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Measurement of carnosine, homocarnosine and anserine by FASI capillary electrophoresis UV detection: Applications on biological samples

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ABSTRACT

A field amplified sample injection (FASI) capillary electrophoresis method with UV detection was developed for the separation and detection of carnosine-related peptides carnosine (Car), anserine (Ans) and homocarnosine (Hcar). The imidazole dipeptides were baseline-separated within 10 min by using 50 mmol/L Tris phosphate pH 2.2 as running buffer. The samples were diluted in water and directly injected on the capillary without complex cleanup and/or sample derivatization procedures. Using the electrokinetic injection, a sensitivity improvement of about 500-fold was achieved without any loss of separation efficiency if compared to the conventional sample injection. The detection limits for carnosine, anserine, and homocarnosine were between 0.4 and 0.5 nmol/L, thus improving of 10–100-fold the LOD of previous described methods based on laser induced fluorescence or chemiluminescence detection. This method has been applied to the analysis of homogenized rat tissue (heart, muscle and brain) and human cerebrospinal fluid (CSF).

1. Introduction

The imidazole dipeptides carnosine, homocarnosine and anserine are histidine-containing dipeptides widely distributed in vertebrate animal tissues, especially in skeletal muscle, heart and the central nervous system [1,2]. In recent years, imidazole dipeptides have been shown to play an important role in a large number of physiological functions in vertebrates, including humans [3]. Examples are buffering action in muscles, antioxidant action, neurotransmitter function, modulation of enzymatic activities, and chelation of heavy metals [3,4]. The therapeutic functions of carnosine and histidine-containing dipeptides include antihypertensive effects, actions as immunomodulating agents, wound healing, and antineoplastic effects [4]. In view of the growing interest and the biological importance of these dipeptides and also to better explore their roles in physiological and pathological conditions, attention has been focused on the development of analytical techniques to separate, characterize and quantify these compounds. HPLC with UV, fluorescence or amperometric detection has been used for determining imidazole dipeptides in tissues [4–8], plasma [9], human CSF [10] and urine samples [11], but analyses are sometimes time-consuming, because a tedious pre-treatment of the biological sample (such as solid-phase extraction or analyte derivatization) is usually necessary prior to its injection into the instrument. In some cases complex gradient systems are also required and long analytical times are needed due to prolonged elution times that add up to equilibration time between runs. Capillary electrophoresis (CE) offers several advantages over chromatographic techniques for the analysis of biological samples. The main advantages of CE are higher separation efficiency, higher selectivity, the smaller sample volume required, and minimal sample processing before injection. Recently, three capillary electrophoresis methods have been described for imidazole dipeptides evaluation, a microchip electrophoresis with chemiluminescence detection [12] and a capillary electrophoresis with laser induced fluorescence detection [13] both characterized by time-consuming sample pre-treatment (derivatization times between 4 and 9 h). An interesting sensitive field-amplified sample injection (FASI) capillary electrophoresis has been also described but the application on biological samples of this method has not been demonstrated [14]. It is well known that for FASI-CE the sample must be prepared in a low conductivity matrix to have an affective sample stacking that allows to

Abbreviations: Ans, anserine; Car, carnosine; Hcar, homocarnosine; CE, capillary electrophoresis; FASI, field amplified sample injection.

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increase sensitivity [15]. This because the FASI methods are based on the conductivity differences between the sample solution and the background electrolyte. Therefore, the application on biological samples, in which the matrix may be complex and heterogeneous (due to sample physiological content and to sample preparation steps), must be tested when a FASI-CE method is developed to verify the real gain in sensitivity. By this work we aim to develop a high sensitive FASI-CE method UV detection for the rapid quantification of Car, Hcar and Ans in real samples avoiding the use of complex and time-consuming sample pre-treatment steps (as cleanup and/or sample derivatization procedures). The developed method has been applied to the detection of histidine-containing dipeptides in homogenized muscle, heart and brain rat and in human CSF samples.

2. Materials and methods

2.1. Chemicals

Carnosine, homocarnosine, anserine, TRIS, and phosphoric acid were obtained from Sigma (St. Louis, USA). 0.45 μm membrane filters (used to filter all buffer solution before CE analysis) were purchased from Millipore (Bedford, USA).

2.2. Preparation of samples

Samples were extracted and deproteinized as previously reported [16]. Briefly, 1 g of sample tissue was homogenized with 3 volumes of 0.01 mol/L HCl in a stomacher (Seward Laboratory) for 8 min and further centrifuged in the cold (4 °C) at 10,000 rpm for 20 min. The homogenized muscle (22 mg/mL protein), heart (31 mg/mL) and brain (prefrontal cortex) (14 mg/mL) derived from one rat was mixed with 999 μL of water before injection on capillary electrophoresis. CSF from a single subject was diluted 10-fold in water before injection.

2.3. Capillary electrophoresis

A MDQ capillary electrophoresis system equipped with a diode array detector was used (Beckman instruments, CA, USA). The system was fitted with a 30 kV power supply with a current limit of 300 μA. Before use, the instrument was programmed to rinse the capillary at high pressure with 0.1 mol/L NaOH (5 min), deionized water (5 min), and finally with the running buffer (5 min). The analysis was performed in an uncoated fused-silica capillary, 75 μm ID and 60.2 cm length (50 cm to the detection window) injecting the sample by electrokinetic injection of 10 kV for 200 s. The separation was carried out in a 50 mmol/L Tris buffer titrated with 1 mol/L phosphoric acid to the pH 2.20, 15 °C and 20 kV (75 μA) at normal polarity. After each run, the capillary was rinsed with 1 min of 0.5 mmol/L NaOH, 1 min of water and 1 min of 0.1 mmol/L HCl and equilibrated with run buffer for 1 min.

3. Results and discussion

3.1. Optimization of the CE separation

Car, Hcar and Ans are basic amino acids that at low pH are positively charged and show a higher electrophoretic mobility than acid or neutral compounds in CE with a normal polarity configuration (anode at the inlet end), when an acidic run buffer is employed. Therefore, we modify our previous CE assay for the measurement of small basic analytes in which a good separation was reached by using the Tris phosphate as effective run buffer [17–22]. In order to optimize the electrophoretic separation, preliminary experiments

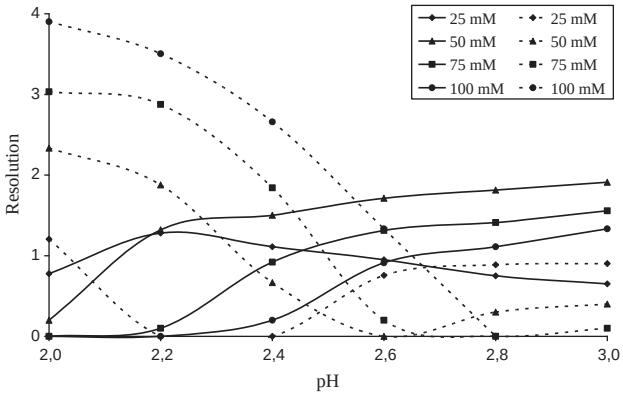

Fig. 1. Effect of concentration and pH of Tris phosphate run buffer on peak resolution of Car and Ans (continuous line) and Ans and Hcar (dotted line) in a standard mixture containing 20 μmol/L of each analyte. *Electrophoretical conditions*: uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV; detection, 207 nm; hydrodynamic injection (under vacuum), 27 nL (0.5 psi × 7 s). Concentrations refer to Tris phosphate running buffer.

were carried out by using a standard mixture containing 20 μmol/L of each analyte. A capillary with an effective length of 50 cm was employed for all the experiments. A Tris phosphate run buffer at different concentrations (from 25 to 100 mmol/L) and different pH values (from 2.0 to 3.0 pH units) was used. All experiments were performed injecting 27 nL (0.5 psi × 7 s) onto the capillary, similarly to our previous described assays [17,18]. The effect of the run buffer composition on the peaks resolution is reported in Fig. 1. By increasing pH values, a rise of resolution between Car and Ans and a decrease in resolution between Ans and Hcar were observed. Moreover an increase in the buffer concentration allowed to obtain better separations between Ans and Hcar while for Ans and Hcar a

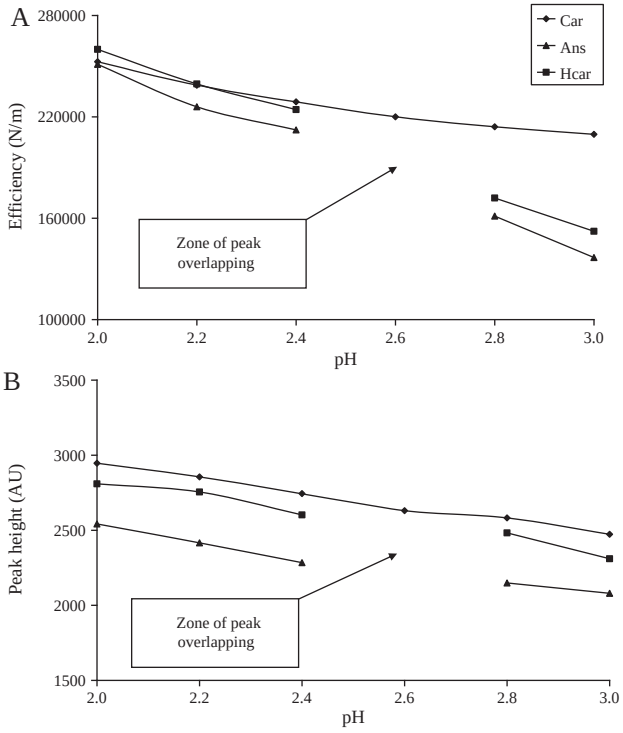

Fig. 2. Effect of concentration of Tris phosphate run buffer pH on efficiency and eight of peak. *Electrophoretical conditions*: a 50 mmol/L Tris phosphate buffer; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV; hydrodynamic injection: 27 nL (0.5 psi × 7 s). Around pH 2.6 Ans and Hcar peak were overlapped therefore peak height and efficiency was not evaluable.

Table 1
Effect of separation voltage on method performance. *Electrophoretical conditions*: 50 mmol/L Tris phosphate buffer pH 2.2; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; hydrodynamic injection: 27 nL (0.5 psi × 7 s). Experimental has been performed on a standard mixture (20 μmol/L).

Voltage (kV)	Current (μA)	Peak height (AU)			Efficiency (N/m)			Resolution		Migration time (min)		
		Car	Ans	Hcar	Car	Ans	Hcar	Rs_1	Rs_2	Car	Ans	Hcar
10	40	1554	1397	1441	169,768	175,287	175,878	1.351	1.902	21.24	21.46	21.82
15	63	1747	1521	1604	174,334	185,359	187,904	1.345	1.889	13.54	13.68	13.9
20	75	2152	1873	1975	180,833	192,029	197,212	1.322	1.877	9.58	9.68	9.73
25	115	1937	1651	1734	179,912	188,464	196,050	1.092	1.783	7.26	7.34	7.46
30	145	1811	1593	1700	178,165	186,338	196,775	0.991	1.623	5.83	5.88	5.97

Rs_1: resolution between Car and Ans; Rs_2: resolution between Ans and Hcar.

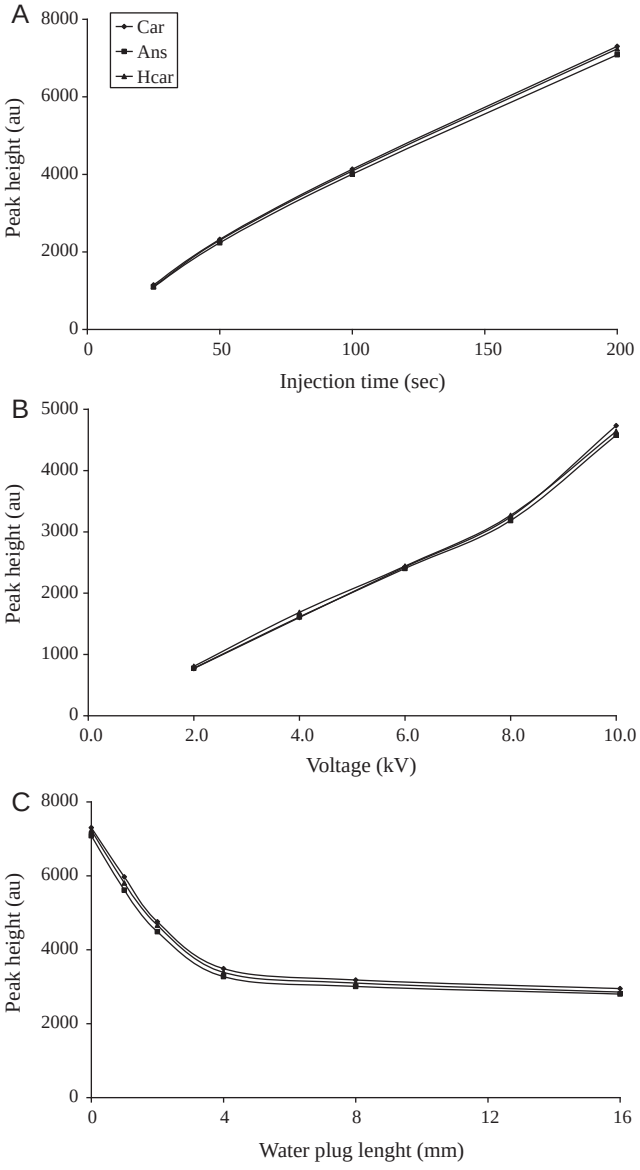

Fig. 3. Factors affecting the peak height. (A) Effect of the injection voltage on peak height (injection time 200 s). (B) Effect of the injection time of the sample on peak height (applied voltage 10 kV). Effect of water plug length on peak height (electrokinetic injection 10 kV × 200 s). *Electrophoretical conditions*: 50 mmol/L Tris phosphate buffer; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV. Experimental has been performed on a standard mixture (200 nmol/L).

better resolution was achieved using 50 mmol/L buffer at pH above 2.2. Efficiency and sensitivity increased with the increasing of the run buffer concentration (data not shown) and the decreasing of pH levels (Fig. 2). The effect of run voltage on electrophoretic parameters has been tested in a range between 10 and 30 kV. As reported in Table 1, higher efficiency and sensitivity, with an acceptable resolution, were obtained at 20 kV separation voltage. Furthermore, we tested the effect of the cartridge temperature on the resolution by increments of 5 °C between 20 and 45 °C. As expected, by rising the temperatures, the current increases and the migration time decreases, this principally due to the run buffer viscosity related to temperature. Although higher temperatures allowed to shorten analysis time, a loss of resolution was observed (data not shown). Thus, a capillary temperature of 15 °C was selected since it yielded a good resolution with acceptable migration times and current values. To evaluate the best wavelength for detection we performed an absorbance spectra between 190 and 400 nm in the selected run buffer. The maximum absorbance at 190 and 207 nm was observed for all analytes (data not shown). Moreover, another maximum peak of absorbance was visible at 282 nm for Car and Hcar and 295 for Ans. 207 nm wavelength was set for the following experiments. To sum up, the most favourable non-stacking separation condition was found using 50 mm Tris phosphate buffer pH 2.2, with an applied voltage of 15 kV, 15 °C cartridge temperature and 207 nm as wavelength.

3.2. FASI optimization

In order to increase the method sensitivity we tried to inject larger sample volumes onto the capillary. However, above 110 nL a significant loss of efficiency and resolution was observed (Table 2). Under the selected electrophoretical conditions and injecting 110 nL of sample, a baseline separation of Car, Ans, and Hcar within 10 min were achieved with LODs between 0.250 and 0.300 μmol/L (S/N = 3). This sensitivity was 10–20-fold higher than the LODs

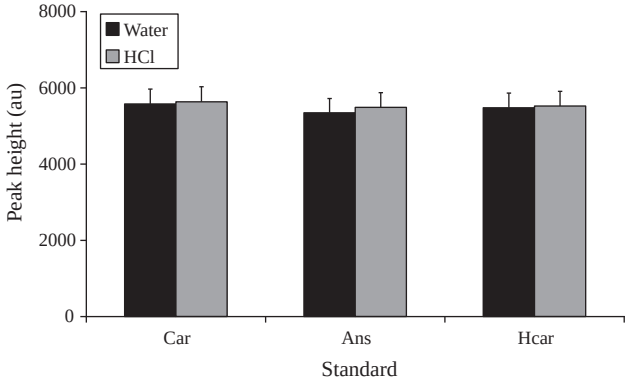

Fig. 4. Evaluation of matrix effect on peak height of analytes. Experimental has been performed in triplicate on a standard mixture (200 nmol/L) dissolved in water or in 10^{-5} mol/L HCl.

Table 2
Effect of sample injection volume on method performance. *Electrophoretical conditions*: 50 mmol/L Tris phosphate buffer pH 2.2; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV; hydrodynamic injection: 27 nL (0.5 psi × 7 s). Experimental has been performed on a standard mixture (20 μmol/L).

Injection			Peak height (AU)			Efficiency (N/m)			Resolution	
psi	s	nL	Car	Ans	Hcar	Car	Ans	Hcar	Rs_1	Rs_2
0.5	7	27	2152	1873	1975	180,833	192,029	197,212	1.322	1.877
1	7	55	3159	2813	2951	167,235	188,421	183,548	1.288	1.798
1.5	7	82	5687	5188	5465	143,517	169,271	163,013	1.212	1.733
2.0	7	110	6754	6214	6562	116,306	131,288	137,738	1.045	1.631
2.5	7	137	7738	7280	7631	90,299	119,767	122,388	0.905	1.544
3.0	7	165	8455	7869	8287	75,412	101,332	104,968	0.812	1.223

Rs_1: resolution between Car and Ans; Rs_2: resolution between Ans and Hcar.

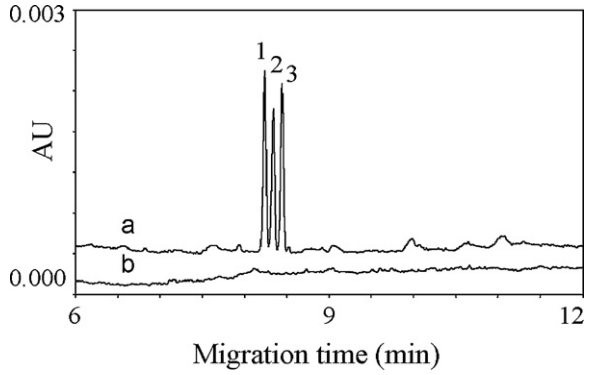

Fig. 5. Electropherograms of a standard mixture containing 0.1 μmol/L of Car (1), Ans (2) and Hcar (3) obtained by FASI-CE (a) and by CE with hydrodynamic sample injection (b). *Electrophoretical conditions*: 50 mmol/L Tris phosphate buffer pH 2.2; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV.

reached by the other CE assays described in literature [12–14]. To improve the detection sensitivity the FASI was applied to the method. By FASI the cations are subjected to a field-amplified enrichment and are subsequently stacked at the interface of the solvent plug and the run buffer. The amount of analyte injected with FASI is dependent on the injection time and voltage under the employed conditions. To determine how these parameters affected the sensitivity we have investigated on the effect of applied voltage and time of injection. By varying the injection time from 25 to 200 s (using a voltage of 10 kV), we found that the peak height raised as the injection time increased (Fig. 3A). Moreover the maximum sensitivity was obtained when a voltage of 10 kV was applied during sample injection (Fig. 3B). It has been reported [23–25] that the introduction of a short plug of water before sample electrokinetic injection can provide a high electric field strength from the beginning of the injection thus yielding a concentration factor improvement. However, in this case, the use of a short water plug before electrokinetic injection induced a decrease of sensitivity (Fig. 3C). Therefore, samples were injected onto the capillary by electrokinetic injection for 200 s at 10 kV without water plug. The effect of matrix composition on the peak signal has also been evaluated. Not significant differences have been observed between standards dissolved in water or in 10^{-5} mol/L HCl (sample matrix composition after 1000-fold dilution of sample) as reported in Fig. 4. Moreover, the concentration values of samples, extrapolated from the calibration curve prepared in 10^{-5} mol/L HCl and from the calibration curve obtained by spiking the samples with pure standards, were similar, thus suggesting the absence of a matrix bias (data not shown). Fig. 5 depicts the comparison of the electropherograms of a standard mixture composed by 0.1 μmol/L of Car, Ans and Hcar obtained by FASI-CE (a) and CE with hydrodynamic sample injection (b) showing the remarkable increase in detection sensitivity obtained by FASI procedure.

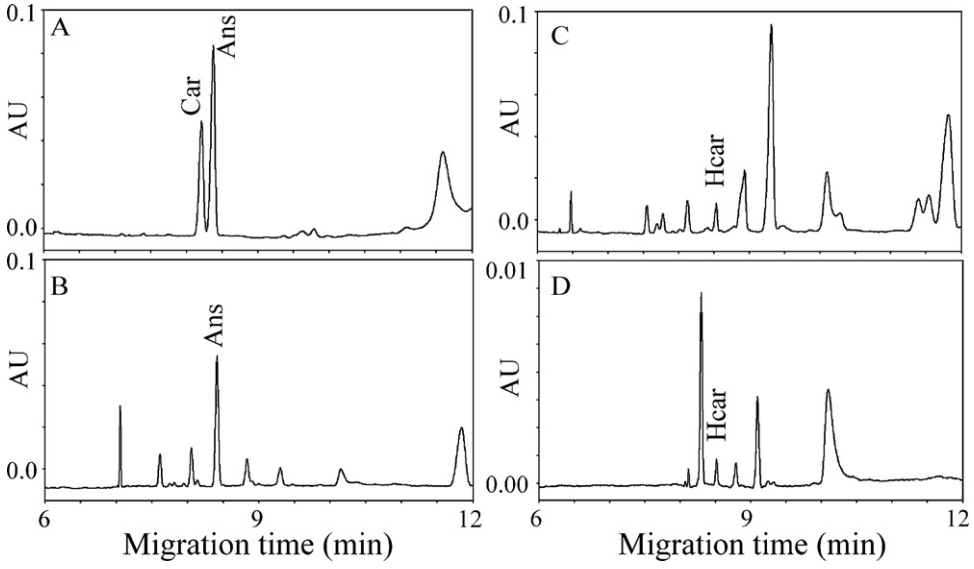

Fig. 6. Electropherograms of homogenized rat muscle (A), heart (B), brain (prefrontal cortex) (C) and human CSF (D). *Electrophoretical conditions*: 50 mmol/L Tris phosphate buffer pH 2.2; uncoated silica capillary, 60.2 cm × 75 μm ID; cartridge temperature, 15 °C; voltage, 20 kV; electrokinetic injection: 10 kV × 200 s. *Analyte concentrations*: (A) Car 6.8 nmol/mg prot, Ans 18.1 nmol/mg prot; (B) Ans 8.0 nmol/mg prot; (C) Hcar 1.9 nmol/mg prot; (D) Hcar 0.23 μmol/L.

3.3. Method validation

Calibration curves, obtained as peak areas of analytes versus concentration, plotted for six concentration levels (5, 50, 200, 500, 2000, 5000 nmol/L) of mixed standard samples (dissolved in 10^{-5} mol/L HCl), were linear in the concentrations range tested: $Y = 12X - 121$ ($R^2 = 0.999996$) for Car; $Y = 6.4X + 46$ ($R^2 = 0.999998$) for Ans; $Y = 8.7X + 52$ ($R^2 = 0.999996$) for Hcar. The injection reproducibility was calculated by injecting ten times consecutively the same standard solution. Within-run precision (intra-assay) of the method was evaluated by injecting the same biological sample 10 times consecutively, while between-run (inter-assay) precision was determined by injecting the same biological sample on 10 consecutive days. Precision tests indicate a good repeatability of our method both for migration times (CV < 1.55%) and areas (CV < 2.67%). Moreover, a good reproducibility of intra-assay and inter-assay tests was obtained (CV < 4.31% and CV < 6.29% respectively). For the assessment of the analytical recovery, the muscle sample was spiked with standard solutions of all the analytes at three different concentrations (400 μmol/L, 100 μmol/L and 25 μmol/L), and the mean of recovery, evaluated by five different experiments, was between 97.8 and 102.4%. Similar data were obtained from rat (recovery range 98.1–101.7%), brain (recovery range 96.9–100.3%) and human CSF (recovery range 97.6–103.1%). The limit of detection (LOD) of method, measured on a real sample, evaluated as signal-to-noise ratio of 3 was 0.45 nmol/L for Car and Hcar and 0.54 nmol/L for Ans.

3.4. Method application

The method application has been proved on biological samples. In particular, Fig. 6 shows electropherograms obtained from homogenized rat muscle (A), heart (B), brain (C) and human CSF (D). All samples were diluted in water before injection without further sample pre-treatement. The analytes identification in samples was performed by spiking with Car, Ans and Hcar standard. Furthermore, diode-array spectroscopy measurements made on standard and sample peaks confirmed the identity and purity of analytes in samples.

4. Concluding remarks

We describe a new FASI-CE UV-detection method by which an improvement of about 500-fold compared to the normal hydrodynamic injection was obtained. The reached sensitivity of 0.5 nmol/L improved of 10–100-fold the LOD reached by the previous described methods on Car, Hcar and Ans measurement [12–14]. This allows to dilute sample in large volumes of water in order to have the needed conductivity difference between the sample solution and the background electrolyte that guarantees the stacking effect. Over the increase sensitivity this procedure allows to reduce all pre-analytical steps (protein elimination and sample derivatization were avoided) to a simple dilution with a noteworthy saving of time. The applicability of this assay to complex sample matrices has been demonstrated.

In conclusion, the short time needed for the sample pre-treatment, the relatively short analysis time (single electrophoretic run time of 12 min) and the low analysis costs makes this method suitable for large-scale applications.

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