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Short communication

Sensitive high-performance liquid chromatography method for the determination of low levels of perchlorate in biological samples

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Abstract

A rapid and sensitive high-performance liquid chromatography (HPLC) method was developed to detect perchlorate in tissues of male and female rats, both pregnant and lactating (including milk) after administration of perchlorate. Supernatants of ethanol precipitated rat fluids and tissues were evaporated to dryness under nitrogen and reconstituted in deionized water. Reconstituted samples were injected into HPLC system coupled with conductivity detection. Isocratic separation of perchlorate was achieved using an anion-exchange column with sodium hydroxide as mobile phase and a conductivity detector. In this method, perchlorate showed a linear response range from 5 to 100 ng/ml. The lower detection limits for perchlorate in fluids and tissues of rats were 3–6 ng/ml and 0.007–0.7 mg/kg, respectively. The described method has the unique advantage over the existing methods of determining low traces of perchlorate in different biological matrices without complex sample preparation.

Keywords: Perchlorate

1. Introduction

Ammonium perchlorate is an oxidizer that has been used as a component of solid rocket fuel [1,2]. The production and storage of ammonium perchlorate (AP) has resulted in contamination of soil and ground and drinking water in a number of states. Perchlorate, the dissociated ion of ammonium perchlorate, has recently been recognized as a persistent and pervasive contaminant of water supplies in a number of major metropolitan areas [1,2]. Current efforts at assessing the health risks of perchlorate have been hampered by a lack of relevant toxicity data and by a lack of understanding of the potential toxic mechanism of action. Perchlorate is known to disrupt thyroid hormone homeostasis in a number of species via an inhibition of iodine uptake into the thyroid gland [3]. Because of perchlorate's potential toxicity and widespread distribution in water supplies, there is an increasing interest in perchlorate metabolism and disposition.

Chow and co-workers [4,5] employed radioactive perchlorate to study the kinetics of distribution of perchlorate in rats and guinea pigs. Our laboratory

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has been unable to obtain radioactive perchlorate with high specific activity. Since available radioactive perchlorate with low specific activity could not be suitable for detecting low concentrations of perchlorate in biological samples, there was a need to develop a new analytical method with a high sensitivity for perchlorate. In the present work, we present such a method which was aimed at investigating perchlorate kinetics in male and female rats, both pregnant and lactating. The method was also used to measure milk from the lactating rats. While perchlorate has been analyzed either in water or environmental samples using available HPLC techniques [6–9], the described method has the unique advantage of determining low levels of perchlorate in different biological samples without complex sample preparation.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium perchlorate (99.9% purity) was obtained from Aldrich (St. Louis, MO, USA). Sodium hydroxide was purchased from Fisher Scientific (Fairlawn, NJ, USA). Ethanol (HPLC grade) was purchased from Sigma (St. Louis, MO, USA). Deionized water with a specific resistance of 18 MΩ-cm or greater was used for the study.

2.2. Animal treatment and sample collection

2.2.1. Preparation of the samples

The following tissues were collected and analyzed for perchlorate in gestational day 20 (GD20) dams: blood, thyroid, skin, amniotic fluid, placenta, mammary gland, gastric tract and gastrointestinal (GI) content. The milk samples were collected and analyzed for perchlorate in postnatal day 10 (PND10) dams. Serum, thyroid and urine samples from intravenously dosed male rats were collected at 24 h following perchlorate administration.

For the HPLC analysis of perchlorate in thyroid samples, rat thyroid samples were homogenized in 250 μl of deionized water, using micro tissue grinders (Kontes Glass Company, Vineland, NJ, USA). Homogenates were centrifuged twice at 31 500 *g* for 30 min at 4 °C. The supernatants were diluted 12.5, 40, 80 and 250 times with deionized water for 0, 0.01, 0.1, 1 and 10 mg/kg perchlorate dose groups, respectively, bringing the final volume to 2 ml with deionized water. The diluted supernatants were then filtered through 0.45-μm non-sterile acrodisc syringe filters provided with versapor (supported acrylic copolymer) membranes (Pall Gelmann Laboratory, Ann Arbor, MI, USA). Then, 1000 μl of each filtrate was injected into the HPLC system, using an autosampler. The mean sample run time for rat fluids and tissues analyzed was 12 min.

For the HPLC analysis of perchlorate in serum and urine matrices, 100 μl of serum and urine samples were precipitated with ice cold 100% ethanol (1:4, v/v); ethanol precipitated serum and urine samples were then centrifuged twice at 31 500 *g* for 30 min at 4 °C. Supernatants were evaporated to dryness under nitrogen at 37 °C and reconstituted in 2 ml of the deionized water. Reconstituted urine and serum samples were diluted 40, 2, 3.3, 6.6 and 40 times with deionized water for 0, 0.01, 0.1, 1 and 10 mg/kg perchlorate dose groups, respectively, bringing the final volume to 2 ml with deionized water. Diluted urine samples were filtered through 0.45-μm non-sterile acrodisc syringe filters provided with versapor (supported acrylic copolymer) membranes (Pall Gelmann Laboratory). Using an autosampler, 1000 μl of each filtrate was injected into the HPLC system. Amniotic fluid samples from GD20 dams and milk samples from PND10 dams were processed in the similar manner as that of the serum samples.

All solid rat tissues were processed to a homogenate in water, and then prepared in a similar manner as serum for HPLC analysis. Solid tissues were weighed in the range 0.2–0.5 g in 4-ml vials. For gastrointestinal contents, gastric tract, and mammary gland tissues, deionized water was added in the ratio 1:3 by weight of the tissue. For placenta and skin tissues, deionized water was added in the ratio 1:3 and 1:8 by weight of the tissue, respectively. Tissues were homogenized separately until a homogenous mixture was attained for all the tissue samples. All the tissue homogenates in water are prepared in the similar manner as that of a serum sample for perchlorate analysis.

To verify the ability of the anion-exchange chromatography system to resolve both, chlorate and perchlorate anions were eluted by gradient analysis. Simultaneous determinations of perchlorate and chlorate anions were performed in chlorate-spiked GD20 dam thyroid and serum samples from control and perchlorate exposed groups. The mobile phase consisted of an initial concentration of 0.05 m*M* sodium hydroxide in deionized water followed by a linear gradient from 0.05 to 100 m*M* sodium hydroxide in deionized water for 30 min.

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 chromatographic 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, an autosampler (AS-3500) with 1000 μl sample loop, computer interface ACI and AutoIon 450 software. Separation by isocratic elution of perchlorate was performed on a Dionex AS11 analytical column (4×250 mm) preceded by a Dionex AG11 guard column (50×4 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 was a solution of 100 m*M* sodium hydroxide in deionized water. The mobile phase was filtered through a 0.45-μm filter provided with nylon membrane (Micron Separations, Westboro, MA, USA). The flow-rate was set at 1 ml/min.

2.5. Preparation of standards

Standards were prepared in deionized water. The perchlorate concentrations of the prepared standards were 2, 5, 10, 20, 50 and 100 ng/ml.

3. Results

The HPLC standard curve constructed for perchlorate was linear in the range of 5–100 ng/ml in deionized water. The average regression coefficient (r^2) for standard curves was greater than 0.99. The calibration line for perchlorate was typically described by the equation $y = 105\,109x$. For the calculation of the detection limit the regression line $y + 3s$ was used for all tissues, where y is the intercept and s is the standard deviation of the signal (noise) at the retention time of perchlorate in different tissue samples ($n = 10$). The method's detection limit was 5 ng/ml for perchlorate in urine and serum samples. The signal-to-noise ratio at 5 ng/ml was greater than 3 for perchlorate in these samples. The detection limits for perchlorate were in the ranges 3–6 ng/ml and 0.007–0.7 mg/kg for rat fluids and tissues, respectively. Method detection limits (MDL) for perchlorate in rat fluids and tissues are given in Table 1. The mean analytical recovery (found/added) over the range 5–100 ng/ml was determined to be 100±1.0% ($n = 10$) in all samples. Day-to-day precision (C.V.) of the method was within 1% ($n = 10$) for perchlorate in all the samples over the 5–100 ng/ml range. The mean between-assay coefficients of variation for perchlorate were lower than 1% over the range 5–100 ng/ml. The within-assay coefficients of variation were lower than 1% as measured at 5 or 20 ng/ml. The criterion of reliability for rat fluids and tissues is presented in Table 2. No interfering endogenous compounds appeared in any of the tissues analyzed from the control or treated animals.

Table 1
Method detection limits for perchlorate in rat fluids and tissues

Rat fluids and tissues	Method detection limits
Water	2 ng/ml
Serum	5 ng/ml
Amniotic fluid	3 ng/ml
Urine	5 ng/ml
Milk	6 ng/ml
Thyroid	0.007 mg/kg
Placenta	0.12 mg/kg
Skin	0.25 mg/kg
Gastric contents	0.25 mg/kg
Gastric tract	0.25 mg/kg
Mammary gland	0.13 mg/kg

Table 2
Recovery and precision of the HPLC determination of perchlorate in rat fluids and tissues

Rat fluids and tissues	Concentration		Recovery[a]	Between-assay C.V. (%)[b]	Within-assay C.V. (%)[c]
Water (ng/ml)	2	5	100±0.9	0.6	0.6
Serum (ng/ml)	5	30	101±2	0.7	0.6
Amniotic fluid (ng/ml)	5	20	103±3	0.5	0.5
Urine (ng/ml)	5	100	100±1	0.7	0.8
Milk (ng/ml)	5	20	100±1	0.6	0.9
Thyroid (mg/kg)	0.030	0.100	100±0.8	0.99	1.0
Placenta (mg/kg)	0.20	0.60	99±0.9	0.89	1.03
Gastric contents (mg/kg)	0.30	1.0	100±1	0.89	0.99
Gastric tract (mg/kg)	0.6	2.0	99±0.7	1.05	1.0
Mammary gland (mg/kg)	0.35	1.0	99±0.3	0.99	0.99

[a] Mean±SD at two concentrations as shown (at each concentration, $n=5$).
[b] Determinations at two concentrations as shown (at each concentration, $n=5$).
[c] Determinations at lowest concentrations ($n=5$).

In spite of the proper maintenance of anion-exchange columns and the instrument in general, perchlorate retention time decreased gradually depending on the number of sample injections made in succession. There were no changes in sensitivity or resolution. The decrease in the retention time after 800 sample injections was 2±0.4%. Perchlorate standard samples and control samples spiked with known perchlorate concentrations were run each time along with the tissue samples from control or treated animals. This ensured the identification of perchlorate at the right retention time in all of the biological samples.

Fig. 1 represents the typical ion chromatogram of 5 ng/ml perchlorate standard prepared in deionized water. Fig. 2 represents the chromatogram obtained for a GD20 dam thyroid sample from the 10 mg/kg/day dose group. The retention times for perchlorate in water and thyroid samples were 8.48 and 9.78 min, respectively. One unidentified peak with a retention time of 2.58 min appeared in the perchlorate 5 ng/ml standard sample. Three unidentified peaks with retention times of 2.75, 2.90 and 5.80 min were observed in the control and treated animals in thyroid samples (Fig. 2). The chromatograms obtained for perchlorate in fluids and tissues of perchlorate exposed rats followed a pattern similar to that of a thyroid sample chromatogram.

Fig. 3 represents the chromatogram illustrating the gradient separation of chlorate and perchlorate an-

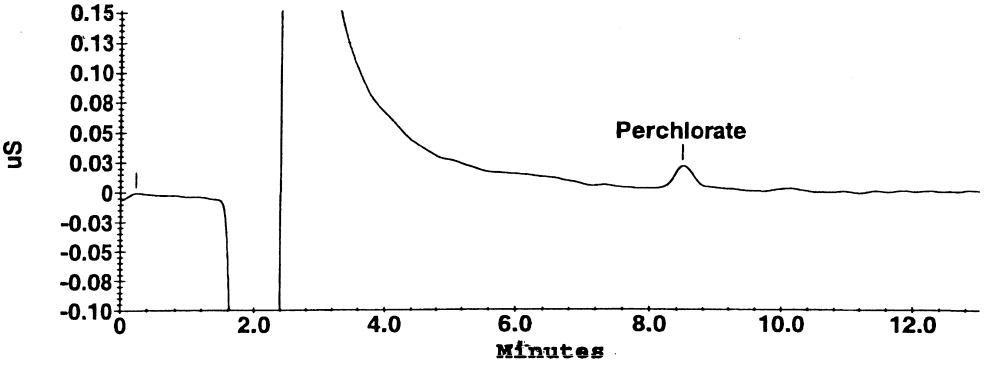

Fig. 1. Typical ion chromatogram of a 5 ng/ml perchlorate standard prepared in deionized water.

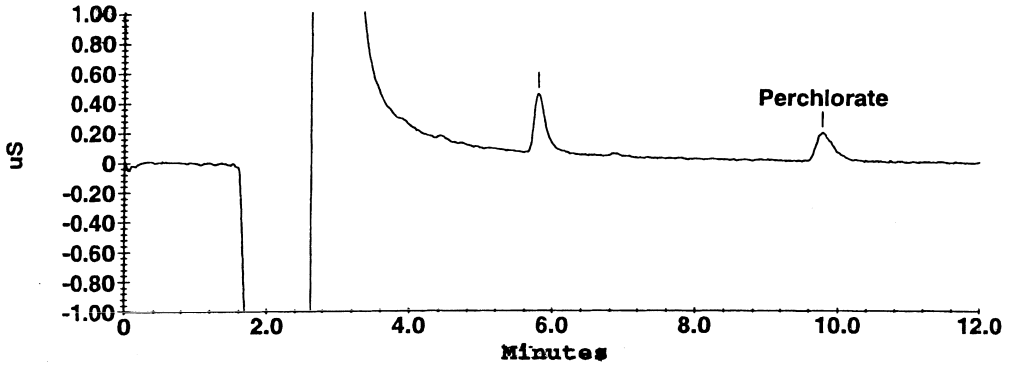

Fig. 2. Chromatogram obtained for a thyroid sample of a GD20 dam from the 10 mg/kg/day dose group.

ions in a perchlorate and chlorate combined standard sample. The retention times for chlorate and perchlorate anions were 2.67 and 8.33 min, respectively (Fig. 3). Simultaneous determinations of chlorate and perchlorate were performed in dam serum and thyroid samples from control and perchlorate exposed groups. No chlorate ion was detected in any of the sample analyzed.

Perchlorate concentrations in different tissues of male and female rats analyzed are given in Tables 3 and 4.

Table 3 illustrates changes of perchlorate in different tissues of pregnant and lactating rats. Pregnant and lactating rats were provided with drinking water containing perchlorate with one of five target doses (0, 0.01, 0.1, 1 and 10 mg/kg/day) from gestation day 2 (GD2) until either gestation day 20 (GD20) or postnatal day 10 (PND10). Rats were sacrificed at the end of drinking water exposures (GD20 or PND10). Perchlorate concentrations were determined in fluids and tissues of pregnant and lactating rats from control and perchlorate exposed dose groups. Perchlorate concentrations were determined only in control and 10 mg/kg dose groups for placenta, gastric tract and mammary gland samples from GD20 dams. Perchlorate concentrations increased with increasing doses of perchlorate in fluids and tissues from pregnant and lactating rats.

Table 4 illustrates perchlorate concentrations in urine, serum, and thyroid glands in intravenously dosed male rats. Male rats were dosed intravenously with perchlorate at concentrations of 0.01, 0.1, 1 and

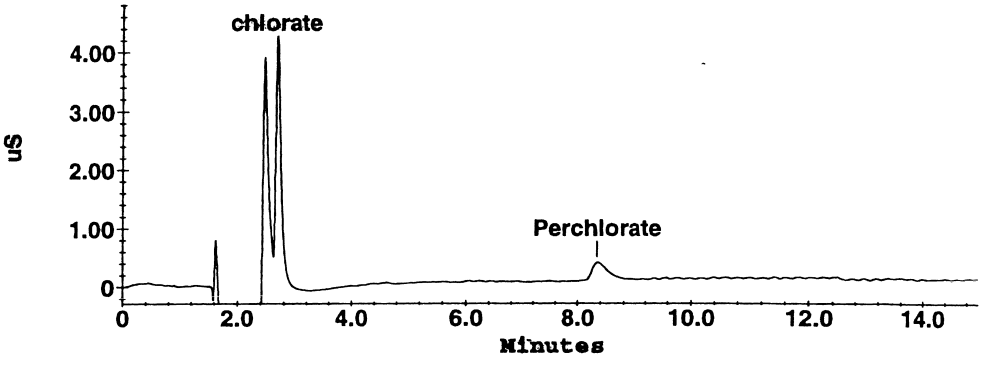

Fig. 3. Chromatogram illustrating the gradient separation of chlorate and perchlorate anions in a combined perchlorate and chlorate standard sample.

Table 3
Changes of perchlorate concentration in different tissues of pregnant and lactating rats (mean±SD)

Tissue		Control	Perchlorate/kg body weight (mg)			
			0.01	0.1	1	10
Amniotic fluid (GD20)	Perchlorate conc. in (mg/l or μg/ml of the amniotic fluid) ($n=6$)	0.00±0.00	0.03±0.002	0.06±0.01	0.75±0.07	5.73±0.44
Placenta (GD20)	Perchlorate conc. in (mg/kg or μg/g placenta) ($n=6$)	0.00±0.00				2.22±0.19
Gastric contents (GD20)	Perchlorate conc. in (mg/kg or μg/g gastric contents) ($n=6$)	0.00±0.00	0.00±0.00	0.33±0.04	1.00±0.10	4.67±0.52
Serum (GD20)	Serum perchlorate conc. in (mg/l or μg/ml serum) ($n=10$)	0.00±0.00	0.06±0.01	0.31±0.03	0.50±0.04	4.72±0.48
Thyroid (GD20)	Thyroid perchlorate conc. in (mg/kg or μg/g thyroid) ($n=10$)	0.00±0.00	0.70±0.09	4.69±0.49	14.29±1.76	61.22±6.61
Gastric tract (GD20)	Perchlorate conc. in (mg/kg or μg/g gastric tract) ($n=6$)	0.00±0.00				4.09±0.42
Skin (GD20)	Perchlorate conc. in (mg/kg or μg/g skin) ($n=6$)	0.00±0.00	0.19±0.02	0.25±0.01	2.78±0.31	4.49±0.50
Mammary glands (GD20)	Perchlorate conc. in (mg/kg or μg/g mammary glands) ($n=6$)	0.00±0.00				3.83±0.25
Milk (PND10)	Perchlorate conc. in (mg/kg or μg/g mammary glands) ($n=6$)	0.00±0.00	0.40±0.04	0.77±0.12	1.57±0.31	6.87±1.97

3 mg/kg, and samples were collected 24 h after perchlorate administration. Perchlorate concentrations increased with increasing doses of perchlorate in urine, serum, and thyroid glands in intravenously dosed male rats.

4. Conclusion

A sensitive method was developed for the analysis of perchlorate in biological samples. Perchlorate analyses in pregnant and lactation rats helped us to develop pregnancy and lactation models for perchlorate in pregnant and lactating rats. Due to perchlorate potential toxicity and widespread distribution in water supplies, there is an increasing interest in perchlorate metabolism and disposition. In order to investigate perchlorate metabolism and disposition in rats, we have developed a highly sensitive method to detect low levels of perchlorate in biological samples. While available HPLC techniques offer sensitive procedures to detect perchlorate either in water or environmental samples, the described method has the unique advantage of

Table 4
Perchlorate concentration in urine, serum and thyroid gland in intravenously dosed male rats and samples collected at 24 h following the perchlorate administration (mean±SD)

Dose (mg) (perchlorate/kg body weight)	Thyroid perchlorate (mg/kg or μg/g thyroid) ($n=6$)	Serum perchlorate (mg/l or μg/ml serum) ($n=6$)	Urine perchlorate (total amount in mg) ($n=6$)
0.01	0.01±0.02	0.000±0.000	0.0034±0.0003
0.1	0.66±0.36	0.06±0.01	0.0217±0.0046
1	5.56±0.01	0.21±0.07	0.2849±0.0666
3	10.76±2.62	0.54±0.21	0.8593±0.1999

determining low traces of perchlorate in different biological matrices without complex sample preparation. The present method is inexpensive and does not involve extensive extraction procedures for sample preparation. The developed method involves a sample preparation, which is followed by a one step HPLC and detection procedure. The method is capable of detecting perchlorate in the range 5–20 ng/ml in biological samples. The detection limit for water was lower than that for biological matrices. However, endogenous compounds do not interfere with perchlorate. The chlorate ion is early eluted with a retention time about 6 min and, therefore, does not interfere with perchlorate analysis. No chlorate was measured in samples from exposed animals.

The ability of the method to detect low traces of perchlorate in biological matrices makes it a suitable method to investigate perchlorate pharmacokinetics or to detect perchlorate in biological samples following clinical trials. The method can be employed to provide data to develop pharmacokinetic models for perchlorate in rodents or humans.

References

[1] E.T. Urbansky, Bioremed. J. 2 (1998) 81.
[2] E.T. Urbansky, M.R. Schock, J. Environ. Manag. 56 (1999) 79.
[3] J.C. Siglin, D.R. Mattie, D.E. Dodd, P.K. Hildebrandt, W.H. Baker, A 90-day drinking water toxicity study in rats of the environmental contaminant, ammonium perchlorate, Toxicol. Sci. 57 (2000) 61.
[4] S.Y. Chow, L.R. Chang, M.S. Yen, J. Endocr. 45 (1969) 1.
[5] S.Y. Chow, D.M. Woodbury, J. Endocr. 47 (1970) 207.
[6] California Department of Health Services, Determination of perchlorate by ion chromatography, Rev. 0: June 3, 1997.
[7] California Department of Health Services, Perchlorate in California drinking water, September, 1997.
[8] D.T. Tsui, R. Clewell, E. Eldridge, D.R. Mattie, Perchlorate Analysis by AS-16 Separation Column, AFRL-HE-WP-1999-024, 1999.
[9] P. Glaser, D. Forest, G. McKee, S. Quave, W. Budde, Environ. Sci. Technol. 12 (1981) 1426.