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Preliminary Study on J-Resolved NMR Method Usability for Toxic Kidney's Injury Assessment*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Nowadays, the Nuclear Magnetic Resonance (NMR) techniques are tested for metabolomic urine profile in order to detect early damage of kidney.
Objectives. The purpose of this investigation was the initial assessment of two-dimensional J-resolved NMR urine spectra analysis usability for early kidney injuries detection. The amino acids (AA) and acids profile change after the exposure to nephrotoxic agent (the cisplatin infusion) was examined.
Material and Methods. The material was the urine of patients with non-small-cell lung cancer, treated with cisplatin in Pulmonology and Lung Cancers Clinic in Wrocław. The urine of healthy volunteers was also examined. The identification of metabolites in urine was based on two-dimensional JRES signals in spectra, described in Human Metabolites Database (HMD). The molar concentration of metabolites was calculated from the volume under the signals. The analysis was focused on amino acids and organic acids (lactid acid and pyruvic acid) profiles.
Results. Any specific amino acids were identified after cisplatin infusion in comparison to the state before infusion. However, the differences in concentration were observed over 2-fold increase in valine, isoleucine and leucine, over 3-fold in alanine. Also, the concentration of pyruvic and lactic acids increased significantly ($p \leq 0.05$, $p \leq 0.01$).
Conclusions. There were no specific amino acids identified in response to the infusion of cisplatin; however, some changes in the concentrations of amino acids and other small molecules were found. The analysis of two-dimensional JRES spectra showed an increase of alanine, leucine, isoleucine and valine concentration after the application of cisplatin. It seems that it is worth developing the JRES method based on special computer program (**Adv Clin Exp Med 2015, 24, 4, 629–635**).

Key words: NMR, JRES, aminoacids, nephrotoxicity, cisplatine.

Toxic kidney injury often results from exposure to nephrotoxic agents or during drug therapy. In this situation early identification of the damage and an implementation of the nephroprotective procedure is very important. The Nuclear Magnetic Resonance techniques are now tested for metabolomic urine profile as a tool to detect early damage of the kidney [1, 2].

The sensitive NMR techniques (500 MHz) and software enable the registration and the analysis of signals in the range from 3 to 13 ppm in steps of 0.001 ppm. The rich database of human metabolites (HMDB – Human Metabolites Database v. 3.5, 41519 metabolites) [3] provides considerable opportunities to quickly identify the agents that have various structures and low concentration. Easy preparation of urine samples (centrifugation and phosphonate buffer addition with 5% D_2O, TSP as an internal standard, NaN_3 to prevent bacterial degradation in urine) and the automatisation of

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the procedure encourage the use of the NMR techniques for the identification changes in urine. Two or three-dimensional NMR techniques in cooperation with computer-program which allows us to separate and analyze each signal of the spectrum seems to provide new tools in diagnosis.

During the last decade, among various nuclear proton resonance techniques, the R-resolved, two--dimensional spectra methods were significantly developed, because of their elimination of overlap signals [4–6].

The purpose of this investigation was an initial assessment of the usability of two-dimensional J-resolved Nuclear Magnetic Resonance (J-RES-NMR) urine spectra analysis for detection of early kidney injuries by the identification of amino acids (AA) and acids profile changes after exposure to the nephrotoxic agent. The analysis of metabolites urine profile was done for the patients (5 men) who were cured by cisplatin (cis-diamminedichloroplatinum(II)). Cisplatin, used in-clinic in cancer therapy, in our experiment was treated as a model nephrotoxic agent. Urine analysis before application of cisplatin, and after intravenous infusion was made as a means of detecting differences in amino-acid profile. The urine from healthy volunteers (5 men) was also analysed. J-RES spectra was made totally for 15 samples, which were analysed by our own software.

Material and Methods

The material for the investigation consisted of the urine from 5 men with diagnosed non-small--cell lung cancer (NSCLC), age 55–74 (the average 63.2), who were cured by cisplatin in the Clinic of Pulmonology and Lung Cancers, Wroclaw Medical University and the urine from 5 healthy volunteers – men in age range 57–71 (the average 60.2). The urine was taken twice: before application of cisplatin (A) and 4 h after application (B). The control sample was the morning urine from healthy volunteers (K). The patients were treated with intravenous cisplatin infusion in concentration 186.37 mg/1000 mL 0.9% NaCl. The study protocol was approved by the Bioethics Committee at Wroclaw Medical University (No 658/2012).

The urine was taken into polyethylene containers without any preservatives. Morphotic elements were removed by centrifugation with 3000 rpm/min during 15 min. Next 1 cm^3 centrifugated urine was lyophilisated and stored in –80°C temperature until the time of research.

Samples for NMR analysis were prepared according to the procedure recommended by Bruker, but with modification for lyophilisate. Lyophilisate was dissolved in 0.6 cm^3 phosphate buffer pH 7.4 included 5% D_2O. Next (trimetylosililo) propioniane natrium (TSP) was added as a reference compound and 0.05% NaN_3. The concentration of TSP in all the samples was 1 mmol/dm^3. The solution was centrifugared 10 min (3000 rpm/min) and put into NMR tube. 1H NMR spectra of 15 urine samples were acquired from a Bruker BioSpin Avance III 500-MHz system equipped with 5-mm cryoprobes, CPTCI (1H-13C/15N/2H + Z--gradients) (Bruker BioSpin). Water signals extinction was achieved by presaturation. Every 1D 1H NMR spectrum consists of 32 scans accumulated with a spectral width of 10,273 Hz. Except one-dimensional spectra (1D 1H) also two-dimensional JRES spectra were obtained using a double spin-echo sequence with 16 transients in 32 steps. Fourier transformed, manually phased, and the TSP internal reference peak was set to 0.000 ppm by use of TopSpin (v. 3.2, Bruker Biospin). All spectra were preprocessed as described earlier [7]. The JRES spectra were transferred to computer program R cran (v. 3.0.1, 64 bit) [8] for further analysis. The identification of compounds was made based on one- and two-dimensional JRES spectra, analysis Human Metabolites Database (HMDB) [3] and R. Bartona papers [9]. Because chemical shifts in HMDB database are referred to DSS (2,2-dimetylo-2-silapentano-5-sulfonowego), spectra with TSP needed 0.016 ppm correction for signals [10]. Identified compounds were confirmed in ^{1}H NMR and COSY spectra. The volume under the signal was used to calculate molar concentration (C_{mx}) of the investigated compound in urine.

$$c_{mx} = \frac{c_{TSP} \cdot V_x \cdot y}{V_{TSP} \cdot 9},$$

where:
c_{TSP} – molar concentration of TSP (for every sample 1 mmol/dm^3),
V_x – volume of the signal for investigated compound,
y – the number of protons, which create the signal,
9 – the number of methyl protons, from TSP signal.

The molar concentration (c_{mx}) was re-counted into weight/volume concentration (c_{px}) and normalized for to the content of creatinine (c_x), in order to eliminate the influence of urine dilution.

V_{TSP} – volume of the signal for reference TSP

$$c_x = \frac{c_{p_x}}{c_{p_{Cre}}},$$

where:
c_x – content of investigated compound in relation to creatinine (mg of the compound/1 mg of the creatinine),

c_{p_x} – weight-volume concentration of investigated compound (mg/dm^3),
$c_{p_{Cre}}$ – weight-volume concentration of creatinine (mg/dm^3).

All statistical analyses were performed with a Student's *t*-test. Differences between the groups were considered significant at $p < 0.05$. Statistical evaluation of results was performed using the program STATISTICA 10 PL, StatSoft Inc., USA.

Results

The analysis of obtained J-RES-NMR was focused on amino acids and organic acids (lactic acid and pyruvic acid) profiles. Table 1 shows the list of identified amino acids, acids glucose and creatinine in urine of control group (K) and patients before cisplatin infusion (A), and after infusion (B).

Some differences in the personal profile of metabolites were found. Also, few differences between A and B like phenylalanine in patient 2(B) or lysine in 5(B), but any characteristic signals common for all treated with cisplatine were found.

The obtained spectra were very rich in compounds signals. The position of signals (chemical shift in ppm), frequency (H_z) and multiplicity (singlet, doublet, quartet etc.) are described in Table 2.

The example of spectrum with signals of alanine is shown at Fig. 1.

The comparative analysis of urine profile before and after cisplatin infusion did not show any specific amino acids signals, which would be common for all groups of patients. No new signals, which were repeatable for all patients, were identified. On the other hand, a significant increase in the molar concentration of some amino acids and acids after cisplatin application was observed.

Table 3 presents the average values of molar concentration of amino acids and organic acids that increased after cisplatin application.

An over 2-fold increase in valine, isoleucine and leucine concentration was observed along with an over 3-fold increase in the concentration of alanine in patients after chemotherapy (Table 3). The concentration of pyruvic acid and lactic increased significantly ($p \leq 0.05$, $p \leq 0.01$) after the application of cisplatin as compared to the value before drug application.

The changes in concentration before and after the application of cisplatin and in the control group are shown graphically in Fig. 2.

Discussion

Cisplatin (*cis*-Diamminedichloroplatinum) is one of the most widely used chemotherapeutic agents, but the risk of nephrotoxicity frequently hinders the use of higher doses to maximize its antineoplastic effects. This nephrotoxic side effect is reported for about 30% of patients [11]. Cisplatin binds with DNA, damages mitochondria increasing the generation of reactive oxygen species (ROS). ROS is the main factor that causes nephrotoxicity. The cisplatin nephotoxiticy was widely described [12, 14] and monitored by several markers like neutrophil gelatinase-associated lipocalin (NGAL) [13].

Table 1. The identified compounds

Symbol of sample	Type of compound
1K	A, L, IL, W, G, KM, KP, KC, Kr
2K	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T, FA
3K	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T, Li
4K	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T
5K	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T, FA
1A	A, L, IL, W, G, KM, KP, KC, Kr, H, MH, FA
2A	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T
3A	A, L, IL, W, G, KH, KM, KP, KC, T, Kr, H, Li
4A	A, L, IL, W, G, KM, KP, KC, Kr
5A	A, L, IL, W, G, KH, KM, KP, KC, Kr, T
1B	A, L, IL, W, G, KM, KP, KC, Kr, H, MH, FA
2B	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T, FA
3B	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, Li, T
4B	A, L, IL, W, G, KH, KM, KP, KC, Kr
5B	A, L, IL, W, G, KH, KM, KP, KC, Kr, H, MH, T, FA, Li

A – alanine, L – leucine, IL – izoleucine, W – valine, G – glucose, KM – lactat, KP – pyruvate, KC – citriate, KH – hippurate, Kr – creatinine, H – histidine, MH – l-methylohistidine, T – tyrosine, FA – phenyloalanine, Li – lysi.

Table 2. The characteristic of signals

Name of the signal	Chemical shift (range) and frequency (range)			
	from [ppm]	to [ppm]	from [Hz]	to [Hz]
TSP singlet – as an internal standard – $(CH_3)_3SiCD_2CD_2COO^-$				
TSP	0.020	–0.020	3.80	–3.80
Lactate– quartet CH_3-CHOH-COO^-				
Lac1	4.145	4.135	9.27	4.00
Lac2	4.137	4.127	4.45	0.00
Lac3	4.129	4.119	0.00	–4.45
Lac4	4.120	4.110	–4.00	–9.27
Pyruvate – singlet – C**H**$_3$-CO-COO^-				
Pyr	2.374	2.363	4.00	–4.00
Alanine – doublet – C**H**$_3$-$CHNH_2$-COOH				
ALA1	1.499	1.485	7.60	0.00
ALA2	1.484	1.470	0.00	–7.60
Valine – doublet – $(CH_3)(CH_3)CHNH_2$-COOH				
VAL1	1.060	1.045	7.06	0.15
VAL2	1.045	1.030	–0.15	–7.06
Izoleucine – doublet – $CH_3CH_2CH(CH_3)CHNH_2$-COOH				
ILE1	1.026	1.014	5.93	0.74
ILE2	1.013	1.001	–0.74	–5.93
Leucine – 2 × doublets – $(CH_3)_2CH_2CHNH_2$-COOH				
LEU1	0.982	0.973	5.50	0.75
LEU2	0.969	0.959	–0.75	–5.50
LEU3	0.971	0.962	5.50	0.75
LEU4	0.958	0.949	–0.75	–5.50
Creatinine – singlet -C**H**$_2$-				
Cre	4.068	4.052	6.00	–6.00

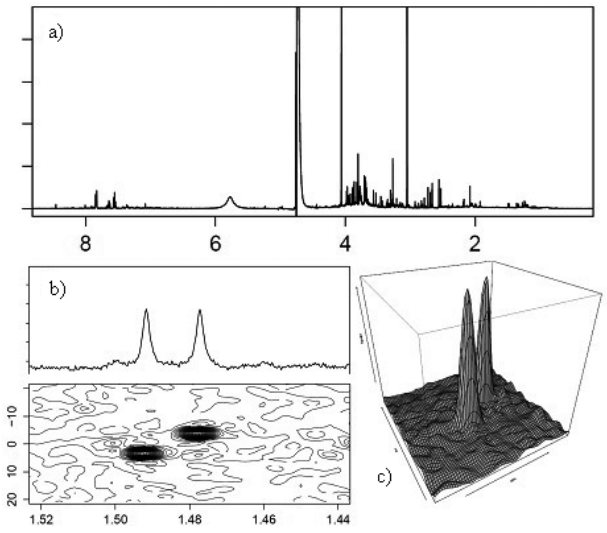

Fig. 1. (a) 500 MHz spectrum of urine; (b) JRES NMR spectrum of region 1.52–1.44 ppm, projection 2D; (c) 3D – signals of alanine

Table 3. The concentration of amino acids and organic acids in urine of patients before (A), and after (B) cisplatine infusion and in control group (K) (mol/mmol of creatinine)

	Lactate	Pyruvate	Ala	Val	Iso	Leu
K						
$\bar{x}$	4.85	8.90	55.33	14.43	5.19	14.41
SD	2.96	2.93	13.19	4.61	1.83	3.39
A						
$\bar{x}$	2.87	5.36	41.36	14.97	6.47	16.65
SD	0.85	0.85	9.53	3.06	1.64	4.49
B						
$\bar{x}$	6.13	11.19	130.48	32.83	11.89	31.45
SD	2.35	3.15	98.59	24.70	5.47	14.99

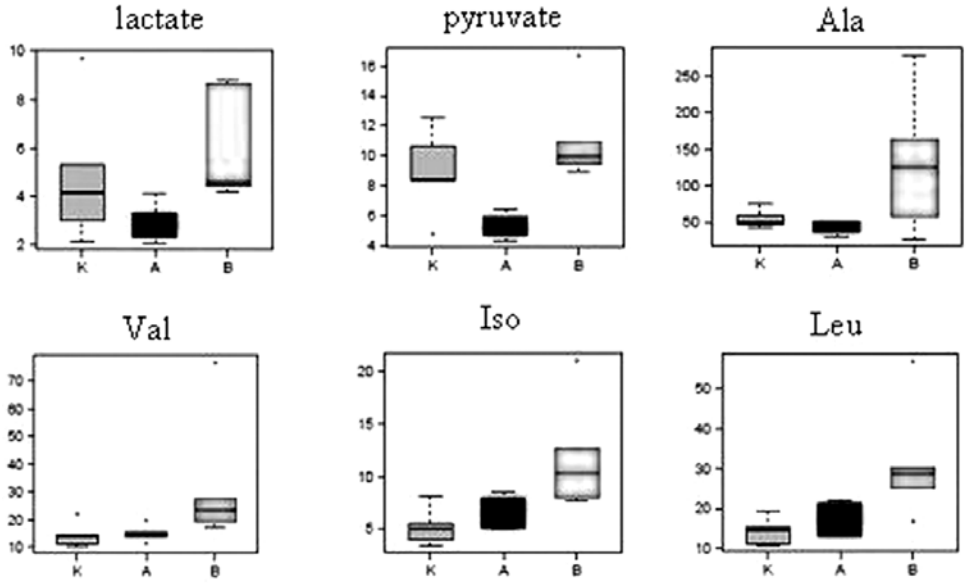

Fig. 2. Diagrams of AA and organic acids molar concentration in urine of patients before (A) and after (B) application of cisplatin, and in the control group (K)

The metabolomic study of cisplatine nephrotoxicity is described by Portilla [14]. The urine samples of mice treated with a single injection of cisplatin (20 mg/kg body weight) were collected for a period of 3 days and every sample analyzed by ^{1}H NMR. The changes in metabolites level were observed in the first 24 h after injection. At first glycosuria, aminoaciduria and increased level of pyruvate and lactate were noted. After 2 days the increased level of aminoacides in urine were also observed, like alanine (12-fold increase), leucine (7-fold increase), methionine (4-fold increase), valine (8-fold increase). The research showed that urine of mice with acute renal failure had special metabolomic profile.

Different investigation of Boudock et al. [15] (materials: rats, method GLC/MS), shows, after one day of treatment with various nephrotoxins the increased concentration of aminoacids like: threonine (3.53-fold), glutamine-(2.52-fold), histidine-(1.6-fold), lysine (1.49-fold), cadevarine (1.64-fold), putrescine (2.68-fold), and other metabolite: glycilproline (3.26), glucosamine (1.95), monoethanoloamine (3.62 fold), phosphate (3.81), glucose (1.2) etc.

Most of cisplatin investigations are based on GLC/MS analysis. Only Portilla et al. [16] used one dimension ^{1}H NMR spectra. In our own investigation two-dimensional JRES spectra were obtained, which eliminate the overlapping of signals in ^{1}H NMR and the separation of multiplets (Fig. 1). Further transformation of two-dimensional spectra were made by TopSpin software. Overlap of the signals and volume calculation under signals (the concentration of compounds) were made automatically by the author's program.

Our study showed high level of glucose, similar to mouse investigation [14, 16]. Also, increased concentrations of alanine, valine, isoleucine and leucine in the urine after the cisplatin infusion were found. The free AAs contained in the plasma are filtrated largely by GFR (glomerular filtration rate). Reabsorption of free AA is very important in maintaining the homeostasis of the organism, by which these valuable components are not lost and not excreted in urine [17]. In the kidney, the main place of reabsorption process occurs in the proximal tubules. Several xenobiotics damage renal proximal tubule, the part of the nephron with the great sensitivity to nephrotoxic effects. The proximal tubule is subdivided into 3 segments (S1, S2, and S3) present in the cortical labyrinth and the medullar rays. More than 90% of AA from glomerular filtrate is reabsorbed in proximal tubules [18, 19]. Reabsorption takes place by the use of transport system [17]. Besides the fact that the proximal tubule is the main transport place of more than 90% AA in the kidneys, this renal tubules, which are in direct contact with the glomerular filtrate, are very susceptible to the nephrotoxic action of xenobiotics, including cisplatin [19].

The harmful effect of cisplatin may lead to disturbances of transport and reabsorption of AA in proximal tubules. The result of these abnormalities is the increased urinary excretion of AA, which in properly functioning kidneys is minimized [1, 16, 20–23].

The amount of AA in urine reported in our study may therefore indicate the potential abnormalities caused by cisplatin within the proximal tubules and could be treated as an early signal of tubular injury. Studies in rodents have shown increased content of alanine, threonine, methionine in the urine after application of various nephotoxines, including cisplatin, while the other conventional indicators of kidney damage (blood urea nitrogen, creatinine concentration) did not show raised values [15, 24].

The raising content of pyruvate can be the result of mitochondria disorder caused by cisplatine, which may induce a distortion of the acid synthesis Krebs cycle. Excretion of significant amounts of lactate may be caused by a disorder of the Cori cycle, which is the metabolic pathway of glucose synthesis from the lactate. The presence of these metabolites in the urine of patients after infusion of cisplatin may also be the result of oxidative stress generated by cytostatic.

In summary, the preliminary study shows the utility of JRES-NMR examination of urine. The great amount of signals in NMR spectrum of urine could be separated and identified, but it needs well-projected computer program and sensitive NMR equipment. Early detection of nephrotoxicity is important to prevent serious and irreversible damage of the kidneys, especially proximal tubules. These will improve the diagnosis of kidney injury. The use of appropriate computer programs for identification of signals with automatic calculation of the concentrations is necessary.

The authors concluded that there were no specific metabolites, common for all groups, identified in response to the infusion of cisplatin; however, some changes in the concentrations of amino acids and other small molecules were found.

The analysis of two-dimensional J-RES spectra showed an increase of alanine, leucine, isoleucine, valine, pyruvic and lactic acids concentration after the application of nephrotoxic agent.

J-RES NMR analysis seems worth developing, but it needs a well-projected computer program and sensitive NMR equipment.

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