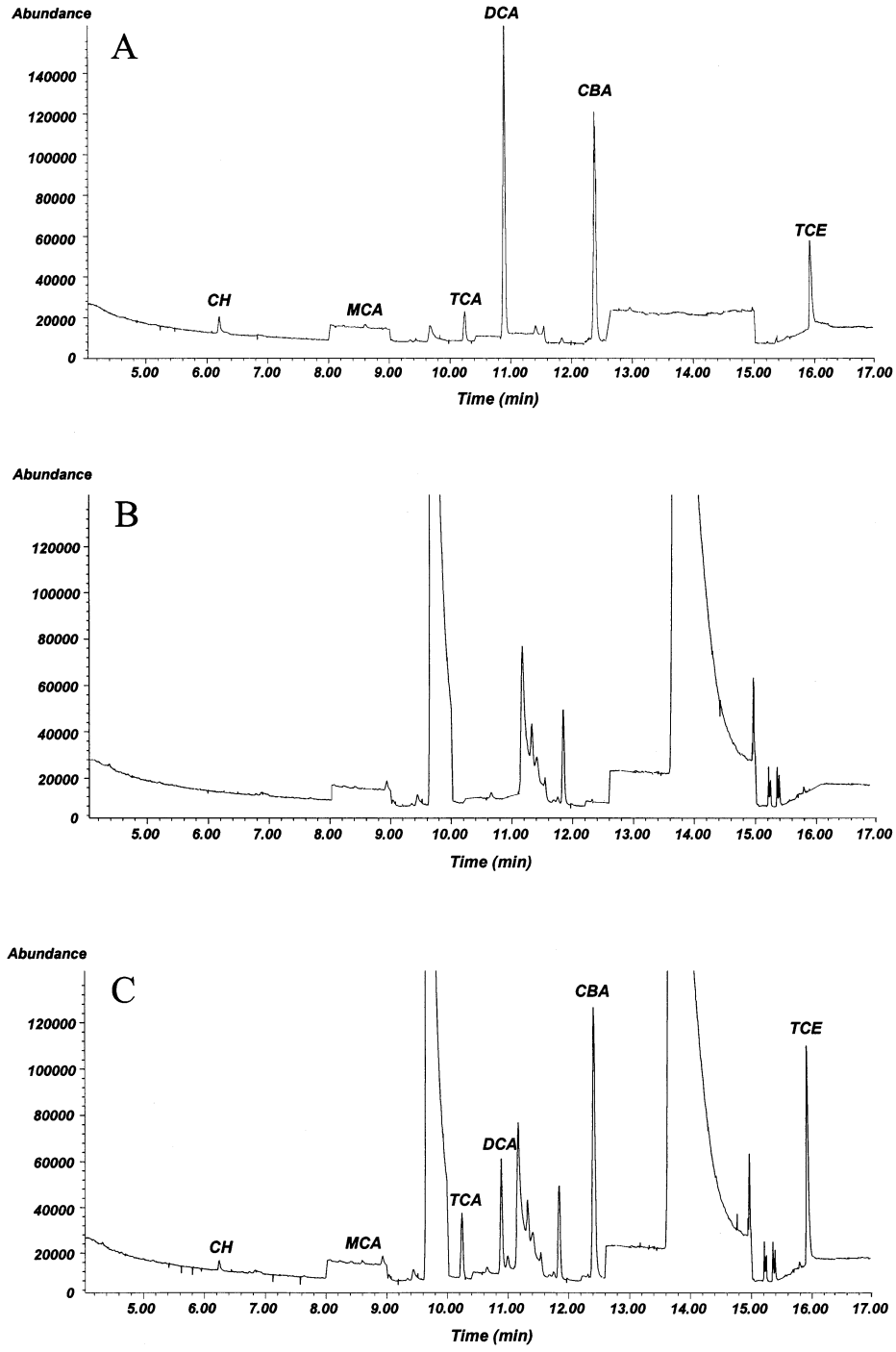

Fig. 1. TIC of GC/MS analysis using the selected ion monitoring mode. Panel A was from the authentic compounds spiked in water, panel B was from a plasma sample collected from a child before CH administration, and panel C was from a plasma sample collected from the child 1 h after an oral dose of CH (50 mg kg^{-1}). MCA, TCA, DCA and CBA are methyl esters of parent molecules and CH is the dimethyl acetal CH-Me_2 TCE includes both free TCE and that derived from TCE-Glu.

Table 3
Recovery of derivatization, extraction efficiency and limit of quantiation of human plasma ($n = 5$)

Compound	Recovery of derivatization[a] mean ± SD (%)	Extraction efficiency[b] mean ± SD (%)	Total recovery mean ± SD (%)	Limit of quantiation mean ± SD (μM)	RSD (%)
CH-Me_2	21.34 ± 0.88[c]	86.05 ± 0.923[c]	18.36 ± 0.97[c]	7.83 ± 0.88	11.25
MCA-Me	78.34 ± 0.21	88.85 ± 0.35	80.71 ± 4.20	0.18 ± 0.02	13.29
DCA-Me	92.43 ± 0.45	84.24 ± 1.34	86.84 ± 5.47	0.12 ± 0.02	12.67
TCA-Me	27.62 ± 1.17	88.56 ± 0.87	24.46 ± 2.21	7.64 ± 1.13	14.83
TCE		70.26 ± 0.41	24.15 ± 1.08	7.11 ± 0.53	7.55

[a] Determined from the derivatization and extraction of the parent compounds from plasma.
[b] Determined from the extraction of methylated standards (except in the case of TCE which is unmethylated) from plasma.
[c] Dichloroacetaldehyde diethyl acetal was used in place of CH-Me_2 because no commercial authentic CH-Me_2 was available.

3.2. Accuracy and precision

Quantitation was based on the peak area ratio of the target ions in SIM of the compound to that of the internal standard. CH and its metabolites were not detected in the blank control samples, the derivatization reagent or the extraction solvent. The calibration curves obtained from intraday determinations were used for quantitation of the plasma samples. Precision and accuracy of the present method were expressed by standard deviation (SD), relative standard deviation (RSD) and bias values listed in Table 2. The linearity was represented by the regression coefficient value r^2. There were no significant differences between the r^2 values from different determinations for each compound.

3.3. Limit of quantitation and recovery of derivatization and extraction

As presented in Table 3, total recoveries from derivatization and extraction are calculated as the ratio of the slope of the calibration curve of each compound from derivatization and extraction versus that of the calibration curve of the corresponding methyl ester directly spiked in methylene chloride. Extraction efficiency was obtained as the ratio of the slope of the calibration curve of each compound through extraction versus that of the calibration curve of original solution without extraction, and multiplied by a correction factor for volume change during extraction. In this case, 0.93 ml of methylene chloride was recovered from 1 ml used in the extraction, i.e. the correction factor for volume change was 93%. The recovery of derivatization reaction was calculated by multiplying the value of the total recovery by the extraction efficiency. The recovery of CH was not obtained, because authentic CH-Me_2 was not commercially available.

The TCE concentrations determined using the current method included free TCE and TCE-Glu. Since only a limited amount of plasma from the children was available for our experiments, only four typical samples collected from four children 12 h after CH administration were assayed to demonstrate that the conversion of TCE-Glu to free TCE occurred during derivatization. The results indicated that 55.09 ± 20.97% (mean ± SD) of the total TCE was derived from TCE-Glu and the remainder originated from free TCE. The high SD value resulted from individual differences in the rate of biotransformation of TCE to TCE-Glu.

The limit of quantitation of each compound was determined based on results obtained from different analyses performed on five consecutive days. The limit of quantitation of each compound was about 10 times higher than the lowest detectable signal. Although the standard deviation levels of limit of quantitation were lower that 15% of the mean, they might be out of the linear range of the calibration. The values for the limit of quantitation were directly related to the recoveries

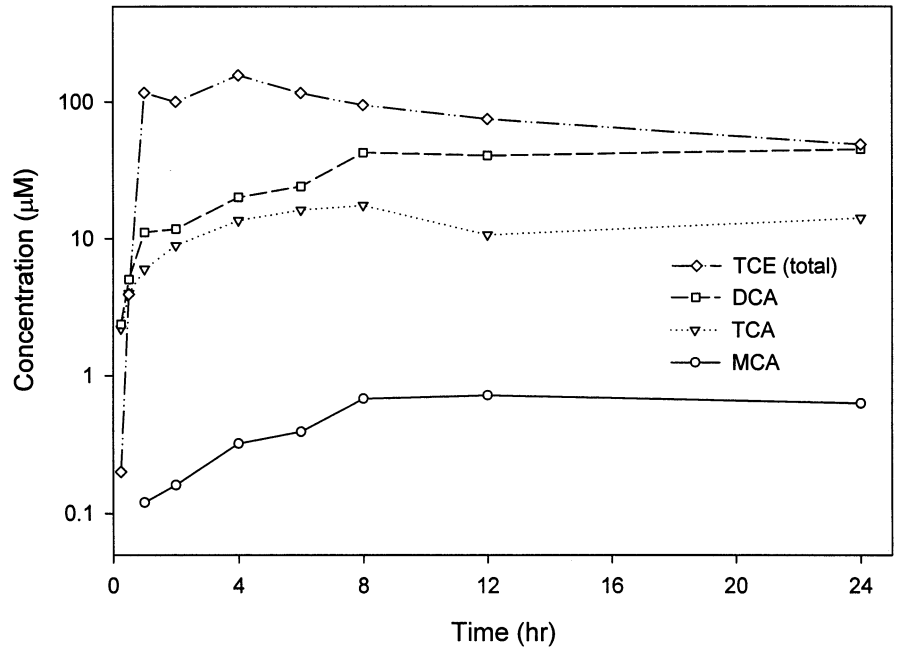

Fig. 2. Plasma concentrations of CH metabolites after a single oral dose of CH (50 mg kg^{-1}) to a child.

of derivatization and extraction, as shown in Table 3.

3.4. Application

The present method was developed to facilitate studies of the kinetics and metabolism of CH in humans, and has been applied to more than 400 plasma samples containing CH and its metabolites. In a typical case, a child received a single dose of CH (50 mg kg^{-1} in syrup) by mouth. The plasma samples collected within 24 h after the dose of CH were assayed using the present GC/MS method, and the plasma concentrations of CH metabolites, i.e. MCA, DCA, TCA and TCE (free and glucuronide) are shown in Fig. 2. This investigation was the first to identify MCA and DCA as CH metabolites in humans. Since CH was cleared from the plasma in a short time, it was detectable only in the first few minutes after administration. Therefore the data for CH concentrations were not presented in Fig. 2. Based on our results and those of previous reports (8–10, 16), a schematic of the probable pathways of CH biotransformation is illustrated in Fig. 3.

4. Discussion

4.1. Derivatization and extraction

Silylation and esterification are commonly used methods to derivatize the carboxylic acid to a non-polar group. Both methods have been applied to the study of TCA formation from CH. The advantage of silylation is that both the carboxylic acid group of TCA and the hydroxy group of TCE can be protected, and the molecular ion or typical fragments of silylated groups may be useful in structure identification [12,14]. However, silylation reagents are sensitive to moisture and so the samples must be dried before they are derivatized. Unreacted silylation reagents may contaminate GC columns and detectors.

In contrast, derivatizing of TCA by esterification can be conducted in an aqueous environment by reacting the sample with BF_3 (or BCl_3)-MeOH [12,15], dimethyl sulfate [9] and diazomethane [10] and no laborious drying or extraction procedures are required for using these reagents. Since these reaction mixtures can be purged with water and

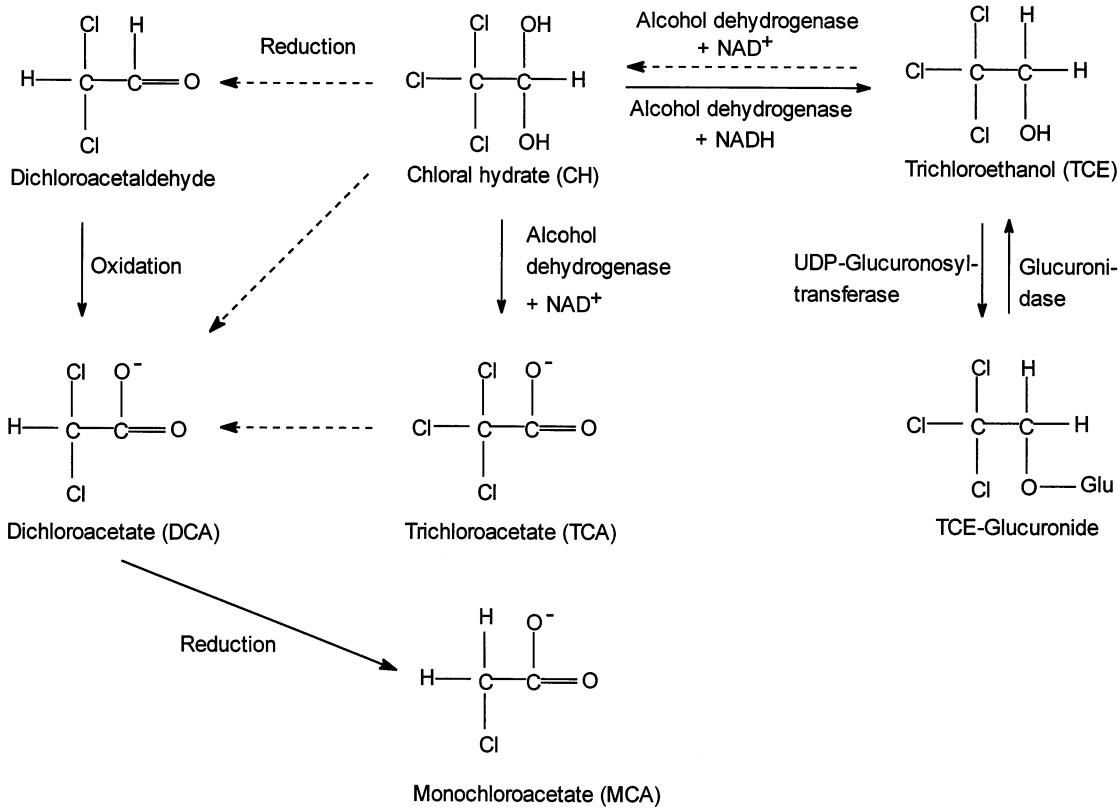

Fig. 3. Proposed pathways of CH biotransformation in humans. Fig. 3 is a modification of that published in [16] and it is presented here with the permission of the copyright owner. Solid arrows indicate established pathways and broken arrows indicate postulated pathways.

extracted with organic solvents, the final extract will be cleaner than those obtained from silylation, and this minimizes background noise and contamination of the column and detector. We chose BF_3-MeOH, because dimethyl sulfate, diazomethane and diazoethane have low solubilities in aqueous samples. BF_3-MeOH can precipitate plasma proteins and dissolve the analytes and it can also be washed out with water after the derivatization reaction.

In addition, since TCE-Glu is too polar to be assayed by GC, converting TCE-Glu to TCE is a required procedure in determining the presence of TCE-Glu as a metabolise of CH. Although TCE-Glu can be degraded by β-glucuronidase [8,10,12] or concentrated sulfuric acid [9], it is known that TCE-Glu can be converted by BF_3 (or BCl_3)-MeOH to free TCE [12]. Therefore, the steps to convert TCE-Glu to TCE and to esterify TCA, DCA and MCA are merged by using BF_3-MeOH as the derivatization reagent.

Aldehydes can also be converted to dimethyl acetals by reacting with BF_3-MeOH [17]. In our method, CH was converted to CH-Me_2 during derivatization, and the latter can be extracted easily with methylene chloride. Nevertheless, were it necessary to quantitate free CH and TCE, plasma samples could be extracted with methylene chloride without prior derivatization, and the extract could be analyzed using the GC/MS conditions of the present method. The retention times of free CH and TCE are 4.71 and 15.99 minutes, respectively. Since the hydroxy group of TCE was not protected during derivatization, almost 30% of the analyte was lost in the aqueous phase (containing about 30% of methanol) during extraction.

4.2. Protein binding

In addition to loss during extraction, protein binding may affect sample recovery. According to previous reports, over 71% of TCA (6-612 μM) and 35% of TCE (20–515 μM) are bound to human plasma proteins [8,18]. The effect of plasma protein binding on the calibration curves of TCA and TCE was also reported by Breimer et al. [9]. Thus, the low recoveries of TCA and TCE from samples in our study may be due to protein binding (as shown in Table 3). Considering that protein binding with TCA and TCE might be broken during the derivatization reaction, another possibility is that TCA and TCE were made very soluble in water by the presence of MeOH from the derivatizing reagent and were lost in the aqueous phase during extraction.

It is interesting that CH has similar analytical characteristics to those of TCA and TCE (Table 3). A possible reason why CH has a high limit of quantitation is that methylation of CH to CH-Me_2 was incomplete, and the unchanged CH was lost in the aqueous phase (containing about 30% methanol) during extraction. It should be noted that the present method can simultaneously quantitate both CH and CH-Me_2. There is no significant difference in extraction efficiencies among dichloroaldehyde diethyl acetal, MCA, DCA and TCA (Table 3).

In conclusion, a GC/MS method was developed and validated for the determination of CH and its in vivo metabolites MCA, DCA, TCA and TCE (free and glucuronidated) in human plasma. Our investigation has also identified two new metabolites of CH (DCA and MCA) in humans. This new method should facilitate future clinical studies of the kinetics and biotransformation of CH.

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