Laboratory Procedure Manual

Analyte: **Perchlorate**

Matrix: **Urine**

Method: **Ion Chromatography with Tandem Mass Spectrometry (IC-MS/MS)**

Method No: **VOC-UP8-1.01**

Revised: **3/21/2005**

as performed by:

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Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

1. Clinical Relevance and Summary of Test Principle

A. Clinical Relevance

Perchlorate has been commonly used as an oxidant in solid fuel propellants for rockets and missiles since the 1950s. Lesser amounts of perchlorate are used in matches, fireworks, and automotive airbags. Chronic exposure to significant amounts of perchlorate can cause thyroid dysfunction in humans[1] which can lead to hypothyroidism[2,3] and birth defects[4]. Although perchlorate contamination is a national problem, the states with the widest contamination are California, Nevada, and Arizona[5]. As of December 2004, 4% of water distribution systems in 29 states have perchlorate levels ranging from 4 to 420 ng/mL[6]. In 2002 the EPA proposed a containment level of perchlorate of 1 ppb in drinking water until official limits are established. Some states have set their own limits ranging from 1 to 18 ppb. Unequivocal exposure assessment through improved biomonitoring methods will assist in identifying levels of perchlorate exposure that do not have measurable health effects. This data will provide important information for state and federal agencies debating appropriate regulatory limits for perchlorate in drinking water, and provide useful baseline information on the scope of perchlorate exposure in the U.S. population.

B. Test Principle

This method is a quantitative procedure for the measurement of perchlorate in human urine using ion chromatography coupled with electrospray tandem mass spectrometry. Chromatographic separation is achieved using an IonPac AS16 column with sodium hydroxide as the eluant. The eluant from the column is ionized using an electrospray interface to generate and transmit negative ions into the mass spectrometer. Comparison of relative response factors (ratio of native analyte to stable isotope labeled internal standard) with known standard concentrations yields individual analyte concentrations. The method is applicable to the determination of perchlorate in 0.5 mL of urine over the range 0.05 to 100 ng/mL.

2. Safety Precautions

A. Reagent toxicity or carcinogenicity

Perchlorate ion can reversibly inhibit thyroid function at doses of µg per kg body weight per day. Therefore perchlorate intake (oral or inhalational) should be avoided. Additionally, some perchlorate salts (e.g. ammonium perchlorate) are strong oxidizers. Special care should be taken to prevent contact of solid ammonium perchlorate salt with combustible or oxidizable material as this constitutes an extreme fire and explosion hazard. However, aqueous solutions of perchlorate do not present a fire or explosion hazard. Perchlorate solutions can irritate skin and mucous membranes, and thus dermal exposure should be avoided.

Observe Universal Precautions (wear gloves, lab coat, and safety glasses) while handling all human urine. Disposable supplies (pipette tips, autosampler tubes, gloves, etc.) contaminated with urine are to be placed in a biohazard autoclave bag. These bags should be kept in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with a 10% dilution of commercial bleach (sodium hypochlorite) or equivalent when work is finished.

Special care should be taken when handling and dispensing sodium hydroxide solution. Always remember to add base to water. This material is a caustic chemical capable of severe eye and skin damage. Always wear gloves, a lab coat, and safety glasses. **If sodium hydroxide comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 minutes.**

B. Radioactive hazards

None.

C. Microbiological hazards

Follow Universal Precautions. Because of the possibility of exposure to various microbiological hazards, appropriate measures should be taken to avoid any direct contact with the urine specimen. Gloves, lab coats and safety glasses must be worn while handling all human urine products. A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues.

D. Mechanical hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should read and follow the manufacturer's information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the mass spectrometer unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair should only be performed by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Precautions should be used when working in these areas.

E. Protective equipment

Standard safety precautions should be followed when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

F. Training

Formal training in the use of the ion chromatograph and mass spectrometer is necessary. Users are required to read the operation manuals and should demonstrate safe techniques in performing the method.

G. Personal hygiene

Follow Universal Precautions. Care should be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing should be practiced. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

H. Disposal of wastes

Waste materials must be disposed of in compliance with laboratory, federal, state, and local regulations. Solvents and reagents should always be disposed of in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. All disposable items that come in direct contact with the biological specimens are to be placed in a biohazard autoclave bag that should be kept in appropriate containers until sealed and autoclaved. The unshielded needles, pipette tips and disposable syringes should be placed immediately into a sharps container and autoclaved when this container becomes full. Wipe down all surfaces with a freshly prepared bleach solution (a 10% dilution of commercial sodium hypochlorite (bleach) or equivalent) when work is finished. Any non-disposable glassware or equipment that comes in contact with biological samples must be washed with bleach solution before reuse or disposal. Any other non-disposable glassware should be washed and recycled or disposed in an appropriate manner.

Observe Universal Precautions. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.

3. Computerization; Data-System Management

A. Software and knowledge requirements

This method has been validated using the Dionex IC system controlled by PeakNet Software coupled with a SCIEX mass spectrometer run with the Analyst 1.4 software. Results are exported from Analyst software to Microsoft Excel files and entered into the ATLIS relational database. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

B. Sample information

Information pertaining to particular specimens is entered into the database either manually or electronically transferred. The result file is transferred electronically into the database. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier.

C. Data maintenance

All sample and analytical data are checked prior to being entered into the ATLIS database for transcription errors and overall validity. The database is routinely backed up locally onto a computer hard drive and CDs through the standard practices of the NCEH network. The local area network manager should be contacted for emergency assistance.

D. Information security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers).

4. Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection

A. Special instructions

No special instructions such as fasting or special diets are required.

B. Sample collection

Urine specimens are collected from subjects in polystyrene cryo tube vials or polypropylene (PP) centrifuge tubes. Specimen collection containers should be lot screened to ensure the absence of perchlorate contamination. Sterile collectors should be used for specimen acquisition.

C. Sample handling

Specimen handling conditions are outlined in the DLS protocol for urine collection and handling (copies available in branch, laboratory and special activities specimen handling offices). Collection, transport, and special requirements are discussed in the division protocol. In general, urine specimens should be transported and stored at 4±3°C. Once received, they can be frozen at -20±5°C until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn should be refrozen at -20±5°C. Samples are not compromised by repeated freeze and thaw cycles. Preliminary experiments indicate that perchlorate is stable in urine samples for > 9 months when stored at temperatures ≤ -20°C.

D. Sample quantity

The minimum amount of specimen required for analysis is 0.5 mL, with the optimal amount being 2 mL.

E. Unacceptable specimens

Specimens are rejected if suspected of contamination due to improper collection procedures or devices. Specimen characteristics that may compromise test results include contamination of urine by contact with dust, dirt, etc. from improper handling. Samples with visible microbiological growth (e.g. mold, bacteria) will also be rejected. In all cases, a second urine specimen should be requested.

A description of reasons for each rejected sample should be recorded on the sample transfer sheet such as low sample volume, leaking or damaged container.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibration Materials, Control Materials, and all Other Materials; Equipment and Instrumentation

A. Reagents and sources

Reagents and sources used during the development, validation, and application of this method are listed in Table 1. All chemicals and solvents are used without further purification. Reagents procured from other sources should meet or exceed these listed requirements.

Table 1. Reagents and Sources.

Reagent	Grade	Source *
Sodium Hydroxide, 50% w/w	Certified	Fisher Scientific, Fairlawn, NJ
Urisub Synthetic Urine	-	CST Technologies, Inc, Great Neck, NY
Sodium Perchlorate	98%	Sigma Aldrich, St. Louis, MO
Ammonium Perchlorate	99.999%	Sigma Aldrich, St. Louis, MO
Labeled Sodium Perchlorate ($^{18}O_4$)	98%	Isotec, Miamisburg, OH
Deionized Water	18 MOhm-cm	Barnstead water purifier

* or equivalent

B. Preparation of Reagents

(1) Sodium Hydroxide 50 mM

The 50 mM sodium hydroxide used as the eluant in the ion chromatograph is prepared by diluting 2.6 mL of sodium hydroxide (50% w/w) in one liter DI water and mixing. The solution is filtered through a 0.45 micron filter and sonicated for 5 minutes.

(2) Sodium Hydroxide 50 mM

Sodium hydroxide 100 mM solution is used to flush the column for maintenance purposes. This action is performed on a weekly basis to prolong the life of the column. The solution is prepared by diluting 5.2 mL of sodium hydroxide (50% w/w) to one liter with DI water.

C. Preparation of Calibration Materials

(1) Stock Solutions and dilutions

(a) *Perchlorate Stock Solution*

Perchlorate stock solution is prepared by weighing 10 mg of ammonium perchlorate neat standard and diluting it to 100 mL of DI water with a target concentration of 100 ppm. Successive dilutions from stock solution are

made with a final target concentration of 1 ppm from which the working standard solutions are prepared as shown in Table 2.

(b) *Labeled Internal Standard Solution, $NaCl^{18}O_4$*

Labeled standard solution is prepared by weighing 2.5 mg of ^{18}O-labeled sodium perchlorate and dissolving it in 25 mL of DI water with a target concentration of 100 ppm. Successive dilutions are made with a final concentration of 1 ppm from which the working solution is prepared. The concentration of the working solution is 0.004 ng/µL from which 500 µL is added to the sample. A solution of 500 mL of labeled internal standard solution is prepared every two weeks. This solution is prepared by taking 2 mL of 1 ppm solution and diluting it to 500 mL with DI water.

(2) Working Standard Solutions

Working standard solutions are prepared as presented in Table 2, below.

Table 2. Perchlorate Calibration Standards

Standard #	Actual perchlorate concentration (ng/mL)	Concentration after spiking 50 µL in 0.5 mL of urine (ng/mL) (ng/mL)	Amount of perchlorate in standard (ng)	Volume of 1 ppm stock solution diluted / Final solution volume
SS*mmyy*01	0.50	0.050	0.025	25 µL / 50 mL
SS*mmyy*02	1.00	0.10	0.050	0.025 mL / 25 mL
SS*mmyy*03	3.33	0.333	0.1665	0.083 mL / 25 mL
SS*mmyy*04	10.0	1.0	0.500	0.250 mL / 25 mL
SS*mmyy*05	33.3	3.33	1.665	0.833 mL / 25 mL
SS*mmyy*06	100	10.0	5.0	2.55 mL / 25 mL
SS*mmyy*07	333.3	33.3	16.7	3.33 mL / 10 mL
SS*mmyy*08	750	75.0	37.5	7.5 mL / 10 mL
SS*mmyy*09	1000	100.0	50	-

* *mmyy* represents the month and year of standard preparation.

Aliquots of these solutions are stored in 1.5 mL vials in the -20±5°C freezer until use. Once the vial is being used, it is stored at 4±3°C.

D. Preparation of Control Materials

(1) Quality Control materials

Quality control materials are prepared by spiking a known amount of perchlorate solution into 500 mL of synthetic urine to achieve the target concentration. Two urine pools are prepared at levels within the linear range of the method: a low (5 ppb) and a high QC (75 ppb). After spiking the synthetic urine with a known amount of perchlorate, the QC solutions are stored overnight at 4±3°C for equilibration. The next day the QC solutions are brought to room temperature and aliquoted into 1.2 mL cryo-vials previously screened for perchlorate and stored at -70±5°C until use.

(2) Proficiency Testing materials

Proficiency Testing materials are prepared from certified perchlorate reference solutions (AccuStandard, New Haven, CT) diluted to final concentration with synthetic urine. Aliquots are blind coded and stored in cryo-vials at -70±5°C until use. Proficiency testing samples are run twice a year as well as following any major maintenance on the instrumentation. Proficiency testing samples are blind coded for analysis; results are evaluated by an external quality control officer.

E. Other materials and supplies

Materials / supplies and sources used during the development, validation, and application of this method are listed below. Materials / supplies procured from other sources should meet or exceed these specifications.

(1) Nalgene 1.8 mL cryo-vials (Fisher Scientific, Fairlawn, NJ).

(2) Eppendorf Repeater Plus Pipette (Brinkmann Instruments Inc., Westbury, NY).

(3) Rainin Electronic Pipettes (Rainin, California)

(4) Pasteur pipettes and bulbs (Kimble Glass, Inc., Vineland, NJ).

(5) VWR Brand Mini vortexer (The Lab Depot, Alpharetta, GA).

(6) 1.5mL Vial Kit with Split Septum (Dionex, Sunnyvale, Ca)

(7) ASRS Ultra II, 2mm Suppresor (Dionex, Sunnyvale, Ca)

(8) Ion Pac ® AS 16 Column (Dionex, Sunnyvale, Ca)

(9) Nalgene Sterilization filter unit (Fisher Scientific, Fairlawn, NJ)

F. Instrumentation

Analyses were conducted with a Dionex ion chromatography system equipped with a GP50 gradient pump, AS50 autosampler, AS50 thermal compartment and a 2-mm anion self-regenerating suppressor (ASRS Ultra II) operated in the external water mode (Dionex Corp, Sunnyvale, CA). PeakNet 6 chromatography software was used for system control. The separation was performed using an IonPac AS16 column (2 x 250mm, Dionex) with a 25 µL injection loop. A SCIEX API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with electrospray interface was used for the detection of perchlorate.

(1) Ion chromatograph configuration

The ion chromatograph configuration is described in Table 3 below. The separation conditions were optimized to obtain resolution between perchlorate and other interferences present in urine (e.g. sulfate).

Table 3. Ion Chromatograph Configuration.

Parameter	Setting
Column type	AS16 (2 x 250 mm)
Column temperature	30 °C
Eluant	50 mM sodium hydroxide
Flow	0.5 mL/min
Injection Loop Volume	25 µL
Suppressor	ASRS Ultra II

(2) Mass spectrometer SRM configuration

The following parameters were optimized for the ions of interest. These parameters should be re-optimized when transferring the method to another instrument. The mass spectrometer was operated under MRM (Multiple Reaction Monitoring) mode. The transitions of interest are presented in Table 4 and typical mass spectrometer parameters are presented in Table 5.

Table 4. Perchlorate MRM Transitions.

Analyte	MRM Transition
Perchlorate ClO_4^-	
Quantification	98.9 / 83.1
Confirmation	100.6 / 85.2
Labeled Perchlorate, $Cl^{18}O_4^-$	106.9 / 88.97

Table 5. Mass Spectrometer Configuration.

Parameter	Setting
Scan type	MRM
Polarity	Negative
Ion Source	Turbo Spray
Temperature	600°C
IS	-4000 V
CAD	12
CUR	10
GSI	40 psi
GS2	50 psi
DP	-40V
EP	-10V
CE	-34
CXP	-5
Dwell Time	400 msec
Probe Y distance	2.0 mm

7. Calibration and Calibration Verification

A. Creation of curve

(1) Calibration Data

A linear calibration curve, using nine standards with a concentration range from 0.005 to 100 ng/mL, is generated using the ratio of the peak area of the analyte to the labeled internal standard. Fresh calibrators are prepared and analyzed with each set of unknowns and QCs to form the calibration curve for that set of samples.

(2) Calculation of curve statistics

The slope, intercept and R-squared value for the nine point calibration curve is generated using a 1/x-weighted linear regression. The slope and intercept of the calibration curve are determined by linear least squares fit using the Analyst 1.4 software.

(3) Evaluation of curve statistics

The R-squared value of the curve must be equal to or greater than 0.990. Linearity of the standard curve should extend over the entire standard range. If the calculated value of one calibrator deviates by greater than 20% from the actual value then that one calibrator can be excluded.

(4) Calibration verification

In order to verify the calibration curve, an external standard blind to the analyst is analyzed. Agreement with certified or accepted values should be within the 95% confidence and range intervals.

B. Use of the calibration curve

The lowest point on the calibration curve is the lowest reportable level and the highest point is above the expected range of results. The remainders of the points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns fall.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of a blank, 9 calibration standards, 2 low level QCs, 2 high level QCs and up to 75 unknown urine samples.

A. Sample preparation

(1) Preliminary sample preparation steps

(a) Allow frozen urine specimens, quality control materials, calibration standards and synthetic urine to reach ambient temperature. Mix thoroughly by inversion or vortexing.

(b) Set up and label a series of 1.5 mL autosampler vials corresponding to the number of blanks, standards, QCs and samples to be analyzed.

(2) Preparation of standards

(a) Transfer 50 µL of the appropriate standard stock solution into the appropriately marked autosampler vial.

(b) Add 450 µL of synthetic urine.

(c) Add 500 µL of the internal standard solution to make a final volume of 1 mL.

(d) Cap the vial and mix for a few seconds using a vortex mixer.

(3) Preparation of the blank

(a) Transfer 500 µL of synthetic urine into the appropriately marked autosampler vial.

(b) Add 500 µL of the internal standard solution to make a final volume of 1 mL.

(c) Cap the vial and mix for a few seconds using a vortex mixer.

(4) Preparation of the low Quality Control sample

(a) Transfer 500 µL of the QC Low stock solution into the appropriately marked autosampler vial.

(b) Add 500 µL of the internal standard solution to make a final volume of 1 mL.

(c) Cap the vial and mix for a few seconds using a vortex mixer.

(5) Preparation of the high Quality Control sample

(a) Transfer 250 µL of the QC High stock solution into the appropriately marked autosampler vial.

(b) Add 250 µL of synthetic urine.

(c) Add 500 µL of the internal standard solution to make a final volume of 1 mL.

(d) Cap the vial and mix for a few seconds using a vortex mixer.

(6) Preparation of the unknown specimens

(a) Mix (either by vortexing or repetitive sample inversion) the unknown sample.

(b) Aliquot 500 µL of unknown into vial.

(c) Add 500 µL of the internal standard solution to make a final volume of 1 mL.

(d) Cap the vial and mix for a few seconds using a vortex mixer.

B. Instrument and software setup for the IC-MS/MS

(1) Preliminary system setup

(a) *Tuning and calibration of the mass spectrometer*

Set the y distance of the probe to 8mm and infuse the PPG 3000 solution at a flow rate of 10 μL/min. Using **Manual Tuning** make sure that the Mass spectrometer is detecting the appropriate ions. Once checked, perform a **Resolution Optimization** with **Calibration** upon success. Make sure that the specified parameters are met. For peak width, the resolution is set to 0.60 ± 0.05 mass units and sensitivity is met using the ion 932 m/z with an intensity of 2.0×10^7 minimum (combined intensity of 10 scans). The tune and mass calibration of the instrument should be checked on a weekly basis.

(b) *IC system setup*

Prepare sodium hydroxide mobile phase and let the system equilibrate for one hour prior to starting a run. Make sure to fill the water reservoir for the suppressor. Once the total conductivity in the system reaches a value less than 3 μSiemens the system is ready.

(c) *Performance evaluation*

First let the system equilibrate with the method to be run (both MS and IC). To check the performance of the system, a standard is injected three times to ensure equilibration of the system. This is checked by obtaining reproducible retention times as well as good signal to noise ratio. Once this is met the system is ready to start a run.

(2) Final setup and operation

(a) *Create the run sequence*

In the PeakNet software of the IC system, create a sequence for the run using the wizard. Make sure that the appropriate number of samples is loaded, the appropriate program is selected (*mmddyy* – UP8.pgm; where *mmddyy* is the most recent date that the program was changed and/or saved).

(b) *Assign the acquisition and quantitation methods*

Import the .csv file obtained from ATLIS into Analyst. The .csv file includes the sequence information of the standards, QCs, and unknowns to be analyzed. Select the acquisition method (*mmddyy*_UP8.dam; where *mmddyy* is the most recent date that the method was changed and / or saved) and the quantitation method (*mmddyy*_UP8.qmf; where *mmddyy* is the most recent date that the method was changed and / or saved). The icons on the right corner of the window should be green in color indicating that the system has equilibrated and is ready to start.

(c) *Submit and start batch in Analyst*

Open and submit **Equilibration** batch as well as the batch of the unknowns to be analyzed. Press the "Start Sample" icon on top of the window to start run. The instrument waits for a sync signal from the IC to start acquisition.

(d) *Start the sequence in IC*

Click **Batch** in the main menu and select edit. Once the window is open select the sequence to be run starting with the equilibration sequence. Once sequences are selected, press **Start** making sure that the MS is ready to start. The system will immediately start by turning green on the first sequence to run.

(3) System shutdown

After the end of an analytical run flush the system with DI water to eliminate any salt residue accumulation. After flushing the system shut down the IC instrument as well as the MS.

C. Processing of data

Once the run has finished make sure that you note the final pressure as well as conductivity in the instrument maintenance book. All raw data files are quantified using the quantitation capabilities of the Analyst software. The peaks are automatically integrated using the quantitation method created for the analysis. The integration of each peak is visually reviewed and manually corrected when needed. A calibration curve is generated from the calibrators; QCs, unknowns and blanks are quantified against the calibration curve. The reviewed data files are saved in a report file and exported as a text file. The text file is opened in the Excel file, mcrSCIEXup8.xls available on the Q drive, and run through the macro "mcrFormatResults". The data is saved as an Excel file and imported into the ATLIS Perchlorate database for further evaluation, including the QC evaluation described in Section 10.b.2.

9. Reportable Range of Results

A. Linearity Limits

The reportable range of results for perchlorate using this method is 0.05 to 100 ng/mL. The lower reportable limit corresponds to the lowest standard 0.05 ng/mL which is greater than the detection limit for the method. The upper reportable limit corresponds to the concentration of the highest standard 100 ng/mL.

B. Limit of Detection

The limit of detection was determined (using Taylor's method[7]) by calculating the standard deviation at different standard concentrations following repeated measurements of the concentration standards in urine. The absolute values of the standard deviations were then plotted versus concentration. The intercept of the least squares fit of this line equals S_0 (value =0.0011) with $3S_0$ (0.0033 ng/mL) being the LOD. The lowest standard (0.05 ng/mL) is used as the method reportable limit, in place of the calculated LOD.

C. Accuracy

The accuracy of the assay is established by analyzing a certified perchlorate standards blind to the analyst (i.e. Proficiency Testing samples), matrix spike samples, and QC materials. The accuracy of the method was obtained by comparing the concentration results to the theoretical concentration. The results of these measurements are given in Table 6.

Table 6. Method Accuracy and Precision.

Sample		N	Theoretical ng/mL	Average	Std Deviation	CV% (stdev/avg)	% Diff
Proficiency Testing							
	PT1	5	0.19	0.19	0.01	7.6	2.1
	PT2	5	2.40	2.29	0.08	3.3	4.4
	PT3	5	12.00	11.42	0.55	4.8	4.8
	PT4	7	72.00	69.14	2.13	3.1	3.8
QC Materials							
	QC Low	20	5.0	4.92	0.21	4.2	1.6
	QC High	20	75	75.09	2.08	2.8	-0.1
Urine Matrix Spike							
	Unspiked Urine	4		4.34	0.03	0.7	
	Spiked 1 ng/mL	4	5.34	5.29	0.03	0.7	5.0
	Spiked 5 ng/mL	4	9.34	9.31	0.02	0.2	0.4
	Spiked 50 ng/mL	4	54.34	54.5	0.20	0.4	-0.3

D. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficient of variation (CV) of the method over an analysis of 20 QC samples is listed in Table 7 below.

E. Analytical specificity

The IC/MSMS system provides excellent specificity. The selection of a confirmation ion and the ratio ^{35}Cl / ^{37}Cl gives additional confirmation of the presence of perchlorate in the sample.

10. Quality Assessment and Proficiency Testing

A. Quality Assessment

Quality assessment procedures follow standard practices[8]. Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are added to each day's run sequence. The QC blank is analyzed at the beginning of each run to check the system for possible contamination or in the spiking solutions and/or reagents. Relative retention times are examined for the internal standard to ensure the choice of the correct chromatographic peak. A calibration curve is developed for the batch using a complete set of calibration standards. The calibration curve must be linear with an R^2 value of at least 0.990. The results from the analysis of a QC standard obtained using this calibration curve and are compared with acceptance criteria to assure the proper operation of the analysis.

B. Quality Control Procedures

(1) Establishing QC limits

Quality control limits are established by characterizing assay precision with 20 distinct analyses of each QC pool. Two different pools of quality control material are used, QC low and QC High. Different variables are included in the analysis (e.g. different analysts, columns, reagents) to capture realistic assay variation over time. The mean, standard deviation, coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy on a daily basis. Typical QC characterization statistics for perchlorate are listed in Table 7.

Table 7. Perchlorate Quality Control Samples.

QC material	CV	Mean - 3σ	Mean - 2σ	Mean	Mean + 2σ	Mean + 3σ
QL0404	4.18%	4.30	4.51	4.92	5.33	5.54
QH0404	2.78%	68.84	70.92	75.09	79.26	81.34

(2) Quality Control evaluation

After the completion of a run, the quality control limits are consulted to determine if the run is "in control". The quality control rules apply to the average of the beginning and ending analyses of each of the QC pools. The quality control results are evaluated according to Westgard[8] rules:

(a) If both the low and the high QC results are within the 2σ limits, then accept the run.

(b) If one of two QC results is outside the 2σ limits, then apply the rules below and reject the run if any condition is met.

i. **$1_{3\sigma}$** – Average of both low QC OR average of both high QC is outside of a 3σ limit.
ii. **$2_{2\sigma}$** – Average of both low QC AND average of both high QC is outside of 2σ limit on the same side of the mean.
iii. **$R_{4\sigma}$ sequential** – Average of both low QC AND average of both high QC is outside of 2σ limit on opposite sides of the mean.
iv. **10_x sequential** – The previous 9 average QC results (for the previous 9 runs) were on the same side of the mean.

If a QC result is declared "out of control", the results for all patient samples analyzed during that run are invalid for reporting.

C. Proficiency Testing

(1) Scope of PT

The proficiency testing (PT) scheme for this method is administered by an in-house Proficiency Testing Coordinator. Aqueous proficiency testing materials were purchased, diluted into synthetic urine, and blind-coded by the in-house PT Coordinator. The samples are analyzed and the results evaluated by the in-house PT coordinator.

(2) Frequency of PT

Five samples of unknown PT concentrations are analyzed twice a year using the same method described for unknown samples.

(3) Documentation of PT

Analytical PT results are reviewed by the analyst and laboratory supervisor, then submitted to the in-house PT Coordinator electronically. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if $\geq$ 80% of the results deviate $\leq$ 20% from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens are not analyzed until the method successfully passes proficiency testing.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If an analyte result for a quality control material falls outside of the 3σ limits for mean or range it fails the QC criteria described in section 10.b.2, then the following steps are taken.

A. If a particular calibration standard is obviously in error, remake a new dilution of that calibration standard (see section 8.a.2), reanalyze it, and reprocess the sample analyses using this new result as part of the calibration curve.

B. Prepare a fresh dilution of the failing QC material (working QC standard) (see sections 8.a.4 & 5) and re-analyze it.

C. Prepare fresh dilutions of the calibration standards (see section 8.a.1), and re-analyze the entire calibration curve using the freshly prepared standards.

If these three steps do not result in correction of the “out of control” values for QC materials, the supervisor should be consulted for other appropriate corrective actions. Analytical results are not reported for runs that are out of statistical control.

12. Limitations of Method, Interfering Substances and Conditions

The described method is highly selective. Due to excellent chromatographic and mass spectrometric resolution, we have not found any substances that have similar chromatographic and mass spectrometric characteristics. In less than 1% of urine samples the presence of an unknown compound does distort perchlorate chromatography. This problem is resolved by diluting the sample 5-fold and re-analyzing it.

13. Reference Ranges (Normal Values)

Reference ranges of perchlorate have not yet been established. We are currently quantifying perchlorate in NHANES 2003 – 2004 urine samples to establish a population-based reference range.

14. Critical Call Results ("Panic Values")

The health effects of chronic exposure to trace levels of perchlorate are unclear. Therefore a definitive panic value has not been established. The National Academy of Sciences has reviewed the toxicological literature for perchlorate, and recommended a reference dose of 0.0007 mg/Kg-day. This dose correlates to a urinary perchlorate excretion rate of 35 µg per g creatinine and would be flagged as a "high exposure level". Greer et al reported possible inhibition of thyroid hormones at a dose of 0.5 mg/Kg-day of perchlorate [9]. This dose correlates to a urinary perchlorate excretion rate of 24000 µg/g creatinine, which would be set as the "Critical Call Value".

15. Specimen Storage and Handling During Testing

Specimens may reach and maintain ambient temperature during analysis. Perchlorate in urine is stable at room temperature. If the measurement is delayed until the next day, samples should be refrigerated at 4±3°C.

16. Alternate Methods for Performing Test or Storing Specimens if Test System Fails

Alternate validated methods have not been evaluated for measuring perchlorate in urine. If the analytical system fails, then samples should be refrigerated (at 4±3°C) until the analytical system is restored to functionality. If long-term interruption (greater that 4 weeks) is anticipated, then store urine specimens at –20±5°C.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)

Results are reported to two significant digits based on assay sensitivity calculations. Study subject data is reported in both concentration units (ng/mL) and adjusted based on creatinine excretion (µg/g creatinine).

Once the validity of the data is established by the QC/QA system outlined above, these results are verified by a DLS statistician, and the data reported in both hard copy and electronic copy. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e.

supervisor, branch chief, division director). After approval at the division level, the report will be sent to the contact person who requested the analyses.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

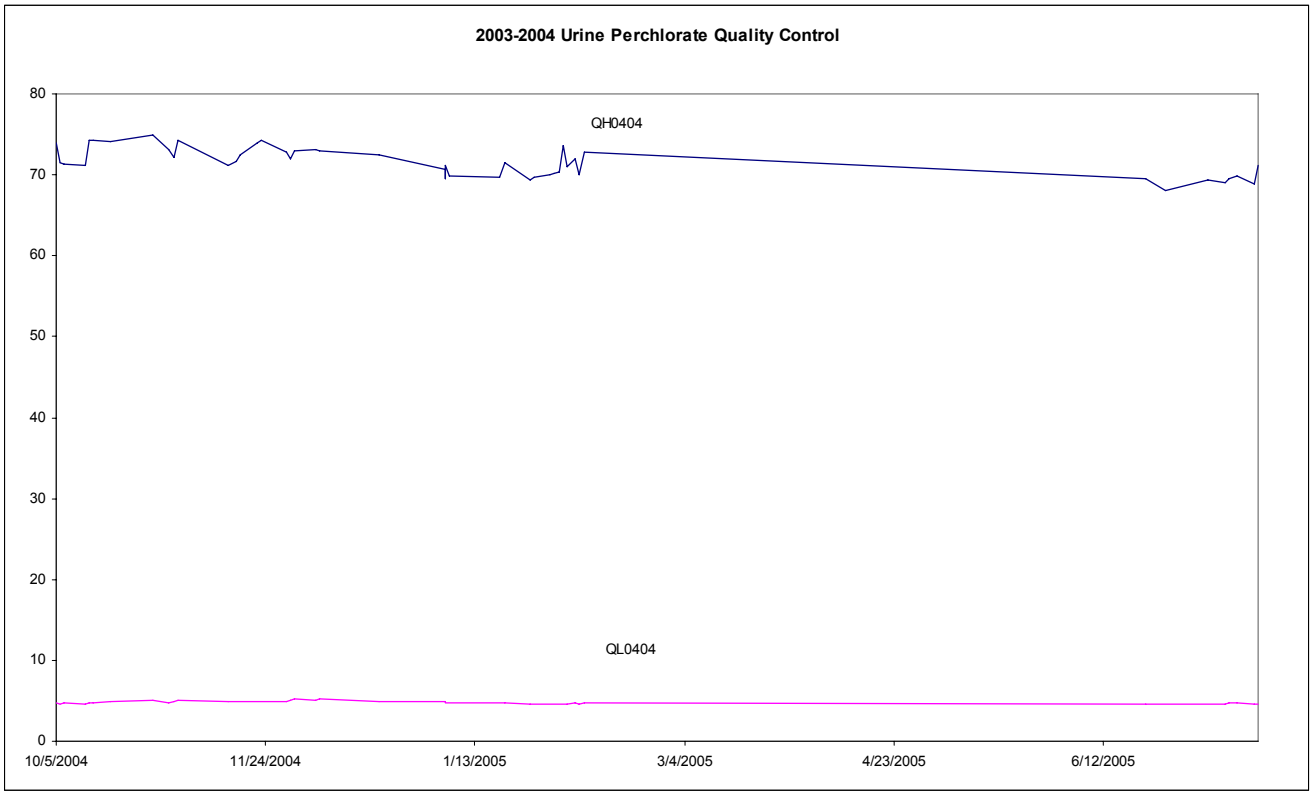
If greater than 1 mL of sample remains following successful completion of analysis, this material should be returned to storage at -20±5°C in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

Standard record keeping (e.g., database, notebooks, data files) is used to track specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer.

19. Summary Statistics and QC Graphs

Summary Statistics for Urine Perchlorate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL0404	47	10/5/2004	7/19/2005	4.76	0.18	3.7
QH0404	47	10/5/2004	7/19/2005	71.53	1.80	2.5



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