sina-pub.ir

Journal of Chromatography B, 879 (2011) 3700–3706

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Direct analysis of carbohydrates in animal plasma by ion chromatography coupled with mass spectrometry and pulsed amperometric detection for use as a non-invasive diagnostic tool

Darja Kotnik, Andrej Šmidovnik*, Petra Jazbec-Križman, Mitja Križman, Mirko Prošek

National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 7 July 2011
Accepted 6 October 2011
Available online 13 October 2011

Keywords:
Carbohydrates
High-performance anion-exchange chromatography
Pulsed amperometric detection
Mass spectrometry
Lactulose–mannitol index

ABSTRACT

The present paper demonstrates that electrochemical detection (ECD) coupled to ion chromatography and electrospray ionization tandem mass spectrometry (IC-ECD–ESI/MS/MS) can be used to rapidly estimate some indications of the health status of organisms. The lactulose to mannitol ratio (L/M) is used as a non-invasive assay to investigate small intestinal absorption pathways and mucosal integrity. In the present study, an evaluation of the negative effects of nonsteroidal anti-inflammatory drug meloxicam perorally administrated to a group of dogs was carried out by determining the lactulose/mannitol index using the IC-ECD–ESI/MS/MS hyphenated technique. According to the results of the study, meloxicam altered gastrointestinal permeability. Coenzyme Q_{10} (CoQ_{10}) was tested to determine if it could prevent meloxicam induced gastrointestinal damage and it was found that CoQ_{10} could be an effective preventive treatment. Furthermore, plasma glucose concentration level was determined to be an indirect indicator of the oxidative state in the blood. To find out the beneficial effects of a double antioxidant combination (α-lipoic acid (ALA) and CoQ_{10}) on the total glucose level in chickens, ALA and CoQ_{10} were provided as food additives in factory farm raised chicken. The results of the pilot study indicate that the glucose level in the plasma of chickens group fed with CoQ_{10} and ALA was significantly decreased compared to the control group. Ion chromatography (IC) utilizing pulsed amperometric detection (PAD) was compared to ion chromatography coupled with tandem mass spectrometry (MS/MS) as an analytical tool for monitoring the carbohydrate level in biological fluids. In electrochemical detection, the newly developed two-pulse waveform successfully withstands matrix effects in biological samples. Continuous on-line desalting of the high salt concentrations used as the eluent for carbohydrate separation from the anion-exchange column allows coupling of IC and MS techniques. A make-up solution (0.5 mM LiCl) was delivered prior to MS detection for efficient ionization of eluted carbohydrates. Method validation showed that both used techniques are practically comparable and some advantages of each are presented.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Oxidative stress is the general term used for the oxidative damage to cells, tissues or organs caused by reactive oxygen species. Cellular oxidative stress results from exogenous sources, such as exposure to alcohol, medications, trauma, infections, and pollutants, and represents the imbalance between the rate of induced oxidative damage and effective antioxidant defense. The body's natural antioxidant protection and that provided by the diet should provide appropriate amounts of antioxidants and micronutrients to animal blood and cells. To provide adequate functioning of the antioxidant defense it is necessary to know and accommodate the nutritional components needed to manage oxidative stress and to evaluate the blood and tissue level of antioxidant capacity. Many degenerative disorders are caused by a depletion of specific cellular antioxidants such as superoxide dismutase (SOD), catalase, vitamin E, vitamin C, carotenoids, coenzyme Q_{10} (CoQ_{10}), bilirubin, α-lipoic acid (ALA), and glutathione [1–5]. Lipoic acid has been reported to reduce oxidative stress in healthy adults and diabetic patients [6]. An important indicator of some degenerative diseases produced by prolonged oxidative stress can be evaluated by determining the plasma levels of certain carbohydrates, for instance glucose. Prolonged hyperglycemia has been reported to cause most long-term diabetes complications, increased oxidative stress, which results in stimulation of the polyol pathway, formation of advanced glycation end products (AGE), and subsequent formation of reactive oxygen radicals [7–10]. Recently, ALA and CoQ_{10} have attracted

* Corresponding author. Tel.: +386 1 47 60266; fax: +386 1 47 60300.
E-mail address: andrej.smidovnik@ki.si (A. Šmidovnik).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.10.010

sina-pub.ir

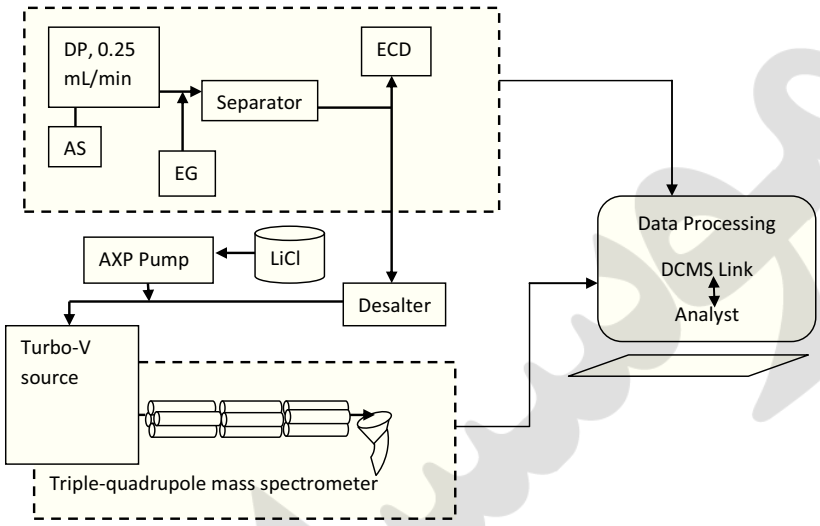

Fig. 1. The employed hyphenated IC-ECD-MS/MS system.

interest because of their diverse biological actions along with their potent antioxidative capacity [11]. Studies have shown that ALA exhibits metabolic effects on glucose transport and utilization [12,13].

Modern industrial production of poultry and eggs is very stressful for the animals involved therefore large-scale factory farming requires new ways to produce healthy poultry. In the present study, the benefits of providing fodder additives with antioxidant activity to chickens during controlled industrial raise were observed. To this end, we fed chickens with CoQ_{10} and ALA as food additives and evaluated the total glucose level in the chicken's plasma. The experiment was carried out in cooperation with the largest poultry producer in Slovenia, Perutnina Ptuj, SI. The synergistic effects of these two antioxidants were studied and we assumed that the plasma glucose level would be an appropriate indicator of the organism's oxidative status [13].

In addition, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) in small animal medicine includes the treatment of acute traumatic injuries of the musculoskeletal system, acute inflammation, and their use as preoperative analgesics in surgical procedures [14]. NSAID use in dogs is frequently limited due to gastric irritation and ulceration after oral administration [15]. NSAIDs cause damage to the gastrointestinal mucosa, which induce a subsequent increase in the permeability of the mucosa to toxins and luminal agents such as bile, pancreatic secretions and bacteria [16]. The administration of site specific permeability probes, such as monosaccharides and disaccharides, to detect permeability defects at different levels of the gastrointestinal tract represents a single screening test for assessment of the functional integrity of the gastrointestinal mucosa [17]. Gastrointestinal permeability tests have been demonstrated to be a hallmark of several disease processes that culminate in epithelial damage. Thus, the determination of the lactulose to mannitol ratio in blood/urine samples is a useful diagnostic procedure in studies of intestinal permeability. Under physiological conditions, monosaccharides are readily adsorbed by the intestinal villi, while disaccharides that are absorbed in the crypt epithelium are excluded. Atrophy of the villi may result in the decrease in monosaccharide absorption and increased exposure of the crypts to luminal contents. The dysfunction of intestinal mucosa is therefore characterized by an increase in disaccharide/monosaccharide ratio [18]. In the present study, the negative effect of the nonsteroidal anti-inflammatory drug meloxicam perorally administrated to a group of dogs was evaluated by applying the lactulose/mannitol index.

Thus, the second goal of the present study was the determination of the effects of meloxicam on gastrointestinal mucosa and the effectiveness of CoQ_{10} (50 mg/day) in preventing meloxicam induced gastrointestinal damage in healthy dogs by means of the lactulose/mannitol (L/M) index [19].

A demand for novel tools facilitating fast, simple screening of compounds with antioxidant properties and the corresponding levels of carbohydrates has resulted in the development of many analytical methods for the determination of carbohydrates in complex biological fluids, some of them introduced by our laboratory [20–22]. For monitoring the carbohydrate level in biological fluids, the on-line IC–MS/MS hyphenated technique is compared with the conventional IC-ECD technique. Both techniques were used in parallel for quantitative determination of polar substances in a complex biological matrix with low concentrations of analytes (Fig. 1). Determination and quantization of different mono- and disaccharides were carried out using different approaches, such as full scan mode (MS), selected ion monitoring (SIM), multiple reaction monitoring (MRM) and two PAD detection modes, as well as the standard quadrupole potential waveform and newly developed two-step potential waveform.

2. Experimental

2.1. Chemicals

Methanol, ethanol, 2-propanol, 1.4-dioxane, acetonitrile, hexane, perchloric acid and acetic acid (LC grade) were supplied by Merck (Darmstadt, BRD). Sugar alcohol and carbohydrate standards (D-glucose, D-lactulose, D-mannitol) were purchased from Sigma–Aldrich (Steinheim, BRD). Deionized (DI) water with a resistivity of 18.2 MΩ cm or greater was obtained from an Aqua Solutions (Jasper, GA) water purification system.

Stock solutions of selected mono- and disaccharides with concentrations of 100 mM were prepared by dissolving in MQ water. Stock solutions were stable for one month if stored in the dark at 4 °C. Calibration standard solutions (CC) were prepared from the stock solutions daily. Quality control samples (QC) were prepared from the pooled chicken plasma a short time before the start of analysis. Known amounts of glucose (100 μM), lactulose (1 μM) and mannitol (5 μM) were spiked and stored together with samples at −80 °C. Accuracy and recovery were determined with the spiked quality controlled sample, QC.

sina-pub.ir

2.2. Samples

2.2.1. Chicken plasma samples

The level of glucose in chicken blood samples collected during the 41 day period of industrial raising was evaluated. Blood plasma samples were collected from two hundred chickens (one day old, provenience ROSS 308) obtained during the regular hatching process. Chickens were distributed into four subgroups each consisting of 50 animals. Animals in all groups were administrated the same basic food. After twelve days, animals in the experimental groups started to eat the fodder additives fortified with CoQ_{10}, ALA, and a combination of both. Concentrations of CoQ_{10} and ALA in feed were calculated from feed instruction tables so that each chicken received an average of approximately 5 mg of CoQ_{10} and (or) 50 mg of ALA per kg of animal weight per day. The experiment was carried out under optimal health and growing conditions according to the prepared protocol. During the 41-day production period, all animals were treated under identical environmental and growing conditions. Health, appearance and behavior of each animal were recorded and blood samples were taken on days 16, 28 and 40. From each group 14 animals were selected randomly, 2 mL of blood were taken and heparinated plasma samples were prepared. After about 24 h the collected frozen samples were transported to the laboratory facility for long-term storage at −80 °C and kept in this way until needed for analysis. Numerous chemical and biochemical parameters were analyzed, but in this study only the result of the concentrations of glucose were processed.

2.2.2. Dog blood samples

Colon damage was provoked by the perorally administrated non-steroidal anti-inflammatory drug Meloxicam. Each test animal was supplied with a food fortified with 300 mg of lactulose and 100 mg of mannitol daily. Six dogs were included in the research. The blood samples were drawn from the jugular vein 120 min after ingestion of the sugar solution into K_3EDTA tubes. The tubes were centrifuged at 1500 *g* for 15 min at 4 °C. Plasma samples were stored at −80 °C prior to lactulose/mannitol (L/M) ratio analysis.

2.3. Extraction of sugars

In both cases sample preparation was carried out in the following way. 400 μL acetonitrile (75%) was added to 100 μL of plasma. The mixture was vortexed for 3 min; followed by centrifugation for 4 min at 10,000 *g* on a tabletop centrifuge (Centrifuge Kendro Lab. Products, Heraeus, Biofuge, Stratos) to remove proteins. The 100 μL aliquot of supernatant was diluted into a 5-mL volumetric flask and injected into the lC–EC–MS/MS system.

2.4. Instrumentation

Chromatographic measurements were performed on a Dionex ICS-3000 liquid chromatograph (Sunnyvale, CA), consisting of a DP dual gradient pump, an AS autosampler with a cooled sample tray, a DC detector compartment equipped with electrochemical detector (Au working electrode), and an EG eluent generator compartment.

2.5. Separation

Carbohydrates were separated using a strong anion-exchange stationary phase Dionex CarboPac PA10 analytical column (2 mm × 250 mm) and guard column (2 mm × 50 mm). The stationary phase was a 6.5 μm diameter ethylvinylbenzene/divinylbenzene substrate (55% cross-linking) agglomerated with 130 nm MicroBead™ difunctional quaternary ammonium ion (5% cross-linked). The carbohydrates were eluted with an isocratic mobile phase of 35 mM potassium hydroxide at a flow rate of 0.25 mL/min.

2.6. Detection

2.6.1. Electrochemical detection

The three-electrode detection cell was a thin-layer type and consisted of a disposable thin-layer gold working electrode (Au DGE, 0.785 mm^2), a pH–Ag/AgCl reference electrode and a titanium counter electrode. A standard four-potential waveform was used and compared to the proposed new two-potential waveform in carbohydrate analysis.

2.6.2. Mass spectrometry

The mobile phase (35 mM KOH) with separated substances was sent through a 2 mm anion self-regenerating suppressor (ASRS Ultra II). For efficient ionization of the eluted carbohydrates, a make-up solution (0.5 mM LiCl) was delivered via a MicroTEE into the eluent flow at a flow rate 50 μL/min. The separated carbohydrates were directed to the TurboIonSpray™ source of the Sciex QTRAP LC/MS/MS system from Applied Biosystems/MDS (Sciex Concord, ON, Canada). The mass spectrometer was tuned in positive mode with a polypropylene glycol (PPG) solution (Applied Biosystems).

Determination of glucose was carried out in MS scanning mode in the mass range from 150 to 500 *m*/*z*. A glucose quasi-molecular ion was formed with the added lithium ion $(180 + 7)^+$ in positive ESI–MS ionization mode, operated under the following conditions: temperature of the turbo gas was 600 °C, the voltage applied to the needle was +4500 V, the nebulizer gas was set to 45, the curtain gas to 10, and the auxiliary gas to 65 units, the pressure of the collision gas in the collision cell during Q_3 MS was set to medium value. The compound-dependent parameters for mannitol and lactulose were optimized as follows: the declustering potential between Q_0 and orifice plate was set to 81 V, entrance potential 10 V, and collision cell exit potential to 20 V.

2.7. Data analysis

Sciex Analyst software was used to perform data analysis and peak integration. Dionex Chromatography Mass spectrometry Link (or DCMSLink) for Analyst software (from Applied Biosystems/MDS Sciex) provides an interface for controlling of Dionex Chromatography instruments with the Analyst software (MS control).

Due to the broad range and different types of detection procedures, linear, weighted linear ($1/x$) and exponential calibration curves were used. The stability of the quantified sugars was confirmed with QC samples during the method development. The calibration samples (CC) were prepared in batches and stored in the refrigerator at 4 °C for nearly twenty days, while the QC control samples were prepared daily. The obtained values confirmed the stability of sugars for at least for two weeks in the refrigerator (4 °C). Calibration curves with seven calibration standards from 0.1 μM to 100 μM of glucose and 0.01 μM to 10 μM of mannitol and lactulose were prepared. The values of slope (b), intercept (a), correlation coefficient (R), and standard deviation of slope $V(b)$ were calculated using a weighted calibration curve. Weight factors were calculated from the equation $fw_i = 1/(100 + 5 \times X_i)^2$. The limit of detection (LOD) and limit of quantization (LOQ) were calculated from the confidence interval.

All statistics were run using Statgraphic plus Ver. 4. An analysis of the variance (ANOVA) and a Student *t*-test were employed to evaluate differences between groups with respect to analyte plasma levels, and the relationship between concentration levels

sina-pub.ir

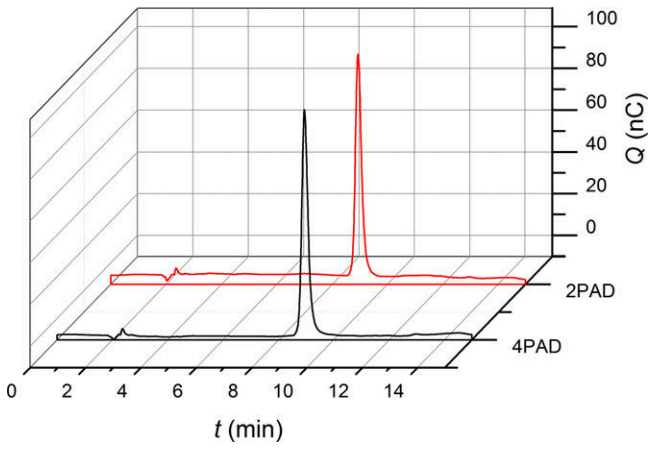

Fig. 2. Typical chromatograms of plasma glucose sample detected by the new two-step potential waveform (red line) and by standard four-step waveform (black line). Analytical column: CarboPac PA10 (2–mm), injection volume: 2.5 μL, isocratic elution: 35 mM KOH, flow rate: 0.25 mL/min.

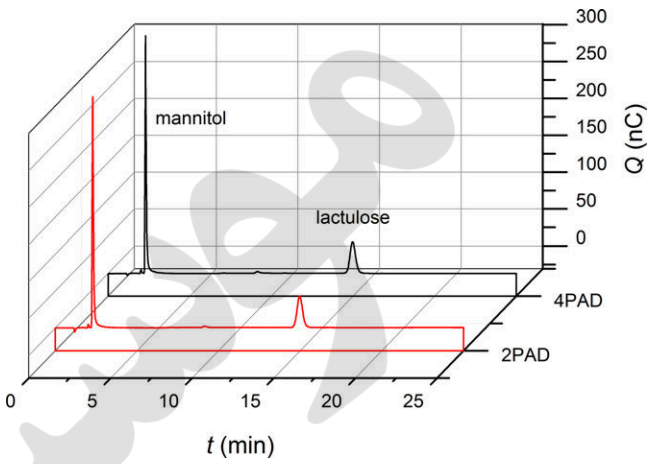

Fig. 3. Comparison of the chromatograms of mannitol and lactulose in plasma samples at different pulsed waveforms: (black line) four-step waveform and (red line) two-step waveform. Separation conditions: see Fig. 2.

and supplementation time (slope). A probability level of $P < 0.05$ was considered statistically significant.

3. Results and discussion

An efficient method of qualifying and quantifying carbohydrates in biological samples and food products was determined based on anion-exchange chromatography coupled with electrochemical detection at gold working electrodes. In electrochemical detection of carbohydrates, a standard quadruple-potential waveform is applied to the Au working electrode, which exploits the electrocatalytic activity of carbohydrates at elevated pH values (greater than 12). The generated current is proportional to the carbohydrate concentration, thus enabling sensitive detection and quantification. This method is relatively inexpensive and therefore well established in many laboratories. In electrochemical detection, the concentrations of selected sugars in plasma samples were determined on the Au electrode using a standard four-step potential waveform and compared to the new two-step waveform, developed in our laboratory. In the two-pulsed potential waveform, cathodic conditioning/re-activation of the electrode surface is applied exclusively and results in slightly increased sensitivity in comparison to the standard waveform. Calibration curves for the injection of 10 μL of glucose solutions (not shown) between 0.1 μM and 1.0 mM concentrations are: for 2PAD: $Q_{max}(nC) = (2.02 \pm 0.05)\,C\ (\mu M) + 39 \pm 18$ ($R^2 = 0.9956$), and for 4PAD: $Q_{max}(nC) = (1.99 \pm 0.05)\,C(\mu M) + 39 \pm 20$ ($R^2 = 0.995$). Both methods result in similar detection limits confirmed by calculated correlation factors. The mechanism of the newly developed two-step potential waveform is not the issue of this paper and is described in detail elsewhere [23]. The amperometric detection cell and the MS detector were placed in parallel (Fig. 1). Fig. 2 shows the typical chromatograms of glucose in plasma samples, obtained by the standard four-step and the new two-step potential waveform. Glucose in plasma samples was separated on CarboPac PA10 in isocratic runs and on-line electrolytically generated 35 mM KOH was used as the eluent. All electrochemical measurements of alditol and carbohydrates in plasma samples of chickens and dogs detected by the newly developed waveform were compared to that obtained by standard potential waveform and MS methods. Fig. 3 shows the comparison of chromatograms of mannitol and lactulose in plasma samples obtained by separation/pulsed amperometric detection with the electrochemical detector equipped with an Au electrode, utilizing the standard and new two-step potential waveforms. The solid line represents the detector output when the classical four-step potential waveform was applied, while the dotted line represents the detector response when the new two-step potential waveform was applied. Completely comparable results obtained by the standard four- and the new two-step waveform are evident, thus confirming that the new simpler potential waveform also works well in cases where a strong matrix effect (adsorption of serum residues) is expected. With the elimination of the oxidative step the two-step waveform does not result in fouling or dissolution of Au electrode surface in a prolonged use thus ensures long term stability of the detector response.

As PAD is not selective it is necessary to use mass spectrometry for sugar identification, which provides a better selectivity for problematic samples and is the tool of choice to elucidate unknown peaks. The huge amount of ions arising from the IC separation process creates unwanted background noise and therefore this technique has not been considered suitable for on-line coupling to MS. Due to the high salinity and low volatility of the potassium/sodium hydroxide used as the eluent in the IC separation of carbohydrates, it cannot be directly injected through the MS interface. The high saline and low volatile hydroxide eluent can cause electrical shortcuts and capillary plugging by salt crystallization, which has to be removed prior to MS. Thus, the eluent and separated substances have to be sent through an anion self-regenerating suppressor to lower the pH value of the eluent to neutral levels [24,25]. The use of a high-capacity suppressor (desalter) enables the coupling of IC and MS detection via an electrospray interface. Electrochemical desalting is an efficient way to convert hydroxide into pure water. The neutralization of KOH/NaOH is obtained by a combination of water electrolysis and ion-exchange: H^+ are produced by the oxidation of water, which are then exchanged with the K^+/Na^+ ions of the eluent, which is therefore neutralized without diluting. The conductivity detector in front of the MS interface was set to monitor the suppression of the high-value pH eluent (Fig. 1). Background noise is significantly decreased and the resulting eluent is compatible with MS detection. However, at this stage the MS detection of carbohydrates becomes challenging, as carbohydrates at neutral pH cannot be directly ionized by ESI. Many strategies in LC–MS interfacing have been discovered for carbohydrate ionization [26,27]. Underivatized carbohydrates are poorly detectable in ESI or APCI due to their low ionization yields. Many authors have described on-line LC–MS analysis of carbohydrates using different ionization methods for carbohydrates molecules in neutral media. [28–33]. Owing to the high polarity and low volatility of carbohydrates, electrospray ionization is preferred to

sina-pub.ir

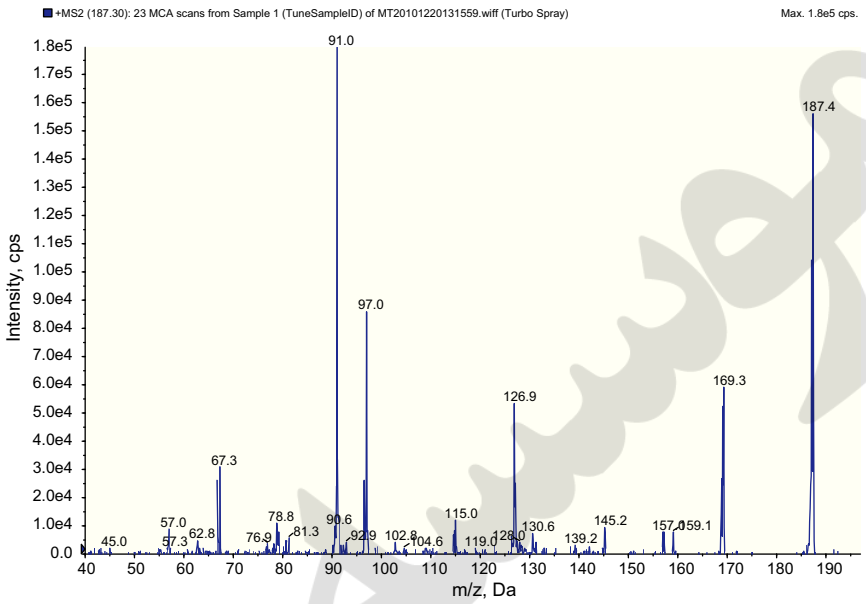

Fig. 4. Typical mass spectrum of glucose detected as a lithium adduct.

APCI. To improve the ionization efficiency, carbohydrates have been analyzed by ESI in positive ion mode as metal ion adducts (including sodium, cobalt, lithium, cesium, lead) and in negative mode as anion adducts (including iodine chloride and acetate) [34–41]. An alternative method to post-column addition could be the partial suppression of the KOH/NaOH eluent in order to form adducts with the remaining K^+/Na^+ ions. As the suppressor current must be maintained at a constant value to avoid baseline drifting this method cannot be applied during gradient elution. In the literature, it was found that the most abundant carbohydrate metal adducts are formed with lithium adducts [24,25]. For that reason, we continuously deliver a make-up solution (0.5 mmol/L LiCl) in front of the ESI interface. Consequently, the response factor is greatly enhanced in positive mode by the formation of $[M+Li]^+$ ions. On the other hand $[M+Cl]^-$ ions are poorly detectable in negative mode. The typical mass spectrum of glucose as an Li-adduct is represented in Fig. 4. Carbohydrate detection as lithium adducts allows the LOD to be obtained at the sub-pmol level. The LOQ for selected carbohydrates was approximately 0.02 μM. The applied IC–MS/MS methods were tested in terms of selectivity, precision and accuracy (recovery) and showed an acceptable range. The stability of the prepared methods was confirmed using QC samples during the method development. Two sets of QC samples were provided, one stored in refrigerator and the other prepared freshly from the standards on a daily basis. The obtained values confirmed the stability of sugars for at least two weeks in the refrigerator (4 °C). There was no measurable change in the intensity of the signal of control samples at room temperature (20 °C) during the analysis time (12 h in the auto-sampler in the dark). The applied scanning parameters and the results of the validation of developed analytical methods are shown in Table 1.

3.1. Method validation

During the IC-ECD–MS/MS method development the methods were validated with basic validated parameters shown in Table 1. As can be seen in Table 1, there is no significant difference

Table 1
Analytical parameters for IC-ECD and IC–MS/MS determination of glucose, mannitol and lactulose.

Parameters	Glucose			Mannitol	Lactulose
Detection mode	SIM (Q_1/Q_3)	2PAD	4PAD	MRM	MRM
m/z parent ion	187.4 $(M+Li)^+$			189.2 $(M+Li)^+$	349 $(M+Li)^+$
m/z fragments	91.0			171.3/96.8/81.1	330.9/187/97.2
Linearity range	0.5–100 μM	0.1–500 μM	0.1–500 μM	0.05–100 μM	0.01–100 μM
R^2	0.9996	0.9956	0.995	0.9994	0.9998
LOD	0.2 μM	0.1 μM	0.1 μM	0.02 μM	0.01 μM
LOQ	0.5 μM	0.4 μM	0.4 μM	0.05 μM	0.02 μM
Precision ($n = 6$)	3.87%	3.52%	3.52%	4.47%	5.05%
Accuracy ($n = 5$)	4.02%	2.79%	2.72%	3.05%	3.32%
Selectivity	RS > 2.0	RS > 2.0	RS > 2.0	No interference	No interference

Table 2
Concentrations of glucose (mg/L), and relative changes expressed in (%) in chicken plasma during the administration of ALA end CoQ_{10} are presented. Measurements were done with IC–MS in SIM mode. Obtained values were normalized to the starting concentrations measured on day 16 (taken as 0%).

Day	Control group		CoQ_{10} 5 mg/kg daily		CoQ_{10} + ALA 5 mg + 50 mg/kg daily		ALA 50 mg/kg daily	
	mg/L	Rel %	mg/L	Rel %	mg/L	Rel %	mg/L	Rel %
16	20.2 ± 1.7	0.0	25.8 ± 0.6	0.0	25.1 ± 0.8	0.0	29.6 ± 1.1	0.0
28	25.2 ± 3.1	25.2	27.2 ± 1.3	5.2	27.3 ± 0.2	8.9	30.7 ± 1.1	3.5
40	24.0 ± 0.8	19.1	27.5 ± 0.5	6.6	25.6 ± 0.3	2.1	27.8 ± 1.4	−6.1
Sum		44.3		11.8		11.0		−2.6

sina-pub.ir

Table 3
Calculation of L/M index during 14-day administration of CoQ_{10} (50 mg/day). Concentrations of lactulose, mannitol, and CoQ_{10} in dog plasma after perorally administrated drug meloxicam. L/M index was calculated on the day 1, 7, and 14.

Day	[a]Mannitol (μM)	[a]Lactulose (μM)	L/M index	[b]CoQ_{10} (mg/L)	"Placebo" L/M index
1	0.70 ± 0.07	1.09 ± 0.17	1.58 ± 0.31	0.28 ± 0.09	0.98 ± 0.33
7	0.68 ± 0.13	0.82 ± 0.11	1.25 ± 0.26	0.53 ± 0.11	1.00 ± 0.30
14	0.70 ± 0.08	0.73 ± 0.13	1.05 ± 0.18	0.65 ± 0.16	1.04 ± 0.27

[a] Concentrations of sugars were measured with IC–MS/MS in MRM mode at $m/z = 865$.
[b] Concentration of CoQ_{10} was measured with LC–MS in SIM mode at $m/z = 865$.

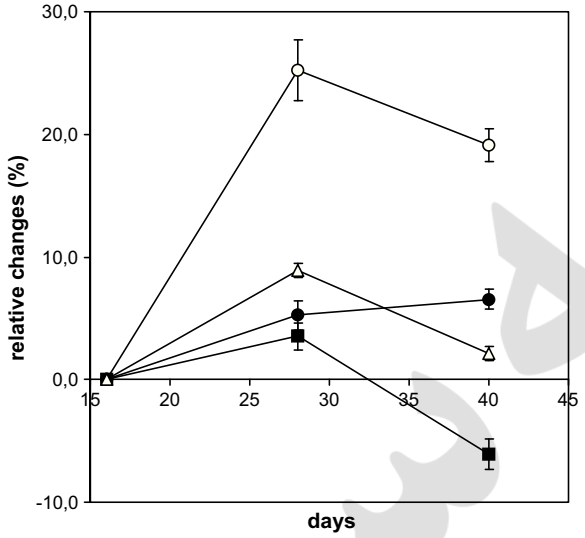

Fig. 5. Relative changes in glucose plasma concentrations measured during the experimental chicken raising period. Concentrations measured on day 16 are taken as the starting point. Experimental groups are labeled as: control group (—○—), fodder fortified with CoQ10 (—●—), with ALA (—■—), and combination of CoQ10 and ALA (—△—).

considering validated data that could prefer one of the methods. The advantages of particular method are considered during the practical use in determination of the authentic samples. Thus, the IC-ECD method is considered to be the most accurate routine method to qualify carbohydrates, whereas IC–MS/MS provides a better selectivity for problematic samples and is the tool of choice to elucidate unknown peaks. Electrochemical detector is not expensive and thus available to each laboratory. In MS detection the selected carbohydrates can be isomers yielding identical mass spectra. Verification of the identity of individual carbohydrates is feasible using a comparison of chromatographic retention times with reference or isotope labeled compounds.

A very high specificity is obtained with single ion monitoring channels, which allows the determination of coeluting compounds and the elimination of background from the non-carbohydrate components of the sample matrix as well as the mobile phase. The characteristic multi-reaction monitoring (MRM) transitions of glucose (187.4/91.0) are used for monitoring carbohydrates in plasma samples. The results in Fig. 5 (Table 2) show that the concentration of glucose in plasma was increasing during the 40 day raising period. In the control group, the final concentration was 19.1% higher on day 40 than the concentration measured on day 16 counting from the first day of the raising period. In the test groups that were administrated the fortified fodder; the increase in the glucose level in plasma was smaller. In the case of administrated CoQ_{10}, the final concentration was approximately 7% higher and in the case of fodder with a combination of ALA and CoQ_{10} the final concentration was only 2% higher than on day 16. The most rewarding result was obtained with pure ALA where the final plasma glucose concentration was 6% lower, thus showing that the glucose level decreased during the administration of fodder fortified with ALA (50 mg/kg). Our results are in accordance with the literature data and likewise show that CoQ_{10} and ALA food additives may reduce the plasma level of glucose and have beneficial effect in the case of diabetes, if taken as food additives.

Tandem mass spectrometry represents an advanced quantitative analysis of complex mixtures performed by creating the

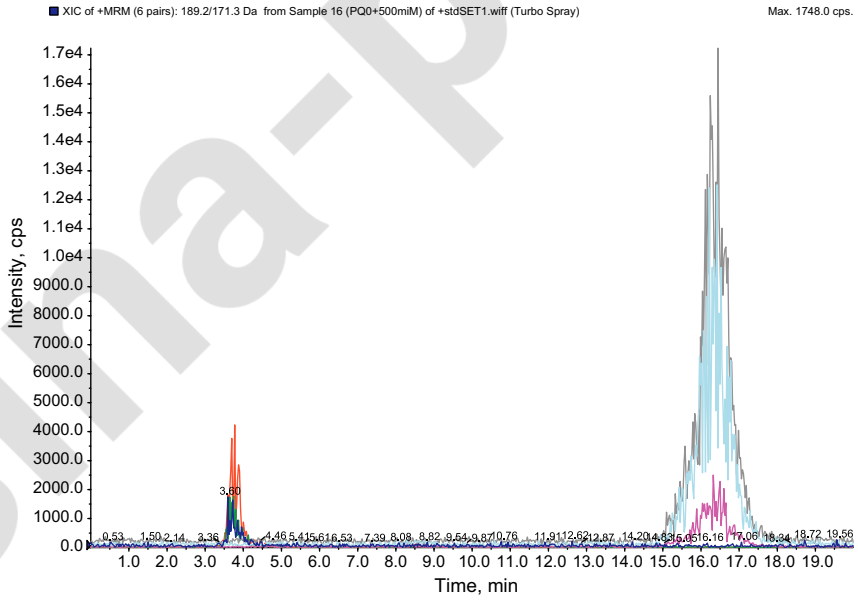

Fig. 6. Chromatogram of mannitol and lactulose in plasma sample detected in MRM mode. Analytical column: CarboPac PA10 (2–mm), injection volume: 2.5 μL, isocratic elution: 35 mM KOH, flow rate: 0.25 mL/min.

sina-pub.ir

fragments of a molecular ion by collisionally activated dissociation (CAD) and thus providing a fingerprint assay. Product ions are generated from the molecular ion by covalent bond cleavage within the carbohydrate backbone and/or losses of water. Mannitol and lactulose were measured in MRM mode. The parent ion for mannitol was 189.2 m/z $(M+Li)^+$ and fragment ions were 171.3, 96.8 and 81.1 m/z. For lactulose, the selected parent ion was 349 m/z $(M+Li)^+$ and fragment ions were 330.9, 187.0 and 97.2 m/z. A typical chromatogram with selected ion transitions of mannitol and lactulose is shown in Fig. 6. In MRM mode, the values of the L/M index could be measured in dog blood even 48 h after the peroral application of the non-steroidal anti-inflammatory drug meloxicam. After drug administration, higher L/M index values were found in plasma. Supplemented CoQ_{10} accelerated the recovery of the colon, which was observed through lower values of L/M index. The concentration of CoQ_{10} in plasma was measured with IC–MS/MS in MRM mode. The obtained results are shown in Table 3.

4. Conclusions

A combination of ion chromatography with MS equipped with an on-line desalter and improved ionization with the post column addition of LiCl gave good results and was successfully applied to real samples. The developed methods are reliable, sensitive and selective for carbohydrate determination in biological fluids. Comparison between the applied methods showed a perfect correlation and the obtained results confirm that IC–EC is a good complimentary method to the advanced IC–MS/MS, especially when small polar molecules have to be separated and quantified in blood and tissue samples. The development of a new two-pulsed technique showed that new optimizations in pulse electrochemical detection are still possible. In the two-step potential waveform cathodic electrode conditioning/re-activation is applied exclusively. The mechanism of the electrode re-activation in the two-pulse waveform thus reveals the novel view of electrochemistry of gold in terms of catalytic sites at gold electrode surface. The two-step waveform with the elimination of the oxidative step allows an increased sampling frequency, which could be of significant importance in faster eluting systems, such as, for example, in the IC systems composed of monolithic columns or in FIA analytical systems.

In the present study, the effect of CoQ_{10} and ALA on the reduction of oxidative stress was observed. In this context the level of particular sugars in chicken's blood after feeding with fodder additives fortified with CoQ_{10}, ALA, and a combination of both were measured. Special emphasis was given to the concentration of glucose in the chicken's blood after administration of ALA. The positive effects of the food additives on blood glucose levels confirmed the hypothesis of the synergism of ALA and CoQ_{10}.

The increased lactulose/mannitol (L/M) index commonly indicates colon damage in humans or animals. Increased L/M index values were found in dog blood even 48 h after the last application of meloxicam. Our industrial chicken farming and dog experiments showed that ALA and CoQ_{10} as food additives have a significant positive influence on the blood level of glucose and on the L/M index.

Acknowledgement

Grateful acknowledgement is made to the Slovenian Research Agency for financial support of the project (L1-2174).

References

[1] M. Prosek, A. Smidovnik, M. Fir, M. Strazisar, JPC: Modern TLC 17 (2004) 181.
[2] M. Prosek, J. Butinar, B. Lukanc, M. Milivojevic Fir, L. Milivojevic, M. Kriman, A. Smidovnik, J. Pharm. Biomed. 47 (2008) 918.
[3] M. Strazisar, M. Fir, A. Golc-Wondra, L. Milivojevic, M. Prosek, V. Abram, J. AOAC Int. 88 (2005) 1020.
[4] K. Tomsic, M. Prosek, B. Lukanc, A. Seliskar, A.N. Svete, Slovenian Vet. Res. 46 (2009) 93.
[5] P. Jazbec, A. Smidovnik, M. Puklavec, M. Krizman, J. Sribar, L. Milivojevic, M. Prosek, JPC: Modern TLC 22 (2009) 395.
[6] V. Borcea, J. Nourooz-Zadeh, S.P. Wolff, M. Klevesath, M. Hofmann, H. Urich, P. Wahl, R. Ziegler, H. Tritschler, B. Halliwell, P.P. Nawroth, Free Radic. Biol. Med. 26 (1999) 1495.
[7] J. Nourooz Zadeh, A. Rahimi, J. Tajaddini Sarmadi, H. Tritschler, P. Rosen, B. Halliwell, D.J. Betteridge, Diabetologia 40 (1997) 647.
[8] L.W. Oberley, Free Radic. Biol. Med. 5 (1988) 113.
[9] J.W. Baynes, Diabetes 40 (1991) 405.
[10] P.S. Vandam, B.S. Vanasbeck, D.W. Erkelens, J.J.M. Marx, W.H. Gispen, B. Bravenboer, Diabetes Metab. Rev. 11 (1995) 181.
[11] L. Packer, E.H. Witt, H.J. Tritschler, Free Radic. Biol. Med. 19 (1995) 227.
[12] D.E. Estrada, H.S. Ewart, T. Tsakiridis, A. Volchuk, T. Ramlal, H. Tritschler, A. Klip, Diabetes 45 (1996) 1798.
[13] A. Rudich, A. Tirosh, R. Potashnik, M. Khamaisi, N. Bashan, Diabetologia 42 (1999) 949.
[14] K.A. Mathews, Can. Vet. J. 37 (1996) 539.
[15] S.F. Forsyth, W.G. Guilford, S.J. Haslett, J. Godfrey, J. Small Anim. Pract. 39 (1998) 421.
[16] I. Bjarnason, T.J. Peters, Baillieres Clin. Rheumatol. 10 (1996) 165.
[17] J.B. Meddings, I. Gibbons, Gastroenterology 114 (1998) 83.
[18] B. Melichar, R. Hyspler, E. Dragounova, J. Dvorak, H. Kalabova, A. Ticha, BMC Cancer 7 (2007).
[19] L. Kompan, B. Kremzar, E. Gadzijev, M. Prosek, Intensive Care Med. 25 (1999) 157.
[20] M. Pukl, M. Prosek, J. Planar Chromatogr. 3 (1990) 173.
[21] I. Vovk, B. Simonovska, L. Kompan, M. Prosek, JPC: Modern TLC 16 (2003) 374.
[22] M. Prosek, B. Simonovska, A. Golc-Wondra, I. Vovk, S. Andrensek, E. Micovic, T. Golob, JPC: Modern TLC 16 (2003) 58.
[23] D. Kotnik, M. Novic, W.R. LaCourse, B. Pihlar, J. Electroanal. Chem. (2011), doi:10.1016/j.jelechem.2011.09.026.
[24] C. Bruggink, R. Maurer, H. Herrmann, S. Cavalli, F. Hoefler, J. Chromatogr. A 1085 (2005) 104.
[25] C. Guignard, L. Jouve, M.B. Bogeat-Triboulot, E. Dreyer, J.F. Hausman, L. Hoffmann, J. Chromatogr. A 1085 (2005) 137.
[26] D. Garozzo, M. Giuffrida, G. Impallomeni, A. Ballistreri, G. Montaudo, Anal. Chem. 62 (1990) 279.
[27] G. Puzo, J.J. Fournie, J.C. Prome, Anal. Chem. 57 (1985) 892.
[28] T.S. McIntosh, H.M. Davis, D.E. Matthews, Anal. Biochem. 300 (2002) 163.
[29] D.S. Wunschel, K.F. Fox, A. Fox, M.L. Nagpal, K. Kim, G.C. Stewart, M. Shahgholi, J. Chromatogr. A 776 (1997) 205.
[30] C.R. Taormina, J.T. Baca, S.A. Asher, J.J. Grabowski, D.N. Finegold, J. Am. Soc. Mass Spectrom. 18 (2007) 332.
[31] M.B. Clarke, D.Z. Bezabeh, C.T. Howard, J. Agric. Food Chem. 54 (2006) 1975.
[32] Z.L. Chen, X.Y. Jin, Q.P. Wang, Y.M. Lin, L. Gan, Chromatographia 69 (2009) 761.
[33] E.C.H. Wan, J.Z. Yu, J. Chromatogr. A 1107 (2006) 175.
[34] E. Rogatsky, V. Tomuta, D.T. Stein, Anal. Chim. Acta 591 (2007) 155.
[35] J.J. Conboy, J. Henion, Biol. Mass Spectrom. 21 (1992) 397.
[36] M. Kohler, J.A. Leary, Anal. Chem. 67 (1995) 3501.
[37] E. Rogatsky, H. Jayatillake, G. Goswami, V. Tomuta, D. Stein, J. Am. Soc. Mass Spectrom. 16 (2005) 1805.
[38] B. Bothner, L. Carmitchel, K. Staniszewski, M. Sonderegger, G. Siuzdak, Spectrosc.: Int. J. 16 (2002) 71.
[39] J.H. Zhu, R.B. Cole, J. Am. Soc. Mass Spectrom. 12 (2001) 1193.
[40] T. Niwa, L. Dewald, J. Sone, T. Miyazaki, M. Kajita, Clin. Chem. 40 (1994) 260.
[41] G.E. Black, A. Fox, J. Chromatogr. A 720 (1996) 51.