

FAST AND RELIABLE DETERMINATION OF ALKALI AND ALKALINE-EARTH ELEMENTS IN BIOLOGICAL FLUIDS USING LASER-INDUCED BREAKDOWN SPECTROSCOPY

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Abstract

This work is focused on the development of a quantitative assay for the determination of Na, K, Ca, Mg, and Li in blood serum, urine, and saliva by a simple procedure using single pulse laser-induced breakdown spectroscopy (LIBS). All analyses were performed on samples in the form of homogeneous glass-supported polyacrylamide gel films containing Rb and Ba as internal standards. No additional sample preparation was necessary with only 20 μL of sample consumption. Limits of detection achieved for Li, Na, K, Ca, and Mg with the proposed setup were 0.2, 6.2, 0.6, 1.9, and 1.7 ppm, respectively. The resulting linearity for the five elements ranged from 3 times below to 2 times above the expected concentration levels variation. Relative standard deviations lower than 10% indicate the excellent precision. The trueness was proved by spike experiments and by comparing the LIBS results to an automated clinical laboratory assay.

Key words: single pulse laser-induced breakdown spectroscopy, LIBS, blood serum, saliva, urine, electrolytes, lithium

Introduction. The analysis of biological fluids has a crucial role in the diagnosis of diseases or their prevention and control. Concentrations of particular biochemical components are considered indicators of different physiological conditions, so medical decisions often rely on results obtained from analyses of biological fluids. Electrolytes in living systems such as Na^+ , K^+ , Ca^{2+} , and Mg^{2+}

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are one of the most common analytes determined in a clinical laboratory because they function in the maintenance of the body homeostasis and their levels are informative for many disease conditions [1]. Lithium medication has been widely used to prevent relapse or recurrence of bipolar disorder and is recommended as “first-line” treatment [2]. The therapeutic level of Li in blood ranges between 0.4 and 1.2 mmol L⁻¹, but the toxic effects are apparent at concentrations of ≥ 1.4 mmol L⁻¹ [3]. In addition, the increasing lithium industrial use led to elevated attention to high lithium concentrations in the working environment and to residents of lithium-polluted areas [4]. For this reason, fast and easy to implement, and possibly inexpensive assays for determination of lithium in biological materials are also needed.

Among the most common analytical methods ensuring quantification of Ca, K, Li, Mg, and Na in biological samples are flame atomic emission/absorption spectrometry [5] and electrochemical methods [6, 7], as well as combined optical and electrochemical techniques [8] incorporated in biochemical analyzers [9]. Inductively coupled plasma (ICP) emission and mass spectrometry are possible alternatives for determination of up to 60 different chemical elements, but at much higher cost and/or exhaustive sample preparation procedures [10, 11]. Laser-induced breakdown spectroscopy (LIBS) is an elemental analysis technique possessing some significant advantages such as very low sample consumption, multi-element capability, fast response, remote sensing, little to no sample treatment, relatively low instrumentation and running costs of analyses, combined with ease of use [12, 13]. Reliable and accurate quantitation of elements in biological fluids is still a challenge due to relatively poorer data reproducibility, low sensitivity, strong matrix effects, narrow linear ranges, and the need of transforming samples into a solid state, especially in single pulse configurations. These drawbacks could be overcome if the method is specifically optimized for a defined matrix with minimal variations in composition, such as biological fluids.

This work is focused on the development of a fast and reliable method for quantitation of Na, K, Li, Ca, and Mg in blood serum, urine and saliva by LIBS. The analytical procedure is based on encapsulation of the sample in a polyacrylamide gel followed by direct measurement with no need of any special sample pretreatment. The proposed method demonstrates several advantages such as easy operation, low sample consumption, rapidity, robustness, good precision and compactness within a modern clinical laboratory setup as an alternative to standard techniques for monitoring electrolytes in the clinical practice.

Experimental. Reagents and materials. All reagents were of analytical-reagent grade and all aqueous solutions were prepared in high-purity (18 MOhm) water (Millipore Corp., Milford, MA, USA). Stock standard solutions for AAS for Li, K, Ca, Mg, and Ba were 1000 mg L⁻¹ in 2% HNO₃; Trace CEPTTM (Sigma-Aldrich, USA). Sodium chloride was purchased from Merck KGaA (Darmstadt, Germany) for preparation of Na standard solution. Analytical grade RbCl salt

(Sigma-Aldrich, USA) was used for internal standard (IS) preparation. Lyophilized human serum (Precicontrol Clin Chem Multi 1, LOT 410037, Cobas, Roche, Switzerland with certified values: $2620 \pm 240 \text{ mg L}^{-1}\text{Na}$; $144 \pm 12 \text{ mg L}^{-1}\text{K}$; $90.2 \pm 10.8 \text{ mg L}^{-1}\text{Ca}$; $18.9 \pm 2.4 \text{ mg L}^{-1}\text{Mg}$) was used for the accuracy assessment. Acrylamide, tetramethylethylenediamine (TEMED), sucrose and ammonium persulphate (APS) were purchased from Fisher Scientific, USA. Microscope glasses with a 2–3 mm silicon glue flat film on top were prepared and used as a planch for sample drops deposition and film preparation. For preparation of the silicon (polysiloxan) substrate two glass slides were covered with silicon and compressed one onto another. The glasses were then separated by pulling slowly in a direction parallel to the surface of the glass so that a flat surface coating was formed. Silicon keeps drops standing during drying and film formation and does not give interfering signals in case the laser punch the sample film. The silicon covered glasses were left under pure water, changing the water several times to keep the substrate free from contamination before use.

Instrumentation. An integrated LIBS 2500+ system from Ocean Optics, consisting of a 1064 nm pulsed laser (CFR200, Quantel) emitting 10 ns, 180 mJ pulses focused to a 300 μm spot on the sample, was used for the experiments. The optical emission from the ablated material was collected by a seven channel fibre bundle (2000 μm diameter), positioned 35 mm from the focus and at 45° from the laser beam. The fibres relayed light to seven spectrometers, spanning 198–969 nm with 0.1 nm resolution (HR2000 + Ocean Optics), each with a 2048-element linear CCD array. The spectrometer was triggered to acquire 2.9 μs after laser firing and with integration time of 1 ms. LIBS was performed in air at normal conditions. The public Atomic Spectra Database of the National Institute of Standards and Technology (NIST) was used for spectral identification and peak annotation as well as spectral interferences evaluation. Sample drops were placed at pre-marked positions on a slide to facilitate rapid sample positioning at the laser focus. To lower variation of the results five to twenty laser pulses were fired in 3 to 6 different positions of the drop spot and the corresponding spectra recorded and automatically averaged by the software. The averaged spectra were integrated and the corresponding ratio between the analyte peak area and the corresponding internal standard peak area was used as analytical signal. Peak areas for Li (670.8 nm), Na (589.0 nm), and K (766.5 nm) were normalized using the peak area of Rb (as an internal standard) at 780.1 nm. Barium at 455.4 nm was applied as an internal standard for normalization of signals for Ca (393.4 nm) and Mg (279.6 nm).

Cobas Integra 400 biochemical analyzer was used for determination of the target analytes in real biological samples. Serum electrolytes Na and K were directly quantified using undiluted specimens by ion-selective electrodes. Calcium and magnesium were determined directly by use of spectrophotometric detection on the same Cobas integra instrument based on methods with o-Cresolphthalein

complexone and with Chlorophosphonazo III, respectively. The instrument was calibrated via the instrumental standard calibration procedure before use with standard calibrator solution (ISE Calibrator direct Cobas Integra, Ref. 20763055, LOT 45180301) and performance checked with normal control serum (PeciControl ClinChem Multi 1). Urine samples were run via urine method using automatically diluted specimens.

Sample collection and treatment. Pooled blood serum, urine and saliva from an adult volunteer were used during method development and previously analysed individual blood serums with known concentrations for validation of the assay. All samples were taken according to the standard operation procedures and good clinical practice of the laboratory and were immediately split into two equal volumes – one for analyzing electrolytes in clinical laboratory and one to be analyzed by LIBS. Urine samples were collected for 24 hours. All samples were either immediately processed or kept at 4 °C in refrigerator. Saliva samples were taken in one use, sterile, capped PET containers after washing teeth and triply rinsing the mouth with Milli-Q water. One hundred μL of saliva were diluted to 500 μL with Milli-Q water before analysis. Diluted samples were centrifuged at 14 000 rpm for 5 min and the supernatant used for further analysis.

Analytical procedure for determination of Na, K, Li, Mg, and Ca. A portion of 40 μL of 15% acrylamide water solution was deposited on the silicon coated planch with a micro pipette. Then 20 μL aqueous solution of the internal standards Rb (900 mg L^{-1}) and Ba (330 mg L^{-1}) were injected into the same drop with a new tip, 20 μL water solution of sucrose (2%) and 20 μL of sample or standard aqueous solution of the analytes were added to the drop with new tips. The mixture was homogenized through multiple sucking and pumping the drop with the pipette. Finally, 10 μL 0.3% TEMED and 10 μL 0.05% APS solutions were added to the drop for initialization of acrylamide polymerization. The substrate (the silicon coated microscope glass) was placed on a hot plate at temperature of 70–80 °C until the water phase from the drop evaporated. A gel spot developed for around 4–8 min and then the analytes were measured by LIBS under optimal instrumental parameters. Up to 10 samples could be prepared simultaneously on the same plate with dimensions of 30 × 70 mm and run together. The diameter of the final thin gel film drop spot obtained by this procedure was around 7–10 mm, permitting many shots per spot and easy aiming the target.

Results and discussion. Sample preparation. Compared to solids, the direct LIBS analysis of liquids presents several inherent drawbacks, such as surface ripples, splashing, and shorter plasma duration. The consequences on LIBS analytical performance are often dramatic, especially with respect to measurement repeatability and sensitivity [14]. A possible strategy for overcoming these obstacles is the conversion of the liquid specimen into a solid or “quasi solid” sample by freezing, sample deposition on a solid substrate (adsorbent), transformation of the liquid samples into a gel deposited as thin film on a substrate [15]. Here,

we preferred to use the last one due to the procedure robustness achieved. The gel was formed by adding a drop of acrylamide as a gel-forming reagent sequentially to the internal standard and sample solution. After water evaporation, a film with a defined and wide enough surface for multiple laser shots was formed. Thus prepared drop spot samples are durable at room temperature and easy to manipulate. Internal standard signals correct any variations during the thin film system formation as well as the signal fluctuations of the instrument. With this system spectral interferences were not observed.

Selection of experimental conditions and internal standards. According to the NIST LIBS spectral database, the most intensive emission lines for the analytes are: Na I 588 nm, K I 766.5 nm, Ca I 393.4 nm, Mg I 279.6 nm, and Li I 670.8 nm. These lines are the most widely used in the emission spectroscopy. Sodium concentration in human blood serum is more than one order of magnitude higher than the amount of the other analytes. The narrow working range of the detector, however, does not permit the determination of Na at its most sensitive line. The second most abundant line for Na (Na I at 568.8 nm) was selected which allows the simultaneous determination of the five elements. No interferences have been observed for these wavelengths and they were used in the measurements.

The choice of internal standards was based on the widely accepted principles for the elements that have similar behaviour to the analytes but are not naturally present in biological fluids. Moreover, the IS should not cause or suffer from spectral interferences. All of these requirements are completely met by rubidium and barium. The signal for the Rb line at 780.1 nm and Ba line at 455.4 nm does not interfere with matrix signals and the sensitivities for the analytes and IS are in the same order of magnitude. For these reasons Rb and Ba were chosen for internal standards in the present study.

LIBS analysis of biological fluids. Linear ranges, limits of detection, and limits of quantification. Serum matrix consists of water, 2–6% dissolved proteins, low molecular metabolites, and salts/electrolytes. Among electrolytes, in healthy people, Na has the highest concentration (3100–3400 mg L⁻¹) and is most likely to cause matrix effects, followed by K (137–200 mg L⁻¹), Ca (82–102 mg L⁻¹), and Mg (17–27 mg L⁻¹) [5]. Nevertheless, blood serum composition is relatively constant. For patients, treated with lithium, expected levels of Li in blood serum are between 2.8–8.4 mg L⁻¹. The high organic content in serum does not cause any interference, as it does in other analytical techniques, which eliminates the need for slow and exhaustive sample preparation and digestion. Urine is composed of water (91–96%), organic acids, hormones, N-containing substances (urea), and other organic compounds but concentrations of electrolytes, especially of Na and K, strongly depend on the particular diet. Saliva is a very dilute fluid, composed of more than 99% water where concentrations of electrolytes are also at ppm levels [16]. In all cases of biological fluids sample volume needed for the analysis was 20 µL.

External calibration with aqueous standard solutions (20 μL) spiked with the appropriate IS was performed. Five concentration points were used to construct the calibration curves for each analyte ranging approximately within 3–5 times the natural variation of the elements in blood serum being more constant matrix than urine and saliva. For each point 3×9 laser shots at different positions on the drop spot were generated, IS normalized and averaged. Taking into account the expected (therapeutic) levels of lithium in blood serum, the working range for Li was selected to be 1–30 mg L^{-1} . As can be seen from Table 1, a good linearity was demonstrated for all analytes ($R^2 > 0.9950$) and although relatively narrow, the obtained working ranges were found satisfactory. Above these ranges linearity is significantly sacrificed, deviations are observed for much higher concentrations within 3 orders of magnitude. Additionally, internal calibration for Li (exogenous element) was done by spiking the same concentrations in blood serum and urine. The obtained values for the determination coefficient R^2 (0.9995 and 0.9989, respectively) confirm the excellent linearity as well as the absence of strong matrix effects. Limit of detection (LOD, 3σ) and limit of quantification (LOQ, 10σ) for each element were determined by diluting and analyzing aqueous standard solutions until reaching peak height corresponding to 3 times (for LODs) and 10 times (for LOQs) the noise of the baseline.

T a b l e 1

Linear range, limits of detection (LOD) and quantification (LOQ) of the method

Parameter	Na	K	Li	Ca	Mg
Linear range, mg L^{-1}	1000–5000	100–550	1–30	50–300	10–60
R^2	0.9965	0.9977	0.9983	0.9984	0.9952
LOD, mg L^{-1}	6.2	0.6	0.2	1.9	1.7
LOQ, mg L^{-1}	20.4	1.9	0.7	6.2	5.5

Although LODs and LOQs are also at ppm-level, the obtained values are absolutely satisfactory for the purposes of the current assay because of the relatively high concentrations of the analytes in biological fluids. Additionally, LOD and LOQ for Li were determined by spiking blood serum and urine with decreasing concentrations of Li standard solutions until reaching signal around 3 times the baseline noise, corresponding to around 100 integration units of peak area. Thus LODs in serum and urine were estimated to be 0.3 mg L^{-1} and 0.2 mg L^{-1} , respectively, while LOQs were 0.9 mg L^{-1} and 0.7 mg L^{-1} Li.

Precision and trueness. Precision of the proposed method, expressed as relative standard deviation (RSD), was established by analysis of spiked samples of biological fluids (blood serum, urine, and saliva) following the analytical procedure described above. It was evaluated by spiking the biological fluids at three concentration levels, considering the defined working ranges (Table 1) for each an-

alyte as well as their natural occurrence. Each concentration level as well as the unspiked samples was replicated five times. The values of RSD obtained for spiked serum samples (Table 2) were less than 7%, which indicates the excellent precision of the developed procedure. The measurement trueness, defined as the closeness of agreement between the average of measured concentrations and the respective reference values, was assessed by calculation of the analytical recovery (R , %). The analytical recoveries between 95–104% approve the very good trueness of LIBS measurements for quantitation of alkali and alkaline-earth elements in biological fluids (Table 2). Very similar results were obtained after spiking the same concentrations of analytes in urine (RSD < 10%, R : 96–110%) and saliva (RSD < 8%, R : 96–107%) samples, confirming the lack of significant matrix interferences and the applicability of the method overall.

T a b l e 2

Precision and analytical recovery of the method (spot serum samples, $n = 5$, mean $\pm$ SD)

Analyte	Added, mg L ⁻¹	Found, mg L ⁻¹	RSD, %	R , %
Na	–	3350 $\pm$ 60	1.8	–
	500	3782 $\pm$ 101	2.7	98 $\pm$ 3
	1000	4439 $\pm$ 116	2.6	103 $\pm$ 3
	2000	5490 $\pm$ 102	1.9	104 $\pm$ 2
K	–	195.3 $\pm$ 3.9	2.0	–
	50	239.0 $\pm$ 4.1	1.7	97 $\pm$ 2
	150	344.7 $\pm$ 6.3	1.8	100 $\pm$ 2
	300	498.4 $\pm$ 12.1	2.4	101 $\pm$ 3
Li	–	< LOQ	–	–
	1	0.95 $\pm$ 0.06	6.3	95 $\pm$ 5
	5	5.2 $\pm$ 0.3	5.8	104 $\pm$ 5
	20	19.8 $\pm$ 0.9	4.5	99 $\pm$ 4
Ca	–	101.8 $\pm$ 4.1	4.0	–
	50	153.5 $\pm$ 5.2	3.4	102 $\pm$ 3
	200	300.1 $\pm$ 11.4	3.8	98 $\pm$ 4
	400	502 $\pm$ 18	3.6	100 $\pm$ 3
Mg	–	16.5 $\pm$ 0.9	5.5	–
	10	25.9 $\pm$ 1.5	5.8	96 $\pm$ 5
	20	36.9 $\pm$ 1.8	4.9	102 $\pm$ 5
	40	56.3 $\pm$ 2.7	4.8	99 $\pm$ 4

Furthermore, the trueness was also evaluated by analysis of lyophilized human serum (Precicontrol Clin Chem Multi 1, certified values: 2620 $\pm$ 240 mg L⁻¹Na; 144 $\pm$ 12 mg L⁻¹K; 90.2 $\pm$ 10.8 mg L⁻¹Ca; 18.9 $\pm$ 2.4 mg L⁻¹ Mg), blood serum and urine samples by the developed method (LIBS measurement) and by standard

procedure from clinical practice (biochemical analyzer). The found concentrations and the calculated values of Student's t -criteria for statistical comparison are presented in Table 3. As can be seen, t_{cal} values are lower than the theoretical value $t_{tab} = 2.31$ (95% confidence level, degree of freedom 8), indicating the lack of statistically significant differences between the results obtained by the developed LIBS procedure and those of the routine clinical assay.

T a b l e 3

Comparison between results obtained by LIBS ($n = 5$, mean $\pm$ SD) and biochemical analyzer ($n = 5$, mean $\pm$ SD)

Sample	Analyte	LIBS mg L ⁻¹	Biochemical analyzer mg L ⁻¹	t_{cal}
Lyophilized human serum	Na	2550 $\pm$ 64	2592 $\pm$ 105	0.78
	K	139.4 $\pm$ 3.1	146.2 $\pm$ 7.3	2.07
	Ca	91.1 $\pm$ 3.9	87.6 $\pm$ 5.4	1.18
	Mg	19.2 $\pm$ 1.1	18.6 $\pm$ 1.7	0.68
Blood serum	Na	3323 $\pm$ 72	3369 $\pm$ 102	0.84
	K	194.4 $\pm$ 4.2	187.7 $\pm$ 7.6	1.80
	Ca	110.1 $\pm$ 3.9	106.2 $\pm$ 6.1	1.23
	Mg	21.8 $\pm$ 1.1	20.5 $\pm$ 1.9	0.69
Urine	Na	3230 $\pm$ 62	3268 $\pm$ 84	0.81
	K	951.2 $\pm$ 15.6*	982.1 $\pm$ 27.6	2.26
	Ca	100.4 $\pm$ 5.2	106.8 $\pm$ 8.2	0.80
	Mg	61.0 $\pm$ 3.3	68.2 $\pm$ 8.4	1.92

*Measured after two-fold dilution

Conclusions. A fast, reliable, and cost-effective method for quantitation of Na, K, Li, Ca, and Mg in biological fluids drop spots was proposed using single pulse LIBS as an analytical technique. The method showed excellent accuracy and applicability in the analysis of human blood serum, urine, and saliva. In general, LIBS offers several advantages, such as very fast and easy sample preparation, small sample consumption, high sample throughput, and compact design for possibly mobile remote applications. Multiple shots per drop spot necessary for accuracy improvement may be fully automated, and in this case, the analytical time could be reduced to several seconds per sample or several minutes for a batch of many samples. Being clearly multi-elemental, this technique could be used for other elements determination as well, once some instrumental improvements in sensitivity and precision are achieved.

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