Published in final edited form as:
Anal Methods. 2014 June 21; 6(12): 3990–3994. doi:10.1039/C4AY00340C.

Determination of Methylarginines in Infant Plasma by CE-LIF

Thomas H. Linz[1,3,**] and **Susan M. Lunte**[1,2,3,*]

[1]Department of Chemistry, University of Kansas, Lawrence, KS

[2]Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS

[3]Ralph N. Adams Institute for Bioanalytical Chemistry, University of Kansas, Lawrence, KS

Abstract

Methylarginines (MAs) are a class of nitric oxide synthase inhibitors that have been implicated in respiratory complications of critically ill infants. This paper describes the development of an analytical method to measure these compounds in the plasma of newborns using capillary electrophoresis (CE). The CE separation method was optimized to enable complete baseline resolution of the four MA analogues of interest. Sample preparation concerns for infant-derived samples were addressed by validating a heat-assisted extraction method for the analysis of low volume (≤100 μL) samples from a plasma matrix. It was determined that the sample matrix (plasma versus serum) did not affect the measured MA concentrations, while extracting smaller volumes of plasma that underwent heat-induced gelation afforded higher MA recoveries than larger volume samples. These methods were then applied to blood samples collected from infants housed in the neonatal intensive care unit. It was discovered that these newborns had significantly elevated concentrations of MAs at younger ages (< 6 months) while amounts were similar between infants 6 months old and adults. The data are preliminary, but demonstrate an interesting age dependence on the concentrations of these nitric oxide inhibitors, which has not been previously reported.

Introduction

A common characteristic of most clinical diagnostic tests is that they rely on technology that can be implemented on a large scale and in a cost-effective manner.[1] Assays that produce colorimetric changes are ideal since they can be readily discerned either by using absorbance detection with conventional multiwell-plate technology or visually with simple point-of-care devices.[2] Each facilitates rapid analysis to achieve high sample throughput. However, in order to be effective, these assays must be highly selective for a given analyte and demonstrate very low cross-reactivity. This typically necessitates that only a single analyte be measured per assay; therefore, multiple assays must be employed to measure a range of biomarkers. Although this is not problematic in most routine analyses, in situations where an immediate response is necessary, waiting hours to days for a hospital lab to measure all the markers of interest can jeopardize the health of that patient. This problem is further

*Corresponding author. Dr. Susan Lunte, Ralph N. Adams Institute for Bioanalytical Chemistry, University of Kansas, 2030 Becker Dr. MRB 220, Lawrence, KS 66047, slunte@ku.edu, Fax: 785-864-1916.
**Current address: Dr. Thomas Linz, Department of Chemistry, University of North Carolina, Chapel Hill, NC

exacerbated in the analysis of samples derived from infants, especially those in the neonatal intensive care unit (NICU). Given the poor health of infants in the NICU and considering that many of them were born prematurely, limitations exist in the volume of blood that can be collected. This sample volume restriction may prevent all of the desired assays from being conducted, and may also restrict the assays to the analysis of plasma instead of less complex serum.[3] Considering the severe time constraints placed on diagnosing newborns in the NICU, it would be highly beneficial for a method to rapidly measure multiple analytes within a single assay from a small volume of plasma.

Capillary electrophoresis (CE) is an analytical technique that can be used to separate multiple analytes in a single assay from a sub-microliter volume sample. However, prior to the analysis of a biological sample, sample preparation steps must frequently be undertaken to allow the sample to be compatible with the analysis technique.[4] Most often this is accomplished by protein precipitation followed by solid-phase extraction (SPE) or affinity-based separation methods.[5] However, such procedures are generally complicated and labor-intensive. A recently reported heat-assisted extraction method was developed by our group to alleviate these concerns by expediting the sample preparation to enable higher sample throughput than SPE with far lower cost.[6]

This paper describes the development of a CE-based method to measure methylarginines (MAs) in infant plasma. MAs (Figure 1) are a class of compounds that inhibit the synthesis of nitric oxide (NO) *in vivo*. The concentrations of monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) (and their endogenous ratios) have been shown to serve as biomarkers for a multitude of NO-related pathologies, including cardiovascular diseases and pulmonary hypoxia.[7–10] In this study, blood samples obtained from critically ill newborns were analyzed to determine the correlation between NO deficiency and their severe medical condition. Given the central role that NO plays in the pulmonary system and the fact that respiratory distress is prevalent in newborns in the NICU, we hypothesized that these infants would exhibit diminished NO production due to elevated MA concentrations. The desire to analyze infant-derived samples necessitated that the analytical method be optimized and validated for the analysis of low-volume samples (≤100 μL) from a plasma matrix, which has not been previously described.

Materials and methods

Reagents

Standards of MMA, ADMA, SDMA, and propylarginine (PA) were purchased from Enzo Life Sciences (Farmingdale, NY). Sodium tetraborate and sodium cyanide were obtained from Sigma Aldrich (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde (NDA) and sulfobutylether-β-cyclodextrin (SBEC) were acquired from Invitrogen (Carlsbad, CA) and Cydex Pharmaceuticals (Lenexa, KS), respectively. HPLC-grade dimethylsulfoxide, acetonitrile, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). All solutions were prepared with 18.2 MΩ•cm deionized water (Millipore, Billerica, MA). Serum samples from anonymous adults were acquired at Lawrence Memorial Hospital (Lawrence, KS). Scavenged plasma samples were collected from infants in the NICU at Children's Mercy Hospital (CMH) (Kansas City, MO). The procedure was approved by the

CMH Institutional Review Board (IRB) and collected under the direction of Michael Norberg and Dr. William Truog at CMH. All samples were stored at −80 °C until ready for analysis at KU.

Heat-assisted extraction procedure

To prepare the adult serum samples, aliquots of pooled serum (25–100 μL) were transferred into 2 mL polypropylene microcentrifuge tubes (Fisher Scientific) to which 5% (v/v) of 10 μM PA (1.25–5 μL) was added. For the infant samples, 25 μL of plasma was mixed with 1.25 μL of 10 μM PA. All tubes were immersed in boiling water (100 °C) for 1.5 min. During the heating process, the liquid serum quickly congealed to form a solid gel. Once the serum gel was formed, water was added to each vial at a volume identical to the initial serum volume (*e.g.*, 25 μL water into 25 μL serum gel) and the vials were vortexed for ~20 s for a near no-net-dilution extraction. Samples were allowed to incubate for 5 min at room temperature and were then centrifuged to sediment the aggregated proteins.[6] The supernatants were decanted into separate tubes for subsequent CE analysis.

Capillary electrophoresis

A Beckman P/ACE MDQ CE instrument (Brea, CA) with a 50 μm i.d. capillary (Polymicro Technologies; Phoenix, AZ) 65 cm in length (50 cm to window) was utilized in this study. The run buffer consisted of 15 mM sodium tetraborate, 10 mM SBEC, and 28% (v/v) DMSO. Samples were injected hydrodynamically at 1.0 psi for 5.0 s, and separations were carried out at an applied field strength of 460 V/cm. A 445 nm diode laser (CrystaLaser; Reno, NV) was used to stimulate fluorescence emission which was measured with an external laser-induced fluorescence (LIF) detector (Picometrics; Ramonville, France). 32 Karat software (Beckman) was used to operate both CE operation and LIF detection.

Samples analyzed by CE were first derivatized with NDA/CN. NDA was dissolved in 1 : 1 acetonitrile : water; all other solutions were prepared in deionized water. The derivatization procedure entailed combining equal volumes of sample, 50 mM sodium tetraborate, NDA, and 5 mM NaCN and allowing the mixture to react for 10 min prior to injection. The initial NDA concentration was 1 mM when derivatizing standards and 5 mM when derivatizing blood samples. Peak areas from both standards and serum samples were normalized to the area of the non-endogenous internal standard, PA, for quantitation. All standards were measured in triplicate. Serum samples from adults were analyzed by CE-LIF once each, while infant samples were analyzed in duplicate.

Results and discussion

CE separation optimization

A CE-LIF method for the determination of the four MAs involved in NO synthase regulation using NDA/CN derivatization was previously reported by our group.[6, 11] Baseline resolution between the analytes of interest was obtained with a run buffer containing borate, SBEC, and DMSO. This method was capable of quantifying endogenous MA concentrations in adult serum; however, the reproducibility of the separation was somewhat variable. Specifically, for a few of the analyses, the resolution between the peaks for MMA and an

unknown endogenous analyte was diminished to the point where quantitation of MMA was precluded. To prevent this issue from affecting the study described here, the CE method was further optimized using pooled serum from unidentified adult donors.

In an attempt to improve separation resolution, several run buffer modifiers were investigated, including modified cyclodextrins, surfactants, and organic solvents. Following these studies, it was ultimately determined that the best and most reproducible results were obtained by simply adjusting the DMSO content. Baseline resolution between MMA and the unknown compound was achieved by increasing the amount of DMSO in the run buffer from the previous optimum of 25% to 28%. While the resolution between MMA and PA was slightly reduced under this set of conditions, it permitted more precise peak integration. Under these conditions, the unknown peak in serum samples split into two distinct peaks. Increasing the percentage of DMSO in the run buffer caused one of them to migrate earlier in the electropherogram, while the other migrated after PA (Figure 2). Therefore, the MA peaks of interest were no longer obscured by the interfering compounds, allowing more precise quantitation. The migration times also increased as a result of the additional DMSO; therefore, the separation voltage was increased to 460 V/cm, which generated a similar total analysis time without diminishing resolution. This optimal separation was utilized for all future analyses. Peak identities in clinical samples were verified by spiking experiments (Figure SI1).

Effect of sample volume on recovery

In an effort to make the heat-assisted extraction method more amenable to the analysis of samples from infants, a reduction in the required sample volume was sought. First, validation of the extraction recovery was required to ensure that the utilization of smaller volumes of serum would not be detrimental to the analysis. Therefore, aliquots of 100 μL, 50 μL, and 25 μL from a pooled lot of adult serum were dispensed into separate vials into which 5 μL, 2.5 μL, and 1.25 μL of 10 μM PA was added (each sample was prepared in triplicate), respectively, to hold the ratio of serum-to-water constant for each sample. Samples were heated until a serum gel formed and were then extracted into a volume of water identical to the original volume of serum for a near no-net-dilution extraction prior to analysis.

It was determined from this experiment that the volume of extracted serum impacted the recoveries of the MAs. Figure 3 illustrates that the recovered fraction for each MA analogue was higher on average for the smaller volume samples. This improvement can be attributed to a larger solvent-accessible surface area of the serum gel. Thinner serum-clots better allowed the extraction solvent to penetrate into the gel and resolubilize the analytes trapped within the aggregated protein framework. Smaller initial serum volumes (25 μL) enabled recoveries of ~90% as opposed to the ~60% recovery observed with larger serum volumes (100 μL). This finding demonstrates not only the analytical benefit of conducting experiments with smaller sample volumes (*i.e.*, more efficient extractions) but also the clinical benefit of requiring less blood to be drawn from infants. The high variance observed for the recoveries can be primarily attributed to inconsistency in the serum gel. The post-extraction sample volume differed due to variability in gel water retention by as much as

±20% from the volume incubated with the gel. These volume discrepancies exhibited an appreciable effect on the extracted analyte concentration. This effect was further accentuated after normalization of the data to the standard curve for recovery determination where the errors of the analyses were compounded. However, given that the recovered volume impacted all analytes of interest to similar extents, the differences in average recoveries were found to be significant.

Effect of sample matrix

The method described in our previous report utilized serum for MA analysis.[6] However, when conducting studies involving infants in the NICU, insufficient blood volumes are collected to allow whole blood to be effectively converted into serum using standard clot-activating vials. Therefore, the studies here were limited to the use of plasma. While serum and plasma matrices are similar in their pH, ionic strengths, and high protein content, serum is a less complex matrix compared to plasma, which contains additional clotting proteins. Additionally, it has been shown that the concentrations of various analytes may not be identical between the two matrices.[12, 13] Therefore, an investigation was performed into whether the specific blood matrix (*i.e.*, plasma or serum) impacted MA recovery following the heat-prep method.

To determine if a difference would be observed between serum and plasma in MA quantitation, whole blood was drawn from a single adult and split into two fractions—one aliquot was converted to plasma and the other to serum. Each sample underwent heat-assisted extraction followed by CE-LIF analysis under the optimized separation conditions. Table 1 compares the concentrations of MAs present in serum versus plasma. The results indicated that no significant difference existed between the two matrices (based on a two-tailed t-test; $p>0.45$ for each analyte). This finding was anticipated since the additional proteins from the plasma should have denatured and incorporated into the gel framework without impacting the extraction of small molecules from the gel. The similarity between the matrices will allow plasma samples from newborns to be analyzed without fear of matrix-related bias.

Determination of MAs in clinical samples

Upon validating that the analytical method permitted the analysis of low volume plasma samples, an investigation was performed to determine the concentrations of MAs in the plasma of critically ill infants housed in the NICU. The results showed that these newborns had very high amounts of MAs (Figure 4a), particularly during the first month of life. These high concentrations were consistent with the few existing reports in the literature concerning MA concentrations in newborn plasma.[14–16] Significant differences were observed ($p<0.05$ for each analyte) between patients of different ages, especially with SDMA, which first increased in concentration and then diminished. ADMA and MMA maintained similar concentrations initially but then exhibited a significant concentration reduction as the infants aged. Further correlation with disease state could not be performed due to the fact that samples were supplied to us from unidentified newborns with only their ages made available. Future experiments will explore the link between the elevations of MA

concentrations and the etiology of the disease to elucidate the biochemical pathways involved.

For comparison purposes, blood samples were also analyzed from anonymous adult donors (n = 17). The average ADMA and SDMA concentrations from these adult samples were <0.5 μM; these were substantially lower than the values obtained from infants, which exceeded 1 μM in the first 1+ month of life (Figure 4b). The MA concentrations decreased significantly as a function of age. Once infants reached 6 months of age, their MA concentrations were similar to those observed in adults.

The higher MA concentrations found in infants are in line with values that have been previously reported.[14–16] However, this is the first report demonstrating the age-dependence of the amount of endogenous MAs. These findings offer interesting insight into the NO pathway of infants. Given the inter-dependence of many compounds on the amount of NO produced, the increased MA concentrations and potential NO inhibition beget the question of whether other NO pathway-related molecules are also at atypical levels in critically ill infants. Disregulation of dimethylarginine dimethylaminohydrolase (DDAH) or protein arginine methyltransferase (PRMT) could lead to higher MA concentrations.[17] It has been reported that DDAH expression and activity increases before birth and then declines thereafter, which may help explain the observed trend.[10] A more thorough investigation of this pathway could provide better information regarding the role that MAs play in neonatal diseases related to NO. This could potentially lead to the design of medications to target the underlying problems affecting these infants.

Other alternative potential reasons for the observation of elevated MA concentrations in newborn plasma could stem from an artifact of sample collection. All newborn-derived samples had some evidence of hemolysis. Reports have shown that red blood cells (RBCs) contain a clinically relevant amount of MAs.[18, 19] If the RBCs in the samples lysed during collection, they could have artificially increased the observed MA concentrations and biased the results; however, not to the extent observed in Figure 4a.

Conclusions

This report demonstrates the application of a new sample preparation and separation method to the analysis of clinical samples. CE-LIF separation and heat-assisted extraction methods were developed to analyze low volume plasma samples, which were then applied to a small-scale study analyzing samples collected from newborns in the NICU. The results from this preliminary study indicated that these infants had extremely high MA concentrations compared to adults. The elevated concentrations in the newborns indicate that they may suffer from NO-related pathologies, which may be the reason for their hospitalization. However, at six months of age, it was shown that the MA concentrations diminished to those found in adults. This is the first study to chart the amounts of MAs over the first several months of life. Future work will delve into the involvement of MAs in disease onset. The analytical method will also be modified to conduct the CE separation in a microfluidic platform to enable more rapid point-of-care diagnostics in a clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Dr. Roger Dreiling, Connie Broers, and Faith Nilhas at Lawrence Memorial Hospital and Dr. William Truog and Michael Norberg at Children's Mercy Hospital for obtaining IRB permission and collecting the blood samples for this study. The authors would also like to acknowledge the American Heart Association for a predoctoral fellowship, NIH R21NS061202, and the Adams Institute for funding. We would also like to thank Nancy Harmony for her editorial assistance with the manuscript.

References

1. Bamforth FJ. Clin. Biochem. 1994; 27:333–342. [PubMed: 7867213]
2. Sapan CV, Lundblad RL, Price NC. Biotechnol. Appl. Biochem. 1999; 29:99–108. [PubMed: 10075906]
3. Hyman SJ, Novoa Y, Holzman I. Pediatr. Clin. N. Am. 2011; 58:1083–1098.
4. Chiu ML, Lawi W, Snyder ST, Wong PK, Liao JC, Gau V. JALA. 2010; 15:233–242.
5. Tulipani S, Llorach R, Urpi-Sarda M, Andres-Lacueva C. Anal. Chem. 2013; 85:341–348. [PubMed: 23190300]
6. Linz TH, Lunte SM. Electrophoresis. 2013; 34:1693–1700. [PubMed: 23417924]
7. Wang Z, Tang WHW, Cho L, Brennan DM, Hazen SL. Arterioscler., Thromb., Vasc. Biol. 2009; 29:1383–1391. [PubMed: 19542023]
8. Zakrzewicz D, Eickelberg O. BMC Pulm. Med. 2009; 9:5. [PubMed: 19178698]
9. Dweik RA. Am. J. Physiol. Lung Cell Mol. Physiol. 2007; 292:L15–L17. [PubMed: 16980373]
10. Arrigoni FI, Vallance P, Haworth SG, Leiper JM. Circulation. 2003; 107:1195–1201. [PubMed: 12615801]
11. Linz TH, Snyder CM, Lunte SM. J. Lab. Autom. 2012; 17:24–31. [PubMed: 22357605]
12. Wedge DC, Allwood JW, Dunn W, Vaughan AA, Simpson K, Brown M, Priest L, Blackhall FH, Whetton AD, Dive C, Goodacre R. Anal. Chem. 2011; 83:6689–6697. [PubMed: 21766834]
13. Teahan O, Gamble S, Holmes E, Waxman J, Nicholson JK, Bevan C, Keun HC. Anal. Chem. 2006; 78:4307–4318. [PubMed: 16808437]
14. Di GIM, Chiandetti L, Gucciardi A, Moret V, Naturale M, Giordano G. Anal. Chim. Acta. 2010; 677:140–148. [PubMed: 20837180]
15. Vida G, Sulyok E, Ertl T, Martens-Lobenhoffer J, Bode-Boeger SM. Pediatr. Int. 2012; 54:476–479. [PubMed: 22375624]
16. Bordigato MA, Piva D, Di GIM, Giordano G, Chiandetti L, Filippone M. Early Hum. Dev. 2011; 87:143–145. [PubMed: 21138787]
17. Bulau P, Zakrzewicz D, Kitowska K, Leiper J, Gunther A, Grimminger F, Eickelberg O. Am. J. Physiol. Lung Cell Mol. Physiol. 2007; 292:L18–L24. [PubMed: 16891395]
18. Davids M, van Hell AJ, Visser M, Nijveldt RJ, van Leeuwen PAM, Teerlink T. Am. J. Physiol. Heart Circ. Physiol. 2012; 302:H1762–H1770. [PubMed: 22367507]
19. Billecke SS, Kitzmiller LA, Northrup JJ, Whitesall SE, Kimoto M, Hinz AV, D'Alecy LG. Am. J. Physiol. 2006; 291:H1788–H1796.

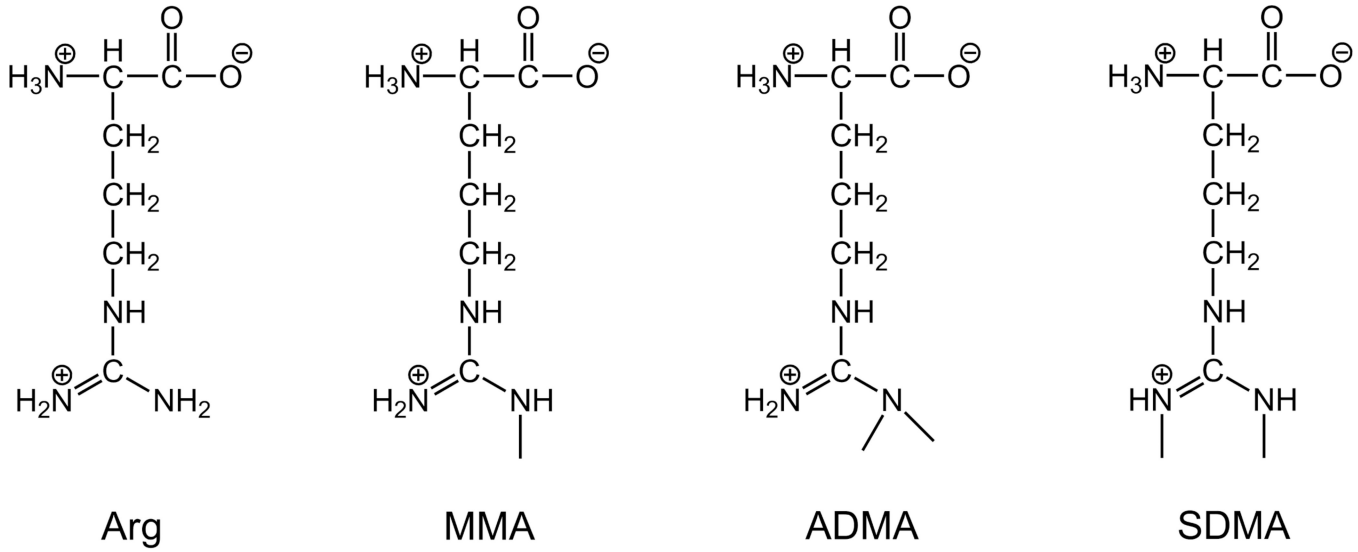


Figure 1.

Structures of the methylarginines involved in nitric oxide synthase inhibition pathways. Arg: arginine; MMA: monomethylarginine; ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine.

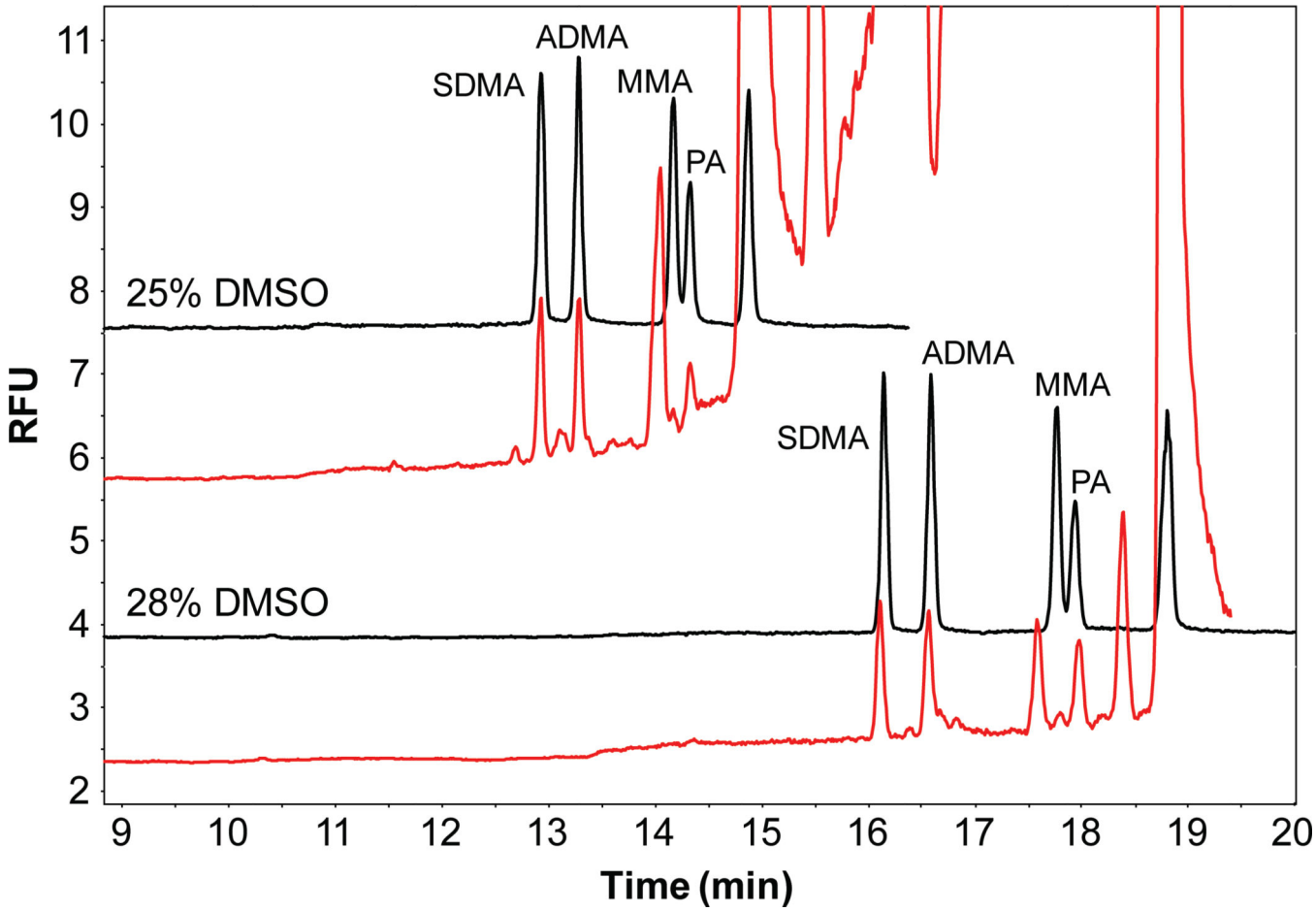


Figure 2.

CE separation (430 V/cm) of standards (black) and adult serum samples (red) with 25% and 28% DMSO in the run buffer. Two unknown peaks resolve from the MMA peak when DMSO is increased to 28%. All MA peak identities in blood samples were verified based on spiking the serum with the authentic compound.

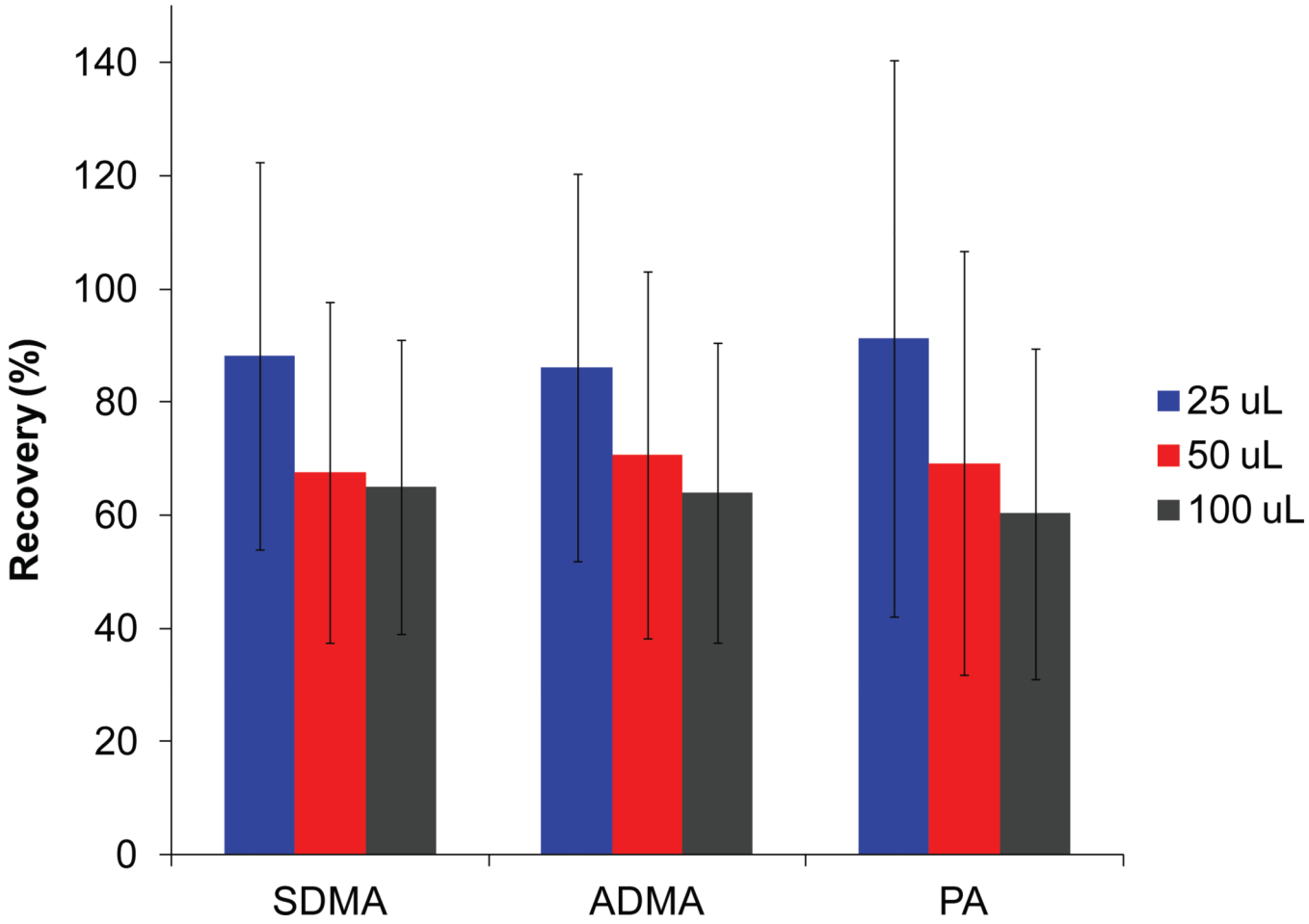
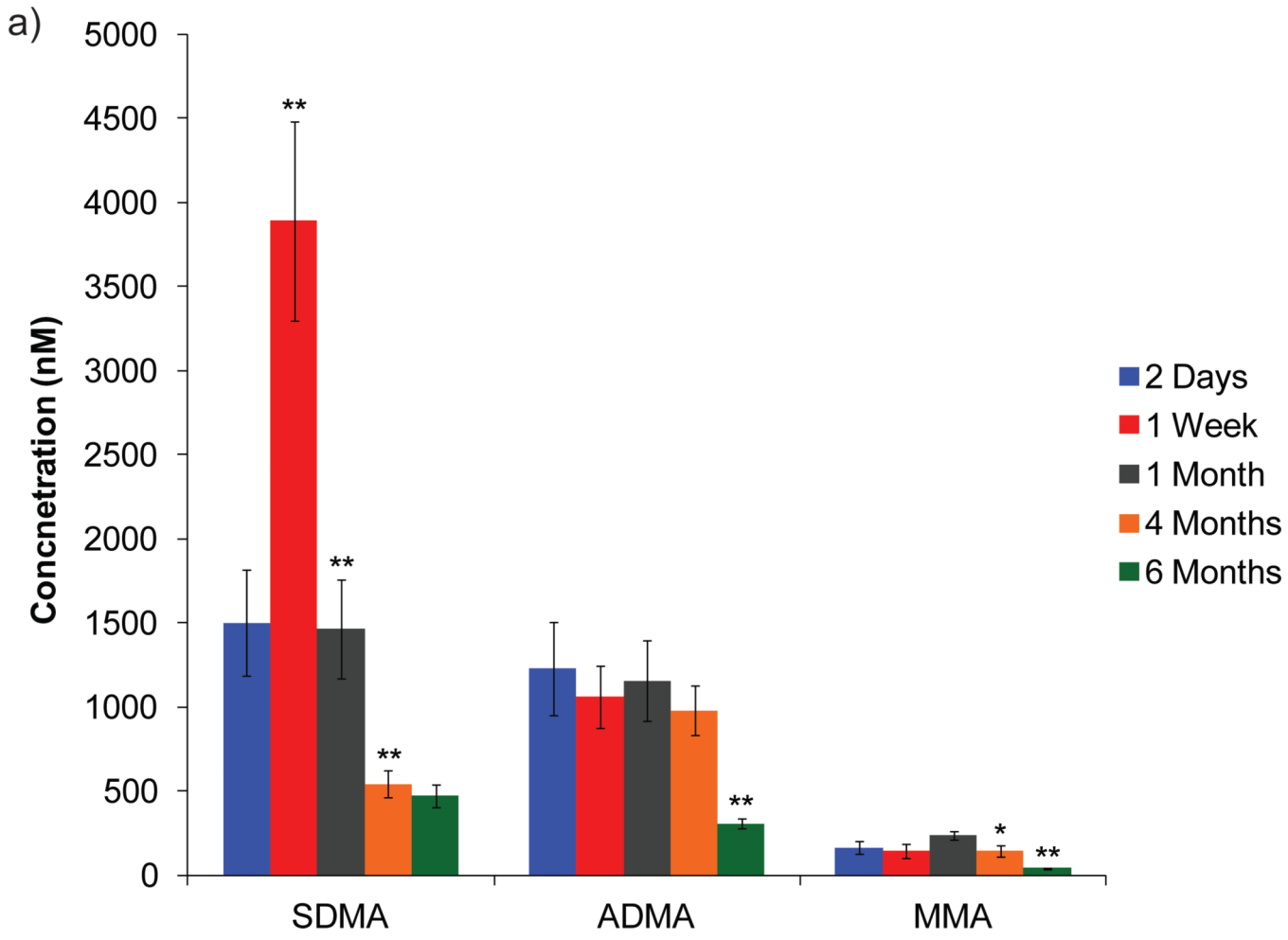


Figure 3.
Effects of initial serum volume on extraction recoveries of MAs from serum gel.



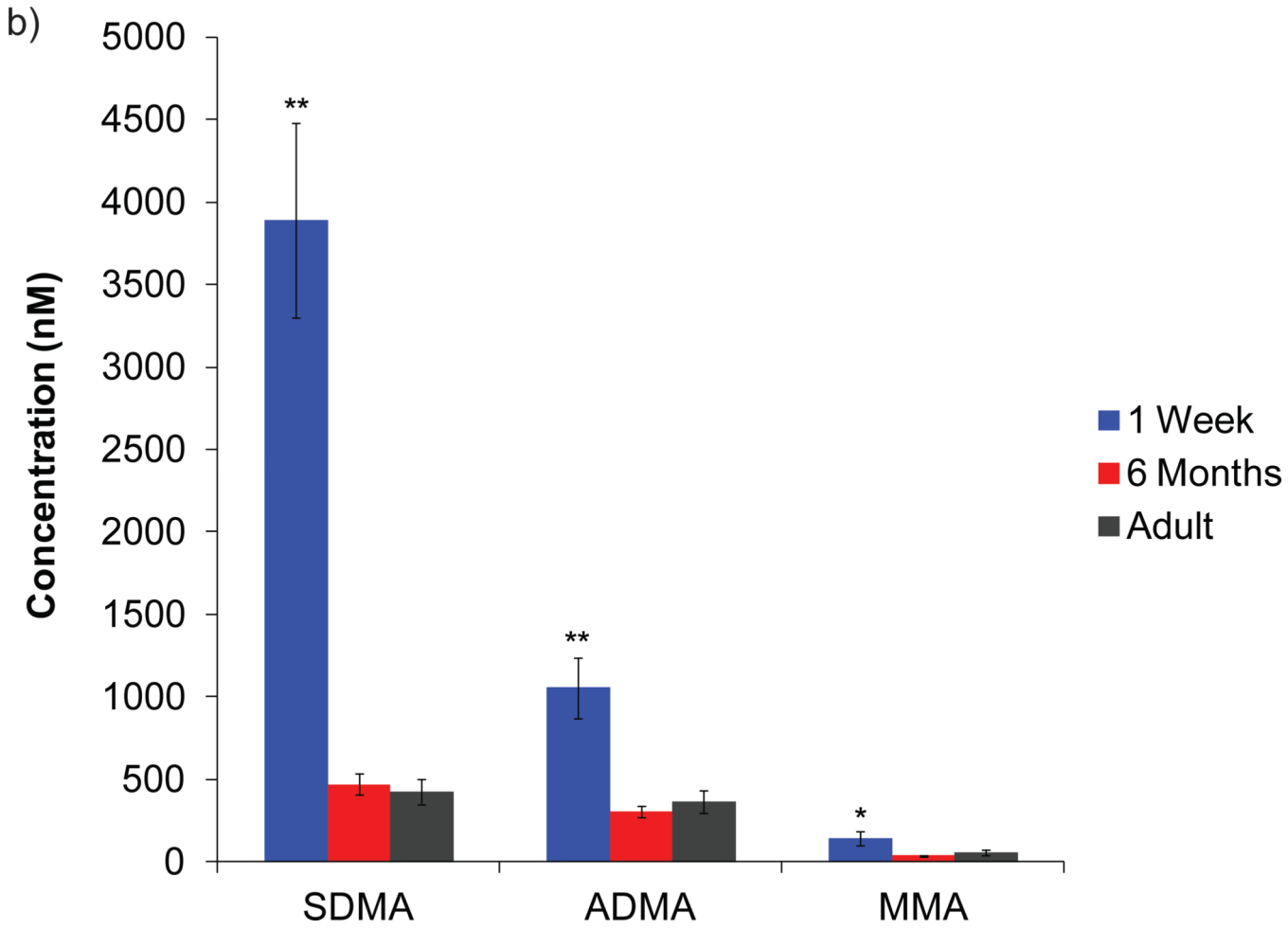


Figure 4.

(a) Concentrations of MAs in the plasma of newborns of various ages. (b) Comparison of the concentrations of MAs between newborns and adults.

*$p < 0.05$; **$p < 0.001$

Table 1

Comparison of the effect of blood sample matrix on MA quantitation

Analyte	Serum (nM)	Plasma (nM)
SDMA	414 ± 41	393 ± 53
ADMA	342 ± 28	354 ± 60
MMA	64 ± 36	57 ± 19