Rapid LC-MS/MS assay for the evaluation of hydroxyl radical generation and oxidative stress induction *in vivo* in rats

Tehnică rapidă LC-MS/MS pentru evaluarea generării de radicali hydroxyl şi a stresului oxidativ indus *in vivo* la şobolani

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Abstract

A new high-throughput liquid chromatography coupled with mass spectrometry (LC-MS/MS) method for the quantification of 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5- dihydroxybenzoic acid (2,5-DHBA) in rat plasma was developed and validated. The separation was performed on a Luna HILIC column under isocratic conditions using a 94:6 (v/v) mixture of acetonitrile and 50 mM ammonium acetate in water (pH 4,5) at 15ºC with a flow rate of 0.5 mL/min. The detection was performed in MRM mode using an ion trap mass spectrometer with electrospray negative ionization. The ion transition monitored was m/z 153 → m/z (109) for both analytes. The rat plasma samples were precipitated using acetonitrile and aliquots from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method showed a good linearity (r > 0.998), precision and accuracy (< 9.1%) over the concentration ranges of 20 - 2420 ng/mL for both analytes. The recoveries were between 85.7% – 103.0%. The method is not expensive, it needs a minimal time for plasma sample preparation and has a run-time of 4 min for instrument analysis (retention times were of 1.5 min for 2,3-DHBA, and of 2.0 min for 2,5-DHBA, respectively). The developed and validated LC-MS/MS method is very simple and more time-saving compared to other similar published methods. It was used in a toxicological assay for the study of the in vivo hydroxyl radical generation in rat after exposure to some xenobiotics and oxidative stress generation.

Keywords: *2,3-DHBA, hydroxyl radical, LC-MS/MS, oxidative stress, rat plasma*

Rezumat

S-a elaborat şi s-a validat o nouă metodă de cromatografie de lichide de înaltă performanţă cuplată cu spectrometria de masă (LC-MS/MS) pentru cuantificarea acidului 2,3-dihidroxibenzoic (2,3-DHBA) şi a acidului 2,5-dihidroxibenzoic (2,5-DHBA) în plasma de şobolan. Separarea s-a realizat pe o coloană Luna HILIC în condiţii izocratice folosind un amestec de acetonitril şi acetat de amoniu 50 mM în apă, pH 4,5 (94:6, v/v) la 15ºC cu un debit de 0,5 mL/min. Detecţia s-a realizat în modul MRM folosind un spectrometru cu trapă ionică, cu io-

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nizare electrospray negativă. S-a monitorizat tranziția ionilor m/z 153 → m/z (109) pentru ambii analiți. Plasma de şobolan s-a deproteinizat folosind acetonitril şi s-au injectat direct în cromatograf alicote din supernatantul obținut după centrifugare. Metoda a prezentat linearitate bună (r > 0.998), precizie şi acurateţe (< 9.1%) pe domeniile de concentrații 20 - 2420 ng/mL pentru ambii analiți. Regăsirile au avut valori cuprinse între 85.7% – 103.0%. Metoda nu este costisitoare, necesită un timp minim pentru prelucrarea probelor de plasmă şi timpul de analiză este de 4 min pentru analiza instrumentală (timpi de retenție de 1.5 min pentru 2,3-DHBA şi de 2.0 min pentru 2,5-DHBA). Metoda LC-MS/MS elaborată şi validată este foarte simplă şi mult mai rapidă decât alte metode similare publicate. S-a aplicat cu succes într-un studiu toxicologic de formare a radicalilor hidroxil in vivo la şobolan după expunere la anumite xenobiotice şi de generare a stresului oxidativ.

Cuvinte cheie: *acid 2,5-dihidroxibenzoic, radical hidroxil, LC-MS/MS, stress oxidativ, plasmă de şobolan*

Introduction

At present, it is well known that oxidative stress is involved in the pathophysiology of several diseases including cancer (1), diabetes, cardiovascular (2), renal (3) and neuronal degeneration diseases (4), but also in the physiological aging process (5). Moreover, the toxicological effects induced by some drugs, such as cocaine, alcohol (6), fluoroquinolones (7), can be explained by oxidative stress generation.

Free radicals are generally very reactive molecules, continuously produced in cells as by-products of metabolism or by leakage from mitochondrial respiration. They can attack biomolecules. Cell death and tissue injury can appear when their formation exceeds the antioxidant defense systems of the organism. Because of the short lifetime of these species, the direct detection of free radicals in biological systems is extremely difficult and requires sensitive technology (8).

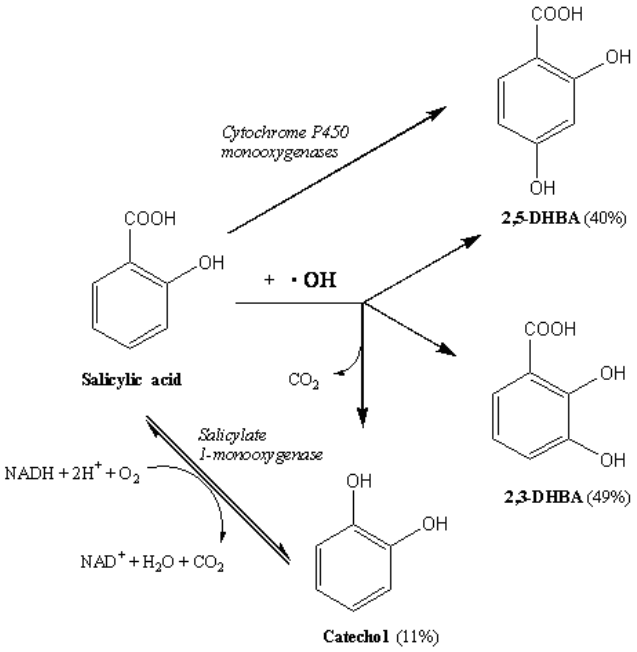

Figure 1. Oxidative and enzymatic pathways of salicylic acid in vitro in the presence of hydroxyl radicals [11]

The hydroxyl radical (·OH) is the most reactive and toxic entity among reactive oxygen species. It has a high reactivity, a very short lifetime and it is present in vivo in extremely low concentrations. Spin resonance spectroscopy (ESR) was used for the detecting of hydroxyl radical formation but it has low sensitivity and cannot be employed in quantitative measurements (9). Several methods used scavengers to trap the hydroxyl radicals in vivo, followed by the determination of reaction products as biomarkers. Endogenous compounds, such as glutathione, vitamin C, vitamin E, or some xenobiotics, such as aromatic compounds including benzoate or salicylate, that produce stable hydroxylation products, were used as hydroxyl radical trap (8, 9).

Salicylic acid (SA) is metabolized to salicyluric acid, gentisic acid, phenolic and acyl glucuronides (10). In humans, 60% of the SA remains unchanged and can trap free hydroxyl radicals. 2,3-DHBA is a metabolite of SA formed only when hydroxyl radicals are present. Grootveld et al. (1986) identified three reaction products after in vitro exposure of salicylic acid to hydroxyl radicals: catechol (11%), 2,3-DHBA (49%) and 2,5-DHBA (40%) (*Figure 1*) (11). In vivo, the formation

of 2,5-DHBA also occurs following an enzymatic pathway catalyzed by cytochrome P450 monooxygenases, while 2,3-DHBA is produced by a nonenzymatic reaction (12). Thus, 2,3-DHBA is analysed as a biomarker of in vivo hydroxyl radical production (13, 14).

Several methods such as gas chromatography (15, 16), high-performance liquid-chromatography (HPLC) with fluorescence (17), electrochemical (EChD) (10, 12, 16, 18) or mass spectrometry detection (19) and capillary electrophoresis (CE) (13) have been reported to determine levels of 2,3-DHBA in biological samples. GC analysis requires an isolation-step and derivatization (16). This sample pretreatment renders the analysis longer and more expensive. The classical HPLC methods also require isolation of analytes prior to instrumental analysis, performed generally by liquid-liquid extraction (10, 12, 16) or solid phase extraction (19), a time-consuming step that increases the cost of the assay and can influence the recovery.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) offers considerable advantages through its powerful performances: speed, selectivity, sensitivity and robustness. Sample preparation is simpler and more rapid and often includes precipitation of proteins (PP) and/or extraction before chromatographic analysis. The aim of this paper was to develop and validate a new simple and efficient high throughput LC-MS/MS assay for the simultaneous quantification of 2,3-DHBA and 2,5-DHBA in rat plasma, which could be applied in toxicological studies of stress oxidative generation.

Material and methods

Reagents and materials

Standard references of 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5- dihydroxybenzoic acid (2,5-DHBA) were purchased from Fluka (Buchs, Switzerland). All chemicals were of analytical-reagent grade. HPLC-grade acetonitrile, HPLC-grade methanol and ammonium acetate were purchased from Merck (Darmstadt, Germany). Bidistilled, deionised pro injectiones water was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca, Romania. The rat blank plasma was supplied from Wistar female rats (with a body weight of 154g ± 20g) by the Practical Skills and Experimental Medicine Centre of University of Medicine and Pharmacy Iuliu Haţieganu Cluj-Napoca, Romania.

Apparatus

The following equipment was used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of a G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 SL (Darmstadt, Germany).

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Luna HILIC (100 x 2.0 mm, 4 μm) column (Phenomenex Inc., USA) under isocratic conditions, using a mobile phase of a 94:6 (v/v) mixture of acetonitrile and 50 mM ammonium acetate in water (pH 4.5) at 15ºC with a flow rate of 0.5 mL/min. Chromatograms were processed using Quant Analysis software (Agilent Technologies). The detection was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an electrospray ionization ion source (ESI), in negative mode: dry gas nitrogen at 12 L/min, dry gas temperature 350ºC, nebulizer 60 psi (nitrogen), capillary 3500 V. Helium was used as collision gas and the collision potential was 1.1 V. The ion transition monitored was m/z 153 → m/z (109) for both analytes.

Standard solutions

Stock solutions of 2,3-DHBA (1.008 mg/mL) and 2,5-DHBA (1.010 mg/mL) were prepared by dissolving appropriate quantities in methanol. Two working solutions (20.16 μg/mL and 2016 ng/mL for 2,3-DHBA, and 20.20 μg/mL and 2020 ng/mL for 2,5-DHBA, respectively) were prepared by appropriate dilutions in drug-free rat plasma. These solutions were used to prepare plasma standards with the concentrations of 20.16, 40.32, 80.64, 161.28, 322.56, 1209.60, and 2419.20 ng/mL for 2,3-DHBA, and 20.20, 40.40, 80.80, 161.60, 323.20, 606.00, 1212.00, and 2424.00 ng/mL for 2,5-DHBA, respectively. Quality control (QC) samples of 80.64 ng/mL (lower), 322.56 ng/mL (medium) and 1209.60 ng/mL (higher) for 2,3-DHBA, and 80.80 ng/mL (lower), 323.20 ng/mL (medium) and 1212.00 ng/mL (higher) for 2,5-DHBA, respectively, were prepared by adding the appropriate volumes of working solutions to drug-free rat plasma. The resultant plasma standards and quality control standards were transferred into 15 mL polypropylene tubes and stored -20ºC until analysis.

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinized with acetonitrile (0.6 mL). After vortex-mixture (10 s) and centrifugation (2 min at 12000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 4 μL were injected into the HPLC system.

Table 1. The intra-day accuracy, precision and recovery data for the measurements of 2,3-DHBA and 2,5-DHBA in rat plasma (n = 5)

Analyte	Nominal concentration (ng/ml)	Found concentration mean		Precision (%)	Accuracy (%)	Recovery	
		ng/ml	± SD			(%)	± SD
2,3-DHBA	20.16	18.57	1.38	7.4	-7.9	93.9	5.4
	80.64	76.29	0.91	1.2	-5.4	97.7	1.1
	322.56	318.32	5.27	1.7	-1.3	96.0	1.5
	1209.60	1117.21	11.18	1.0	-7.6	99.7	0.8
2,5-DHBA	20.20	20.68	1.54	7.5	2.4	86.0	8.8
	80.80	81.08	7.42	9.1	0.3	101.5	9.9
	323.20	321.84	8.68	2.7	-0.4	103.0	2.7
	1212.00	1181.47	48.36	4.1	-2.5	94.1	3.3

Table 2. The inter-day accuracy, precision and recovery data for the measurements of 2,3-DHBA and 2,5-DHBA in rat plasma (n = 5)

Analyte	Nominal concentration (ng/ml)	Measured concentration		Precision (%)	Accuracy (%)	Recovery	
		ng/ml	± SD			(%)	± SD
2,3-DHBA	20.16	19.37	1.36	7.0	-3.9	92.5	2.7
	80.64	82.19	4.90	6.0	1.9	102.2	6.7
	322.56	332.60	11.84	3.6	3.1	98.5	5.6
	1209.60	1143.81	48.94	4.3	-5.4	97.7	2.5
2,5-DHBA	20.20	20.05	1.21	6.0	-0.7	85.7	8.4
	80.80	81.75	5.21	6.4	1.2	95.0	4.9
	323.20	338.38	26.17	7.7	4.7	100.6	2.2
	1212.00	1230.33	42.48	3.5	1.5	97.2	1.7

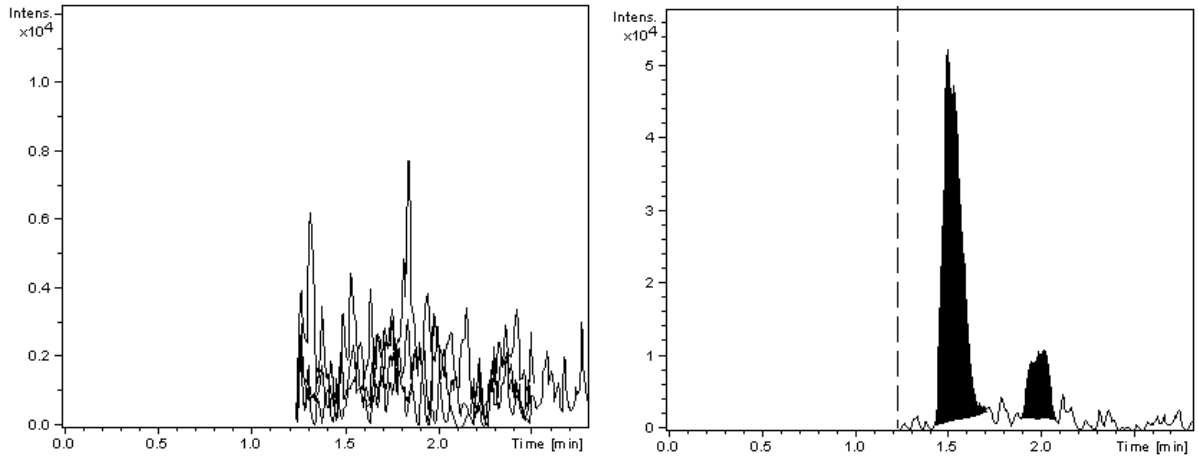

Figure 2. Representative chromatograms of rat blank plasma (left) and rat plasma spiked with 2,3-DHBA and 2,5-DHBA (right) at lower limits of quantification (20.16 ng/ml for 2,3-DHBA and MDA, and 20.20 ng/ml for 2,5-DHBA, respectively) (retention times: 2,3-DHBA - 1.5 min, 2,5-DHBA – 2.0 min)

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing the two analytes with those obtained from different plasma blank samples (n=6).

The concentrations of 2,3-DHBA and 2,5-DHBA were automatically determined by the instrument data system using peak areas and the external standard method. The calibration curve model was determined for five calibration series (n = 5) by the least squares analysis, $y = c + bx + ax^2$, weighted (1/y) quadratic regression, where y - peak area and x - analyte concentration (ng/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by the analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day (*Table 1*). The inter-day precision and accuracy were determined by the analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels) (*Table 2*).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

Absolute recoveries were measured by comparing the response of each analyte from spiked plasma samples with the response from a standard solution with the same analyte concentration, prepared in mobile phase and processed in the same manner with plasma sample.

Statistical analysis was performed by using an in-house customized and validated Microsoft Excel file, which automatically calculates all the method validation parameters.

Results

The chromatographic conditions, especially the composition of the mobile phase, were optimized in several trials to achieve good MS signals, short retention times of analytes and consequently a high-throughput analysis. The best results were obtained with the mixture of acetonitrile and 50 mM ammonium acetate in water (pH 4.5) under isocratic conditions, at 15ºC. In the selected chromatographic conditions, the retention times of 2,3-DHBA and 2,5-DHBA were 1.5 and 2.0 min, respectively, and the analytical run-time was 4 min for instrument analysis. No interfering peaks from the endogenous plasma compon-

ents were observed at the retention times of the two analytes. Representative chromatograms of plasma spiked with 2,3-DHBA and 2,5-DHBA at LLOQ are shown in *Figure 2.*

The detection was performed in MRM mode. The ion transition monitored was m/z 153 → m/z (109) for both analytes (*Figure 3* and *Figure 4*).

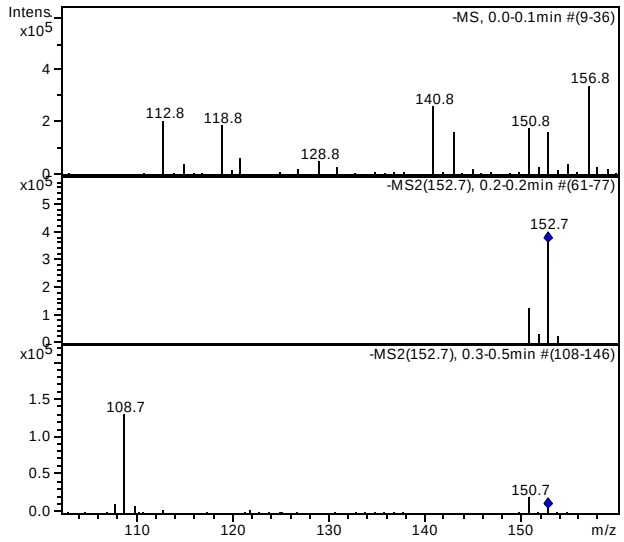

Figure 3. Full-scan spectra (up), MS/MS non-reactiv spectra (middle) and MS/MS reactive spectra (down) of 2,3-DHBA

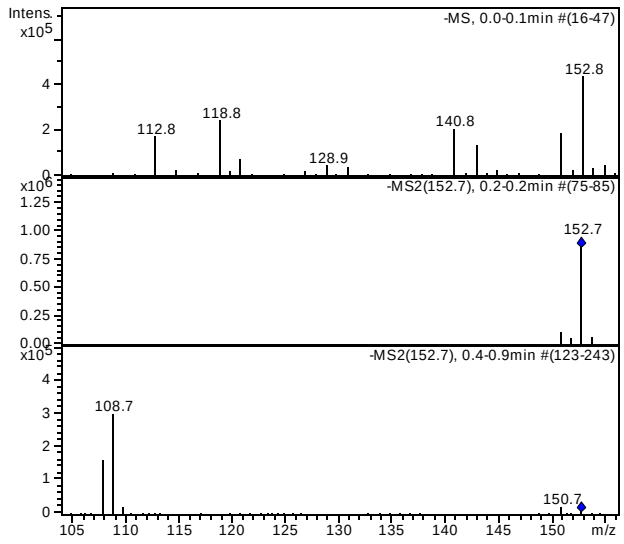

Figure4. Full-scan spectra (up), MS/MS non-reactiv spectra (middle) and MS/MS reactive spectra (down) of 2,5-DHBA

The calibration curves were linear over all the studied concentration ranges in rat plasma, with the correlation coefficients greater than 0.998. The LLOQ were 20.16 ng/mL for 2,3-DHBA, and 20.20 ng/mL for 2,5-DHBA, respectively. The values obtained for intra-day and inter-day precision and accuracy during the validation for plasma are shown in *Table 1* and *Table 2*, respectively.

Discussion

LC/MS/MS assay

In LC-MS/MS assays the sensitivity depends on MS detection mode, but the method used for sample preparation may influence the chromatographic background level and can generate matrix suppression effect. We propose a very simple and rapid pretreatment of plasma samples including only PP with acetonitrile and direct injection into the chromatographic system from the supernatant obtained after centrifugation, with good recoveries (between 85.7% – 103.0%). The other methods reported in the literature (*Table 3*) include an isolation step in the pretreatment of samples, but this operation increases the time of analysis, increases the costs and can affect the recovery.

The chromatographic separation was performed on a HILIC column under isocratic conditions using a mobile phase containing 94% acetonitrile and 6% aqueous solution of 50 mM ammonium acetate (pH 4.5) at 15ºC. We chose the HILIC chromatography because it allows increasing the retention of hydrophilic analytes using reverse phase solvents. In the selected chromatographic conditions, 2,3-DHBA eluted in 1.5 min and 2,5-DHBA in 2.0 min, respectively. The elaborated method ensures a high-throughput analysis, with an analytical run-time of 4 min.

As salicylate hydroxylation products have high electron affinities, they can easily form negative ions at low pH. The detection of these compounds is more sensitive in the neg-

Table 3. Analytical characteristics of reported HPLC methods for the determination of 2,3-DHBA in plasma or serum:

Reference	Matrix	Detection mode[a]	Mobile phase constituents	Sample prepa-ration[b]	LOQ/ LOD[c,d]	Run time (min)	Absolute recovery[d] (%)
Tabatabaei [19]	Rat plasma	LC/MS, ESI, NI	acetonitrile / 0.3% formic acid in water, gradient elution	SPE	0.5 nmol/mL (LOQ)	18	NA
Coudray [10]	Plasma	HPLC-EChD	85% phosphate buffer 100 mM, 0.1 mM SDS, pH 3.3, and 15% methanol, v/v	ELL	0.37 nmol/mL (LOD)	38	86-102
Paterson [16]	Serum	HPLC-EChD	citrate buffer 30 mM, pH 5.25, and methanol, gradient elution	ELL	NA	10	NA
Liu [12]	Plasma	HPLC-EChD	97.2% sodium citrate (30 mM) in acetate buffer (27.7 mM, pH 4.75) and 2.8% methanol	ELL	20 pmol (LOQ)	> 8	NA

[a]ESI, electrospray ionisation; NI, negative ionization mode; EChD, electrochemical detection; [b]SPE, solid-phase extrac tion; LLE, liquid–liquid extraction; [c]LOQ, limit of quantification; LOD, limit of detection; [d]NA, not available.

ative mode than in the positive mode. Therefore, the detection was performed with an electrospray ion source in negative mode by multiple reaction monitoring. Due to structural similitude of 2,3-DHBA and 2,5-DHBA, their mass spectrometry fragmentation pattern is the same and the ion transition monitored for both analytes was m/z 153 → m/z (109).

Assay validation

The method was validated in accordance with international regulations [20-24]. The obtained results proved a good linearity ($r > 0.998$), sensitivity (LLOQ of ~20 ng/mL for both analytes), accuracy and precision over the studied concentration ranges of 20 - 2420 ng/mL. All values for accuracy and precision were within recommended limits (*Table 1* and *Table 2*). The recovery values were between 85.7% – 103.0%, which means no analyte loss during sample preparation due to adsorption on precipitated proteins.

Our developed LC-MS/MS assay is simple, rapid, accurate and not expensive. In comparison with other published assays for determination of 2,3-DHBA levels in plasma (*Table 3*) our method performs better in terms of speed (both sample preparation and chromatographic run-time) and costs, which are essential attributes for methods used in routine analysis. Liu et al. (1997) reported a better sensitivity with electrochemical detection (20 pmol), but it is not clearly specified if this concentration corresponds to 1 mL plasma or it represents the amount of analyte injected in HPLC system. However, the method was not validated on the studied concentration range (12). The LLOQ of our method is equivalent to 0.13 nmol/mL and it shows enough sensitivity for the detection of in vivo hydroxyl radical formation.

Method application

The validated method was successfully applied in a toxicological study of in vivo hydroxyl radical generation in rat after exposure to bisphenol A and/or methyl parabene. The protocol of this study has been approved by the Ethics Committee

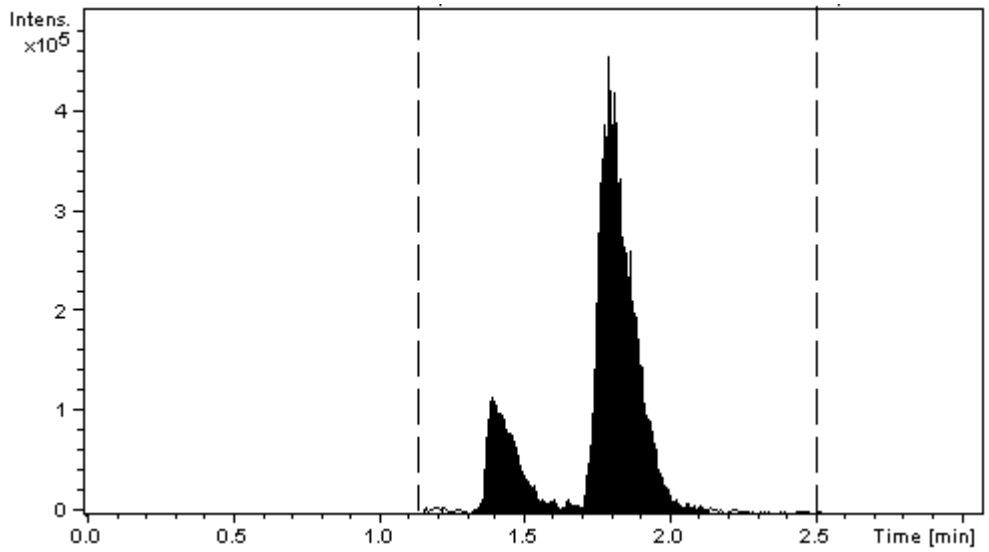

Figure 5. Chromatogram of sample plasma from a rat after oral administration of 50 mg/kg/day bisphenol A for 9 days and 500 mg salicylic acid / kg 90 min before the blood sampling; concentrations found: 106.63 ng/ml 2,3-DHBA and 982.7 ng/ml 2,5-DHBA

of "Iuliu Haţieganu" University, in accordance with institutional and national guidelines for laboratory animal experiments. The animals were kept in temperature, humidity, day/night cycle standard conditions and they had access to food and water ad libitum throughout the experiment in the Practical Skills and Experimental Medicine Centre of the University. A representative chromatogram obtained from a rat treated orally (by gastric intubation) with 50 mg/kg/day bisphenol A for 9 days and 500 mg salicylic acid / kg 90 min before the blood sampling is shown in *Figure 5*. Blood (0.2 mL) was collected from the retro-orbital sinus in the presence of sodium fluoride as anticoagulant. Plasma separated by centrifugation (3000 g / 6 min) was stored at -20°C until the time of analysis.

Conclusion

The developed and validated LC-MS/MS method allows the quantification of 2,3-DHBA in rat plasma as a marker of in vivo hydroxyl radical generation. It is very simple and more rapid than other similar methods reported in the literature and shows enough sensitivity for the detection of in vivo hydroxyl radical formation. It also can easily be adapted for the assay of 2,3-DHBA in human plasma. The method was successfully applied in a toxicological study of in vivo hydroxyl radical generation in rat after exposure to bisphenol A and/or methyl parabene and can be useful in other toxicological or biochemical studies for oxidative stress assays.

Acknowledgement

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Abbreviations

2,3-DHBA – 2,3-dihydroxybenzoic acid
2,5-DHBA – 2,5-dihydroxybenzoic acid
SA – salicylic acid
HPLC - high-performance liquid-chromatography
EChD – electrochemical detection
CE - capillary electrophoresis
LLE - liquid-liquid extraction
SPE – solid-phase extraction
LC-MS/MS - liquid chromatography tandem mass spectrometry
PP - precipitation of proteins
MRM - multiple reaction monitoring

QC - quality control
LLOQ - lower limit of quantification
LOD – limit of detection

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