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Matrix interference in LC-ESI-MS/MS analysis of metanephrines in protein precipitated plasma samples

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Abstract

Metanephrine and normetanephrine are measured in blood plasma to diagnose different diseases. Simpler sample preparation procedures are preferred but tend to yield less purified extracts. Therefore, thorough investigation of matrix effects is required. In this work, several sample preparation methods and chromatographic modes were compared for liquid chromatography tandem mass spectrometric (with electrospray ionization; LC-ESI-MS/MS) analysis of metanephrine and normetanephrine in blood plasma. Protein precipitation with methanol was found to be sufficient for sample preparation and pentafluorophenyl column provided adequate chromatographic separation. A new cheaper and less labor-intensive approach is proposed where necessary quantitation limits are achieved through a sample preparation containing only protein precipitation and dilution of the sample extract. Matrix effects for different sample preparation methods and the use of isotope-labeled internal standards were evaluated. Unusual interference to D_3-labeled internal standard of normetanephrine was discovered – signal of interfering compound increased while the matrix effects were reduced by dilution, e.g. dilution eliminates matrix suppression on interfering compound. The results stress the need to monitor interfering compounds and evaluate matrix effects at every step of method development. Matrix effects and interferences can be different for analytes and their corresponding isotopically labeled internal standards. This means that the use of isotopically labeled internal standards cannot guarantee accuracy of obtained results. New method allows quantification of the low nanomolar concentrations of metanephrine and normetanephrine in plasma samples.

Keywords

Metanephrines, matrix effects, liquid chromatography, mass spectrometry, isotopically labeled internal standard

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Introduction

Metanephrine (MN) and normetanephrine (NMN) are measured in blood plasma for diagnosis of pheochromocytoma. Due to their low concentrations in samples, sensitive and specific methods are needed.[1–3] Search for an optimal method has been ongoing for past ten years. Due to the difficult blood plasma matrix and small relatively polar analytes, both sample preparation as well as chromatographic separation need to be optimized accordingly. Sample preparation is needed to isolate analytes from the interfering matrix components and sometimes also for preconcentration. For MN and NMN analysis in plasma the most commonly used sample preparation method has been some type of solid phase extraction using either HLB[1,4] or weak cation exchange cartridges.[1–3,5] On one occasion, only a protein precipitation with isopropanol has been used without any other extraction techniques.[1]

Due to polarity MN and NMN are difficult to retain and separate on C18 stationary phase. In order to reduce matrix effects due to early eluting matrix components other chromatographic separation modes have been used, mostly HILIC,[1,3,5] but also cyano[4] and pentafluorophenyl[6] columns. To overcome the issue of chromatographic separation, derivatization with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-Cl)[7] and phenylisothiocyanate[8] have been used in order to increase the retention in reversed phase chromatography. Moreover, ion chromatography has been successfully applied.[9] Most commonly mass spectrometric detection is used with electrospray ionization

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(ESI),[2,3,5,6,9] but also applications utilizing atmospheric pressure chemical ionization (APCI)[4,10] have been demonstrated.

Limit of quantification for analyzing MN and NMN in plasma samples vary depending on the method, being the lowest for the most recently developed methods and in the range of 30 pmol L^{-1} for MN and 80 pmol L^{-1} for NMN[2,11] while most of the methods target limits of quantification around 100 pmol L^{-1} for either of the compounds.[1,3,4] Most methods targeting low detection limits use solid phase extraction with weak cation exchange and then the choice of chromatographic columns vary (including HILIC[11] and pentafluorophenyl columns[2]).

When developing methods for LC-MS/MS, matrix effects (ME) can influence the analysis. When there is no analyte-free matrix (blank) available as is the case for plasma samples, ME cannot be evaluated using the post-extraction addition[12] or the post-column infusion experiments[13] and the sample dilution approach should be used for estimating the presence of ME instead.[14] A logarithmic relationship has been shown between ME and matrix concentration,[15] meaning that if the analyte signal is affected by ME, it could be reduced or eliminated with sample dilution. ME has been studied in case of using solid phase extraction for sample clean-up and consist of monitoring the signal of internal standards during the infusion studies and it was concluded that there were no significant ME.[3] To the best of our knowledge, there has not been any investigations of matrix effects in case of protein precipitation methods as there are only few such methods available.

Almost all LC-MS methods that are used for analysis of MN and NMN use isotopically labeled internal standards (ILIS) in order to take into account any analyte losses during sample preparation or ME. In different publications, isotope-labeled standards (deuterated) from different vendors with different labelling have been used, for example labelling in positions (α-D_2, β-D_1) have been used,[2,4,11] but also (α-D_1, β-D_2)[3] and (α-D_2, β-D_2).[5] In case of Fmoc-Cl derivatization, 3,4-dihydroxybenzylamine has been used as an internal standard.[7] In these publications, there is no further discussion about the use of internal standards, their fragmentation profiles or of the matrix influence on their signal.

Until now, most developed applications are labor intensive, expensive and therefore a new method that is optimal, fast, cost-effective and with low matrix effects, is still needed. In present work sample preparation methods with and without solid phase extraction (SPE) and derivatization and different chromatographic modes are tested to minimize matrix effects of the analysis.

Materials and methods

Chemicals, reagents and materials

Chromatographic solvents were of HPLC grade: acetonitrile and methanol purchased from Sigma-Aldrich and 2-propanol from Merck. Metanephrine hydrochloride was purchased from Toronto Research Chemicals Inc (Canada), DL-normetanephrine hydrochloride from Sigma. Deuterium-labeled standards were purchased as solutions from Cerilliant (USA): (±)-Metanephrine-D_3 hydrochloride (100 µg mL^{-1} in methanol, $C_{10}H_{12}D_3NO_3{\cdot}HCl$, $M = 236.72\,g\,mol^{-1}$, CAS 1215507-88-2) and (±)-Normetanephrine-D_3 hydrochloride solution 100 µg mL^{-1} in methanol ($C_9H_{10}D_3NO_3{\cdot}HCl$, $M = 222.68\,g\,mol^{-1}$, CAS 1085333-97-6).

Derivatization reagent DEEMM was purchased from Fluka. Sodium hydroxide was purchased from Chemapol; ammonium hydroxide, boric acid and hydrochloric acid were from Reakhim. Derivatization reagent 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF) was synthesized in-house as in Rebane et al.[16] and also dibenzyl ethoxymethylene malonate (DBEMM) was synthesized as in Rebane et al.[17]

Aqueous solutions were prepared using ultrapure water purified by Millipore Milli-Q Advantage A10 (Millipore). All reagents were of analytical grade if not otherwise stated. Solid phase extraction cartridges HLB (Oasis HLB, 30 mg, 1 mL, Waters) and DSC-WCX (Discovery, 3 mL, Supelco). Syringe filters (regenerated cellulose, pore size 0.2 µm, Minisart RC 4, Sartorius) were used.

Stock solutions, internal standard mixture, and calibration standards

Individual stock solutions of each standard were prepared in methanol (approximately 2 mg mL^{-1}). These were used to prepare a mixed stock solution of metanephrine and normetanephrine (approximately 20 ng mL^{-1}) in methanol. Working standard solutions were prepared by dilution with ultrapure water (MilliQ) in the concentration range of 1 to 2000 pg mL^{-1} and this was also the linear range of the method.

Sample preparation

Used samples were a pooled human plasma bought from the Tartu University Hospital Bloodbank. Multiple of pooled samples were used over time. To each sample 5 µL of internal standard was added (100 ng mL^{-1}).

Derivatization procedures. Derivatization with diethyl ethoxymethylenemalonate (DEEMM) was used as previously published in Oldekop et al.,[18] with 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF) as in Rebane et al.[16] and with dibenzyl ethoxymethylene malonate (DBEMM) as in Rebane et al.[17]

Protein precipitation. To 500 µL plasma 500 µL of methanol was added and then vortexed for 5 min with Eppendorf MixMate and then centrifuged at

17,500 r/min (30,130 g) for 10 min (Eppendorf Centrifuge 5430R). Supernatant was filtered through the syringe filter and used as is for further analyses. For matrix effect investigations, dilutions with MilliQ water were done.

Solid phase extraction. (1) Oasis HLB cartridge was conditioned with 4 mL methanol and 4 mL MilliQ and 500 µL plasma was used. Then cartridge was washed with 4 mL MilliQ and eluted with 4 mL methanol. Extract was dried under nitrogen flow and then redissolved in 1 mL MilliQ. (2) WCX^3 cartridges were conditioned with 500 µL of methanol and 500 µL ultrapure water. Then 500 µL of plasma sample was added to the cartridge, followed by 500 µL of ultrapure water and 500 µL of methanol. For elution 500 µL of 0.1% formic acid in acetonitrile and 2 mL of 2% formic acid in acetonitrile was used. Extract was dried under nitrogen flow and then dissolved in 500 µL of ultrapure water for analysis.

Samples were filtered through syringe filters before analysis.

LC-MS/MS method

Agilent 1290 UHPLC with Agilent 6495 Triple Quad LC/MS equipped with Jet Stream ion source was used.

Columns used: HILIC Phenomenex Kinetex (150 mm × 4.6 mm, particle size of 2.6 µm) with mobile phase components A (0.1% formic acid in water) and B (100 mL of water in 2.5 L of acetonitrile), flow rate: 0.4 mL/min, gradient program: 0–15 min 100–40% B, injection volume 10 µL.

Reversed phase column for derivatized compounds: Phenomenex Kinetex C18 (50 mm × 2.1 mm, particle size of 1.7 µm), mobile phase component A (0.1% formic acid in water) and B (100 mL of water in 2.5 L of acetonitrile), flow rate: 0.4 mL/min, gradient program: 0–5 min 10–50% B, 5–6 min 50–100% B, injection volume 10 µL.

Final method column with pentafluorophenyl stationary phase: Pursuit 3 PFP 150 × 2.0 mm, particle size of 3 µm and guard column 10 × 2.0 mm (Agilent Technologies), mobile phase component A (0.1% formic acid in water) and B (methanol), flow rate: 0.3 mL/min, gradient program: 0–0.5 min 5% B, 0.5–1.5 min 5–60% B, 1.5–4 min 60% B, 4–5 min 60–95% B, injection volume 20 µL.

Derivatized and underivatized MN and NMN and respective internal standards were detected in positive ion mode mass spectrometry in multiple reaction monitoring (MRM) mode with transitions that are presented in Table 1.

The following ionization source parameters were used: nebulizer gas (nitrogen) pressure 40 psi (275.8 kPa), drying gas (nitrogen) flow rate 14 L min^{-1}, temperature 290°C; sheath gas (nitrogen) flow rate was 12 L/min and temperature 400°C. Capillary voltage was 3500 V, nozzle voltage 500 V. iFunnel parameters used were high pressure RF 150 V and low pressure RF 60 V. Cell accelerator voltage was 5 V and collision energy 10 V. Multiple reaction monitoring (MRM) mode was used for MS/MS analysis. The effluent of the analytical column was directed to waste by column switching valve for the first 2 min of the chromatographic run.

Method validation

Limit of detection (LoD) and limit of quantitation (LoQ) were estimated, respectively, as 3 and 10 times the standard deviation (n = 3 to 6) injecting the sample.[19] LoD and LoQ were evaluated at different days over two months. Matrix effects were evaluated using the sample dilution approach[14] with 2-, 5-, 7- and 10-fold dilutions. Additionally, ILIS were used as well as spiking studies and comparison of signal intensities of standard solutions and spiked samples. Recoveries of the solid phase extractions were investigated using spiking at limit of quantitation concentrations as well as ILIS.

Before the ME investigation, it was confirmed that the linear range of the method was 1 to 2000 pg mL^{-1} and repeatability RSD was 5% to 27% for NMNef and

Table 1. Retention times and MRM transitions for DEEMM derivatized and underivatized normetanephrine, metanephrine, and corresponding internal standards.

	DEEMM derivatives			Without derivatization		
Compound	Ret. time (min)	Precursor, *m/z*	Product, *m/z*	Ret. time (min)	Precursor, *m/z*	Product, *m/z*
Metanephrine	2.88	368	322	2.84	180	165
					180	148
Metanephrine-D_3	2.88	371	325	2.84	183	151
Normetanephrine	2.74	354	308	2.20	166	106
					166	134
Normetanephrine-D_3	2.74	357	311	2.20	169	137

Positive ion mode was used.

1% to 32% for MNef with lower RSD values for MeOH and MeCN precipitation and larger for isopropanol precipitation. Intermediate precision was 33% to 76% for NMNef and 36% to 42% for MNef and similarly, larger values were obtained with isopropanol precipitation (explained in the results part of the manuscript).

Results and discussion

Method development from the ME point of view

The aim of the method development was to develop a fast and an efficient method. Due to the complex matrix of plasma, the main purpose of the method development was to find a method with the lowest matrix effects in order to obtain more accurate method. Due to the relatively high polarity of MN and NMN, derivatization was tested as a first approach in order to increase the retention on the C18 reversed phase column. Since derivatization has shown promising results for MN and NMN analysis, diethyl ethoxymethylenemalonate (DEEMM) was tested for the applications since it is known to be less influenced by matrix effects in comparison to previously applied Fmoc-Cl.[18] However, derivatization was not suitable due to complex matrix of plasma.

It has been shown that specialized derivatization reagents for LC-ESI-MS can provide lower detection limits and therefore two in-house developed derivatization reagents were tested for suitability. Although, in case of DBEMM,[17] longer retention times than for DEEMM derivatives were achieved, neither better chromatographic separation nor sensitivity improvement were observed. Additionally, strong matrix effects were observed for DBEMM. In case of FOSF,[16] it was observed that derivatization in a complex matrix of plasma for MN and NMN was problematic. Therefore, method development with derivatization was discarded due to the complex matrix.

Since the second option was analysis of the underivatized compounds, conventional C18 column had to be changed to a column suitable for more polar compounds since even though it was suitable for standard solutions, for samples strong matrix effects occurred. As one of the most popular chromatographic modes used in the literature was HILIC,[1,3,5] this was tested as the first alternative. However, results showed that the sensitivity with HILIC column was not as good as with reversed phase column. Moreover, similar or even worse matrix effects were observed from the preliminary experiments and therefore HILIC column was discarded from the method development.

Finally, a PFP column was chosen, which has also been used in previous works[2,4] and provides more similar sensitivities in comparison to C18 column. Additionally, longer retention times provided better separation from the interfering compounds and therefore less matrix effects were present.

In conclusion, since in positive mode, NMN (M = 183) and MN (M = 197) are protonated to produce molecular ions of m/z 184 and 198, respectively. The spontaneous loss of water from the protonated molecular ions during ionization gives fragments with m/z, 166 and 180, respectively.[4] Transitions used for MN analysis were 180 → 165, 180 → 148 and 180 → 120. However, for quantification 180 → 165 was chosen since in case of protein precipitation sample preparation it had the highest peak intensity, 180 → 120 transition was not sensitive enough and 180 → 148 was chosen as qualifier. As for NMN, 166 → 106 and 166 → 134 were tested but 166 → 106 provided better sensitivity and chosen as quantifier and the second transition was qualifier. Observed transitions are the same as in most works published earlier, but the choice for the qualifier depends on the publication.[2,3,5,11] Chromatograms of standard and sample solutions are presented in Figure 1.

ME in sample preparation

For sample preparation, two approaches were tested, protein precipitation and SPE. For SPE, HLB[1,4] and WCX[2,3,5] phases were tested. Preliminary testing showed that extraction with WCX had poor recoveries as previously observed by Marney et al.[1] and therefore for SPE, HLB was used. However, it was observed that after the sample preparation and drying under the nitrogen, extract was not clean and the resulting slimy residue did not allow concentrating and was just dissolved in 500 µL ultrapure water (hence, exact concentration remained unknown). Even though exactly the same SPE cartridges and conditions were used, these issues have not been mentioned by the previous authors and remains unclear how this issue was addressed.[1,4] However, HLB method had a good process efficiency (combination of recovery and matrix effect) of 108% for NMN and 110% for MN in case of samples without using internal standards. Repeated analyses of samples ($n = 5$) were carried out using solid phase extraction and analyte concentrations in the sample with and without the isotope-labeled standard correction were calculated and compared. The results obtained were virtually identical. Meaning that when using solid phase extraction, it is not necessary to use internal standards as there were no recovery nor matrix effect issues present. Concluding, since the sample extract needed dilution for analysis, the concentrating effect was lost and therefore it was decided to test a method with just protein precipitation since solid phase extraction is time-consuming and also has a higher cost than protein precipitation.

Therefore, the main focus of the work was on the protein precipitation sample preparation and the matrix influence on the analysis. Solvents tested for precipitation were methanol, acetonitrile and also isopropanol, the latter has been suggested by Marney et al.[1] The aim was to find the optimal method and

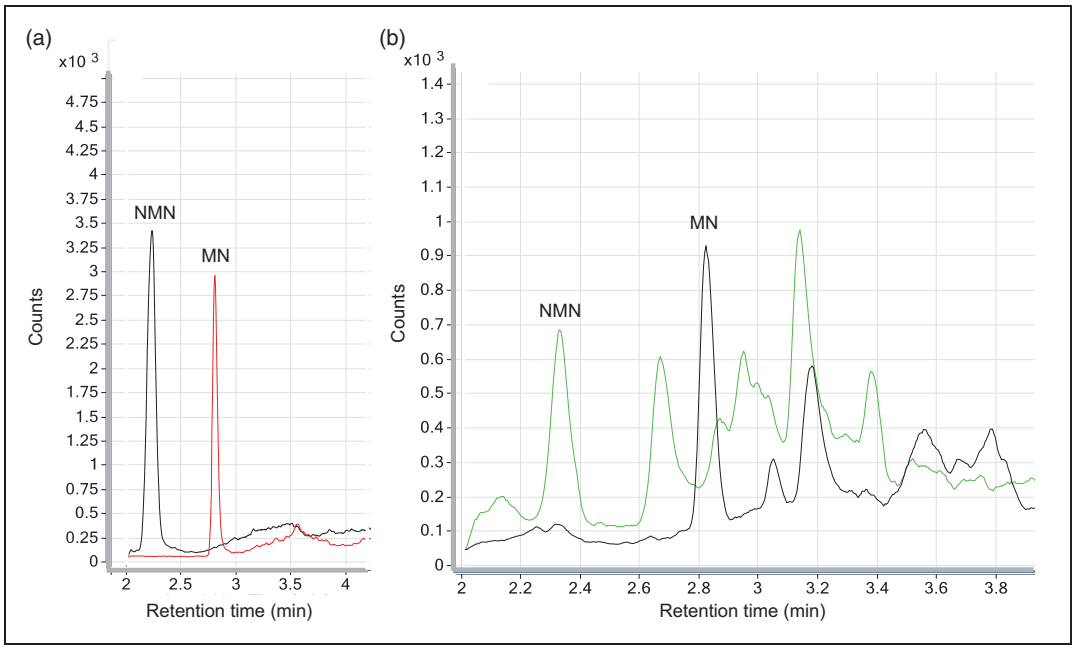

Figure 1. Extracted ion chromatograms of NMN and MN in (a) standard solution of 100 pg mL^{-1} and (b) in protein precipitated (with methanol; no dilution) sample extract of.

therefore different conditions for precipitation were tested as well as different dilution options since it has been shown that dilution can reduce the matrix effects.[14] The sample preparation method development was evaluated through isotope-labeled standards, dilutions and limit of quantifications.

The use of ILIS

For MN and NMN analysis, isotope-labeled standards are commonly used in order to take into account the possible losses during the sample preparation as well as matrix effects in the ESI source. Since protein precipitation yields relatively "dirty" sample extract, ILIS were used to evaluate the matrix effect with dilution of sample.[14]

It has been shown that with sample dilutions, the matrix effect on the analyte signal can be lowered or even eliminated. As blood plasma is even after protein precipitation a complex matrix and MN and NMN elute early on the chromatogram, it was expected that the dilution of the extracts would improve chromatographic peak shape, hence the signal intensity, as well as reduce matrix effects. In the beginning of the validation, absolute signals of MN, NMN and their ILIS were looked at separately without correcting results with the signal of internal standard. Signal intensities of the MN, NMN and MN-D_3 did increase with sample dilution showing the positive effect of dilution.

Rather extraordinary situation was observed for the NMN-D_3 when different dilutions were analyzed. Firstly, when an undiluted sample extract was analyzed, a chromatographic peak corresponding to MRM transition of NMN-D_3 was observed at the same retention time as NMN standard (2.2 min) (Figure 2(a)) as would be expected. However, once the sample was two-fold diluted, an extra peak appeared in the chromatogram at 2.07 min. When further diluted, five-fold, a larger interfering peak appeared and made it impossible to identify or integrate the NMN-D_3 peak. This situation was repeatable (Figure 2(b)) for undiluted and two-fold dilutions but it can be seen that for the largest five-fold dilution due to the strong matrix effects, a variation occurs but is still following the same trend. It was also present for all protein precipitated samples and did not depend on the solvent used for protein precipitation. In addition, modifications in eluent compositions nor gradient did not eliminate the situation.

Nevertheless, the previous situation was not observed in case of SPE sample preparation where chromatograms of undiluted and diluted samples were the same. This effect on the NMN-D_3 signal caused by the dilution was probably due to the fact that an interfering compound's signal is suppressed by the matrix components and when matrix is diluted, matrix effect is reduced, and the interfering peak appears. But with SPE sample preparation the compound causing this effect is removed with SPE and therefore the situation is not present.

To conclude, there is an unknown compound X, which has the same transition (169 → 137) as NMN-D_3 and elutes close to it. Ionization of X is strongly suppressed by the matrix components. Upon sample dilution ionization suppression of X is reduced and X becomes an interference to determination of NMN-D_3. To the best of our knowledge, there has been no report of this type of problem with NMN-D_3 internal standard or with any other analysis for that matter. This might be due to the fact that in most articles, sample

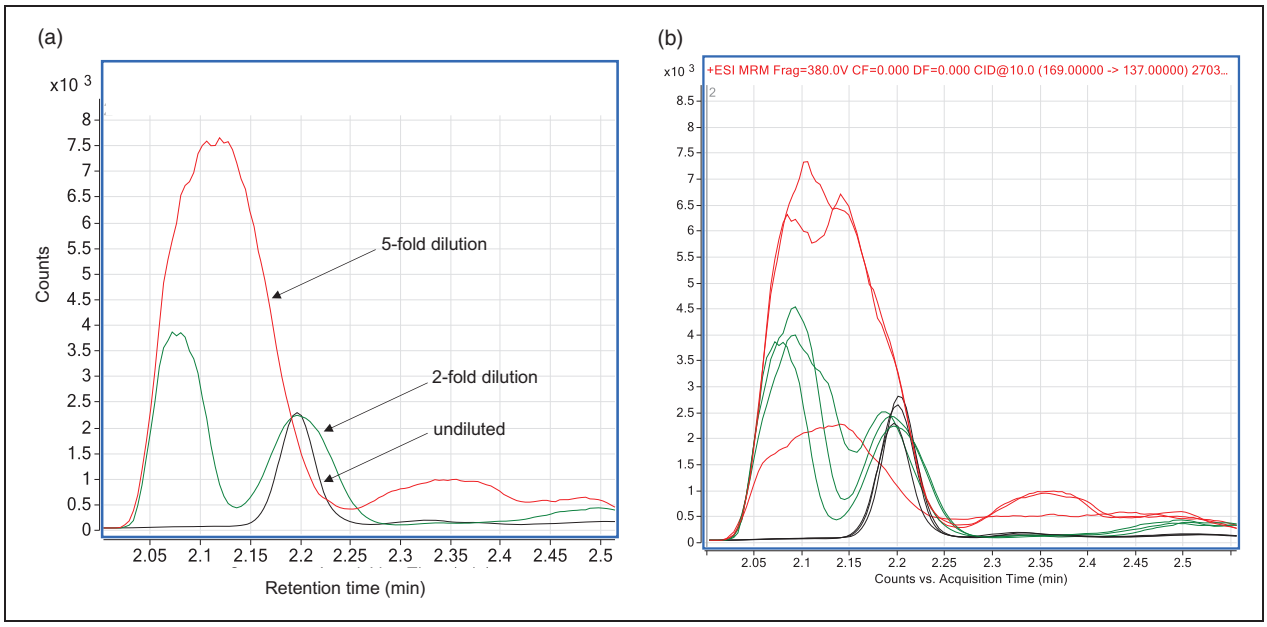

Figure 2. Normetanephrine-D_3 ($C = 500\,pg\,mL^{-1}$) extracted ion chromatograms (169 → 137) of protein precipitated (with acetonitrile) sample extracts analyzed undiluted and with dilutions (a) and (b) shows the triplicates of the same analysis.

extracts are concentrated rather than diluted. Often isotope-labeled standards are considered "magic cure-all" against matrix effects when using mass spectrometric detection. However, these findings indicate, that there are situations when ILIS might not behave the same way as the analyte, having a different interfering matrix compounds.

In conclusion, in order to evaluate the method performance with dilutions, results without internal standard correction were used and additionally, since for MN-D_3 this was not a problem, comparison with the internal standard corrected results was also possible. Moreover, results obtained with solid phase extraction were used for comparison.

Protein precipitation and matrix effects

For protein precipitation, three types of solvents were used, methanol, acetonitrile and isopropanol.[1] In addition to different solvents, different dilutions were tested: 2-, 5-, 7- and 10-fold dilution of the extract with MilliQ water. Results showed that all dilutions provided analyte signals and were therefore useful for quantification. All experiments were done with real plasma samples and therefore results discussed are for pooled samples done in triplicate each time and repeated on different days. Therefore, absolute values vary slightly between days due to different samples, but observed trends were the same. Where available, two analyte concentrations are obtained for each experiment, one with isotope standard correction and the other one without. Table 2 presents MN and NMN concentrations calculated with different dilutions, as well as an average for the ILIS corrected result.

Overall, it can be seen that a sample without dilution has too much of an interference and cannot be integrated for NMN in case of methanol. Results for isopropanol show poor reproducibility as well as stronger matrix effects without using ILIS correction and is therefore not suitable for protein precipitation. It can be noted that it was also visually observed in case of isopropanol that the extracts were cloudier in comparison to extracts that were precipitated with methanol or acetonitrile. In most cases, clogging of the syringe filter occurred.

For NMN, where ILIS was not applicable for dilution, results are compared (Table 2) to the ILIS corrected results obtained for undiluted sample ($0.1\,nmol\,L^{-1}$), as well as to the result obtained for SPE extract ($0.08\,nmol\,L^{-1}$). And it can be seen that in this case without dilution matrix effects are present but after dilutions, methanol precipitation provides the results closest to SPE (Table 2).

For MN results obtained with and without ILIS and SPE purification were compared (Table 2). It was found that protein precipitation with following dilution is just as effective for removing matrix effects as SPE. However, when higher dilutions are used, the MN concentrations calculated without ILIS correction tend to be higher than with ILIS correction. The same tendency was observed for multiple samples over few months. For example, similar MN concentrations were found in five-fold diluted extracts with and without using ILIS (Table 2). In case of 7- and 10-fold dilutions, elevated concentrations were obtained without ILIS. This indicates enhancement of MN and MN-D_3 signals in presence of sample matrix. The influence is more pronounced with acetonitrile precipitation, as in case of methanol and isopropanol results are more similar to ILIS corrected results.

Although, ILIS usually provides adequate correction for matrix effects its chromatographic peak must be

Table 2. Concentrations obtained ($n = 3$) with different protein precipitation solvents with different dilution factors.

	N-fold dilution	NMN (nmol L^{-1})	MN (nmol L^{-1})	MN (nmol L^{-1}) ILIS corrected
SPE (HLB)		0.08	0.20	–
Protein precipitation with methanol	–	NA	0.23	0.20
	2	0.09	0.24	0.21
	5	0.11	0.23	0.20
	7	0.09	0.24	0.19
	10	0.11	0.28	0.20
Protein precipitation with acetonitrile	–	0.06[a]	0.28	0.21
	2	0.14	0.16	0.23
	5	0.15	0.39	0.28
	7	0.11	0.36	0.26
	10	0.09	0.40	0.26
Protein precipitation with isopropanol	–	0.18	0.18	0.24
	2	0.31	0.16	0.21
	5	0.04	0.06	0.20
	7	0.11	0.20	0.24
	10	0.11	0.23	0.23

NA indicates interfering peaks.
MN: metanephrine; NMN: normetanephrine; ILIS: isotopically labeled internal standards; SPE: solid phase extraction.
[a]ILIS corrected result 0.10 nmol L^{-1}.

checked for interferences (in addition to analyte peak). Instead of ILIS correction sample dilution may be used to minimize matrix effects. Based on the tendencies observed in Table 2, a five-fold dilution would be enough to remove the matrix effects and obtain the accurate concentration without use of internal standards. However, to account for sample to sample variability, a 10-fold dilution is advised.

Limit of quantification

Limit of quantification (LoQ) was calculated for blood plasma samples for three batches over a month period and analysis was carried out in triplicate. Calculation of LoQ was 10 times the standard deviation of the concentrations in sample.[19] Since the absolute standard deviation is larger for analytes with higher concentration, a higher LoQ is expected and it can be seen from Table 3, that the LoQ values are higher for MN which has a higher concentration in the sample. Moreover, results were not calculated for isopropanol precipitation due to the poor reproducibility. It can be seen that LoQ values are quite similar with different dilution factors only being somewhat larger for higher dilutions. This is most likely related to the fact that for diluted samples, peak areas are smaller, and this influences the repeatability. But in comparison to SPE, results are very similar. And as expected, LoQ values with ILIS correction are somewhat smaller due to the fact, that in case of ILIS systematic deviances of signals cancel out.

Table 3. LoQ values for different sample preparation methods and dilution factors ($n = 3$).

Sample preparation	*N*-fold dilution	NMN (nmol L^{-1})	MN (nmol L^{-1})
SPE (HLB)	–	0.19	0.66
Protein precipitation with methanol	–	–	0.28
	2	0.11	0.13
	5	0.14	0.71
	7	0.17	0.58
	10	0.35	0.35
Protein precipitation with acetonitrile	–	0.07	0.13
	2	0.16	0.24
	5	0.14	0.61
	7	0.31	0.48
	10	0.29	0.68
Protein precipitation with ILIS	–	0.13	0.13

MN: metanephrine; NMN: normetanephrine; ILIS: isotopically labeled internal standards; SPE: solid phase extraction.

Method throughput

The throughput of different methods has been compared by Zheng et al.[8] where it was shown that method with derivatization had five steps and required 3.5 h per 96 samples, weak cation exchange SPE 4 steps and 1 h per 96 samples and ion pairing SPE 7 steps and

4 h per 96 samples. Method with just precipitation has five steps: (1) addition of precipitating solvent; (2) mixing; (3) centrifugation; (4) solvent evaporation; (5) reconstitution, out of which only two need human interaction. Compared to methods with derivatization and solid phase extraction, the price per sample preparation is significantly lower since the only thing needed is solvent for precipitation and there is no need for derivatization reagents or solid phase extraction cartridges.

Additionally, since the method applies dilution rather than concentrating of the samples, it allows to reduce the sample amount needed for sample analysis.

Conclusions

LC-ESI-MS/MS method was developed for analyzing MN and NMN in blood plasma. After a sample preparation containing only protein precipitation analysis of underivatized MN and NMN was carried out on pentafluorophenyl (PFP) stationary phase. Matrix effects were investigated with sample dilutions, as well as using ILIS. It was shown that extracts obtained with SPE (HLB) exhibit less matrix effects; however, dilution of the protein precipitated extract provided similar results for MN and NMN.

Interesting phenomenon was observed for NMN-D_3 where interfering peak appeared after sample dilution. This unusual appearance of interference rendered signal of NMN-D_3 useless for quantification. To the best of our knowledge it has not been demonstrated earlier, that dilution might eliminate matrix suppression on interfering compound. Nevertheless, the method provided adequate results for NMN—even without using ILIS. This case clearly demonstrates that neither sample dilution nor ILIS can guarantee accurate measurement results. All the steps and possible effects must be carefully considered.

Matrix effect evaluation showed that it is possible to develop a method without solid phase extraction and the use of ILIS if appropriate sample dilution is used. Best results were observed for protein precipitation with methanol followed by (at least five-fold) dilution with water. This approach is less labor intensive, cheaper, less influenced by the matrix components and also provides comparable limit of quantitation to methods that apply solid phase extraction and internal standard correction.

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