Metabolic profiling of target organic acids in Parkinson's Disease using LC-ESI-MS

Author name: Rowan M.Y. Karg
Name of institute: Leiden University, LACDR, SBP
E-mail address: rowankarg@hotmail.com

Supervisors: Dr. Edinson Lucumi Moreno & Cornelius Willacey

ABSTRACT:
To date, the exact cause of Parkinson's disease (PD) is still unclear. Therefore, detection of newer targets which can be linked to the pathogenesis or progression of PD is vital. In this study, liquid chromatography-electrospray ionization-mass spectrometry was used (LC-ESI-MS) to detect target organic acid metabolites. In addition, metabolite levels were determined in three cell lines namely a healthy control, gene-corrected PD and PINK1 mutant PD cell line. Several acids were successfully detected and results showed elevated levels of citric- and propanoic acid in the PINK1 mutant PD cell line compared to control groups. Further method improvement however is still necessary.

Keywords:
PD, metabolomics, LC-ESI-MS, target organic acid metabolites, PINK1 mutation.

INTRODUCTION:
The exact cause of the progressive neurodegenarative disorder Parkinson's disease (PD) is still unclear and knowledge regarding its progression is also lacking. However, several hypotheses such as mitochondrial dysfunction, oxidative stress and genetic factors have been suggested as the most probable causes of PD [1]. In 5-10% of patient's, genetic risk factors have been identified [2]. Mutations in the PTEN-induced kinase 1 (PINK1) gene can result in mitochondrial dysfunction and oxidative stress [3].

Patients seldomly experience PD symptoms such as tremor in early stages of the disease [4]. Therefore, early diagnosis of PD is essential to improve the quality of life at a younger age for patients. Earlier treatment also helps slow the progression of PD. This is however restricted as no biomarkers are available for the early identification of PD [5]. For this reason, identification of promising targets is essential as this enables the development of newer and improved treatments and specific biomarkers which may be used for the early diagnosis of PD [1]. Various approaches such as metabolomics can be used to investigate novel targets which may be linked to the diagnosis, pathogenesis and progression of PD [6]. The field of metabolomics is described as a wide-ranged analysis and thorough characterization of up to thousands of metabolites.

Current used analytical techniques in this field include liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) for the analysis of complex biological samples [6]. The coupling of LC and ESI-MS ensures separation and ionization of metabolites prior to detection [6]. In addition, metabolites can be derivatized to increase specificity and sensitivity [7]. The aim of this research is to optimize and validate a LC-ESI-MS method for the detection of various target organic acid metabolites. The gathered metabolomics data will then be used to study the effect of a PINK1 gene mutation on the metabolite levels in three different cell lines namely a healthy control, gene-corrected PD (PD without the PINK1 mutation) and PINK1 mutant PD cell line. Analyzing organic acid levels is vital as this can provide insight on underlying causes and symptoms of various illnesses and neurodegenerative disorders [8]. The following metabolites will be focused on during this research: acetic-, arachidonic-, citric-, dodecanoic-, formic-, fumaric-, lactic-, oxalic-, propanoic- and uric acid. These acids are implicated in either the mitochondrial dysfunction or oxidative stress hypothesis and are therefore promising targets.

HYPOTHESIS:
Uric acid has been associated with a decreased risk and slower progression of PD as it is believed to reduce oxidative stress [9]. Fumaric acid esters and fatty acids such as arachidonic- and dodecanoic acid have a neuroprotective role and are therefore capable of preventing mitochondrial dysfunction and reducing oxidative stress in PD [10]. Therefore, it is expected that levels of these four metabolites will be lower in the PD cell lines compared to the control group. The remaining acids have been linked to the impairment of the mitochondrial oxidative metabolism, leading to mitochondrial dysfunction and oxidative stress. Therefore, levels of these acids are expected to be higher in the PD cell lines compared to the control group . [11], [12]. This

elevation could partially be triggered by the PINK1-mutation, as this mutation has been a suspected cause of both mitochondrial dysfunction and the oxidative stress in PD [3].

MATERIAL AND METHODS:

Standard stock solutions and buffer preparation: For each metabolite of interest, a 1 mg/ ml or 10 mg/ standard stock solution was prepared and further diluted using 50% DMSO/ 50% DMF. Furthermore, 750 mM (100 ml) triethanolamine (TEOA) and 0,1 M (1 ml) sodium carbonate (Na_2CO_3) were prepared. The pH of TEOA and Na_2CO_3 was 10,0 and 12,5 respectively. A 30 mg/ ml formic acid solution was also made to quench the derivatization reaction.

Derivatization reaction: 10 µL of either TEOA or Na_2CO_3 was added to diluted samples. Subsequently, 10 µL of the derivatization reagent dimethylamino phenacyl bromide (DmPaBr) was added (40 mg/ ml for TEOA samples; 60 mg/ ml for Na_2CO_3 samples). All samples were incubated for 1 hour (65°C). Afterwards, 20 µL formic acid (30 mg/ ml) was added, followed by incubation for another 30 minutes. Lastly, 50 µL acetonitrile (ACN) was added and samples were centrifuged for 5 minutes. After derivatization, all samples were transferred to glass vials for LC-MS measurements.

LC-ESI-MS: The Acquity UPLC system (Waters) and Triple Quad LC-MS series 6460 (Agilent Technologies were used. The mobile phase consisted of 0,1% formic acid 10 mM ammonium formate (A) and 100% ACN (B). Flow rate was gradually increased in 0,100 ml/ min increments up to 0,700 ml/ min (10%A, 90%B). At 0,400 ml/ min the column temperature was switched from off to 60°C. Metabolites were detected using multiple reaction monitoring (MRM), a specific triple quadrupole MS technique which targets ions corresponding to the metabolites of interest. The principle of the MS is to detect ions based on their mass-to-charge (m/z) ratio. This data is then translated into the retention time (RT) which is the time ions need to pass through the column.

Method validation: For each detected metabolite, the parameter linearity was validated using a seven-point calibration curve with a concentration range of 0,156 – 10 µg /ml (n = 1). The repeatability (%RSD) and limits of detection (LOD) were also determined (n = 4).

PINK1 mutated PD stem cells: Cell lines were generated from human neuroepithelial stem cells (hNESCs) using induced pluripotent stem cells (iPSCs) technology. The hNESCs were then differentiated into dopaminergic neurons with- and without a PINK1 gene mutation. During this research three different cell lines were studied namely the K7 healthy control line, 2122 gene-corrected PD (PD without the PINK1 mutation) and 2122 PD mutant cell line (PD with the PINK1 mutation). Using the distributed calibration curves, the concentration of the detected metabolites was then calculated.

RESULTS:

Detected metabolites:
Acetic-, citric- and propanoic acid were detected using TEOA as base. The RT of these acids was 3.35, 5.12 and 3.88 minutes respectively. No considerable differences were seen with Na_2CO_3 for acetic- and propanoic acid. However, it was evident that citric acid was only detectable when TEOA is used, and not Na_2CO_3. Later on, three new metabolites were included namely arachidonic-, dodecanoic- and fumaric acid. These were also detected using TEOA as base and the observed RT was 6.72, 6.32 and 4.43 minutes respectively.

Undetected metabolites:
Unfortunately, no usable results were produced for formic-, lactic-, oxalic- and uric acid. Detection of formic acid failed several times with both bases as high blanks were observed. Uric acid did not dissolve in DMSO/ DMF. When different solvents were used, it was observable that uric acid was soluble in KOH, however poorly soluble in water. Peaks were seen when using water as solvent; however, separation should be improved.

Optimization of LC-ESI-MS method:
The MRM method was optimized to solely include the precursor ion (m/z) of the detected metabolites as shown in Table 1. Using this optimized method, the RT was slighly different from previous measurements. Derivatization was done using DmPaBr and TEOA as base. The following MS parameters were used: product ion (m/z): 134 and 180, Dwell: 50ms, Fragmentor: 135V, Collision energy: 20 eV, positive polarity, Gas Temp: 300°C.

Method validation:
The R_2-value was calculated after the distribution of the calibration curves (Table 1). Propanoic acid had the highest R_2 of 0,9965, whereas acetic acid had the lowest i.e. 0,8824. The parameters repeatability (%RSD) and limits of detection (LOD) were also validated (n = 4). Repeatability ranged from 8% - 21%, with acetic acid having the lowest % and

fumaric the highest (Table 1). The LOD ranged from 2,0 – 6,4 nM, with arachidonic acid having the lowest LOD and fumaric acid the highest (Table 1

Table 1. Optimized MRM-method and validated parameters. The precursur ion (m/z) is the expected metabolite mass after being charged with ESI divided by the ion charge. The validated parameters linearity (R^2), repeatibility (%) and LOD (nM) are also shown.

Metabolite	Precursor ion (m/z)	Ret (min)	R^2	Repeatibility (%)	LOD (nM)
Acetic acid	222.26	3.47	0.882	8%	4.6
Arachidonic acid	466.68	7.35	0.988	16%	2.0
Citric acid	676.74	5.38	0.989	17%	3.3
Dodecanoic acid	362.53	6.77	0.985	16%	3.9
Fumaric acid	439.48	5.21	0.985	21%	6.4
Propanoic acid	236.28	4.08	0.997	10%	6.0

PINK1 mutant PD cell line:

The concentration of acetic acid in the three cell lines was not calculated due to a low R_2-value. Arachidonic acid was not present in the cell media; thus, the concentration of this metabolite was also not calculated. The concentration of citric-, fumaric- and propanoic acid appears to be elevated in the PINK1 mutant PD cell line compared to control groups (Figure 1). In contrast, the concentration of dodecanoic acid appears to be elevated the most in the control group, rather than the PINK1 mutated PD cell line.

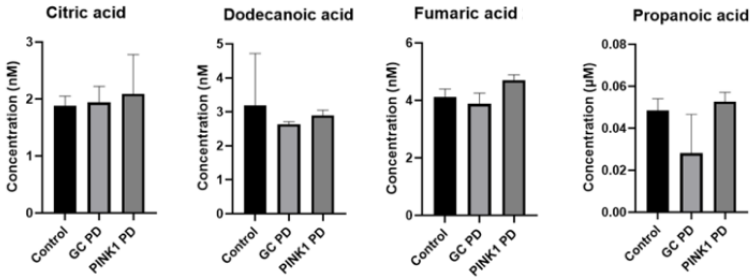

Figure 1. Elevated levels of citric- and propanoic acid in PINK1 mutant PD cell line. The average conc. of each metabolite (n = 3) is plotted on the y-axis. The three cell lines are depicted on the x-axis. Used error bars are an indication of the uncertainty within the used sample size. It is observable that the conc. of citric-, fumaric- and propanoic acid is the highest in the PINK1 mutant PD cell line compared to the healthy control group.

DISCUSSION:

Uric acid proved insoluble in DMSO/ DMF. When dissolved in water, peaks were seen with LC-MS, however separation still needs improvement. The used composition of the mobile phase could have resulted in a bad separation and peak resolution of uric acid. In order to obtain a better resolution, the number of theoretical plates should be increased by extending the length of the column or by using a column with a smaller particle size [13]. Future LC-MS measurements with uric acid should also be done preferably using solvents such as KOH [14]. Furthermore, it is possible that the undetected metabolites never hit the MS detector after being ionized [15]. Therefore, this could mean that the ion never passed through from the first quadrupole to the second, as only ions with the selected targeted mass are able to pass through when using a triple quadrupole MS [15]. To confirm this statement, a full scan with the MS or single ion monitoring (SIM) should be performed prior to using the MRM method.

With the optimized LC-ESI-MS method, six metabolites were detected using TEOA as base. Good linearity was seen as an R_2-value of > 0,98 was observed, except for acetic acid. However, measurements were not done in triplicates. Therefore, the number of samples per data point should be increased to at least n = 3 to gain a more accurate representation of the sample group and thus reliable validation. The repeatability was also validated and the limits of detection (LOD) was calculated for each metabolite (n = 4). The LOD is accepted when this is 15-20% below the lowest limits of quantitation (LLOQ) [16]. However, the LLOQ could not be calculated due to the used small sample size. Therefore no conclusion can be made regarding the LOD of the studied metabolites.

Lastly, the concentration of the detected metabolites was calculated in a healthy control, gene-corrected PD (PD without the PINK1 mutation) and PINK1 mutant PD cell line. It was observed that the concentration of both citric- and propanoic acid was elevated in the PINK1 mutated PD cell line compared to control groups. This was expected, as previous studies had already linked elevated levels of citric acid to the impairment of the mitochondrial oxidative metabolism [11]. In this case, it is possible that the increased concentration of propanoic acid may have led to elevated levels of citric acid as more of succinyl-CoA is produced. When more of succinyl-CoA is produced, enzymes responsible for the metabolization of citric acid are inhibited, leading to elevated levels of this metabolite [17]. The PINK1 mutation could have negatively affected enzymes of the TCA cycle [18], hereby also inhibiting metabolization of citric acid, resulting in elevated levels. As less of citric acid is metabolized, less of NADH and FADH2 is produced [19]. Depleted levels of NADH and FADH2 could lead to an impaired complex 1 activity of the electron chain transport in PD [3]. This can lead to or further advance mitochondrial dysfunction.

CONCLUSION:

Based on the current research done, it can be concluded that a LC-ESI-MS method was optimized for the detection of five metabolites namely arachidonic-, citric-, dodecanoic-, fumaric- and propanoic acid using DmPaBr as derivatization reagent and TEOA as base. Furthermore, it can be concluded that method validation is still incomplete

as a small sample size was used. Therefore, a larger sample size should be used and more parameters should be validated. This method should also be further expanded to include and detect more metabolites such as uric acid. These can then be studied to gain a better understanding of pathogenic mechanisms involved in disorders such as PD. By including more metabolites in the cell media, the potential role of these metabolites on the pathogenesis and progression of PD can be further studied in a broader context at cellular level. Based on the current results from the cell media it can be said that a mutation or loss of PINK1 leads to elevated concentrations of both citric- and propanoic acid. This enables future possibilities for continuing the investigation of a potential correlation between these two acids and the PINK1 mutation.

ROLE OF THE STUDENT:

Rowan Karg is an undergraduate student whom conducted his bachelor research project at the Leiden Academic Centre for Drug Research at the division Systems Biomedicine & Pharmacology. The topic of this paper is part of the current research field of supervisors Dr. Edinson Lucumi Moreno and Cornelius Willacey. The student Rowan Karg worked in a group with five others whom also used LC-ESI-MS as their main technique. Research on the various organic acid metabolites and the writing of this paper and all of its contents were done by the student alone.

REFERENCES:

1. Shao, Y., & Le, W. (2019). Recent advances and perspectives of metabolomics-based investigations in Parkinson's disease. Molecular Neurodegeneration, 14(1).
2. Tysnes, O., & Storstein, A. (2017). Epidemiology of Parkinson's disease. Journal of Neural Transmission, 124(8), 901-905.
3. Thomas, K., & Cookson, M. (2009). The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics. The International Journal of Biochemistry & Cell Biology, 41(10), 2025-2035.
4. Hughes AJ, e. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinicopathological study of 100 cases.
5. Burté, F., Houghton, D., Lowes, H., Pyle, A., Nesbitt, S., & Yarnall, A. et al. (2017). Metabolic profiling of Parkinson's disease and mild cognitive impairment. Movement Disorders, 32(6), 927-932.
6. Xiao, J., Zhou, B., & Ressom, H. (2012). Metabolite identification and quantitation in LC-MS/MSbased metabolomics. Trac Trends in Analytical Chemistry, 32, 1-14.
7. C.C.W. Willacey, M. Naaktgeboren, E. Lucumi Moreno, D. van der Es, N. Karu, R.M.T. Fleming, A.C. Harms, T. Hankemeier. (2019). LC-MS/MS analysis of the central energy and carbon metabolites in biological samples following derivatization by dimethylaminophenacyl bromide, Journal of Chromatography.
8. Tucker, I., & Tucker, I. (2018). The Clinical Significance of Organic Acids Testing to Mental Health – How Fungal, Bacterial, Mitochondrial, and Other Test Markers Influence the Brain. The Great Plains Laboratory, Inc.
9. Yu, Z., Zhang, S., Wang, D., Fan, M., Gao, F., & Sun, W. et al. (2017). The significance of uric acid in the diagnosis and treatment of Parkinson disease. Medicine, 96(45), e8502.
10. Novel Fumarate Esters as Neuroprotective Agents in Parkinson's Disease. (2019). The Michael J. Fox Foundation for Parkinson's Research | Parkinson's Disease.
11. Melo, D., Kowaltowski, A., Wajner, M., & Castilho, R. (2011). Mitochondrial energy metabolism in neurodegeneration associated with methylmalonic acidemia. Journal of Bioenergetics and Biomembranes, 43(1), 39-46.
12. Mendhekar DN, e. (2005). Parkinsonism and elevated lactic acid with sertraline.
13. Samanidou, V. (2015). Basic LC Method Development and Optimization. Analytical Separation Science, 25-42.
14. Xinhua, D. (2006). Preparation of Uric Acid Standard Stock Solution. Clinical Chemistry, 52(11), 2117-2118.
15. Dass, C. (2006). Tandem Mass Spectrometry. Fundamentals of Contemporary Mass Spectrometry, 119-150.
16. Taverniers, I., De Loose, M., & Van Bockstaele, E. (2004). Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trac Trends in Analytical Chemistry, 23(8), 535-552.
17. Frye, R., Rose, S., Slattery, J., & MacFabe, D. (2015). Gastrointestinal dysfunction in autism spectrum disorder: the role of the mitochondria and the enteric microbiome. Microbial Ecology in Health & Disease, 26(0).
18. Heeman, B., Van den Haute, C., Aelvoet, S., Valsecchi, F., Rodenburg, R., & Reumers, V. et al. (2011). Depletion of PINK1 affects mitochondrial metabolism, calcium homeostasis and energy maintenance. Journal of Cell Science, 124(7), 1115-1125.
19. Akram, M. (2013). Citric Acid Cycle and Role of its Intermediates in Metabolism. Cell Biochemistry and Biophysics, 68(3), 475-478.