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High-sensitivity capillary electrophoresis determination of inorganic anions in serum and urine using on-line preconcentration by transient isotachophoresis

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Abstract

Concentrations of inorganic anions, both as individual species and biotransformation products, in physiological fluids are of strong concern in clinical studies. To date, analytical methodologies have either required different analytical procedures to determine these analytes in plasma and urine, or extensive sample preparation, or unconventional and often expensive detection schemes, or both. A simple and sensitive capillary electrophoresis (CE) method with direct UV detection was developed for the simultaneous determination of iodide, bromide and nitrate in human plasma and urine, with a special focus on reliable quantification of the trace serum iodide. With the latter objective, the method incorporates a transient isotachophoresis (tITP) procedure enabling an efficient on-line preconcentration of iodide (limit of detection, 1.4 $\mu g\,l^{-1}$) as well as other moderately mobile analytes that fall into the tITP range. The analyses of both types of biofluids were performed using an acidic electrolyte system composed of 0.25 $mol\,l^{-1}$ sodium chloride and 7.5 $mmol\,l^{-1}$ cetyltrimethylammonium chloride at pH 2.2 and 0.5 $mol\,l^{-1}$ 2-(*N*-morpholino)ethanesulfonate (pH 6.0) as terminating electrolyte. Relative standard deviations (R.S.D.) below 3.0% and 9.2% were obtained for within-day and between-day precision, respectively. Resolution and quantification of oxalic acid was also feasible under optimized tITP-CE conditions. Sample preparation required only ultrafiltration (serum) and dilution (urine). A number of plasma and urine samples were evaluated with this assay and the iodide, bromide and nitrate concentrations were in the expected clinical concentration ranges.

Keywords: Inorganic ion analysis; Human serum; Urine; Capillary electrophoresis; Transient isotachophoresis

1. Introduction

Over the past decade, there is growing apprehension regarding the impact of inorganic ionic species on biological systems, with an enhanced awareness that numerous biochemical processes are influenced by the species nature and contents. Much attention arises from the desire of clinical chemists to understand what inorganic ions (and at which concentration levels) are necessary for proper functioning of living organism and what are responsible for toxicological effects on human beings. For instance, iodide analysis is important in assessing the risk of developing iodine deficiency disorders and occupational exposure to its radionuclides. Clinical application of iodophores (i.e., antiseptics containing iodine) should also be controlled in view of its toxicological relevance. Last but not least, in its elemental form, iodide is a micronutrient which is essential, e.g., for the biosynthesis of the thyroid hormones playing an important role in immune defence, and exists at different concentrations in serum

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proteins, blood plasma and thyroid hydrolyzates. Bromide is an oxidative metabolite of a widely used halothane anaesthetic and can also be found in plasma and urine when workers were occupationally exposed by alkyl bromides or subjects taken bromine-containing drugs. Likewise, measurement of nitrate levels in biological fluids is critical to improve understanding of the in vivo formation and oxidative metabolism of nitric oxide, a regulator of various aspects of cellular physiology. Needless to stress, body fluid levels of these ions are indicative of an increased risk of toxicity, species resorption and excretion, malfunction of the internal organs, occurrence of several diseases, drugs metabolism and side effects, etc.

The determination of inorganic ions in biological fluids can be approached in many different ways. However, because of the complexity and variable composition of such matrices and existence of several relevant analytes simultaneously, only in a few cases can their analysis be performed directly, e.g., by high-performance liquid chromatography [1]. Alternatively, capillary electrophoresis (CE) can be implicated [1,2], as providing a number of advantages in analytical situations where extensive sample clean-up is not welcome, complex mixtures of analytes (and their different chemical species) are encountered, shorter analysis times required, the sample amount limited, material and operational costs restricted or the results of analysis are to be less compromised with regard to analyte species and matrix stability. However, the CE procedures for assaying biologically important inorganic anions reported to date [2] remain scarce and aimed on only one or two ions simultaneously, mainly nitrate and nitrite [3–5] (see also [6] for an overview of earlier related work). Part of the reasons is that the sub-mg l^{-1} concentrations at which several such analytes occur in biomatrices make UV absorbance detection not enough sensitive [7,8]. Therefore, as concerns the least abundant anion of interest, iodide, its CE determination (along with other species of iodine) in human serum and urine was only accounted for in a single report by Michalke and Schramel [9]. However, in order to quantify the trace iodide levels, a conjunction of CE with inductively coupled plasma mass spectrometry was necessary.

Advantageously, the required sensitivity thresholds in CE can be attained in a simpler and cost-effective way, by the use of an efficient on-capillary preconcentration method, e.g., transient isotachophoresis (tITP). Recently reported was the application of CE to the separation of anionic trace analytes from untreated human serum based on their enhanced focusing by virtue of the tITP stacking effect [10]. The high matrix chloride, exceeding considerably all the analyte concentrations (by a factor 100 and more), was involved in the tITP migration mode and in this way ceased its deteriorating impact on separation. Only one inorganic anion, phosphate, which also played the role of a leading co-ion, was however, concerned in that study. In our previous research [11,12], a highly sensitive quantification of iodide (down to 1 μg l^{-1}) in another sample of high-chloride content, seawater, was achieved by implementation of a thoughtfully optimized tITP procedure.

The aim of the present study was merely to develop a combined tITP-CE method capable of analyzing inorganic anions of various physiological importances. The encompassed analytes were iodide, bromide and nitrate, as well as one of the principal organic anions in biofluids, oxalate, each having appreciable UV absorbance. Special emphasis was put on the preconcentration and detection of the former less abundant anion. Effects of the separation electrolyte pH and composition and some operational variables, such as the UV detection wavelength, were fine-screened in order to optimize the resolution and direct signal response of the analytes. Successful application of the method to the determination of all target anions in several plasma and urine samples was demonstrated. Further developments of the tITP-CE technique toward extending its potential to a wider range of serum and urine anionic analytes, and hence, for high-throughput anionic profiling were discussed.

2. Experimental

2.1. Equipment

A CAPI-3100 capillary electrophoresis analyzer (Otsuka Electronics, Osaka, Japan) was used for all CE measurements. The instrument had a photodiode array detector (200–600 nm) and was equipped with a negative polarity high-voltage power supply. The optimal voltage was −12 kV across the 75 μm i.d. × 100 cm (87.7 cm effective length) long fused-silica capillary (Otsuka Electronics). The temperature was set at 25 °C for capillary chamber. The negative pressure injection mode (0.5 kg cm^{-2}) was used for sample and terminating electrolyte (TE) introduction.

For serum deproteinization experiments, a centrifugal separator Chibitan-II (Millipore, Tokyo, Japan), equipped with an Ultrafree-MC 5000 NMWL filter unit (Millipore), was employed.

2.2. Chemicals and materials

All chemicals used in this study were of the highest purity available. Water was distilled and deionized prior to use by means of a Millipore Labo-system (Tokyo, Japan). Sodium salts of Br^-, I^- and NO_3^-, and oxalic acid were purchased from Katayamakagaku (Osaka, Japan). Standard solutions were made up by serial dilutions of 10–100 mmol l^{-1} stock solutions in 90% serum and 25% urine. 2-(*N*-Morpholino)ethanesulfonate (MES) (>99.5%) (Fluka, Buchs, Switzerland), cetyltrimethylammonium chloride (CTAC) (Tokyo Kasei Kogyo, Japan), NaCl and HCl (both from Sigma-Aldrich Japan, Tokyo) were used for electrolyte preparations.

Human serum (S-7023) was obtained from Sigma-Aldrich. It was stored frozen at −18 °C and thawed out immediately before examination. Urine samples obtained from 3 healthy volunteers were filtered through a 0.45 μm membrane

filter and divided into 2-ml portions and also stored at −18 °C.

2.3. Experiments

A separation electrolyte (SE) containing 250 mmol l^{-1} NaCl and 7.5 mmol l^{-1} CTAC with the pH adjusted to 2.2 with HCl (see Section 3.1 for the optimization trials) was prepared to separate the analyte anions. The high-chloride concentration not only offered the possibility of circumventing interference from biofluid matrix salinity (ca. 100 mmol l^{-1} of chloride) but also ensured a certain stacking effect. Furthermore, such high acidic/cationic conditions resulted in a greatly reduced electroosmotic flow (EOF) and consequently, reasonably fast migration times of anionic analytes; CTAC functioned, therefore, as an anion selector (by selectively decreasing the mobility of iodide) rather than a modifier of the EOF. In addition, the surfactant may protect the capillary walls against remaining protein adsorption. As the TE, MES was employed at a highest achievable concentration of 0.5 mol l^{-1} and pH 6.0, which is in a close proximity of its pK_a (6.03). In a combination with the highly mobile matrix chloride as the leading ion, MES works fairly effective to focus isotachophoretically the moderately mobile anions, as was proved by our earlier computer simulations [11].

The pre-treatment of the capillary involved flushing with 0.1 mol l^{-1} NaOH for 30 min and with water for another 30 min. It was then conditioned with the SE for 3 min before sample injection. Prior to each run, the capillary was rinsed with the SE for 3 min.

Urine samples were subjected to CE analysis after an appropriate dilution. The direct injection of serum was found inappropriate because of denaturation and subsequent precipitation of sample proteins at low pH of SE. To remove proteins, the serum sample was ultrafiltrated by a two-step centrifugation procedure. After a preliminary centrifuging for 30 min at 10,000 rpm, six filtering vials with 0.5 ml of supernatant in each vial were set in the centrifugal separator and further centrifuged for 60 min (10,000 rpm). Such a procedure yielded ca. 1 ml of a pooled ultrafiltrate, containing 35% (w/w) of the original serum sample, suitable for CE analysis.

3. Results and discussion

3.1. Optimization of separation electrolyte composition

Biological fluids are often complex mixtures of analytes displaying pH-dependent anionic functions. Therefore, a key parameter for resolving the analyte peaks when dealing with serum and urine matrices is the pH of the SE. To decrease the number of peaks due to (weak) organic acid anions for the sake of separation of the target inorganic anions, low pH chloride electrolytes were tested. Fig. 1 shows two sets of electropherograms recorded in the pH range of 1.8–2.4 at

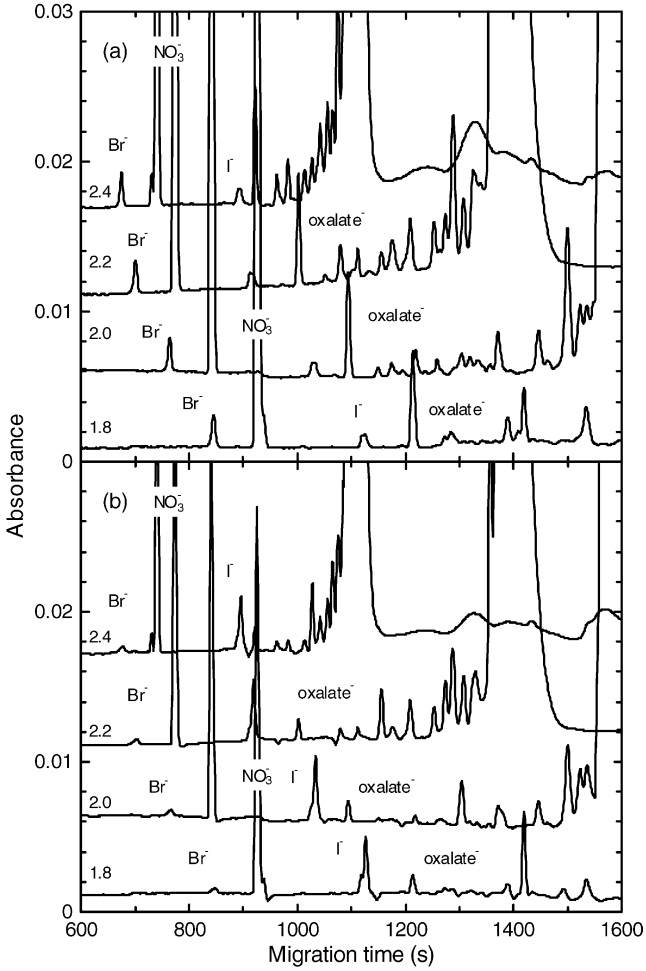

Fig. 1. Effect of the pH of SE on the separation behavior of urine anions. tITP-CE conditions: capillary, fused-silica, 100 cm × 75 μm i.d.; SE, 250 mmol l^{-1} NaCl, 7.5 mmol l^{-1} CTAC, pH adjusted with HCl to the values shown on electropherograms; TE, 0.5 mol l^{-1} MES, pH 6.0 (NaOH); sample and TE introduction, negative pressure at 0.5 kg cm^{-2} for 3 and 8 s, respectively; applied voltage, −12 kV; detection at (a) 210 nm and (b) 226 nm. Sample: four-fold diluted urine.

two principal UV wavelengths (see Section 3.3). It can be seen that the number of peaks detected tends to lower when going from pH 2.4 to 1.8. Next observation is that the higher the pH, the worse is the resolution that implies the concomitant effect of many peaks stacked and the separation window narrowed at larger EOF. On the other hand, decreasing the electrolyte pH improves the resolution but this takes place at the expense of longer migration times. From the standpoint of an optimum between separation performance and analysis time, an electrolyte of pH 2.2 was accepted as a suitable selection. Note that whether the analysis of organic anions with small abundances is a task, SEs of lower pH seems more preferable.

To investigate the influence of CTAC on separation resolution, the surfactant was added at increased concentrations to 250 mmol l^{-1} sodium chloride electrolyte of pH 2.2. As shown in Fig. 2, a delay in migration of iodide was evident.

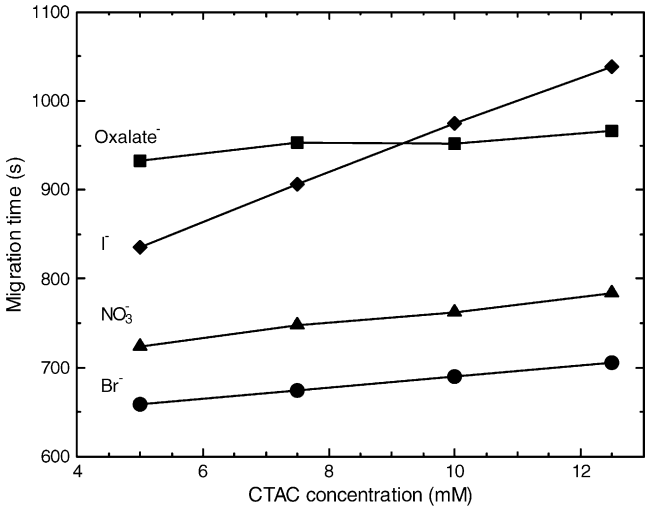

Fig. 2. Migration of anions vs. CTAC concentration. SE, 250 mmol l^{-1} NaCl (pH 2.2) containing CTAC as different concentrations. Detection at 226 nm. Other conditions as in Fig. 1.

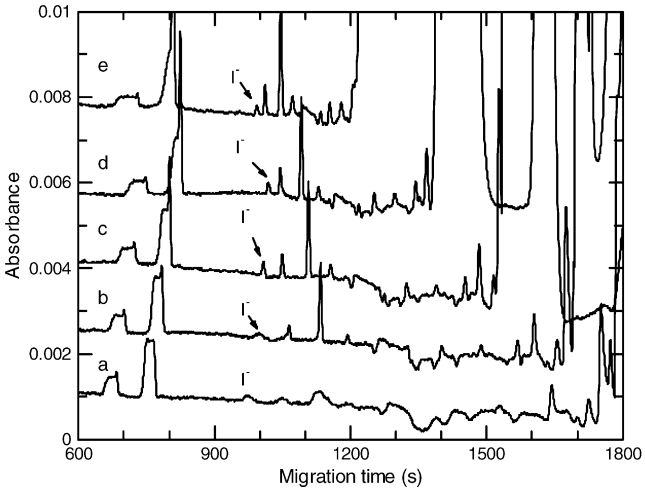

Fig. 3. Detection of serum iodide influenced by the loading of TE. SE, 250 mmol l^{-1} NaCl, 7.5 mmol l^{-1} CTAC, pH 2.2. TE introduction time (s): (a) 0; (b) 2; (c) 4; (d) 6; (e) 8. Sample introduction, 6 s. Other conditions as in Fig. 2.

In contrast, other test anions exhibited only minor changes in migration times as a function of CTAC concentration. Such a dissimilarity in the migration behavior is customarily interpreted in terms of different abilities of analyte ions to ion-pair formation with the cationic surfactant [13,14]. Iodide, possessing a higher polarizability and hydrophobicity, inclines to a stronger ion-association interaction, the asset that was successfully exploited in the related published work on tITP-CE [11,12,15–17]. A surfactant concentration of 7.5 mmol l^{-1} was recognized as a better choice to separate iodide from the associated anions (see also Fig. 4).

3.2. tITP preconcentration

Analytes in biological fluids may differ significantly in abundances, often being present at extremely low concentrations. In the frames of this work, trace serum iodide is such particular case. Indeed, whereas other target inorganic anions are encountered in urine and serum from the low- to sub-mg l^{-1} level (including the urine iodide), iodide usually occurs in serum in concentrations less than 20 μg l^{-1}. It is this circumstance, as well as the projected extension of the tITP-CE method to the determination of minor biofluid organic ions, that was the stimulus for incorporation of the tITP preconcentration step into the overall analytical scheme.

The amount of terminating ion is known to greatly affect the tITP system performance. Therefore, at a fixed concentration of MES, the loaded volume of TE should be considered in order to attain the highest enrichment factors with compromising no resolution, and potentially, sensitivity. Increasing the TE injection time, all else system variables remaining constant, gives rise to the iodide peak, as readily seen in Fig. 3. In practice, there is an optimum loading of TE that can be employed without a trade-off in resolution and detectability regarding other analyte peaks; that is, a loading time of 8 s in our case. It is also important to recognize that if one did not implement the tITP stacking, the detection of iodide in serum becomes unfeasible (see Fig. 3; a lower trace).

3.3. Limit of detection, calibration range and reproducibility

Another operational parameter that intrinsically influences direct UV detectability is the detection wavelength. The possibility of sensitivity improvement by a virtue of recording the analyte signals at a wavelength where they have maximum absorbance (or simultaneously at several wavelengths) is emphasized by a selection of electropherograms given in Fig. 4. The two wavelengths, corresponding to the optimum detectability for Br^-, NO_3^- and oxalate (210 nm) and I^-

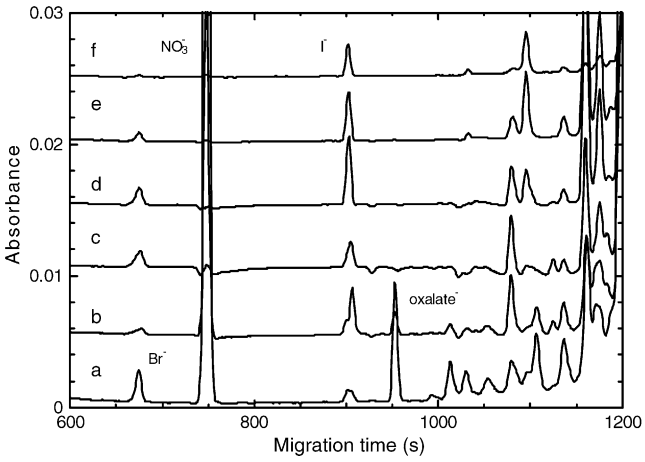

Fig. 4. Anion signal responses at different wavelengths. SE, 250 mmol l^{-1} NaCl, 7.5 mmol l^{-1} CTAC, pH 2.2. Detection wavelength (nm): (a) 210; (b) 226; (c) 245; (d) 260; (e) 275; (f) 295. Sample: undiluted urine. Other conditions as in Fig. 1.

Table 1
Figures of merit of the tITP-CE method

Analyte	Detection limit[a] (mg l^{-1})		Repeatability (R.S.D. (%))		Reproducibility (R.S.D. (%))		Working range[b] (mg l^{-1})	R^{2c}	
	Serum	Urine	Migration time	Peak area	Migration time	Peak area		Serum	Urine
Br^-	0.54	0.44	0.12	1.2	2.4	9.2	1.5–800	0.988	0.989
NO_3^-	0.09	0.13	0.14	2.0	2.1	6.2	0.4–400	0.985	0.999
I^-[d]	1.5	1.4	0.30	3.0	2.4	8.6	4.5–120	0.987	0.995
Oxalate	0.04	0.12	0.16	2.4	2.1	2.1	0.3–30	0.996	0.994

[a] Obtained for the diluted samples.
[b] External calibration.
[c] Correlation coefficient (n = 5).
[d] All concentrations are in μg l^{-1}.

(226 nm), were therefore, used in parallel for all quantitative measurements. (Note that although the peak of I^- was strongest at 260 nm without dilution (see Fig. 4d), this wavelength is less suitable for detecting lower iodide concentrations [14].) Differences in the UV spectral patterns of analytes also make diode-array detection valuable for adding extra selectivity dimension to CE data. Along with spiking experiments, this permitted unambiguous identification of peaks. The limits of detection for the analyte ions are listed in Table 1. Since the matrices of interest typically encounter comparatively high concentrations for most of the analytes, the sensitivity thresholds were evaluated from the translation of actual sample responses to a signal-to-noise ratio of 3.

The external calibration results are also summarized in Table 1. Correlation coefficients were all 0.985 or better. Intra-day and inter-day precision measurements were performed with a four-fold diluted urine sample. The results indicate excellent repeatability obtained for migration time (R.S.D., 0.12–0.30%; n = 15). The repeatability R.S.D.s for peak area were below 3.0%. The values of the reproducibility testing, varied between 2.1 and 9.2% R.S.D. (see Table 1), show a satisfactory long-term stability of the method. Such precision thresholds are low enough to make the procedure acceptable for routine analysis purposes.

3.4. Applicability of the developed method

The proposed method was applied to determine the concentrations of Br^-, NO_3^-, I^- and oxalate in serum and urine samples. Typical electropherograms are presented in Fig. 5. Baseline resolution both for the target analytes and from matrix anions was obtained with direct injection of the protein-free serum and the urine samples prepared without deproteinization. In the CTAC-mediated electrophoretic system, iodide is known [14] to suffer from rather poor peak shapes at its fairly high concentrations what is true for the urine matrix. Therefore, to improve precision and to put into effect an additional stacking concentration, urine was appropriately diluted with deionized water (the concentration of iodide should be less than 120 μg l^{-1} under present injection conditions). The measured concentrations are shown in Table 2. All these values are quite similar to those reported by CE, HPLC and other methods (Table 3). Other urine samples contained iodide at significantly higher levels (8.8 ± 0.7 and 3.2 ± 0.2 mg l^{-1}, respectively). In order to further validate the method's accuracy, the recoveries

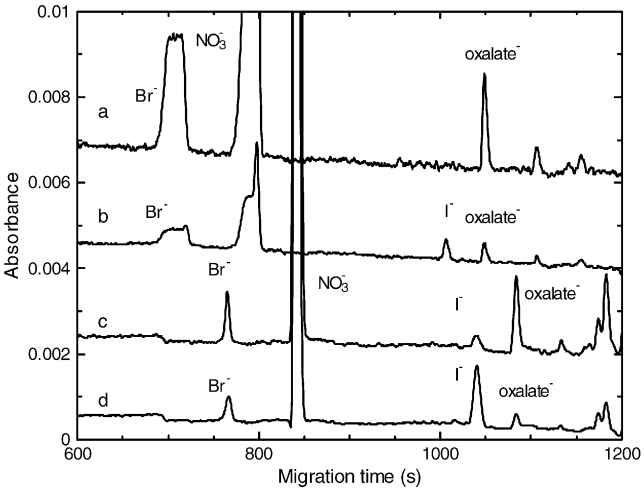

Fig. 5. Electropherograms of: (a) and (b) serum; (c) and (d) four-fold diluted urine samples. Detection at: (a) and (c) 210 nm; (b) and (d) 226 nm. Other conditions as in Fig. 4.

Table 2
Concentrations of anions[a] in biological fluids determined by the tITP-CE method

Analyte	Serum[b]				Urine[b]			
	Mean value ± S.D.	Spiked	Found	Recovery (%)	Mean value ± S.D.	Spiked	Found	Recovery (%)
Br^-	3.51 ± 0.10	4.47	7.88	98.7	2.19 ± 0.04	1.33	3.49	99.1
NO_3^-	2.07 ± 0.07	2.05	4.11	99.8	77.3 ± 3.7	51.7	127.2	98.6
I^-	3.5 ± 0.3	2.5	6.1	101.7	130 ± 5	69.8	199.1	99.6
Oxalate	2.25 ± 0.19	1.80	3.93	93.1	2.79 ± 0.04	1.80	4.57	98.8

[a] In mg l^{-1}, except iodide (μg l^{-1}).
[b] n = 3.

Table 3
Comparison of analyte anion concentrations in normal serum and urine determined by different methods

Serum	Urine	Method
Bromide (mg l^{-1})		
3.51 ± 0.10	2.19 ± 0.04	This work
4	–	HPLC [18]
–	2.7	HPLC [19]
–	4.7–12.9 [20]	Others
5–25 [21]	–	
Nitrate (mg l^{-1})		
2.07 ± 0.07	77.3 ± 3.7	This work
2.1 ± 1.1	–	CE [22]
–	36.6 ± 7.1	CE [3]
2.3 ± 1.0 [23]	39.3 ± 16.4 [23]	Others
2.2 ± 0.8 [24]	–	
Iodide (μg l^{-1})		
3.5 ± 0.3	130 ± 5	This work
13 ± 2.5	–	CE [9]
2.9 ± 1.7	–	HPLC [25]
–	124 [26]	Others
–	91 ± 37 [27]	

of anions being analyzed were determined by adding their known standards to the serum and urine samples. The reasonable results were obtained (Table 2).

4. Conclusions

Thousands of samples, containing hundreds of ionic analytes, must be routinely analyzed in clinical laboratories. A huge amount of analytical information to be acquired places strict requirements on the analytical methodology utilized regarding ease, low cost and speed of use and amenability for automation. Needless to say, a preference should at any circumstance be given to techniques capable to determine reliably as many analytes as possible. Such an array of benefits is distinctive to capillary electrophoretic methods, as has been demonstrated in this study for the profiling of several inorganic anions in the most important types of biological fluids, serum and urine. Samples required no complex pretreatment prior to analysis, and near-trace level analytes can as well be quantified by taking advantage of a simple and rapid on-line tITP procedure. The proposed method is well suited for medically important assays involving the majority of inorganic anions but still not evolved to the identification and determination of most organic acids known as diagnostic metabolites and pathologic markers. Future work planned for this laboratory is, therefore, adapting this simple method to monitor the organic acid profiles of body fluids. Such an CE assay is expected to find a wide range applications in the field of cellular physiology, biomedical research and therapeutic diagnostics.

Acknowledgments

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