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Sensitive high-performance liquid chromatography method for the simultaneous determination of low levels of dichloroacetic acid and its metabolites in blood and urine

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Abstract

Dichloroacetic acid (DCA) is a contaminant found in treated drinking water due to chlorination. DCA has been shown to be a complete hepatocarcinogen in both mice and rats. In this study we developed a rapid and sensitive high-performance liquid chromatography (HPLC) method to simultaneously detect DCA and its metabolites, oxalic acid, glyoxylic acid and glycolic acid in blood and urine samples of animals sub-chronically administered with DCA (2 g/l) in drinking water. Both urine and plasma samples were treated minimally before HPLC analysis. Separation and detection of DCA and its metabolites were achieved using an anion-exchange column and a conductivity detector. The mobile phase consisted of an initial concentration of 0.01 m*M* sodium hydroxide in 40% methanol followed by a linear gradient from 0.01 m*M* to 60 m*M* sodium hydroxide in 40% methanol for 30 min. The lower detection limit for DCA and each of its three major metabolites was 0.05 μg/ml. DCA and its metabolites gave a linear response range from 0.05 to 100 μg/ml. Plasma DCA was also analyzed by gas chromatography (GC), and the results obtained correlated with those from the HPLC method (correlation coefficient=0.999). While available HPLC techniques offer sensitive procedures to detect either glycolic acid or oxalic acid, the described HPLC method has the unique advantage of determining simultaneously the parent compound (DCA) and its three major metabolites (oxalic acid, glyoxylic acid and glycolic acid) in biological samples, without complex sample preparation.

Keywords: Dichloroacetic acid; Oxalic acid; Glyoxylic acid; Glycolic acid

1. Introduction

Dichloroacetic acid (DCA) is a by-product of drinking water chlorination. DCA concentrations of over 100 μg/ml have been reported in chlorine disinfected water [1]. DCA is also a minor metabolite of trichloroethylene (TRI) in mice [17]. Moreover, DCA has been used therapeutically for treatment of congenital lactic acidosis [2], diabetes [3] and hypercholesterolemia [4]. However, its therapeutic use has been limited due to its adverse effect reported in laboratory animals [5].

DCA has been associated with central nervous

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system toxicity in dogs and rats [5], developmental toxicity in rats [6,7], and has been shown to be an inducer of hepatic tumors in mice and rats [8–10]. In recent years DCA has been closely associated with carcinogenicity of TRI, which has been shown to induce hepatic tumors in mice [11,12], but not rats [13], whereas DCA has been reported to induce hepatic tumors in both mice [9] and rats [14].

Because of DCA's potential carcinogenicity and widespread distribution in water supplies, there is an increasing interest in DCA metabolism and disposition. DCA is metabolized to several metabolites including chloroacetic acid, glyoxylic acid, glycolic acid, oxalic acid, glycine, hippuric acid and carbon dioxide [9,13–15]. Oxygenation of DCA to glyoxylic acid is carried out by the newly discovered Zeta class glutathione transfereses [16]. DCA has been detected and measured in biological samples using gas chromatography (GC) [8,18] and mass spectrometry (MS) with electrospray ionization [19]. The lower limits of detection (LOD) have been reported to be 1 μg/ml and 4 ng/ml for GC and MS methods, respectively. Although both methods can detect low levels of DCA, derivatization and extraction procedures are needed for sample preparation and neither method is able to separate and quantify the major metabolites of DCA. DCA and its metabolites, glycolate, oxalate and glyoxylate, have been separated and quantified using [^{14}C] DCA and multiple HPLC runs in DCA pharmacokinetics studies [19,20]. In order to investigate the DCA metabolism and disposition in B6C3F$_1$ mice, we have developed an HPLC method which does not require the labor intensive derivatization and extraction procedures. The sensitivity of this HPLC method for quantification of DCA is comparable to or exceeds that of previously reported GC methods and is considerably less labor intensive.

2. Materials and methods

2.1. Reagents and chemicals

DCA (99.9% purity) and its metabolites glycolic acid (99.9% purity), glyoxylic acid (99.9% purity) and oxalic acid (99.9% purity) were obtained from Sigma (St. Louis, MO, USA). Sodium hydroxide and 70% perchloric acid (ACS reagent) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Methanol (HPLC Grade) was purchased from Sigma. The water for the study was deionized and distilled in our laboratory.

2.2. Animal treatment and sample collection

Male hybrid mice, B6C3F$_1$/CrlBR (25–35 g) were maintained in polycarbonate cages with hardwood chip bedding. The animals were housed one per cage and provided with food and water ad libitum. Treated animals were provided with drinking water containing 2.0 g/l of DCA, neutralized to pH 7 with NaOH to account for possible decreases in the water consumption. Control animals were provided with drinking water containing 2.0 g/l NaCl. Endogenous levels of metabolites of DCA were not detected with the method in control animals. Following 26 days of treatment animals were placed in metabolism cages for a 24 h period and urine samples were collected. All animals were killed after 28 days of treatment by CO_2 asphyxiation at 0, 2, 4 and 8 h post-treatment ($n=8$). Blood samples were collected via the inferior vena cava using heparinized syringes. For the GC analysis of DCA in plasma, samples were quenched with an equal volume of 20% lead acetate (0.2 ml) and stored at −80°C pending analysis. For the HPLC analysis of DCA and its metabolites, plasma samples were precipitated with ice cold 0.17 *M* perchloric acid; urine samples and acid precipitated plasma samples were then centrifuged at 31 500 *g* for 30 min at 4°C. Supernatants were filtered through 0.45 μm nonsterile acrodisc syringe filters provided with versapor (supported acrylic copolymer) membranes (Pall Gelmann Laboratory, Ann Arbor, MI, USA) and stored at −80°C for further analysis. Supernatants from urine samples were diluted with deionized water (1:100) before use.

2.3. Instrumentation and conditions

2.3.1. HPLC

HPLC determinations were performed with a Model Dx-300 (Dionex, Sunnyvale, CA, USA) liquid chromatographic system equipped with background conductivity suppressor. The chromatograph-

ic system consisted of an advanced gradient pump (AGP standard size), conductivity detector (CDM-3), anion self regenerating suppressor (ASRS 4 mm), for the reduction of the background conductivity of the eluent, autosampler (AS-3500), 100 μl sample loop, computer interface ACI and software AutoIon 450. Separation of DCA and its metabolites was performed on a Dionex AS11 analytical column (250*4 mm) preceded by a Dionex AG11 guard column (4×50 mm). The sensitivity of the detector was maintained between 0.5 and 100 μs depending on the concentration.

2.4. Mobile phase

The mobile phase consisted of an initial concentration of 0.01 m*M* sodium hydroxide in 40% methanol and a linear gradient from 0.01 m*M* to 60 m*M* sodium hydroxide in 40% methanol in 30 min. The mobile phase was filtered through a 0.45 μm filter provided with nylon membrane (Micron Separations Inc., Westboro, MA, and USA) and then degassed under vacuum before use. The flow-rate was set at 1 ml/min.

2.5. Preparation of standards

The standards were prepared in the way as that of the samples, using spiked urine and blood samples obtained from untreated control rats. The concentration of the prepared standards were in the range 0.05–100 μg/ml.

2.5.1. GC

DCA was quantified in the blood samples by a previously described gas chromatography method [17].

3. Results

HPLC standard curves constructed for DCA, glycolate, oxalate and glyoxalate were linear in the range of 0.05–100 μg/ml in both plasma and urine matrices. The regression coefficient (r^2) for all the standard curves was greater than 0.999. The equations of the calibration lines were $y=2387794.30x$ for glycolate, $y=80752.69x$ for glyoxalate, $y=2166574.96x$ for DCA and $y=3423662.42x$ for oxalate. For the lower limit of detection the regression line $y+3$ s was used for all compounds where y is the intercept and s is the standard deviation of the signal (noise) at the retention time of the compounds in both plasma and urine ($n=8$). The lower limit of detection (LOD) was 0.05 μg/ml for all four compounds in plasma and urine. Signal-to-noise ratio at 0.05 μg/ml was greater than 3 for all four compounds in plasma and urine. For DCA and its major metabolites, the mean analytical recovery (found/added) over the range 0.05–100 μg/ml was determined to be 100±1.8% ($n=8$) in both plasma and urine samples. Day-to-day precision (C.V.) of the method was within 1% ($n=10$) for all four compounds in both plasma and urine over the range 0.05–100 μg/ml. The mean between-assay coefficients of variation for all these compounds were lower than 1% over the range 0.05 μg–100 μg/ml. The within-assay coefficients of variation were lower than 1% measured at 0.05 or 10 μg/ml.

Figs. 1 and 2 represent the chromatograms obtained for urine and plasma samples spiked with DCA and its metabolites, respectively. The retention times for DCA, glycolic acid, glyoxylic acid and oxalic acid in plasma samples were 12.80, 10.46, 11.36, and 15.00 min, and in urine samples they were 12.95, 10.45, 11.25, and 15.27 min, respectively. No interfering endogenous compounds appeared in samples from the control or treated animals. Two unidentified peaks with retention times of 11.72 and 14.78 min were observed in the control and treated animals in both plasma and urine samples, and a small peak with a retention time of 10.61 was observed in all urine samples (Figs. 1 and 2). An unidentified peak with a retention time of 13.78 min was observed in control urine sample (Fig. 3).

Based on the average daily water consumption, the average daily dose of DCA was estimated to be 300 mg kg^{-1} day^{-1}. Neither DCA nor its major metabolites were found in detectable concentrations in control animals. The peak blood DCA concentration after 28 days of treatment (0 h) as determined by the HPLC method was 17.9±9.8 μg/ml (Fig. 4). Systemic clearance of DCA was rapid: after 28 days of treatment and 8 h suspension from treatment, no

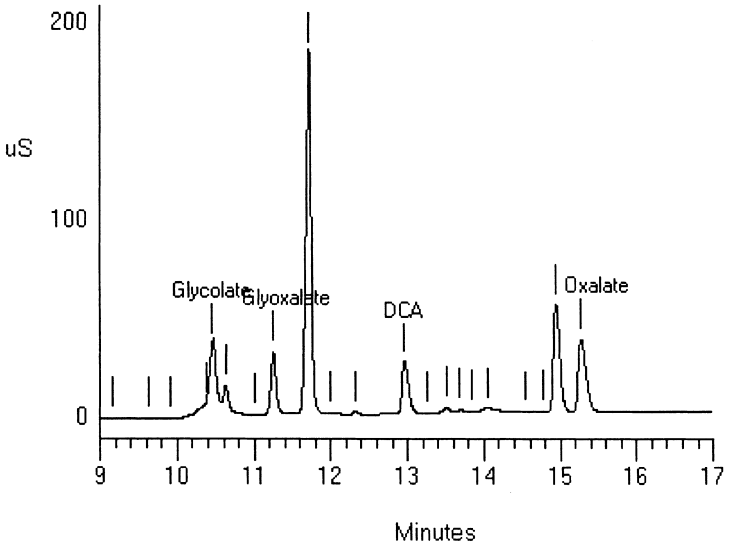

Fig. 1. Representative chromatogram obtained for DCA, and its metabolites glycolate, oxalate and glyoxalate prepared in urine samples from control animals. The *X*-axis of the chromatogram represents the peak retention time in minutes and the *Y*-axis of the chromatogram represents the conductivity in microseimens (μs) measured by the conductivity detector. The actual amounts of DCA and each of its metabolites injected into the HPLC system were 1 μg. The concentrations of glycolate, glyoxalate, oxalate and DCA were 1.31, 1.08, 1.11 and 0.78 μmol/l per each injection.

DCA was detected in the plasma. Plasma samples were also analyzed by a GC method for DCA.

Glycolate, oxalate and glyoxalate were also detected in the blood (Fig. 2) and urine (Table 1) of all treated animals. Glycolate concentration was higher in blood and urine than glyoxalate or oxalate. In blood samples, all three DCA major metabolites were present well above their LOD 8 h after the DCA treatment stopped. The concentration of DCA and it metabolites in urine samples were about 3–4

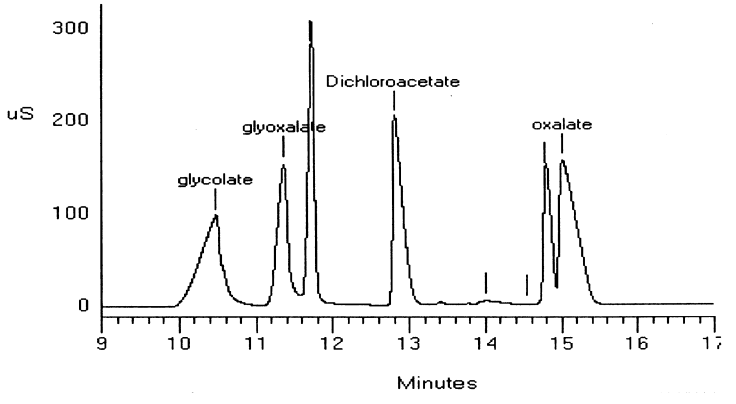

Fig. 2. Representative chromatogram obtained for DCA and its metabolites, glycolate, oxalate and glyoxalate prepared in the plasma obtained from the control animals. The *X*-axis of the chromatogram represents the peak retention time in minutes and the *Y*-axis of the chromatogram represents the conductivity in microseimens (μs) measured by the conductivity detector. The actual amounts of DCA and each of its metabolites injected into the HPLC system were 5 μg. The concentrations of glycolate, glyoxalate, oxalate and DCA were 1.31, 1.08, 1.11 and 0.78 μmol/l per each injection.

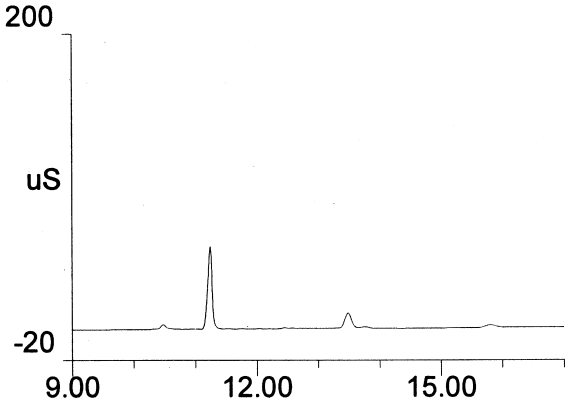

Fig. 3. Blank chromatogram of a urine sample without any additions.

orders of magnitude greater than the LOD. Glycolate, oxalate, glyoxalate were not detectable in control urine samples, since control urine samples were also diluted with deionized water (1:100) before use.

Table 1
Concentration of DCA and its metabolites in urine samples from control and DCA-treated mice collected in a 24-h period following 26 days of treatment (Mean±SD for $n=6$)

Compound	Urine concentration (mg/ml)	
	Control	DCA-treated
DCA	Not detected	0.74±0.6
Glycolate	Not detected	3.59±1.4
Glyoxalate	Not detected	0.46±0.25
Oxalate	Not detected	1.50±0.69

4. Discussion

A sensitive HPLC method was developed for the analysis of DCA and its three major metabolites, glycolate, glyoxalate and oxalate. Previously published methods to separate and detect DCA and its metabolites in biological samples have used extraction procedures and multi-step separations by HPLC [20,21]. In one study, radiolabeled DCA was used to detect only plasma DCA levels slightly greater than

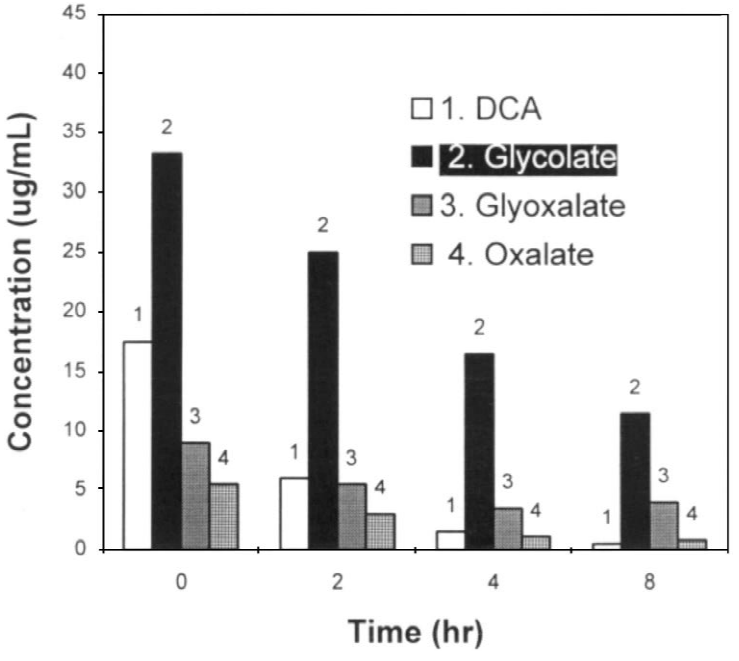

Fig. 4. Systemic clearance of DCA and its metabolites in the blood of mice treated with DCA for 28 days (Mean±SD, $n=8$). Significantly ($p=<0.05$) different than the 0 h time point.

1 μg/ml [20]. In another study using radioactive DCA, Lin et al reported a more labor intensive method that required a two step separation process [21]. In the first step DCA was separated using a reversed-phase liquid chromatography method, and in the second step the metabolites were separated using an anion-exchange liquid chromatography method DCA has also been analyzed by a GC method (LOD=1 μg/ml) which involves extensive sample preparation (acidification, derivatization and extraction) without the ability to detect any of its major metabolites. The present method involves a simple sample preparation which is followed by a one step HPLC separation and detection procedure. The LOD for this method was determined to be 0.05 μg/ml for DCA (as well as its three major metabolites). DCA has been detected and measured in biological samples using GC [8,17] and MS with electrospray ionization [18]. The lower limits of detection have been reported to be 1 μg/ml and 4 ng/ml for GC and MS methods, respectively. Although both methods can detect low levels of DCA, extensive derivatization and extraction procedures are needed for sample preparation and neither method is able to separate and quantify the major metabolites of DCA. While available HPLC techniques offer sensitive procedures to detect either glycolic acid or oxalic acid, the developed HPLC method has a unique advantage over the others [22–24]. The unique advantage is its capability for the simultaneous determination of the parent compound (DCA) and its three major metabolites; oxalic acid, glyoxylic acid and glycolic acid in biological samples, without long sample preparation time. The ability to detect low levels of DCA and its major metabolites (0.05 μg/ml) in biological samples makes this a suitable method to investigate DCA pharmacokinetics or to detect the presence of DCA and its metabolites in biological samples following clinical trials.

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References

[1] P.C. Uden, J.W. Miller, J. Am. Water Works Assoc. 75 (1983) 524–527.
[2] P.W. Stacpoole, Metabolism 38 (1989) 1124–1144.
[3] P.W. Stacpoole, Y.J. Green, Diabetes Care 15 (1992) 785–791.
[4] G.W. Moore, L.L. Swift, D. Rabinowitz, O.B. Crofford, J.A. Oates, P.W. Stacpoole, Atherosclerosis 33 (1979) 285–293.
[5] P.W. Stacpoole, G.W. Moore, D.M. Komhauser, N. Eng. J. Med. 300 (1979) 372.
[6] M.K. Smith, J.L. Randall, E.J. Reed, J.A. Stobber, Teratology 46 (1992) 217–233.
[7] D.L. Epstein, G.A. Nolin, J.L. Randall, S.A. Christ, E.J. Reed, J.A. Stober, M.K. Smith, Teratology 46 (1992) 225–235.
[8] R.J. Bull, I.M. Sanchez, M.A. Nelson, J.L. Larson, A.J. Lansing, Toxicology 63 (1990) 341–359.
[9] A.B. DeAngelo, F.B. Daniel, J.A. Stober, G.R. Olson, Fundam. Appl. Toxicol. 16 (1991) 337–347.
[10] R.E. Richmond, J.H. Carter, H.W. Carter, F.B. Daniel, A.B. DeAngelo, Cancer Lett. 92 (1995) 67–76.
[11] National Cancer Institute, Carcinogenesis Bioassay of Trichloroethylene, DHEW Publication (US NIH) (1976) PB-264-122, NCI-CG-TR-2, pp. 1–196.
[12] C. Maltoni, G. Lefemine, G. Cotti, G. Perino, Ann. NY Acad. Sci. 534 (1988) 316–342.
[13] National Toxicology Program, NTP TR 273, US DHHS, Public Health Services, National Institute of Health, Research Triangle Park, NC, 1988.
[14] A.B. DeAngelo, F.B. Daniel, M.W. Most, G.R. Olson, Fundam. Appl. Toxicol. 114 (1996) 207–221.
[15] Z. Tong, P.G. Board, M.W. Anders, Res. Commun. 331 (1998) 371–374.
[16] M.O. James, R. Cornett, Z. Yan, G.N. Henderson, P.W. Stacpoole, Accel. Commun. 25 (1997) 1223–1227.
[17] R. Abbas, J.W. Fisher, Toxicol. Appl. Pharmacol. 147 (1997) 15–30.
[18] M.M. Ketcha, D.K. Stevens, D.A. Warren, C.T. Bishop, W.T. Brashear, J. Anal. Toxicol. 20 (1996) 236–241.
[19] W.T. Brashear, C.T. Bishop, R. Abbas, J. Anal. Toxicol. 21 (1997) 330–334.
[20] A. Gonzalez, I. Schultz, G. Xu, R. Bull, Toxicol. Appl. Pharmacol. 146 (1997) 189–195.

[21] E. Lin, J. Mattox, B. Daniel, J. Toxicol. Env. Hlth. 38 (1993) 19–32.
[22] A. Hesse, D. Bongartz, H. Heynck, W. Berg, Clin. Biochem. 29 (1996) 467–472.
[23] L. Hagen, V. Walker, R. Sutton, Clin. Chem. 39 (1993) 134–138.
[24] M. Petrarulo, O. Bianco, D. Cosseddu, M. Marangella, S. Pellegrino, F. Linari, J. Chromatogr. 532 (1990) 130–134.