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Capillary Ion Electrophoresis of Endogenous Anions and Anionic Adulterants in Human Urine*

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ABSTRACT: Normal human urine contains many anions and cations. Ionic concentrations in urine have classically been determined by spectrophotometry of color reactions, flame emission spectrophotometry, atomic absorption spectrophotometry, high performance liquid chromatography, or potentiometry with ion-specific electrodes. Capillary ion electrophoresis (CIE) is a form of capillary electrophoresis which uses the differential electrophoretic mobility of ions to perform a separation of an ionic mixture. Various salts can be added to urine specimens to abnormally elevate ionic concentrations and interfere with either immunoassay urine drug screening procedures or gas chromatographic/mass spectrometric confirmation techniques. Application of CIE for the direct detection of endogenous anions and anionic adulterants in human urine specimens was the purpose of this investigation. CIE was performed using a Waters Quanta 4000 Capillary Electrophoresis System with either direct or indirect ultraviolet absorption detection at 254 nm. CIE of 30 random normal urine specimens and 21 urine specimens suspected of adulteration was performed. Duplicate aliquots were assayed by CIE and by colorimetric technique for nitrite. Sixteen specimens had elevated concentrations of nitrite and/or nitrate. The correlation coefficient between nitrite CIE and colorimetric results was 0.9895. Three specimens had detectable concentrations of chromate and were suspected of being adulterated with "Urine Luck," an adulterant found to contain chromate. Two specimens suspected of being adulterated with bleach were found to only contain chloride, sulfate, and phosphate. CIE is applicable to forensic analysis of urine anion concentrations. CIE can easily quantitate numerous endogenous anions and offers a method to detect and/or confirm anion adulteration of urine specimens.

KEYWORDS: forensic science, forensic toxicology, urine drug testing, capillary ion electrophoresis, urine adulterants, chromates, nitrites, nitrates, anion analysis

Normal human urine contains many ions, both anions and cations. Ionic concentrations in urine have classically been determined by spectrophotometry of color reactions, flame emission spectrophotometry, atomic absorption spectrophotometry, high performance liquid chromatography, or potentiometry with ion-specific electrodes (1,2). Capillary ion electrophoresis (CIE) is a form of capillary electrophoresis which uses the differential electrophoretic mobility of ions to perform a separation of an ionic mixture. The concentrations of ions and other endogenous solutes determine the physical chemical nature of urine. Various chemicals can be added to urine specimens to abnormally elevate ionic concentrations and/or interfere with either immunoassay urine drug screening procedures or gas chromatographic/mass spectrometric confirmation techniques (3–6). It is therefore important to differentiate natural endogenous anion concentrations from adulterated concentrations. Addition of an adulterant to the urine will often produce an aberration in the classical urinalysis results, i.e., pH, specific gravity, creatinine concentration, or presence of nitrite (7–10). In an effort to determine if a urine specimen has been adulterated, routine urinalysis, chemical analysis, or use of a dipstick test procedure may be performed (7,8,11). If an abnormal urine is detected, then further analysis may be warranted to determine the cause of the abnormality. This gives credence to the need for specific, selective, and sensitive methodologies to detect and/or confirm ionic adulterants. The purpose of this investigation was to test the application of CIE for the direct detection of anionic concentrations in normal human urine and urine specimens suspected of adulteration.

Materials and Methods

Capillary Ion Electrophoresis

CIE was performed using a Waters Quanta 4000 Capillary Electrophoresis System (Waters Corporation, Milford, MA) with a 745 Data Module using an uncoated 75 μm (i.d.) by 375 μm (o.d.) by 60 cm (length) capillary with a voltage of 16.5 kV, a current of 14 μamps, and either direct ($Cr0_4$ only) or indirect (all other anions) ultraviolet absorption detection at 254 nm (mercury lamp). Bromide (internal standard, 10 μL of 1000 μg/mL (12.52 mM) was added to 1 mL aliquots of urine which had been diluted 1 to 100 with 18 Mohm deionized water in 0.5 mL polypropylene sample vials (Waters), vortexed, and analyzed using a preformulated run electrolyte of 4.7 mM sodium chromate 4.0 mM tetradecyltrimethylammonium hydroxide (OFM-OH), 10 mM 2-[N-cyclohexylamino]-ethane sulfonic acid (CHES), 0.1 mM calcium gluconate at pH of 9.1 (Waters Corporation, Milford, MA) with indirect ultraviolet detection for chloride, nitrite, sulfate, nitrate, fluoride, and phosphate. Chromate was detected by direct ultraviolet absorption in aliquots of urine diluted 1 to 10 with 18 Mohm deionized water in 0.5 mL polypropylene sample vials (Waters), vortexed, and analyzed using a run electrolyte of 12.5 mM monosodium phosphate, 12.5 mM disodium phosphate (Fisher Scientific, Fairlawn, NJ) and 3.5 mM tetradecyltrimethylammonium hydroxide (OFM-OH) (Waters Corporation, Milford, MA) at pH of 7.0. Anions detected and their migration times were: chloride 2.89 min; bromide (internal standard), 2.97 min; nitrite, 3.01 min; sulfate, 3.11 min; nitrate

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3.21 min, fluoride, 3.4 min; and phosphate, 3.57 min. Chromate was eluted and detected separately at 3.12 min.

Analytical standards of the anions of interest; bromide, chloride, fluoride, nitrate, nitrite, phosphate, and sulfate, were prepared from stock standards (1000 μg/mL, Spex CertiPrep, Metuchen, NJ) over a concentration range of 1–20 μg/mL with 18 Mohm deionized water. Chromate standard at a concentration of 4.6 μg/mL was prepared with 18 Mohm deionized water from pyridium chlorochromate (Sigma Chemical Co., St. Louis, MO). Nitrite concentrations were determined by interpolation of the standard curves. Chromate concentrations were determined by cross multiplication of the standard. Aliquoted samples of each anion were analyzed to determine within day ($n = 5$) and between day ($n = 5$) coefficients of variation (CV).

Colorimetric Nitrite Procedure

Urine nitrite concentrations were determined by the sulfanilic acid/ N,N-dimethylnaphthylamine colorimetric diazotization technique (12). Nitrite is converted to nitrous acid which then diazotizes sulfanilic acid. Sulfanilic acid is coupled with N,N-dimethy-1-naphthylamine to yield a colored complex that is spectrophotometrically measured at a wavelength of 500 nm. Analyses were performed on a Wako 30R random access automated chemistry analyzer (Wako 30R, (a.k.a. Syva 30R) Syva Co. (Behring Diagnostics, Inc., San Jose, CA)). Urine (4 μL) is mixed with 200 μL of 0.8 g/L sulfanilic acid in 1.0 M glacial acetic acid (reagent 1) and 200 μL of 0.2 mL/L N,N di-methyl-1-napthylamine in 1.0 M glacial acetic acid (reagent 2) in a reaction cuvette on-board

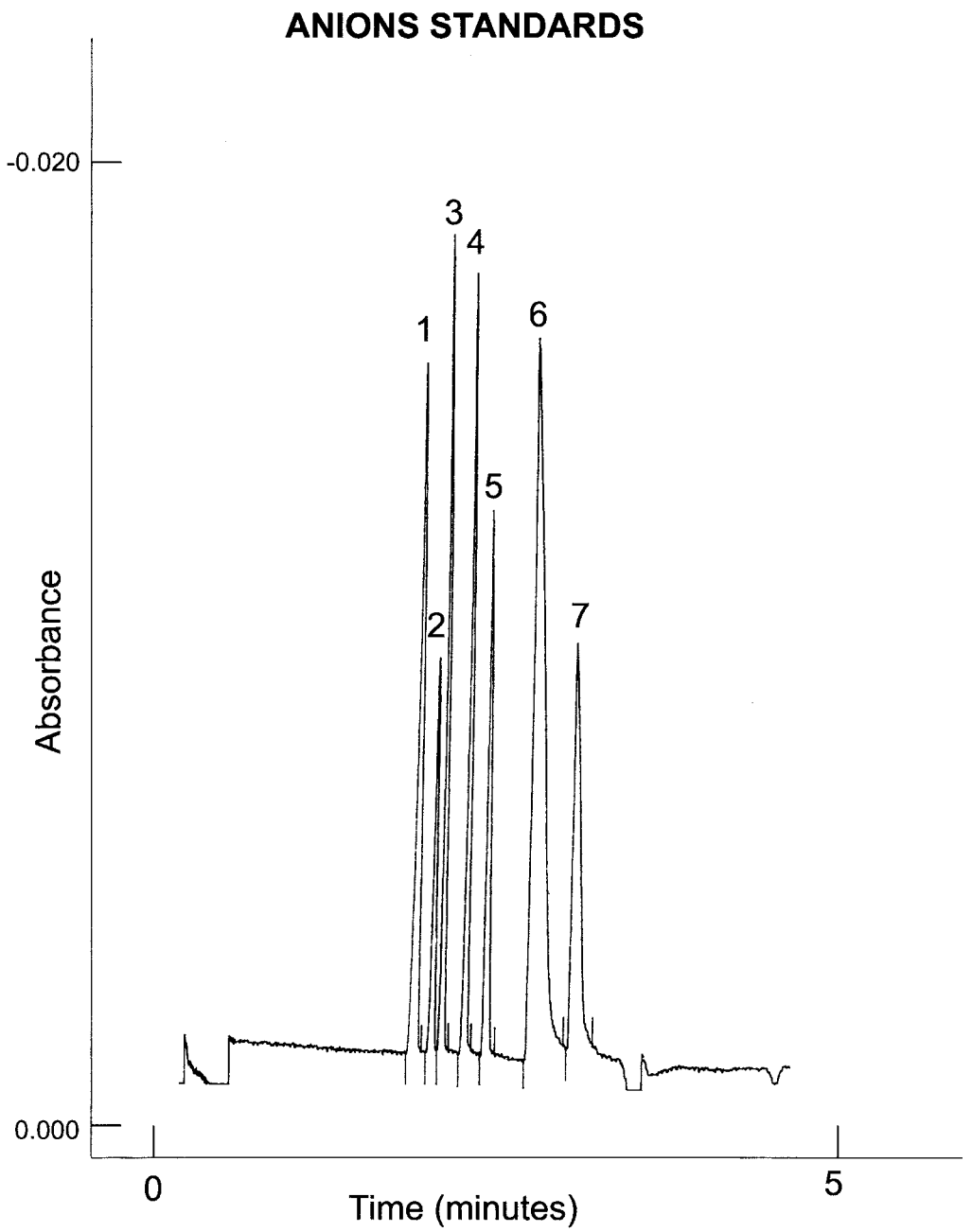

FIG. 1—*A typical electropherogram of anionic standards: 1, Cl; 2, Br; 3, NO_2; 4, SO_4; 5, NO_3; 6, F; 7, PO_4.*

the instrument. Nitrite standards (0, 100, 250, and 500 μg/mL) are made from sodium nitrite by serial dilution of the 500 μg/mL standard with deionized water. All chemical reagents were obtained from Sigma Chemical Co., St. Louis, MO. Nitrite concentrations are calculated from a single endpoint reaction by determining the absorbance change at 500 nm of the reaction mixture after 10 min from an initial reading. Nitrite concentrations are proportional to the increased absorbance of the chromaphore. Specimen concentrations are determined by interpolation of the absorbance change of the specimen from those of the nitrite standards.

Spectrophotometry

Spectrophotometric analysis was performed using a Cary 300 Bio UV-Visible Spectrophotometer (Varian Instruments, Sugar Land, TX) equipped with a Dell OptiPlex GN+ Personal Computer (Dell Computer Corporation, Round Rock, TX) and a Hewlett-Packard Deskjet 692C Color Printer (Hewlett-Packard, Palo Alto, CA). Samples were scanned in matched QS-Hellma, semimicro 1.0 mL, 10 mm optical path, quartz cuvettes (Fisher Scientific, Pittsburgh, PA) from 600 nm to 200 nm at a scan rate of 1 nm/s and a bandwidth of 2 nm in double beam mode with water as a reference. Potassium dichromate (Fisher Scientific, Pittsburgh, PA) was used as a standard at a concentration of 0.1 mg/mL in 18 Mohm deionized water. Specimen concentration was determined by direct comparison to the standard.

Specimens

Normal urine specimens from healthy adults ($n = 30$) were selected from specimens submitted to the lab for analysis. Bromide (internal standard, 10 μl of 1000 μg/mL (12.52 mM)) was added to 1 mL aliquots of urine which had been diluted 1 to 100 with 18 Mohm deionized water in 0.5 mL polypropylene sample vials (Waters), vortexed, and analyzed. Twenty-one urine specimens submitted for standard urine drug screening for drugs of abuse, which were suspected of adulteration, were analyzed. Duplicate urine aliquots were assayed by CIE for chloride, nitrite, sulfate, nitrate, fluoride, phosphate, and chromate ions and by the colorimetric nitrite procedure. A commercially available sample of "Urine Luck," a urine additive, (Spectrum Laboratories, Cincinnati, OH), believed to have been the adulterant of several specimens, was scanned spectrophotometrically following dilution with 18 Mohm deionized water (1:150) and analyzed for anions by CIE following dilution with 18 Mohm deionized water (1:1000).

Results

A typical electropherogram of anionic standards is presented in Fig. 1. Migration times were: chloride 2.89 min; bromide (internal standard), 2.97 min; nitrite, 3.01 min; sulfate, 3.11 min; nitrate 3.21 min, fluoride, 3.4 min; and phosphate, 3.57 min. Relative retention time coefficients of variation (RRT-CV, $n = 20$) ranged from 0.16% to 0.90% (Table 1). Correlation coefficients (r) between peak area ratios and concentrations over the range of 1 to 20 μg/mL were from 0.9994 to 0.9999. Repeated analysis ($n = 5$) of 2 μg/mL quality control specimens resulted in coefficients of variation (CV) ranging from 1.87 to 2.01% within day and from 1.88 to 2.03% between day (Table 2). A typical electropherogram of a normal urine specimen is presented in Fig. 2. Application of this methodology to the analysis of 30 random normal urine specimens revealed the anionic concentrations given in Table 3. Minimal detectable concentration of all anions was 0.1 μg/mL. No nitrite or fluoride concentrations were detected in any of the specimens.

Twenty-one urine specimens suspected of adulteration were analyzed. The results of the CIE and the colorimetric nitrite analysis are presented in Table 4. Sixteen specimens were found to contain elevated concentrations of nitrite and/or nitrate. A typical electro-

TABLE 1—*Relative retention time data.*

Ion	Cl	NO_2	SO_4	NO_3	F	PO_4
Mean	0.9733	1.0145	1.0487	1.0793	1.1431	1.2025
SEM	0.0035	0.0015	0.0033	0.0030	0.0102	0.0049
CV	0.36%	0.16%	0.32%	0.29%	0.90%	0.41%

NOTE: Retention times are relative to the internal standard, bromide. $n = 20$ for each anion.

TABLE 2—*Linearity within and between day statistics.*

Ion	Cl	NO_2	SO_4	NO_3	F	PO_4
r	0.9997	0.9999	0.9997	0.9999	0.9999	0.9994
Within Day						
Mean	1.87	2.02	1.94	1.99	1.95	1.88
SEM	0.006	0.012	0.008	0.011	0.011	0.013
CV	0.76%	1.28%	0.90%	1.25%	1.31%	1.47%
Between Day						
Mean	1.88	2.00	1.99	2.03	1.98	1.90
SEM	0.030	0.020	0.014	0.016	0.028	0.015
CV	3.70%	2.26%	1.57%	1.79%	3.24%	1.78%

NOTE: $n = 5$ determinations for within and between day statistics.

TABLE 3—*Anionic concentrations in 30 normal urine specimens.*

Ion	Cl	NO_2	SO_4	NO_3	F	PO_4
μg/mL						
Mean	5137	ND	1115	29	ND	1649
SEM	456	ND	118	8	ND	193
Range	750–11456	ND	194–2411	0–146	ND	100–3856
mmol/L						
Mean	144.9	ND	11.6	0.6	ND	17.4
SEM	12.9	ND	1.2	0.2	ND	2.0
Range	21–323	ND	2.0–25.1	0–3.2	ND	1.1–40.6

ND: None Detected.

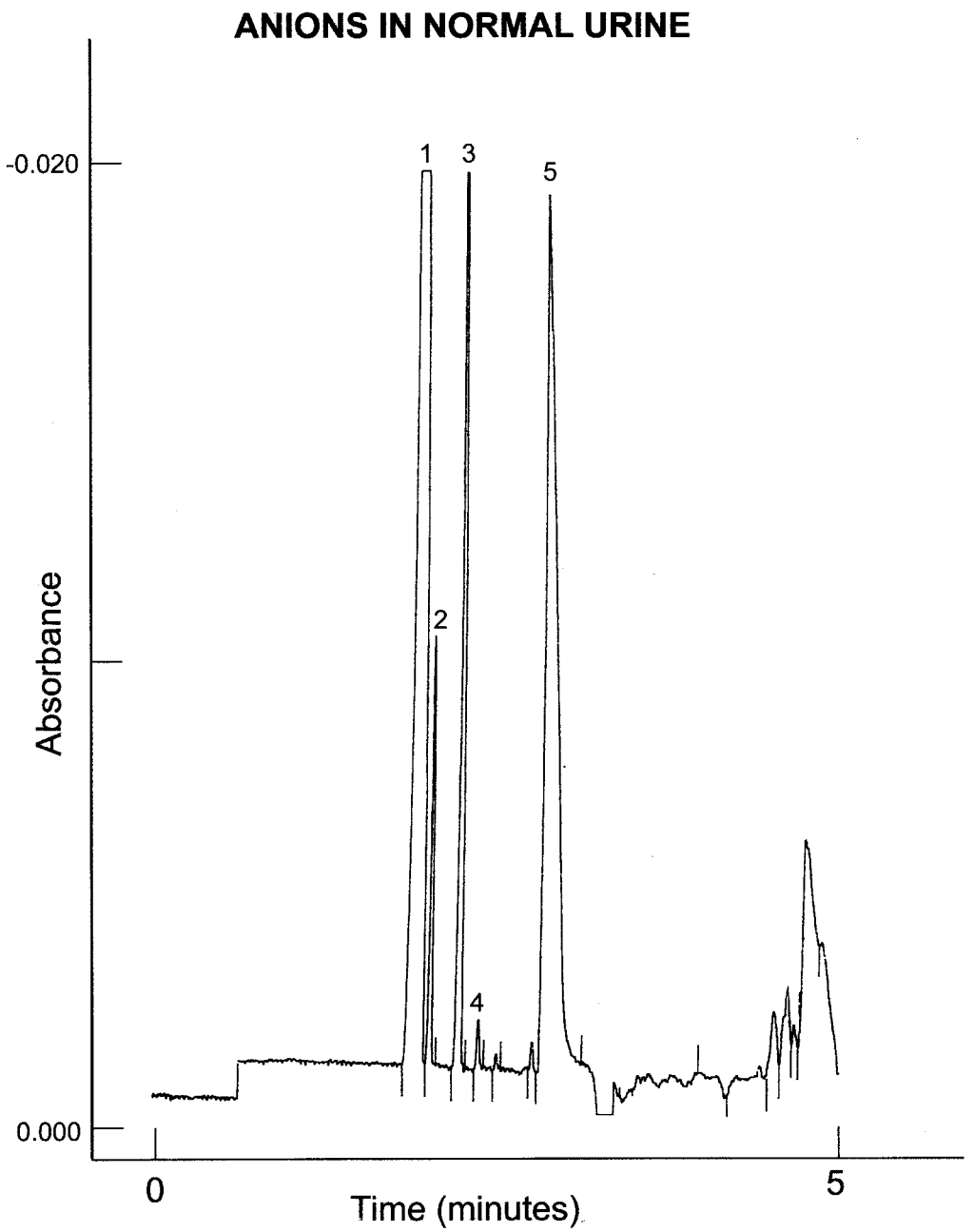

FIG. 2—*A typical electropherogram of a normal urine specimen: 1, Cl; 2, Br; 3, SO_4; 4, NO_3; 5, PO_4.*

TABLE 4—*Results of analyses of suspected adulterated specimens.*

Group	ID #	Cl	NO_2	SO_4	NO_3	F	PO_4	Cr_2O_7	Color NO_2
Nitrite	570143	4419	5565	92	574	ND	6995	ND	6700
	570174	1365	9342	158	182	ND	ND	ND	10635
	550100	6533	3182	2050	233	ND	3133	ND	3496
	559343	9060	10088	1001	361	ND	7262	ND	10090
	577585	1155	13	102	ND	ND	771	ND	31
	569918	4676	7953	2407	269	ND	6045	ND	7665
	566403	2996	4785	656	154	ND	622	ND	4179
	550555	2839	5634	927	132	ND	2032	ND	5354
	577587	7979	6192	1708	205	ND	4659	ND	5805
	494017	7688	8212	2970	312	ND	5556	ND	7837
	587498	4438	373	2099	108	ND	760	ND	358
	556865	3323	5823	2980	395	ND	7309	ND	5736
	559636	2510	72	1978	ND	ND	3692	ND	115
	579543	430	4355	226	100	ND	555	ND	4459
	590625	4277	8	1615	ND	ND	9622	ND	50
	557399	6738	2000	1352	395	ND	7536	ND	2029
Cr_2O_7	587499	2537	ND	3496	115	ND	10863	11.2	ND
	587501	7334	ND	2511	35	ND	1427	31.1	ND
	587505	5028	ND	1878	105	ND	884	44.1	ND
Bleach	586454	938	ND	61	ND	ND	31	ND	ND
	556517	2340	ND	81	ND	ND	19	ND	ND

NOTE: Concentrations are in μg/mL.
ND: None detected.

pherogram of a nitrite contaminated urine is presented in Fig. 3. Nitrite concentrations were 4600 ± 847 (mean ± SE, μg/mL). Correlation of the urinary nitrite concentrations as determined by capillary ion analysis and colorimetric analysis is presented in Fig. 4. The correlation coefficient (r) between nitrite CIE and colorimetric results was 0.9895. Anion analyte concentrations (mean ± SE, μg/mL) in the contaminated urines were chloride, 4401 ± 646; sulfate, 1452 ± 233; nitrate, 214 ± 41; and phosphate, 4159 ± 781. No fluoride concentrations were detected in any of the specimens. Three specimens had detectable concentrations of chromate, 29 ± 10 (mean ± SE, μg/mL) and were suspected of being adulterated with "Urine Luck," a commercially available urine adulterant. Electropherograms of a chromate standard (4.6 μg/mL), a 1:1000 dilution of "Urine Luck," a normal urine, and an adulterated urine with chromate are presented in Figs. 5–8. Results of the UV-visible spectral scan of a 1:150 dilution of "Urine Luck" and a 0.1 mg/mL standard of potassium dichromate are presented in Fig. 9. Two peak absorbances at 352 nm and 257 nm were noted for the "Urine Luck" and the potassium dichromate. Spectral analysis revealed that the concentration of potassium dichromate in the "Urine Luck" specimen was 17.1 mg/mL. Two urine specimens suspected of being adulterated with bleach were found to only contain chloride (938 and 2340 μg/mL), sulfate (61 and 81 μg/mL), and phosphate (19 and 31 μg/mL).

Discussion

Normal urines contain many ions, both anions and cations, at various concentration ranges. Urine contains significant endogenous concentrations of chloride, phosphate, and sulfate excreted by the kidney (1,13). There is an age dependent increase in urinary chloride concentration which correlates with dietary intake (14). Urine chloride and phosphate concentrations can be unnaturally elevated by addition of salts or soaps to the specimen (3). Minimal concentrations of fluoride may be present in the urine due to dietary intake of fluorinated water (13). Fluoride concentrations can be significantly elevated in cases of environmental and occupational exposure or poisoning with fluorinated pesticides (15). Nitrite and nitrate are used as food preservatives which are thus ingested and absorbed (4). Blood nitrite is converted to nitrate and excreted in the urine. Normal urine does not contain nitrite unless there is a urinary tract infection in which bacteria in the bladder and urinary tract convert nitrate which is then excreted in the urine (4,16). Elevated concentrations of nitrite and nitrate can also occur in the urine due to use of nitrogenous medications (e.g., nitroglycerin, nitrofurantoin) as well as the result of nitrite and/or nitrate poisoning. Extremely high concentrations of nitrites can be detected in urine specimens which have been adulterated with nitrite salts in attempts to hinder urine drug analysis (4). Normally there is not a detectable concentration of bromide in the urine which is why we selected bromide as an internal standard. Urine bromide concentrations can occur following the medicinal use of bromide salts as antiepileptics or sedative/hypnotics (16). If bromide was present or is the desired analyte then an alternative internal standard would be needed, i.e., fluoride. It is apparent that a broad range of anion concentrations in the urine can be encountered and need to be considered analytically.

Application of CIE to the analysis of 30 normal random urines revealed detectable concentrations of chloride, phosphate, sulfate, and nitrate (Table 3). The concentrations detected were comparable to normal concentrations reported in the literature by other methods of analysis (1,4,13–16). No fluoride or nitrite concentrations were detected in any of the specimens analyzed thus indicating that the individuals contributing the specimens did not have significant fluoride ingestion or any urinary tract infections. The capillary anion electrophoretic separations were very reproducible (Table 1) and the method demonstrated good accuracy, precision, and linearity (Table 2). The method is direct (simple dilution and addition of in-

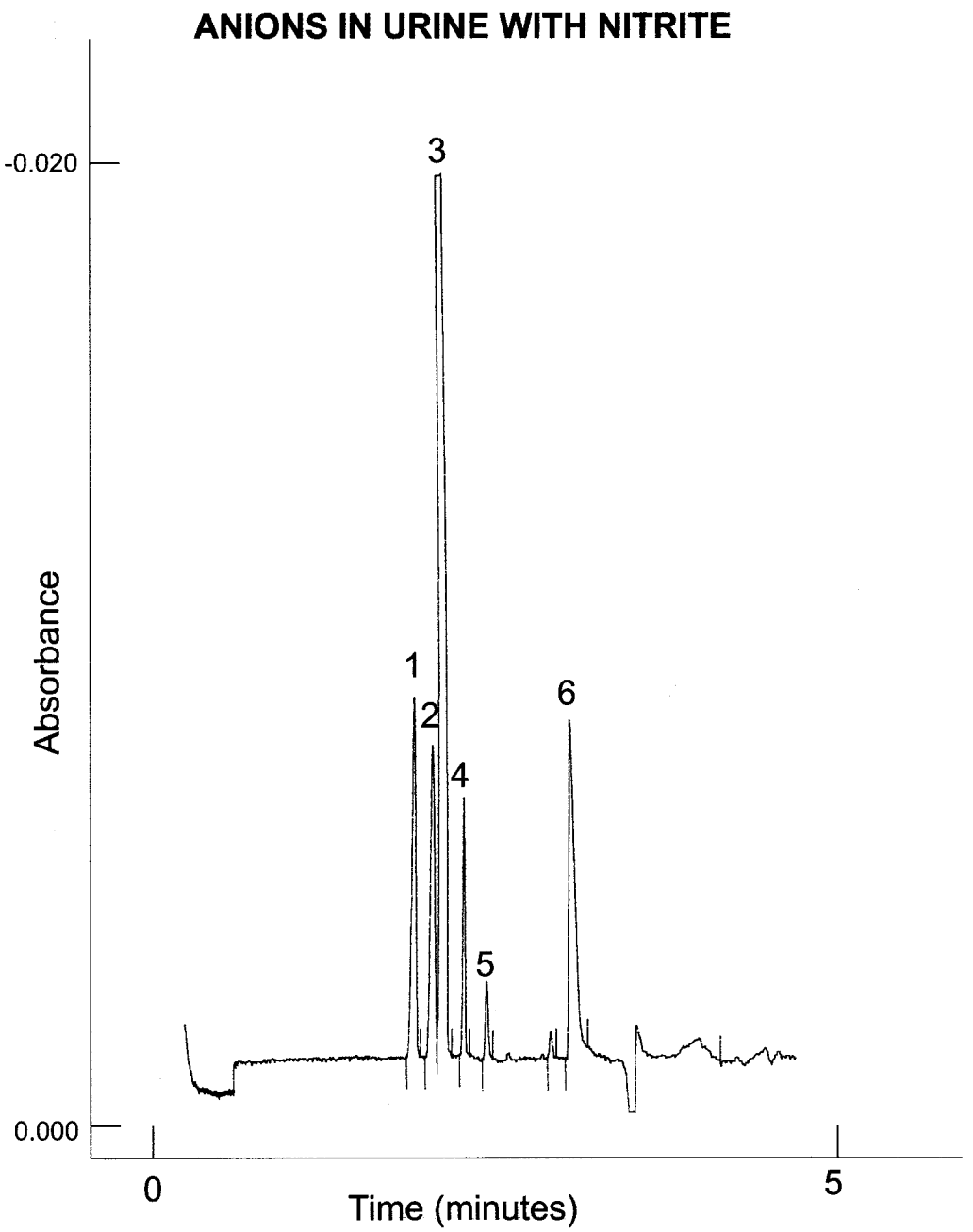

FIG. 3—*A typical electropherogram of a urine adulterated with nitrite: 1, Cl; 2, Br; 3, NO_2; 4, SO_4; 5, NO_3; 6, PO_4.*

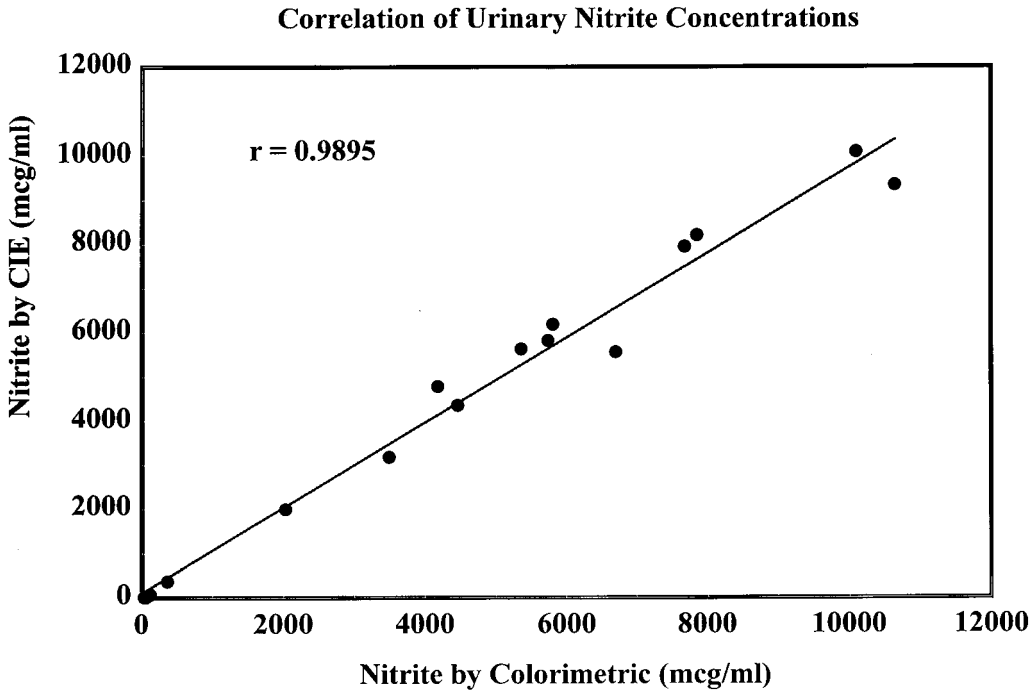

FIG. 4—*Correlation of urinary nitrite concentrations by capillary ion electrophoresis and by colorimetric analysis.*

ternal standard) and allows for the determination of multiple anions in a single analysis versus several alternative methodologies (spectrophotometry of color reactions, flame emission spectrophotometry, atomic absorption spectrophotometry, or potentiometry with ion-specific electrodes) which are designed for analysis of a single anion or group of anions. Multiple dilutions of the specimen can be performed to cover an extensive range in concentration. The procedure offers a more specific, selective, and sensitive method of analysis for confirmation of simple colorimetric or dipstick methods of testing urine for anionic adulterants. CIE is simpler and less expensive than high performance liquid chromatographic or gas chromatograph/mass spectrometric techniques (2,17).

Many different chemicals can be added to urine specimens to abnormally elevate ionic concentrations and/or chemically interfere with either immunoassay urine drug screening procedures or gas chromatograph/mass spectrometric confirmation techniques (3–6). These anions may be either natural endogenous constituents in human urine, e.g., chloride, sulfate, phosphate; exogenous anions which are excreted following dietary intake, e.g., fluoride, nitrate; or anions which are the result of bacterial contamination of the urinary system, i.e., nitrite (1,4,18). Abnormal elevation of an anion above its normal physiological or pathological range would be indicative of an adulterant being added to the specimen. Addition of an adulterant to the urine will often produce an aberration in the classical urinalysis results, i.e., pH, specific gravity, creatinine concentration or presence of nitrite (7–10). In an effort to determine if a urine specimen has been adulterated either routine urinalysis, chemical analysis, or use of a dipstick test procedure may be performed (7,8,11). If an abnormal urine is detected then further analysis may be warranted to determine the cause of the abnormality. This gives credence to the need for specific, selective and sensitive methodologies to detect and/or confirm ionic adulterants.

Results of the CIE of 21 urine specimens suspected of adulteration revealed elevated concentrations of several anions. Sixteen specimens were found to contain concentrations of nitrite and/or nitrate. Three specimens (559636, 577585, and 590625) had relatively low concentrations of nitrite (72, 13, and 8 μg/mL) and no detectable nitrate which may have been from urinary nitrate reducing microorganisms or medications that are biotransformed to nitrite. The nitrite concentrations in all the specimens found to contain nitrite are comparable to those reported in the literature for both adulterated specimens and potentially pathological specimens by other methods of analysis (2,4,18,19). Specimens are considered adulterated if the nitrite concentration is above 200 μg/mL.

Comparison of the capillary ion electrophoretic determination of nitrite to the sulfanilic acid/N,N-dimethylnapthylamine colorimetric diazotization technique revealed an excellent correlation over the concentration range determined (Fig. 4, $r = 0.9895$). The capillary ion electrophoretic method offers an alternative methodology to detect and confirm urinary nitrite concentrations. It also offers the benefit of determining other anion concentrations which may indicate or confirm other adulterants to the urine, i.e., chloride, sulfate or phosphate, or gross dilution.

Three specimens did not have detectable concentrations of nitrite or nitrate nor elevated concentrations of the other anions. They were suspected of being adulterated with "Urine Luck." Spectral analysis of the commercially available sample of "Urine Luck" revealed that it contained potassium dichromate at a concentration of 17.1 mg/mL. An alternative low-mobility electrolyte system with direct ultraviolet detection was used for analysis of chromate ion. Analysis of the "Urine Luck" confirmed the presence of the chromate ion (Fig. 6). Analysis of the three specimens revealed detectable concentrations of chromate ion (Fig. 8, Table 4) as well as normal concentrations of chloride, sulfate, and phosphate. It is more probable than not that these specimens were adulterated with chromate from the "Urine Luck."

Two specimens did not have detectable concentrations of nitrite, nitrate, or chromate. These specimens had normal concentrations

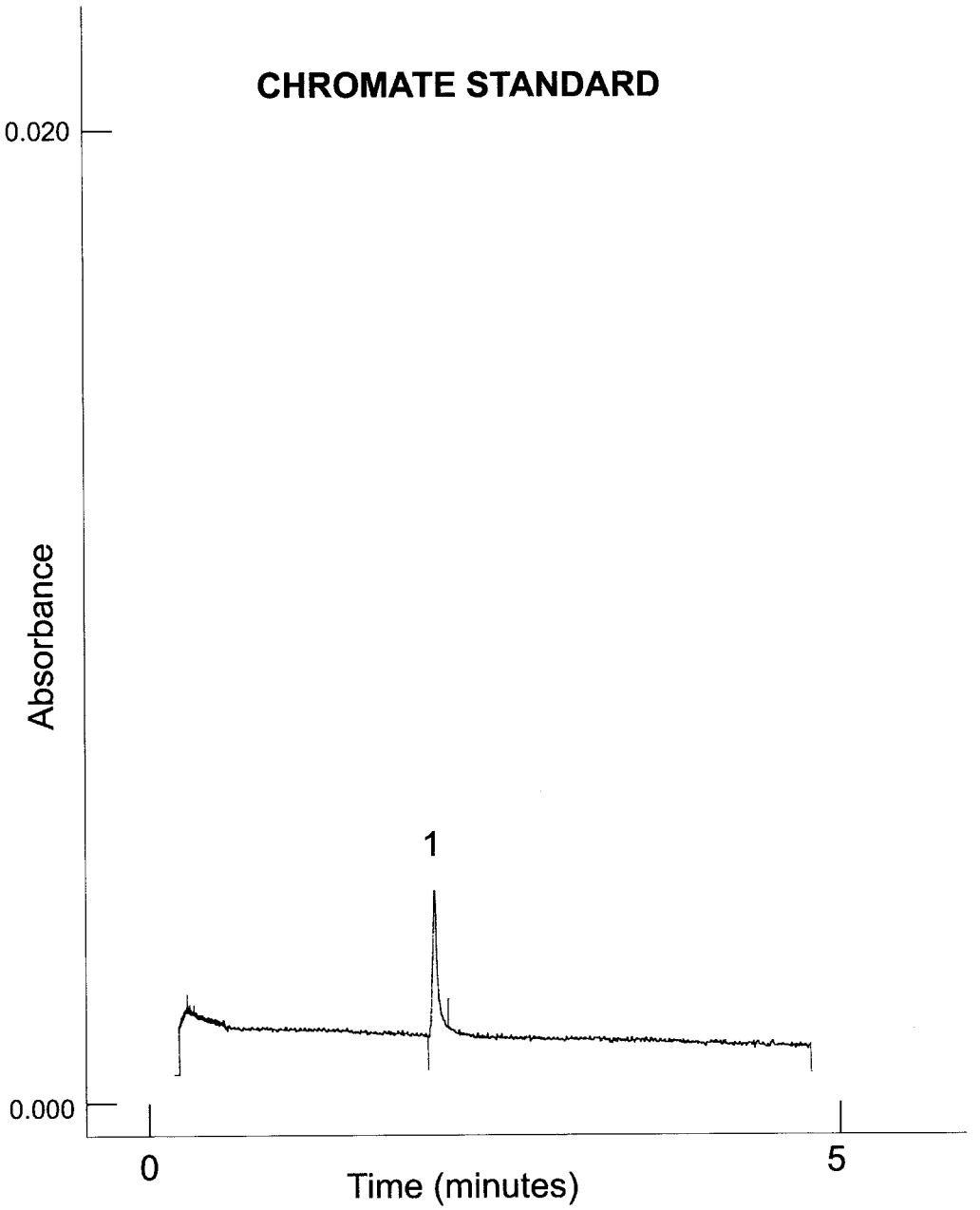

FIG. 5—*An electropherogram of a chromate standard: 1, CrO_4 (4.6 μg/mL).*

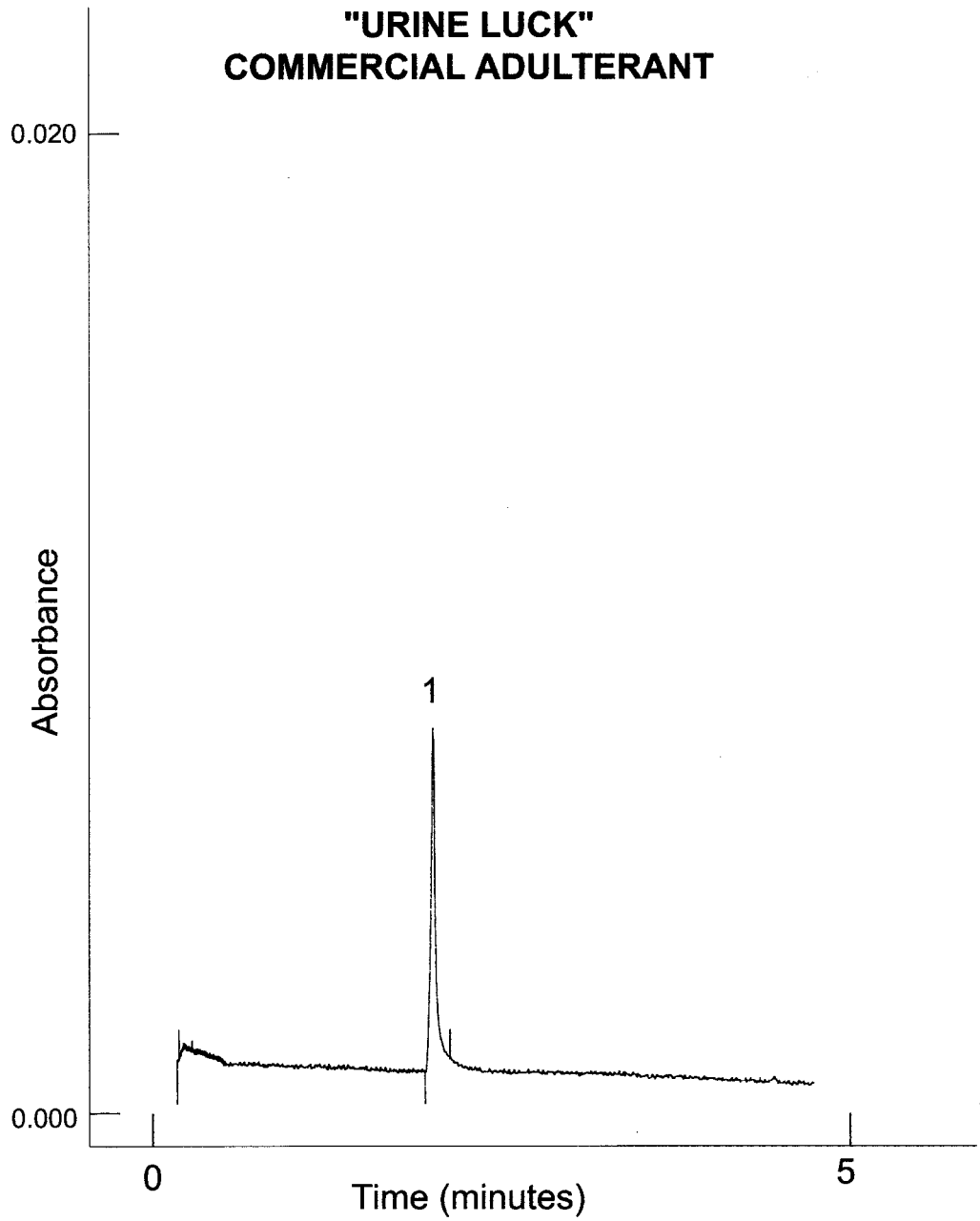

FIG. 6—*An electropherogram of "Urine Luck" (1:1000 dilution).*

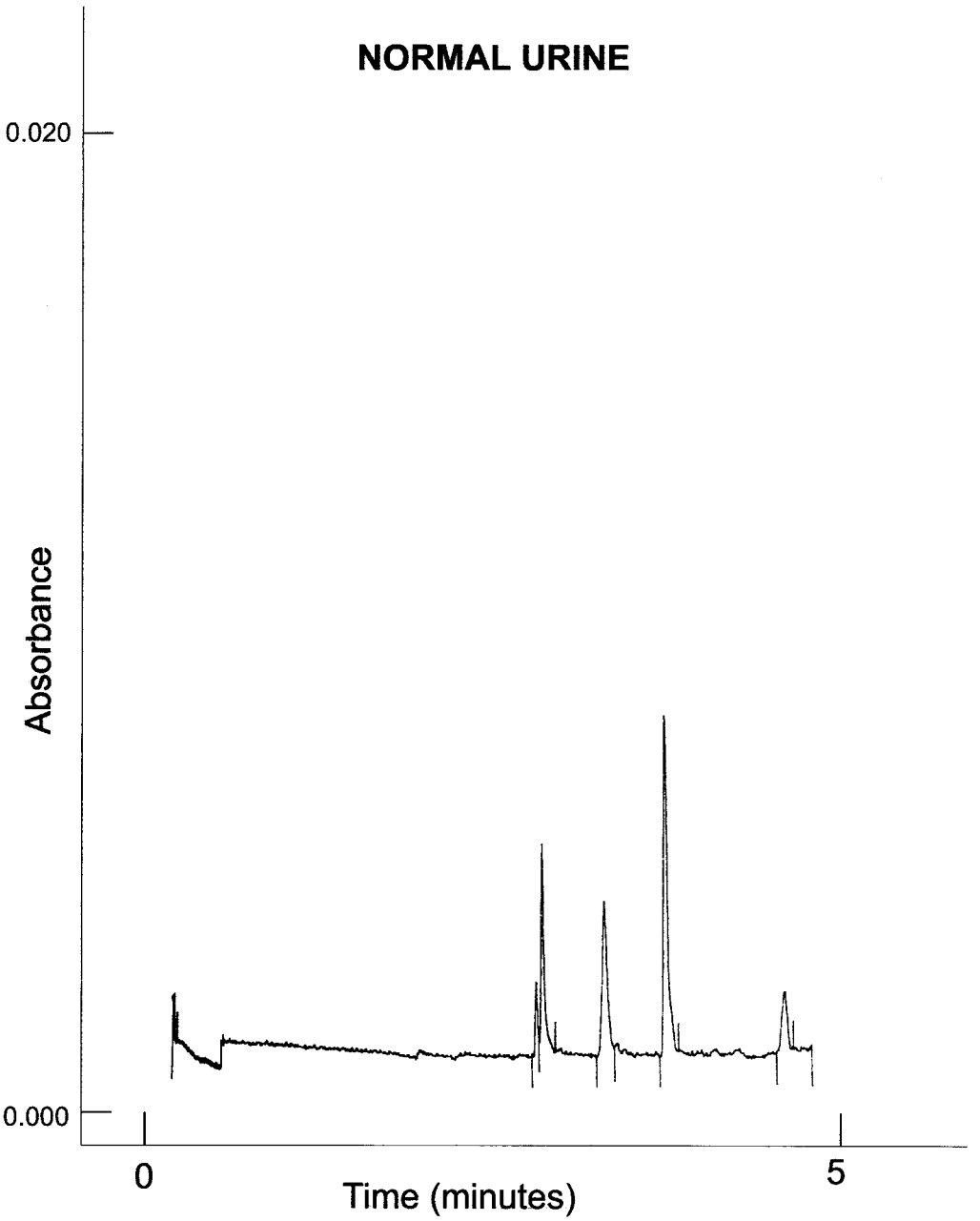

FIG. 7—*An electropherogram of a normal urine.*

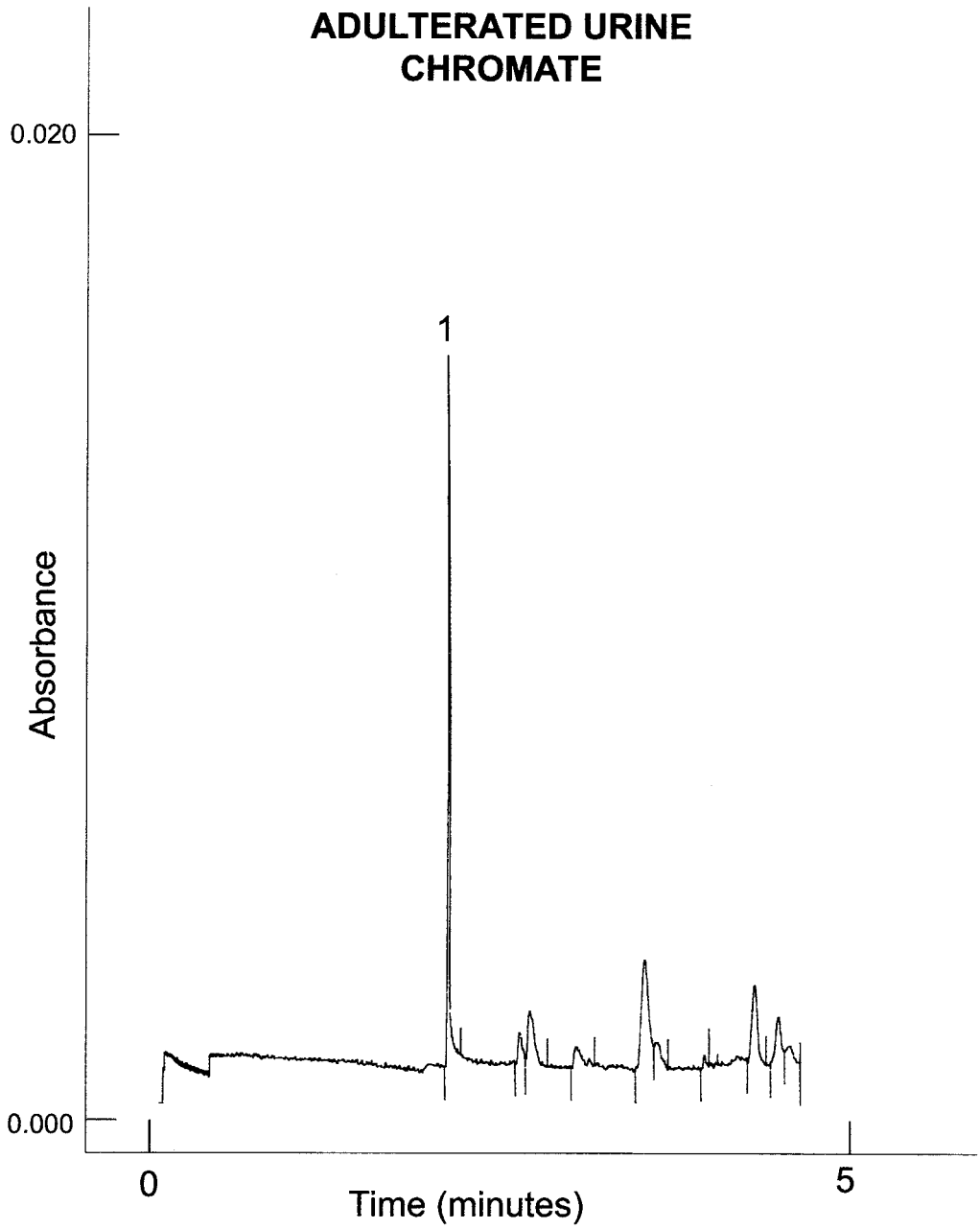

FIG. 8—*A typical electropherogram of a chromate adulterated urine: 1, CrO_4.*

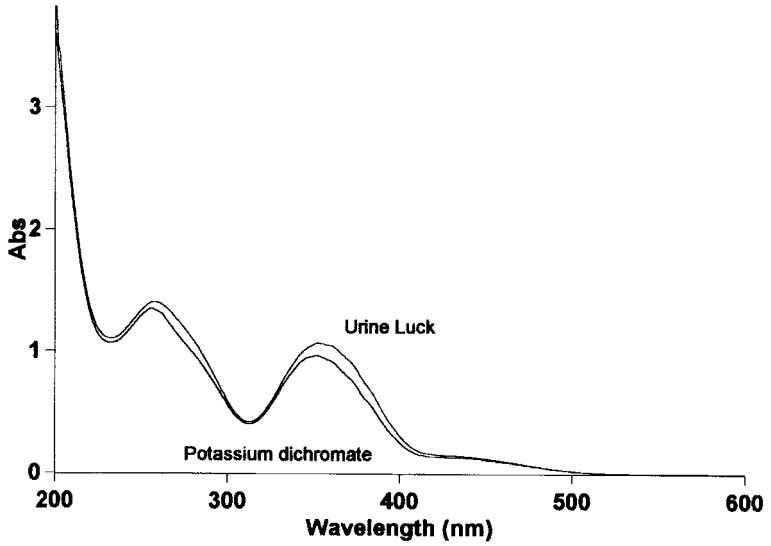

FIG. 9—*Ultraviolet-visible spectra of "Urine Luck" and potassium dichromate, (1:150 dilution of "Urine Luck" and 0.1 mg/mL $K_2Cr_2O_7$).*

of chloride but abnormally low sulfate and phosphate concentrations. These specimens were suspected to have been adulterated with bleach (sodium hypochlorite) or may have been just dilute bleach samples substituted for urine specimens. Further investigation will be needed to detect hypochlorite concentrations in urine by capillary electrophoresis.

Conclusions

CIE is applicable to forensic analysis of anion concentrations in urine specimens. The technique can easily determine endogenous concentrations of anions in urine, anionic concentrations resulting from intoxication, and concentrations of several anionic adulterants. Capillary ion electrophoretic determination of nitrite correlates well with results of the sulfanilic acid N,N-dimethylnaphthylamine colorimetric diazotization technique. Adulteration of urine with chromate ion as from potassium dichromate in "Urine Luck" can be detected by CIE using the appropriate run electrolyte. Quantitation of several anion concentrations is possible by direct CIE of urine. CIE of urine offers a method to detect and/or confirm numerous anion adulterants in urine specimens.

Acknowledgments

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