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Determination of chlorobutanol in mouse serum, urine and embryos by capillary gas chromatography with electron capture detection

Terry J. Amiss*, Ida W. Smoak

Department of Anatomy, Physiological Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA

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Abstract

A sensitive and specific method for the determination of chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol) in mouse serum, urine, and embryos by capillary gas chromatography with electron capture detection is described. For sample preparation *n*-hexane was used to extract chlorobutanol and the internal standard 2,2,2-trichloroethanol (TCE) from each matrix. Following extraction chromatographic separation of the samples was achieved with a fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness). The method as described has the required sensitivity to quantitate chlorobutanol in individual embryos following administration of a single oral dose of the drug to a pregnant mouse. The limit of detection was 1 pg on column and the detector response was linear from 1 to 100 μg/ml for serum, 0.2 to 20 μg/ml for urine, and 1 to 10 ng/embryo.

1. Introduction

Chlorobutanol is used predominantly as an antibacterial and antifungal preservative in over-the-counter (OTC) and prescription drugs. OTC preparations which contain chlorobutanol include nasal decongestant sprays, ophthalmic artificial tear solutions, and contact lens solutions. It is also used as a preservative in prescription drugs such as desmopressin, a synthetic antidiuretic hormone. In addition to chlorobutanol's preservative properties, it is also the active ingredient in certain oral sedatives and topical anesthetics.

Studies of biological effects of chlorobutanol began more than a decade ago after a fatal poisoning and a case of dependence were reported [1]. Since then, pharmacokinetic studies have shown that chlorobutanol is eliminated slowly from humans. In 1982, Tung et al. [2] reported an elimination half life of 10.3 ± 1.3 days, a volume of distribution of 233 ± 141 l and a plasma clearance of 11.6 ± 1 ml/min after a 600 mg oral dose. Urinary excretion was very low, with only 10% of the total dose eliminated in urine over 17 days. The investigators suggested that other pathways of metabolism and excretion might include conversion of the drug to its glucuronide metabolite in the liver with subsequent kidney excretion and also exhalation of the unchanged drug by the lungs.

Studies thus far have investigated effects of

* Corresponding author.

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chlorobutanol on myocardial cells, corneal epithelial cells, aldosterone production, and platelet aggregation and release. In myocardial cell cultures, chlorobutanol decreases the heart's isometric tension causing a decrease in pumping ability [3–5]. When corneal epithelial cells are exposed to a 0.5% solution of chlorobutanol they cease normal cytokinesis and mitotic activity [6,7]. In the adrenal gland, chlorobutanol inhibits the production of the mineralocorticoid aldosterone [8]. Another effect of chlorobutanol is its inhibition of the arachidonic acid pathway, causing decreased blood platelet aggregation and release [9].

Chlorobutanol's presence in many daily-use OTC preparations, its long pharmacokinetic half-life, and its physiologic effects prompted an investigation of its influence on the developing embryo. Recently, Smoak [10] used the technique of whole-embryo culture and reported that chlorobutanol produces dysmorphogenesis in mouse embryos. Significantly, the concentrations of the drug that interfered with normal embryonic development are within the range of human blood levels measured following multiple doses of chlorobutanol.

Chromatographic methods have been published for quantitation of chlorobutanol in serum, tissue, urine, and milk using gas chromatography with electron capture detection (GC–ECD) [2,11,12]. However, an assay sensitive enough for the determination of chlorobutanol in individual embryos has, to our knowledge, never been published. This report describes a sensitive and specific GC–ECD assay for chlorobutanol, following a single oral administration of the drug to a pregnant mouse, in serum, urine, and embryos. The limit of detection was 1 pg on column and the detector response was linear from 1 to 100 μg/ml for serum, 0.2 to 20 μg/ml for urine, and 1 to 10 ng/embryo.

2. Experimental

2.1. Apparatus

The gas chromatograph was equipped with a split–splitless inlet, a ^{63}Ni electron-capture detector Model 2100, and a Laboratory Computing Integrator, Model LC-100 (Perkin Elmer, Norwalk, CO, USA). A Heliflex AT-WAX fused-silica column, 30 m × 0.25 mm I.D. with a 0.25 μm film thickness (Alltech Associates, Deerfield, IL, USA) was used. A Microson ultrasonic cell disrupter (Model MS 50, Heat Systems Ultrasonics, Farmingday, NY, USA), a Rototorque rotary mixer (Model 7637-20, Cole-Parmer Instrument Company, Chicago, IL, USA), and an Eppendorf centrifuge (Model 5415, Brinkman Instruments, Westbury, NY, USA) were used during sample preparation.

2.2. Reagents

Chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol) (Sigma, St. Louis, MO, USA) and the internal standard 2,2,2-trichloroethanol (TCE) (Sigma) were both analytical grade. For the gas chromatographic separation, helium (National Welders, Raleigh, NC, USA) was used as the carrier gas and an argon–methane mixture (95:5) (National Welders) was used as the make-up gas. Both were 99.9995% pure. The helium and argon–methane gasses were passed through an Oxisorb filter (Model 60680, Alltech Associates) to remove oxygen and moisture contamination.

Other supplies included reagent grade potassium phosphate monobasic (Sigma), sodium hydroxide (Fisher Scientific, Pittsburgh, PA, USA), and HPLC grade *n*-hexane (Fisher Scientific).

2.3. Chromatographic conditions

The carrier gas (helium) had a flow-rate of 1 ml/min and the make-up gas (argon–methane, 95:5) a flow-rate of 60 ml/min. The GC was operated in the split mode at a ratio of 1:10. The operating conditions were: injection port temperature 260°C; detector temperature 280°C; oven temperature 125°C.

2.4. Animals

Randomly bred CD-1 mice were maintained on a 12-hour-light, 12-hour-dark cycle and provided with food and water ad libitum. Females were checked for vaginal plugs the morning after

overnight mating, and this day was designated day 0.5 of pregnancy.

2.5. Drug administration and sample collection

Pregnant mice on day 9.5 of gestation (a critical period of organogenesis when teratogenic effects are produced) were dosed with chlorobutanol by gavage at 40 or 80 mg/kg. For the pharmacokinetic study, serum samples were taken at 0, 0.167, 0.333, 1, 2, 4, 8, 12, 24, 36, and 48 h. Urine samples were also collected. To retrieve embryos, mice were anesthetized with halothane and a laparotomy was performed at 2 h post-treatment with chlorobutanol. Uterine horns were removed and embryos were dissected from the surrounding membranes. All samples were immediately frozen at −70°C until analysis.

2.6. Stock, calibration and spiking solutions

Stock solutions of chlorobutanol and the internal standard TCE were prepared by dissolving each separately in *n*-hexane to produce a concentration of 1 μg/μl. Further dilutions of stock solutions were done in *n*-hexane and these solutions were used as calibration standards. The chlorobutanol and TCE solutions were protected from photodegradation and stored at 4°C. The chlorobutanol solutions were made daily and the TCE solutions were made weekly.

2.7. Preparation of serum samples

To test tubes containing 0.1 ml of either drug-free serum or serum from a mouse taken after administration of chlorobutanol, 10 μl of a 1 μg/μl TCE solution was added. A 10-μl aliquot of 0.01, 0.025, 0.05, 0.10, or 1.00 μg/μl chlorobutanol solution was added to drug-free serum producing a standard curve with concentrations of 1, 2, 5, 10, and 100 μg/ml. All samples were vortex-mixed for 5 s. To each tube, 0.1 ml of phosphate buffer (0.05 *M*, pH 7.4) was added and vortex-mixed for 5 s. Then 0.5 ml of *n*-hexane was added to each tube. The samples were mixed on a rotary mixer for 10 min, then centrifuged at 6000 *g* for 1 min. The organic layer was transferred to a small injection vial and 1 μl was injected onto the GC column.

2.8. Preparation of urine samples

To test tubes containing 50 μl of either drug-free urine or urine from a mouse taken after the administration of chlorobutanol, 10 μl of 1 μg/μl TCE solution was added. A 10-μl aliquot of 1.0, 2.5, 5.0, 10.0, or 100.0 ng/μl chlorobutanol solution was added to the drug-free urine producing a standard curve with concentrations of 0.2, 0.5, 1.0, 2.0, and 20.0 μg/ml. All samples were vortexed for 5 s. To each tube, 0.1 ml of phosphate buffer (0.05 *M*, pH 7.4) was added and vortexed for 5 s. Then 0.25 ml of *n*-hexane was added to each tube. The samples were mixed on a rotary mixer for 10 min, then centrifuged at 6000 *g* for 1 min. The organic layer was transferred to a small injection vial and 1 μl was injected onto the GC column.

2.9. Preparation of embryo samples

To a test tube containing either a drug-free mouse embryo or an embryo removed from a pregnant mouse 9.5 days after chlorobutanol administration, 10 μl of a 1 ng/μl TCE solution was added. A 10-μl aliquot of 0.02, 0.05, 0.1, 0.2, or 2 ng/μl chlorobutanol solution was added to drug-free embryos producing a standard curve with concentrations of 1, 2, 5 and 10 ng/embryo. The samples were vortexed for 5 s. To each tube, 50 μl of *n*-hexane was added. Each sample was homogenized with a cell disruptor for 5 s and centrifuged at 6000 *g* for 1 min. The organic layer was transferred to a small injection vial and 2 μl was injected onto the GC column. Following the chlorobutanol assay, embryos were analyzed for total protein content using the Lowry method [13].

2.10. Calculations and data analysis

Integration of the chlorobutanol and TCE peaks was performed by an integrator. The chlorobutanol/TCE peak-area ratios for the standard curve samples and the clinical samples were then determined. A linear regression analy-

sis of the ratios versus chlorobutanol concentrations was performed for the serum, urine, and embryo standard curves. The concentrations of the unknown serum, urine, and embryo samples were calculated by interpolation from the respective linear regression equations.

3. Results and discussion

Different methods of spiking drug-free samples with chlorobutanol were investigated. In the first method, hexane was used to dissolve chlorobutanol and TCE. Then, 10 μl of this solution was placed in an extraction tube, and a gentle stream of nitrogen was used to evaporate the hexane, then neat hexane was immediately added to the extraction tube. When this solution was injected onto the GC column, it was revealed that substantial losses of chlorobutanol had occurred. This method of spiking was abandoned.

Next ethanol was used to dissolve chlorobutanol and TCE. To avoid precipitating proteins with the addition of ethanol an increase in the amount of phosphate buffer added to each sample was needed. For efficient partitioning of the chlorobutanol into the nonpolar phase, the volume of hexane used for the extraction was also increased. The greater volume of hexane used in this method required an evaporation step before injection onto the GC column, to main-

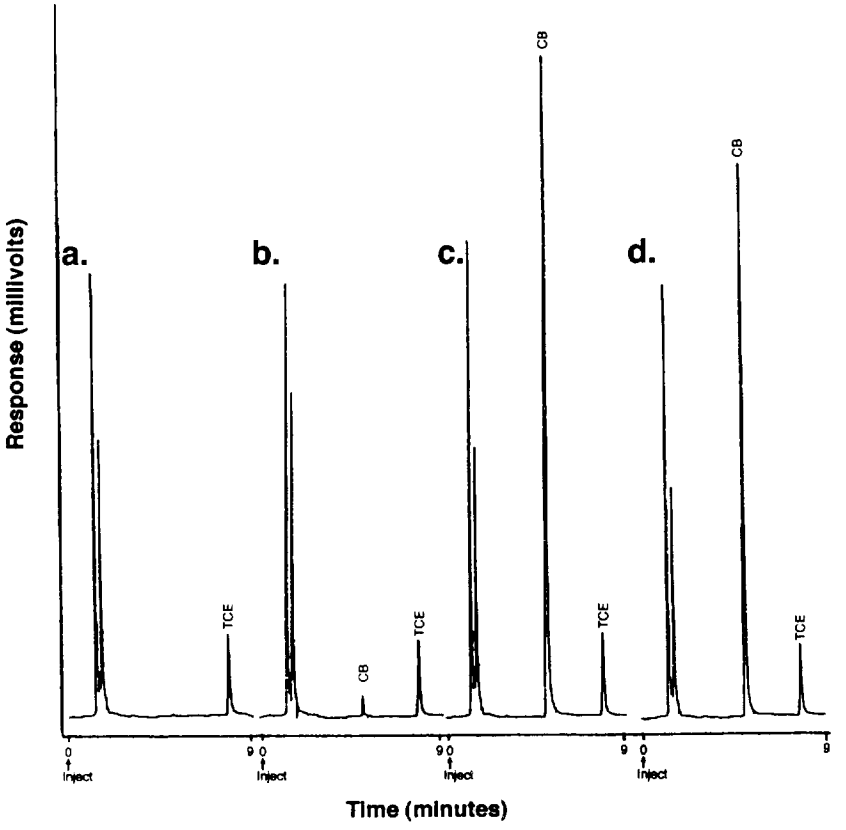

Fig. 1. The four chromatograms show the separation of chlorobutanol and the internal standard TCE from endogenous substances in mouse serum: a = drug-free serum, b = serum spiked at a concentration of 1 μg/ml chlorobutanol, c = serum spiked at a concentration of 100 μg/ml chlorobutanol, and d = serum sample taken at from a mouse 10 min after being given an 80 mg/kg oral dose of chlorobutanol.

tain assay sensitivity. Attempts to evaporate the hexane resulted in losses of chlorobutanol. This spiking method was also abandoned.

The third method of spiking consisted of adding chlorobutanol and TCE dissolved in hexane to each drug-free matrix. To be acceptable, the chlorobutanol and TCE must be distributed throughout the sample. The distribution was tested by adding chlorobutanol and TCE dissolved in hexane to pooled drug-free serum, embryos or urine, and then removing and processing aliquots of the pooled sample. Evaluation of data from the pooled samples revealed that spiking in this manner was acceptable (unreported data).

Figs. 1, 2 and 3 are representative chromatograms obtained from the extraction of serum, urine and embryo samples, respectively. Each figure shows a chromatogram of a drug-free sample, the same sample spiked with chloro-

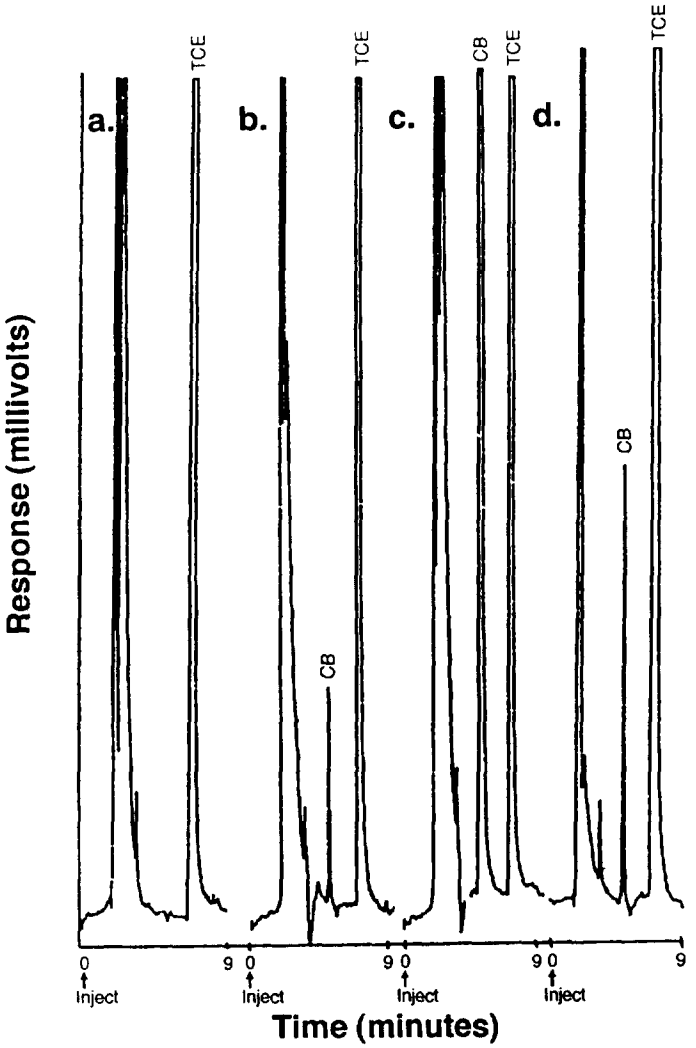

Fig. 2. The four chromatograms show the separation of chlorobutanol and the internal standard TCE from endogenous substances in mouse urine: a = drug-free urine, b = urine spiked at a concentration of 0.2 μg/ml chlorobutanol, c = urine spiked at a concentration of 20 μg/ml chlorobutanol, and d = urine sample collected from a mouse 60 min after being given an 80 mg/kg oral dose of chlorobutanol.

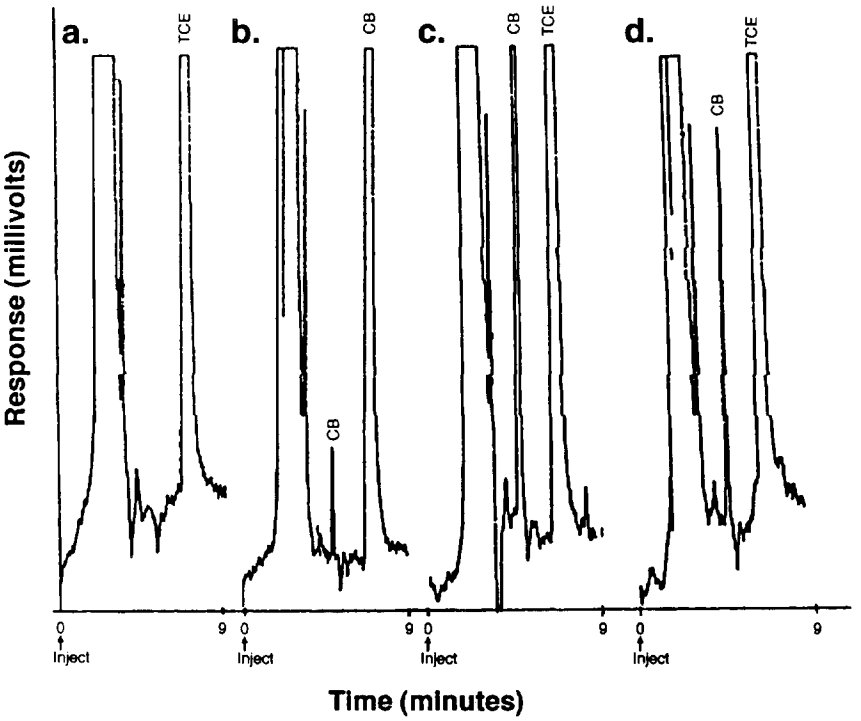

Fig. 3. The four chromatograms show the separation of chlorobutanol and the internal standard TCE from endogenous substances of a mouse embryo: a = drug-free embryo, b = embryo spiked with 1 ng chlorobutanol, c = embryo spiked with 10 ng chlorobutanol, and d = embryo removed from a pregnant mouse 9.5 days after being given an 80 mg/kg oral dose of chlorobutanol.

butanol, and a clinical sample taken from a mouse given 80 mg/kg chlorobutanol orally.

The chromatograms had no interfering peaks from the anesthetic, halothane, or from endogenous substances in serum, urine or embryo samples at the retention time of 5.07 min for chlorobutanol or 7.79 min for TCE.

The correlation coefficients of each standard curve exceeded 0.999.

3.1. Variability

On different days, drug-free serum, urine or embryos were spiked with chlorobutanol and TCE at five concentrations and extracted. The coefficients of variation for the intra-day and inter-day precision range from 1.3% to 16.5% and 1.2% to 12.5%, respectively. Tables 1, 2 and 3 summarize the intra-day and inter-day variability, accuracy, and repeatability for the standard curves.

3.2. Extraction recovery

The extraction recovery was determined by comparing unextracted and extracted peak-area ratios. The average recovery of chlorobutanol added to drug-free serum was $89 \pm 5\%$ for serum, $82 \pm 6\%$ for urine, and $76 \pm 8\%$ for embryos.

3.3. Stability

The stability of chlorobutanol was determined by comparing samples spiked and extracted on the same day to samples spiked and stored at

Table 1
Statistical summary of the analysis of chlorobutanol in serum ($n = 3$)

Concentration spiked (μg/ml)	Intra-day		Inter-day	
	Concentration determined (mean ± S.D.)	Coefficient of variation (%)	Concentration determined (mean ± S.D.)	Coefficient of variation (%)
1	1.17 ± 0.11	9.4	1.12 ± 0.12	10.7
2	2.06 ± 0.15	7.5	2.16 ± 0.09	4.2
5	5.12 ± 0.22	4.3	5.15 ± 0.31	6.0
10	9.89 ± 0.14	1.4	9.84 ± 0.14	1.4
100	99.47 ± 1.27	1.3	98.39 ± 1.43	1.5

Table 2
Statistical summary of the analysis of chlorobutanol in urine ($n = 3$)

Concentration spiked (μg/ml)	Intra-day		Inter-day	
	Concentration determined (mean ± S.D.)	Coefficient of variation (%)	Concentration determined (mean ± S.D.)	Coefficient of variation (%)
0.2	0.19 ± 0.02	8.1	0.24 ± 0.02	8.3
0.5	0.51 ± 0.05	9.8	0.52 ± 0.03	5.8
1	1.06 ± 0.05	4.7	1.02 ± 0.08	7.8
2	1.83 ± 0.11	6.0	1.97 ± 0.07	3.6
20	20.04 ± 0.37	1.9	20.00 ± 0.24	1.2

−70°C for one month and then processed. There was no degradation of chlorobutanol at concentrations of 10 μg/ml for serum, 2 μg/ml for urine, and 5 ng/embryo.

4. Conclusion

Evidence concerning the adverse effects of chlorobutanol in adult [1–9] and isolated em-

Table 3
Statistical summary of the analysis of chlorobutanol in embryos ($n = 3$)

Concentration spiked (ng/ml)	Intra-day		Inter-day	
	Concentration determined (mean ± S.D.)	Coefficient of variation (%)	Concentration determined (mean ± S.D.)	Coefficient of variation (%)
1	1.15 ± 0.19	16.5	1.12 ± 0.14	12.5
2	2.23 ± 0.13	5.8	2.23 ± 0.18	8.1
5	5.08 ± 0.09	1.8	4.87 ± 0.06	1.2
10	10.03 ± 0.20	2.0	9.90 ± 0.14	1.4

bryonic tissues [10] has begun to emerge. Chlorobutanol has been shown to produce dysmorphogenesis during the development of embryonic mice, and therefore may be considered a potential teratogen. Whereas teratogens are often investigated using in vitro methods such as embryonic cell, organ, and whole embryo cultures, it is important to determine the pharmacokinetic properties of these agents in vivo. This paper describes an assay in which chlorobutanol concentrations were determined in serum, urine, and embryos after a single oral dose was given to a pregnant mouse.

The challenging portion of the assay was achieving the sensitivity needed to determine chlorobutanol in a single embryo. Fortunately, a relatively clean extract was produced that could be directly injected onto the GC column. The results suggest that chlorobutanol crosses the murine placenta and is detectable in embryonic tissues.

As presented, the method for the determination of chlorobutanol using GC–ECD is simple and rugged. Determining concentrations of chlorobutanol in mouse serum, urine and embryos contributes to a better understanding of the drug's distribution and potential teratogenicity.

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