
Mass Spectrometric Analysis of Non-canonical Cyclic Nucleotides

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Contents

1	Introduction	294
2	High Performance Liquid Chromatography-Coupled Mass Spectrometry (HPLC-MS/MS)	294
3	Quality Criteria in cNMP Identification by HPLC-MS/MS	296
3.1	Absolute Retention Time	296
3.2	Quantifier/Identifier Ratio	299
4	Minimizing Matrix Effects in HPLC-MS/MS	299
4.1	Appropriate Sample Preparation	300
4.2	Usage of an Appropriate Matrix for Calibration Standards	300
4.3	Inclusion of an Internal Standard	301
5	Examples of cNMP Analyses in Biological Matrices	303
5.1	Identification of Cyclic Nucleotides in Murine Tissues	303
5.2	Identification of Cyclic Nucleotides in Human Urine	304
6	Conclusions and Perspectives	304
	References	305

Abstract

Contemporary investigations regarding the (patho)physiological roles of the non-canonical cyclic nucleoside monophosphates (cNMP) cytidine 3',5'-cyclic monophosphate (cCMP) and uridine 3',5'-cyclic monophosphate (cUMP) have

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been hampered by the lack of highly specific and sensitive analytic methods for these analytes. In addition, the existence of 2',3'-cNMP besides 3',5'-cNMP has been described recently. HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is the method of choice for identification and quantification of low-molecular weight endogenous metabolites. In this chapter, recommendations for an HPLC-MS/MS method for 3',5'- and 2',3'-cNMP are summarized.

Keywords

3',5'-cNMPs • 2',3'-cNMPs • Liquid chromatography • Mass spectrometry

1 Introduction

The well-known cyclic nucleoside monophosphates cAMP and cGMP play key regulatory roles as signaling molecules in all kingdoms of life (Gancedo 2013; Schlossmann and Schinner 2012). In contrast, the non-canonical cyclic nucleotides cCMP and cUMP also occur in vivo (Bähre et al. 2015), but elucidation of their (patho)physiological roles is just at the beginning (Seifert et al. 2015). The availability of reliable detection methods for all cNMP is an absolute requirement for further research in this field. Numerous detection methods for cAMP and cGMP have been described (Berrera et al. 2008; Schmidt 2009) including high performance liquid chromatography (HPLC) with UV or fluorescence detection, immunoassays such as RIA or ELISA, and fluorescence resonance energy transfer (FRET) techniques (Kalia et al. 2013). HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is a powerful tool for the analysis of low-molecular weight signaling molecules (Roux et al. 2011). It is characterized by high sensitivity and selectivity and allows simultaneous identification and quantification of canonical as well as non-canonical cNMP. However, due to their low concentrations in biological fluids as well as rapid metabolism, analysis of cNMP is a highly challenging analytical task. Special care has to be taken regarding the initial sample preparation steps, the robustness of the HPLC method applied, and reliable MS/MS recordings. In this case, HPLC-MS/MS with inclusion of isotope-labeled internal standards and assessment of specific quantifier and qualifier mass transitions is advised. We here present two examples of cNMP analyses by HPLC-MS/MS in biological matrices, i.e., murine tissues and human urine.

2 High Performance Liquid Chromatography-Coupled Mass Spectrometry (HPLC-MS/MS)

First step of the analysis of a sample containing a mixture of small molecules (e.g., cNMP) is the liquid chromatographic separation step. For cyclic nucleotides separation is usually performed on a C18 HPLC column (Zhang et al. 2009; Beste et al. 2012; Jia et al. 2014; Bähre and Kaever 2014) or on porous graphitic carbon

(Martens-Lobenhoffer et al. 2010; Pabst et al. 2010) using reversed phase chromatography. Furthermore, Goutier et al. (2010) performed chromatographic separation of cAMP and ATP in hydrophilic interaction liquid chromatography (HILIC) mode. Since in HPLC molecules are separated due to their chemical structures, those molecules with a high structure similarity may show similar retentional behavior and may therefore coelute from the HPLC column. Whereas in UV detection coeluting molecules with similar absorbance maxima cannot be differentiated from each other, mass spectrometry enables the discrimination of even these molecules.

The mixture of molecules coeluting from the HPLC column is often ionized by electrospray ionization (ESI) in the ion source of the mass spectrometer. A high voltage is applied and induces droplets containing either positive or negative charged ions. Although cNMP were mainly detected in positive ionization mode, in some studies detection in negative ionization mode is reported (Zhang et al. 2009; van Damme et al. 2012). Due to heat-induced desolvation the droplets shrink until the repulsive force inside the droplets becomes too strong resulting in a Coulomb explosion and finally in gaseous ionized molecules. These molecules are accelerated towards the first quadrupole (Q1) of the mass spectrometer where they are separated according to their mass to charge ratio (m/z) and only those molecules with a preset m/z -value will be able to pass the quadrupole on a stable trajectory (Fig. 1). Ions with a differing m/z -value will be discharged at the rods of the quadrupole and will not pass the quadrupole. Normally we expect all ions to have different m/z -ratios and only the one ion we are interested in will pass Q1 resulting in an unequivocal signal in a chromatogram. However, it is possible that the m/z -ratio of at least one coeluting molecule is very similar to the desired analyte. Tandem mass spectrometry (MS/MS) enables the discrimination even between critical molecules by fragmentation. After passing the first quadrupole the so-called precursor ions enter the collision cell (q2) of the mass spectrometer where the fragmentation takes place. The precursor ion collides with an inert collision gas (usually nitrogen or argon) and will dissociate in analyte specific fragment ions. Cyclic nucleotides are usually fragmented by a cleavage of the



Fig. 1 Theoretical background of HPLC-coupled tandem mass spectrometry (HPLC-MS/MS). After chromatographic separation structurally similar molecules (“A,” “B,” and “C,” with “A” being the desired analyte) may coelute from the HPLC column and are ionized in the source of the mass spectrometer at the same time. These compounds enter the first quadrupole (Q1) where ions are selected regarding their m/z -values. And only those ions with a distinct m/z -value are able to pass this quadrupole. But some compounds (e.g., “A” and “B”) may have very similar m/z -values and therefore, cannot be discriminated. Both ions enter the collision cell (q2) and will be fragmented (“a₁–a₃” and “b₁–b₃”). To discriminate between molecule A and B a specific fragment of A (e.g., fragment a₁) is selected in the third quadrupole (Q3) and finally passes through to the detector. Other precursor or fragment ions can be selected by changing quadrupole parameters, which allows a simultaneous detection of numerous analytes in one analytical run

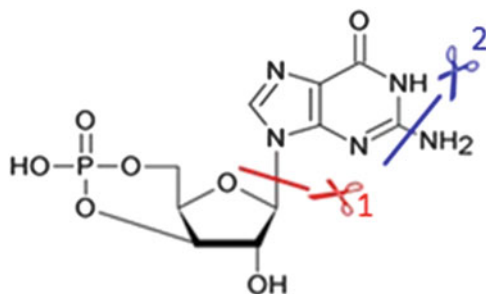


Fig. 2 Structure and fragmentation of cGMP. The dominant fragment m/z 152 results from the cleavage of the bond between the nucleobase and the ribose residue (cleavage 1) and is usually used as quantifier. A further loss of the ammonia group results in a second fragment (m/z 136; cleavage 2), which can function as identifier

glycosidic linkage of the nucleobase and the ribose residue resulting in the base as main fragment (Fig. 2). Additional fragments result, e.g., from further fragmentation of the base or are a result of its rearrangement (Bähre and Kaever 2014).

All created fragments are accelerated and reach the last quadrupole (Q3), where they are sorted again according to their mass to charge ratios. Only selected fragments are enabled to pass through to the detector. As a consequence, the resulting chromatogram only shows signals of the mass transitions of selected analytes. Matrix components or other small molecules with differing mass to charge ratios either of precursor and/or fragment ions are ignored. As a result, an MS/MS-based chromatogram shows a significantly reduced amount of signals in comparison to UV-based methods. This facilitates the unequivocal determination of cNMPs especially when cNMP analysis in biological matrices like tissues is required.

3 Quality Criteria in cNMP Identification by HPLC-MS/MS

Although HPLC-MS/MS is characterized by high sensitivity and, compared to HPLC-UV, high selectivity, analysis may be influenced by matrix components in various ways (Tylor 2005). If a matrix component has (nearly) the same mass transition like a desired cNMP, the resolution of the quadrupoles may not be high enough to discriminate between the matrix component and the cNMP of interest. This can lead to signals in the chromatogram that do not belong to the cNMP and therefore may falsify the result. To ensure a signal really reflects the analyte certain quality criteria should be met.

3.1 Absolute Retention Time

Coupling of tandem mass spectrometry to chromatography allows the discrimination of compounds not only based on their mass transitions but, moreover, based on

their retention times on an HPLC column. In reversed phase chromatography, 3',5'-cyclic nucleotides usually elute in the following order: cXMP, cCMP, cUMP, cIMP, cGMP, cTMP, and cAMP (Fig. 3), with definite retention times depending on the used chromatographic system (e.g., column material and length, flow rate, solvent system, etc.). By knowledge of the retention times of cNMP standards yet “unknown” signals can be assigned to a cNMP.

Not only matrix components but also cNMPs themselves can cause additional signals in a chromatogram. The m/z -values of the cNMP pairs cAMP/cIMP, cCMP/cUMP and cGMP/cXMP only differ in one Dalton (Table 1). As a consequence, the natural isotopes $[(M+1)+H]^+$ of the lighter cNMPs (cAMP, cUMP, and cGMP)

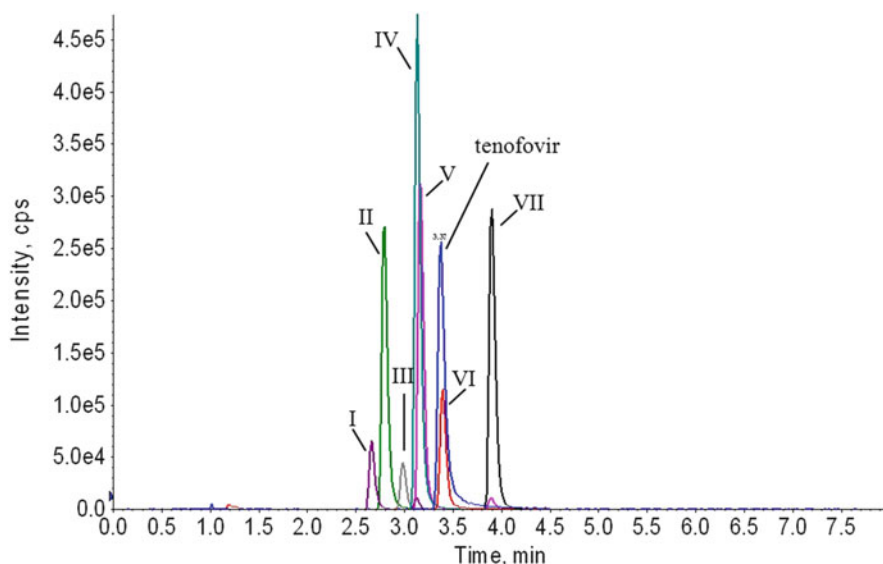


Fig. 3 Typical cNMP chromatogram achieved by reversed phase chromatography. Shown are the quantifier mass transitions of the seven cNMPs cXMP (I), cCMP (II), cUMP (III), cGMP (IV), cIMP (V), cTMP (VI), and cAMP (VII), and the signal of the internal standard tenofovir

Table 1 Masses of precursor ions and fragment ions of cyclic nucleotides found in tandem mass spectrometry using positive ionization mode

Cyclic nucleotide	Neutral mass (Da)	Precursor ion (m/z)	Fragment ion 1 (quantifier) (m/z)	Fragment ion 2 (identifier) (m/z)
cAMP	329	330	136	119
cCMP	305	306	112	95
cGMP	345	346	152	135
cIMP	330	331	137	110
cTMP	304	305	127	81
cUMP	305	306	97	113
cXMP	346	347	153	136

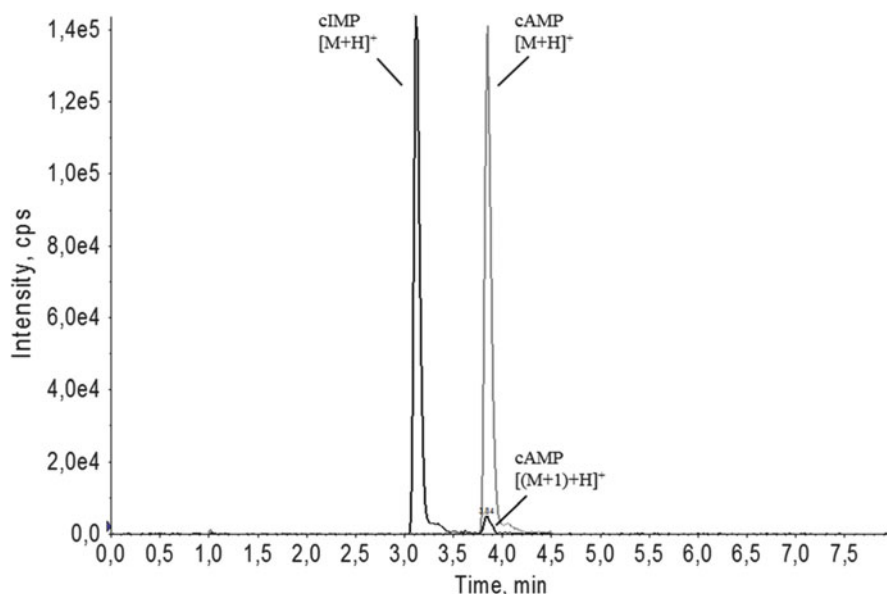


Fig. 4 Chromatogram of the monoisotopic ions of cIMP ($[M+H]^+$; m/z : 331 \rightarrow 137) and cAMP ($[M+H]^+$; m/z : 330 \rightarrow 136) and of the cAMP isotope ($[(M+1)+H]^+$; m/z : 337 \rightarrow 137). Thus, a baseline separation of cIMP and cAMP is essential to ensure that the $[(M+1)+H]^+$ signal of cAMP will not falsify the result for cIMP, especially for high cAMP and low cIMP concentration ranges

show mass transitions with the same m/z -values like the corresponding heavier monoisotopic cNMPs (cIMP, cCMP, and cXMP, respectively). This results in a chromatogram, in which the $[(M+1)+H]^+$ peak of, e.g., cAMP appears as (monoisotopic) cIMP (Fig. 4) at the retention time of cAMP. Without a baseline separation of the cNMP pairs named above and the knowledge of their absolute retention times on the HPLC column, the isotope peaks $[(M+1)+H]^+$ of cAMP, cUMP, and cGMP may falsify the results for the monoisotopic cyclic nucleotides cIMP, cCMP, and cXMP, respectively. This interference becomes most evident in case of high concentrations of the lighter cNMP (cAMP, cUMP, or cGMP) while only low concentration of the heavier cNMP is expected.

Furthermore, in cNMP analysis it has to be considered, that, in addition to the well-established 3',5'-cNMP, 2',3'-cyclic nucleotides do occur in biological samples (Ren et al. 2009; Jackson et al. 2009; van Damme et al. 2012; Bähre and Kaever 2014). Since these metabolites show a high structural similarity to the 3',5'-cNMP, their precursor and main fragment ions are identical (Fig. 5). Therefore a discrimination between 2',3'- and 3',5'-cyclic nucleotides only based on their mass transitions is not possible. As a consequence, a chromatographic baseline separation is an essential criterion for an unequivocal identification of both isobars.

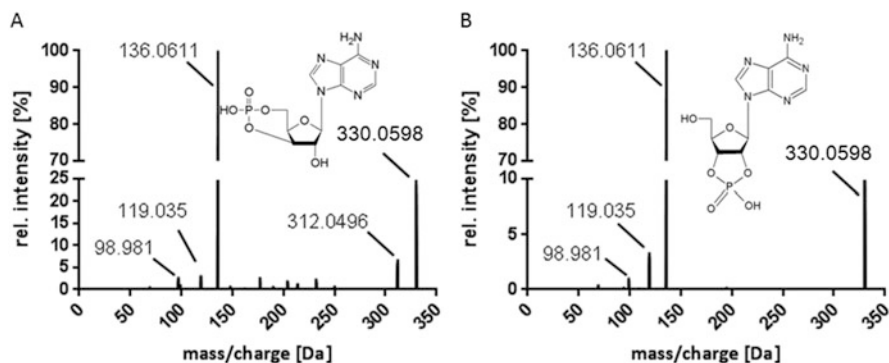


Fig. 5 Fragment spectra of 3',5'-cAMP (a) and 2',3'-cAMP (b)

3.2 Quantifier/Identifier Ratio

In the collision cell (q_2) usually more than one fragment ion of a precursor ion is generated and can be selected in the third quadrupole (Q_3). The main fragment shows highest intensity and is usually used for quantitation. It is therefore called “quantifier.” The detection of additional analyte specific fragments (they are called “identifier” or “qualifier”) significantly improves reliability of an analysis, since the ratio between quantifier and identifier transition is an indicator for proper identification and discrimination of an analyte. Only those signals with a specific quantifier/identifier ratio should be used for quantitation (Fig. 6). This procedure is limited by the intensity of the identifier, which is normally lower than the quantifier intensity. Thus, in low concentration ranges, the identifier signal might be too low or in some cases not even be visible for a ratio calculation.

4 Minimizing Matrix Effects in HPLC-MS/MS

In various samples matrix components do not only result in additional signals but may cause a shift in retention time or influence the ionization efficiency of an analyte. In most cases matrix components lead to a decrease of cNMP ionization efficiency and, as a consequence, to reduced signal intensities. This causes problems especially when only low cNMP concentrations are expected. Furthermore, matrix effects complicate an accurate cNMP quantitation, when there is a differing influence on calibration standards and samples. There are some strategies to minimize the influence of the matrix effects.

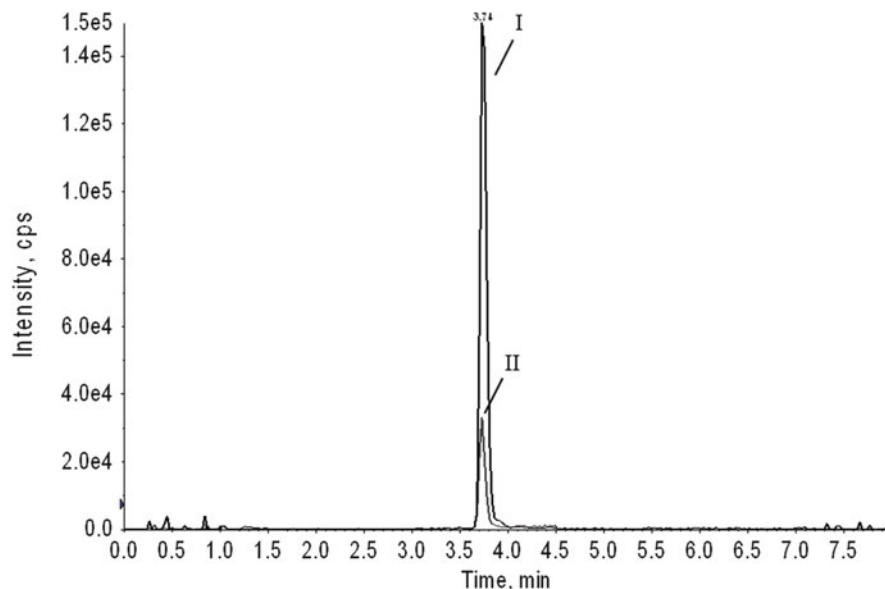


Fig. 6 MS/MS signal of two cAMP mass transitions. The higher signal (signal “I”) reflects the AMP quantifier (m/z 330 \rightarrow 136), the lower one (signal “II”) the cAMP identifier (m/z 330 \rightarrow 119). Since the ratio of both mass transitions is independent from sample matrix, their peak area ratio can be used as indicator for proper identification of cAMP

4.1 Appropriate Sample Preparation

The most efficient way to minimize matrix effects is the removal of interfering matrix components from the sample as complete as possible before HPLC-MS/MS analysis. In cNMP analysis usually an organic protein precipitation step is performed, which also stops cellular metabolism. Afterwards, the protein content in the sample is removed by centrifugation. To improve HPLC performance, the highly organic supernatant fluid should be evaporated to dryness and subsequently be dissolved in HPLC starting conditions (Zhang et al. 2009; Beste et al. 2012; Bähre and Kaever 2014; Jia et al. 2014).

Another sample preparation procedure using of weak anion exchange solid phase extraction (WAX) suitable to remove matrix components from plasma samples is described by Martens-Lobenhoffer et al. (2010).

4.2 Usage of an Appropriate Matrix for Calibration Standards

By preparing the calibration standards in the same matrix as the samples, the matrix influence on, e.g., retention times and ionization efficiency can be determined and corrected. Since cyclic nucleotides are endogenous metabolites, for most

applications it is not trivial to use exactly the same matrix for calibration standards. In those cases an artificial or surrogate matrix, e.g., bovine serum albumin (BSA), may help to compensate for matrix effects (van der Merbel 2008). For example, Oeckl and Ferger (2012) described the usage of artificial cerebrospinal fluid (aCSF) for the quantification cAMP and cGMP in cerebrospinal fluid (CSF).

Martens-Lobenhoffer et al. (2010) compared the slopes of cGMP calibration curves prepared in plasma versus water, finding a significant intercept for the plasma calibration curve (resulting from endogenous cGMP) but only small differences in the slopes of both curves. As a consequence, the quantification of cGMP even in plasma samples can be performed by an aqueous calibration curve, especially when using an isotope-labeled internal standard (see below). Actually, in most cases the calibration curve is prepared in water when quantifying cNMP in biological matrix (Ren et al. 2009; Zhang et al. 2009; Bähre and Kaever 2014; Dittmar et al. 2015).

4.3 Inclusion of an Internal Standard

An internal standard is strongly recommended in HPLC-MS/MS analytic. Its purpose is on the one hand to evaluate the influence of variations in the sample preparation, on the other hand the minimization of matrix effects. The chemical structure of the internal standard should be as similar as possible to the analyte structure to make sure that the retentional behavior and ionization efficiency are most similar to the analyte ones.

The internal standard plays an essential role in quantitative cNMP analysis. For constructing a calibration curve, the peak area ratio of the analyte and the internal standard is calculated and plotted against the analyte concentration. The peak area ratio from an unknown sample can then be related to the results of the calibration standards, and the cNMP amount in the sample can be determined. In this model the internal standard functions as a normalizing factor by compensating, e.g., losses (of analyte and internal standard) due to the sample preparation procedure and by compensating matrix effects as described above. In cNMP analytic various substances are applied as internal standard (Fig. 7):

- The substitution of the cNMP nucleobase with a bromine results in Br-cNMP derivatives, e.g., 8-Br-cAMP, with a similar chemical structure as the native cNMP. Those substances are commercially available, but in HPLC they show longer retention times than native cNMP (Jia et al. 2014)
- Tenofovir is an antiretroviral drug used in treatment of HIV/AIDS and chronic hepatitis B. It is a nucleotide analog, has a nucleobase (adenine) but lacks the ribose sugar. In reversed phase chromatography tenofovir shows similar retentional behavior to cTMP and cGMP and elutes at an intermediate retention time (Fig. 3) (Beste et al. 2012; Bähre and Kaever 2014). Therefore, tenofovir reflects matrix effects for most cyclic nucleotides.

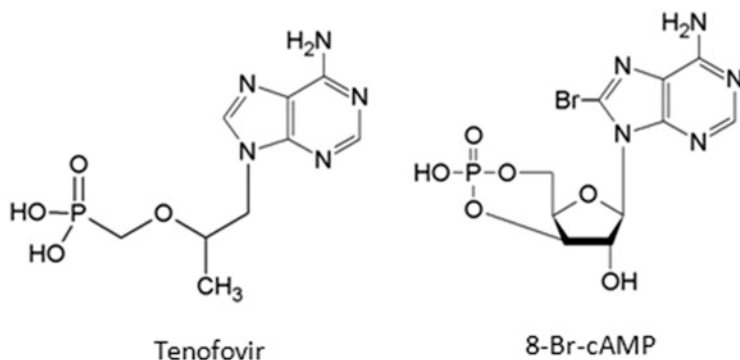


Fig. 7 Chemical structure of tenofovir and 8-bromo-cAMP, two potential internal standards in cNMP analysis

- In some publications the usage of other nucleosides as internal standard is described. For the analysis of rat tissue Ren et al. (2009) found that $^{13}\text{C}_{10}$ -adenosine is suitable for a reliable quantification of cAMP.

Furthermore, Göttle et al. (2010) performed several enzyme assays, adding inosine as internal standard to the samples.

- Stable isotope-labeled cyclic nucleotides show, due to the identical chemical structure, the same retention times as “their” unlabeled analyte and reflect therefore the matrix effect best (Fig. 6). The major problem of stable isotope-labeled internal standards is their purity, since the contamination with the unlabeled molecule would falsify the HPLC-MS/MS result especially in low analyte concentration ranges. Furthermore, stable isotope-labeled NMPs are not available and have to be prepared on custom synthesis. However, the enzymatical preparation of $^{13}\text{C}_{10}$ $^{15}\text{N}_5$ -cGMP has been described by Zhang et al. (2009) and Martens-Lobenhoffer et al. (2010) using the stable isotope $^{13}\text{C}_{10}$ $^{15}\text{N}_5$ -GTP, soluble guanylate cyclase (sGC), and a nitric oxide donor.

This approach can be used for the synthesis of $^{13}\text{C}^{15}\text{N}$ -labeled canonical cNMP, since the required recombinant enzymes are only available for the conversion of labeled GTP to labeled cGMP or labeled ATP to labeled cAMP, respectively. Nevertheless, Göttle et al. (2010) found that edema factor (EF) of *Bacillus anthracis* not only acts as adenylyl cyclase but accepts the substrates CTP, UTP, and GTP as well. Therefore, EF is a universal tool for the synthesis of isotope-labeled canonical and non-canonical cyclic nucleotides (Laue et al. 2014).

In addition to $^{13}\text{C}^{15}\text{N}$ -labeled cyclic nucleotides, Oeckl and Ferger (2012) describe the usage of $^{13}\text{C}_5$ -cAMP and $^{15}\text{N}_5$ -cGMP as internal standards for the quantification of cAMP and cGMP in plasma, CSF, and brain tissues, respectively.

5 Examples of cNMP Analyses in Biological Matrices

5.1 Identification of Cyclic Nucleotides in Murine Tissues

When analyzing complex matrices like animal tissues, it becomes obvious that despite a sample preparation procedure, unequivocal analyte identification can be challenging. Figure 8 shows a typical chromatogram of cGMP in a murine bladder sample, in which a quantifier (black trace) and an identifier (red trace) mass transition of cGMP were selected. The resulting chromatogram displays a complex number of signals, with at least three peaks, for which the expected quantifier/identifier ratio for 3',5'-cGMP is achieved (peak A, B, and C). Only by knowledge of the cGMP retention time in an authentic standard sample, peak C (retention time: 3.1 min) can be assigned to 3',5'-cGMP, whereas peak A can be identified as 2',3'-cGMP. The identity of signal B is still unknown.

In this example the advantage and the need of an appropriate chromatographic step become evident. Without that separation step, the discrimination between the signals A, B, and C would not be possible and, as a consequence, the 3',5'-cGMP amount in this sample would be overestimated.

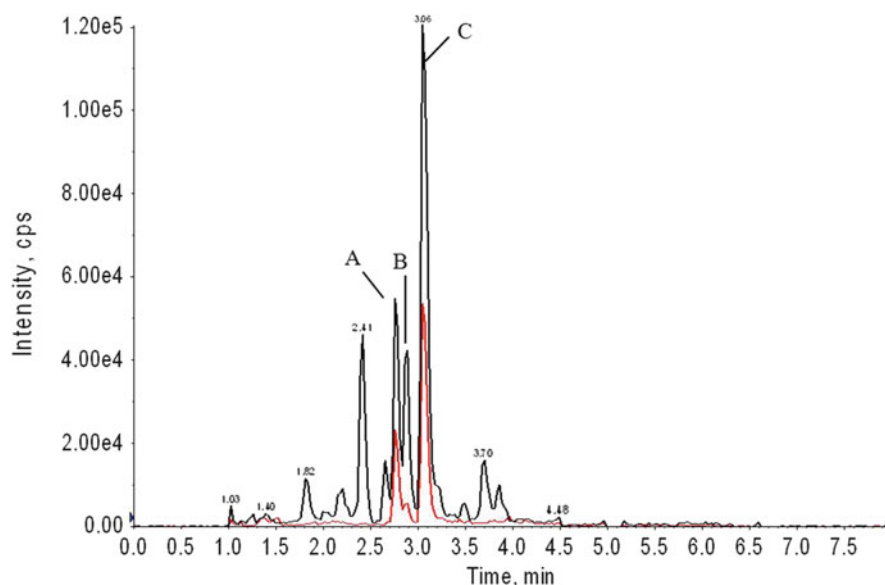


Fig. 8 Chromatogram of the quantifier (*black trace*; m/z 346 \rightarrow 152) and the identifier (*red trace*; m/z 346 \rightarrow 135) of cGMP in a murine bladder sample. Signals A, B, and C show the cGMP-specific quantifier/identifier ratio, with signal C occurring at the expected retention time. Thus signal C can be identified as 3',5'-cGMP, whereas signal A can be assigned to 2',3'-cGMP. The identity of signal B is still unresolved

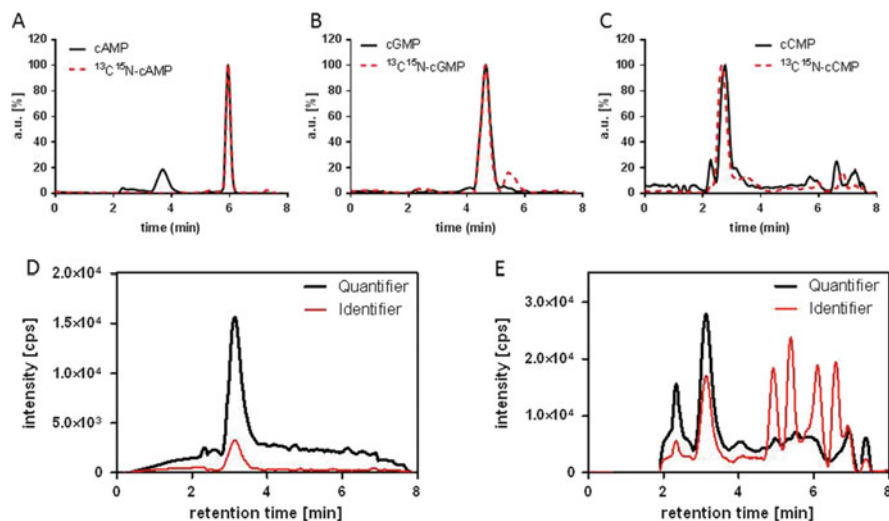


Fig. 9 Identification of cNMPs in human urine. (a) Quantifier mass transition of cAMP and $^{13}\text{C}^{15}\text{N}$ -cAMP, (b) quantifier mass transition of cGMP and $^{13}\text{C}^{15}\text{N}$ -cGMP, (c) quantifier mass transition of cCMP and $^{13}\text{C}^{15}\text{N}$ -cCMP, (d) quantifier and identifier mass transition of cAMP in a standard sample, (e) quantifier and identifier mass transition of cCMP in a human urine sample

5.2 Identification of Cyclic Nucleotides in Human Urine

This analysis was carried out after a sample preparation procedure using the isotope-labeled internal standards $^{13}\text{C}_{10}^{15}\text{N}_5$ -cAMP, $^{13}\text{C}_{10}^{15}\text{N}_5$ -cGMP, and $^{13}\text{C}_9^{15}\text{N}_3$ -cCMP. For cAMP and cGMP the peaks of the cNMP and the corresponding internal standard do overlap (Fig. 9) allowing the unequivocal identification of those cNMPs. On the other hand, the $^{13}\text{C}_9^{15}\text{N}_3$ -cCMP- and the cCMP-signal show a difference in retention time of 0.1 min, which gives a first indication that this “cCMP”-signal does not belong to cCMP. In those cases, a comparison of the quantifier/identifier ratios is highly recommended. Figure 9 shows that for putative cCMP signal in the urine sample this ratio amounts to 1:4, whereas the ratio in a cCMP standards sample is 1:2. Both findings, the mismatch of the retention time and the differing quantifier/identifier ratios, suggest that cCMP is not present in human urine at detectable amounts.

6 Conclusions and Perspectives

The main recommendations regarding critical steps in cNMP analysis by HPLC-MS/MS are (1) adequate sample preparation steps resulting in an instant stop of cellular metabolism and extensive removal of interfering matrix components, (2) selection of robust and reproducible HPLC conditions, and (3) implementation

of reliable MS/MS recordings. However, due to the comparatively low mass accuracy of triple quadrupole mass spectrometers, high resolution but less sensitive mass spectrometers such as time-of-flight systems should be additionally applied in cNMP research (Bähre and Kaever 2014; Bähre et al. 2015; Dittmar et al. 2015). The described HPLC-MS/MS methods for cNMP can easily be upgraded with respect to further nucleoside metabolites.

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