Determination and quantification of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by HILIC SPE in combination with LC-MS/MS

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INTRODUCTION

3’,5’-cyclic guanosine monophosphate (cGMP) is a crucial second messenger molecule involved in signal transduction within cells and diverse physiological functions such as neurotransmission. 3’,5’-cyclic adenosine monophosphate (cAMP) is another related second messenger which modulates cell growth and cellular differentiation in organisms.

Presently the enzyme-linked immunosorbent assay (ELISA) is the most commonly used quantitative method for this type of nucleotides. ELISA methods offer high sensitivity and excellent throughput capacity. However, the high cost of an ELISA kit and the lack of sufficient specificity are considered a drawback. More important problems according to matrix interference with ELISA can occur, leading to lower precision and accuracy when measuring cGMP and cAMP in several biological samples.

HPLC/MS methods have been documented [1,2]. In this work a new more sensitive HILIC SPE method prior to analysis by negative electrospray ionization and tandem mass spectrometry was developed and validated to quantify cGMP and cAMP in human blood and animal tissues. Stable isotope-labeled internal standards, $^{15}N_3$-cGMP and $^{13}C_{10}$,$^{15}N_5$-cAMP were added prior to the sample preparation to compensate for all sources of variability in the analytical methodology used.

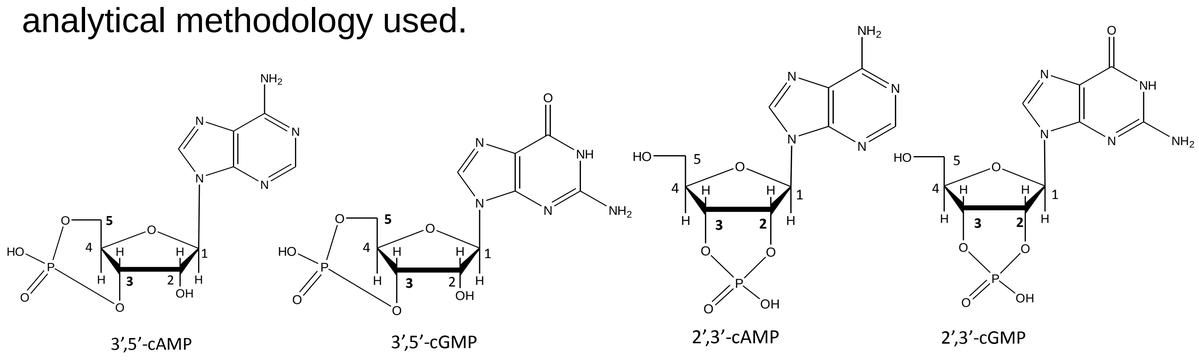

Figure 1: Structures of cyclic nucleotides measured in human blood and animal tissues.

EXPERIMENTAL

Instrumentation

HPLC – Waters 2690 Separation Module

MS – API 3000 triple quadrupole mass spectrometer with Turbo Ion Spray

Chromatographic conditions

Column – Zorbax SB-C18, 150 mm x 3.0 mm I.D., 3.5 μm particles

SPE cartridges – HyperSep Silica SPE Column 500 mg/3 mL

Mobile phase composition – A: water (0.1% formic acid)
– B: 1:acetonitrile/2:methanol/4:water

Gradient – 0% A to 50% A in 10 min

Flow rate – 0.35 mL/min

Column Temperature – 25° C

Injection volume – 50 µL

RESULTS AND DISCUSSION

Development of a new HILIC SPE sample preparation method

The hydrophilic nature of the cyclic nucleotides (log P ≤≤ 1) suggests the use of an SPE method under aqueous normal phase conditions, i.e. pure polar silica particles packed in a cartridge with water as strong eluent. The polar cGMP and cAMP will be retained on the column when high amounts of organic modifier are used. Elution of the compounds can subsequently be done with water, leading to complete metabolite recovery.

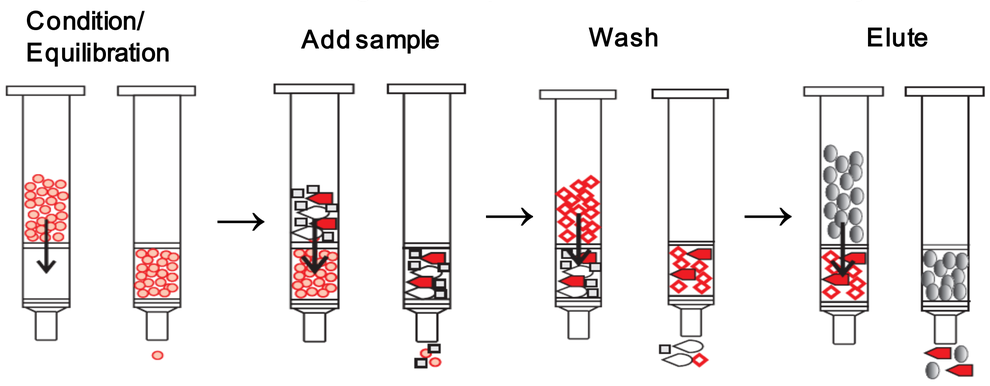

Figure 2: Different steps during HILIC SPE: (A) Condition: 5 mL water; (B) Equilibration: 5 mL buffer (5/95 100 mM ammonium formate/acetonitrile); (C) Loading: 4.2 mL sample/extract; (D) Wash step: 1 mL 10/90 water/acetonitrile; (E) Desorption: 2 mL water; evaporation and reconstitution in 60 µL water.

Application: human blood

200 µL of human blood was spiked with 5 ng/mL of IS 3’,5’-cAMP and IS 3’,5’-cGMP in a plastic vial. 600 µL of water (2% acetic acid), 200 µL of buffer (5/95 100mM ammonium formate/acetonitrile) and 3200 µL of acetonitrile was added. The resulting high acetonitrile percentage induced protein precipitation. These samples were vortexed for 2 min and centrifuged for 5 min at 3000 rpm. The supernatant was transferred to the HILIC SPE Column.

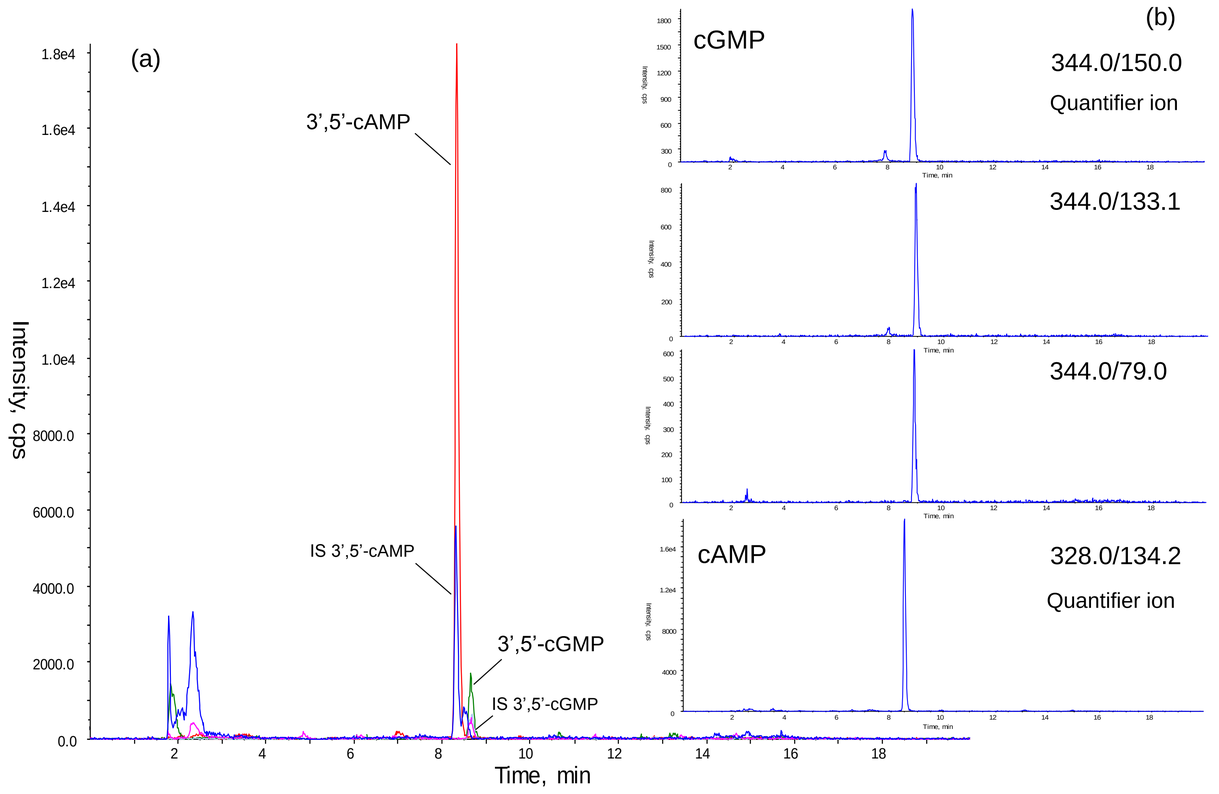

Figure 3: (a) Representative MRM chromatogram of a human blood sample (200 µL) after HILIC SPE. Spiked with 5 ng/mL internal standard of 3’,5’-cAMP and 3’,5’-cGMP. (b) Specific MRM transitions for cGMP and cAMP.

The concentration of 3’,5’-cGMP and 3’,5’-cAMP in human blood was measured for fifteen healthy patients with the HILIC SPE LC-MS/MS method. cGMP concentrations varied between 4.7 – 9.2 ng/mL and cAMP between 0.3 – 5.4 ng/mL, in good accordance with the literature.

Application: animal tissue

Also several animal tissues were investigated with this HILIC SPE LC-MS/MS method: pancreas, kidney and heart of a rabbit. Two extra peaks appeared for the same MRM transitions as 3’,5’-cGMP and 3’,5’-cAMP, assuming to be two isomers. Animal extracts were also analysed with a high resolution mass spectrometer, confirming these peaks to be respectively 2’,3’-cGMP and 2’,3’-cAMP.

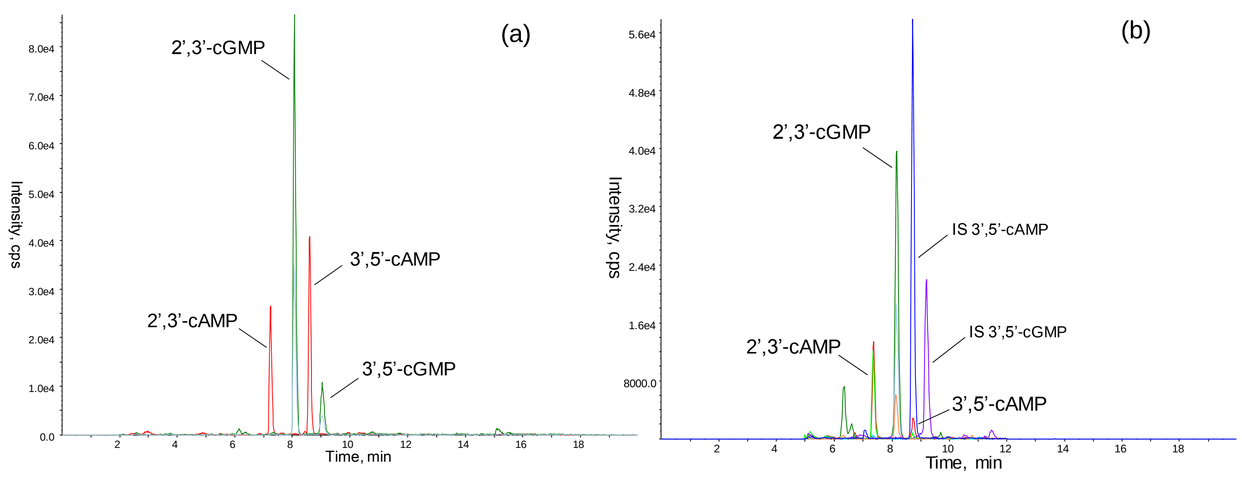

Figure 4: MRM chromatograms of: (a) 160 mg rabbit pancreas; (b) 150 mg rabbit kidney. Spiked with 50 ng/mL internal standard of 3’,5’-cAMP and 3’,5’-cGMP.

CONCLUSION

The combination of this new high recovery HILIC SPE sample preparation method with sensitive LC-MS/MS analysis allows for the quantification of these, from a pharmaceutical point of view, highly relevant messenger molecules in human blood and animal tissues. 2’,3’-isomers were detected and confirmed with high resolution mass spectrometry. Other real samples like urine and plant extracts can be analysed with minimal changes to this method.

References

[1] Y. Zhang et al. J. Chromatogr. B 877 (2009) 513.
[2] J. Martens-Lobenhoffer et al. J. Chromatogr. B 878 (2010) 487.