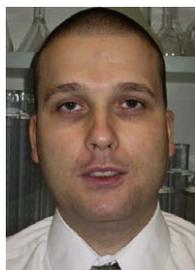




Review

Approaches to measuring the activities of protein arginine *N*-methyltransferasesTed M. Lakowski^a, Cecilia Zurita-Lopez^b, Steven G. Clarke^b, Adam Frankel^{a,*}^aThe Division of Biomolecular and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, BC, Canada V6T 1Z3^bDepartment of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, CA 90095-1569, USA

Ted M. Lakowski



Adam Frankel



Cecilia Zurita-Lopez



Steven G. Clarke

Protein arginine *N*-methyltransferases (PRMTs)¹ are a family of bisubstrate enzymes that transfer methyl groups from the substrate *S*-adenosyl-*L*-methionine (AdoMet) to nitrogen atoms on the guanidine groups of arginine residues within substrate proteins, forming methylarginine residues and *S*-adenosyl-*L*-homocysteine (AdoHcy) [1–4]. Mammalian PRMTs modify the terminal nitrogen atoms to produce ω -*N*^G-monomethylarginine (MMA) residues as a final product or as an intermediate to the formation of one of two types of dimethylarginine (DMA); these enzymes are classified by type of DMA produced. PRMTs that produce asymmetric ω -*N*^G,*N*^G-dimethylarginine (aDMA) residues are classified as type I, and those that produce symmetric ω -*N*^G,*N*^G-dimethylarginine (sDMA) residues are classified as type II [1] (Fig. 1). PRMTs known to exhibit type I activity include PRMT1 [5], PRMT2 [6], PRMT3 [7], coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4) [8], PRMT6 [9], and PRMT8 [10], while PRMT5 is capable of type II activity [11]. Finally, in some cases PRMT7 has been shown to catalyze the formation of

¹ Abbreviations used: [methyl-¹⁴C]AdoMet, *S*-adenosyl-*L*-[methyl-¹⁴C]methionine; [methyl-³H]AdoMet, *S*-adenosyl-*L*-[methyl-³H]methionine; aDMA, asymmetric ω -*N*^G,*N*^G-dimethylarginine; AdoMet, *S*-adenosyl-*L*-methionine; AdoHcy, *S*-adenosyl-*L*-homocysteine; CID, collision-induced dissociation; DMA, dimethylarginine; DTT, dithiothreitol; ESI, electrospray ionization; ETD, electron-transfer dissociation; FPLC, fast performance liquid chromatography; GAR, glycine- and arginine-rich; GST, glutathione *S*-transferase; HEPES, 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MMA, ω -*N*^G-monomethylarginine; OPA, *o*-phthalaldehyde; PRMT, protein arginine *N*-methyltransferase; *R*_f, retardation factor; sDMA, symmetric ω -*N*^G,*N*^G-dimethylarginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIR, selected ion recording; *S*/*N*, signal-to-noise; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin layer chromatography; UPLC, ultrahigh performance liquid chromatography.

* Corresponding author. Address: 2146 East Mall Vancouver, BC, Canada V6T 1Z3. Fax: +1 (604) 822 3035.

E-mail address: afrankel@interchange.ubc.ca (A. Frankel).

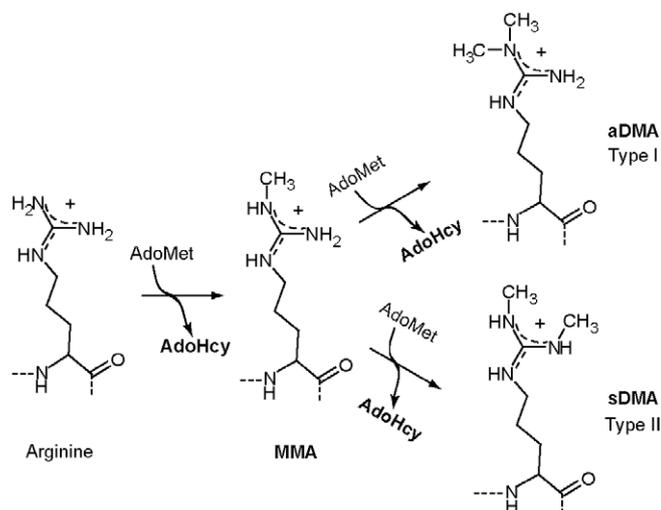


Fig. 1. The major types of mammalian protein arginine methylation. The detectable products of PRMT methylation include AdoHcy, as well as protein-MMA, -aDMA, and -sDMA.

MMA only [12], and in other cases it has been reported to produce MMA, aDMA, and sDMA [13]. The addition of methyl groups by PRMTs does not affect the charge of the substrate arginine residue while it does reduce the ability of the residue to serve as a hydrogen bond donor. The role of arginine methylation in a variety of pathologies including viral replication, cancer, and cardiovascular disease justifies the intense interest in this family of enzymes [1–4].

PRMTs present some unique challenges with respect to the measurement of their enzymatic activities. Since these enzymes catalyze a bisubstrate reaction, a wide variation in both substrate concentrations is needed to accurately measure kinetic parameters. In the case where the K_m for a substrate is known, measurement of apparent kinetic parameters is achieved with saturating concentrations of this substrate and varying subsaturating concentrations of the other substrate. Furthermore, the bisubstrate PRMT kinetic mechanism is complicated because the product of a single methylation (i.e., an MMA residue) can be the substrate for a second methylation. The reaction complexity is increased if a protein substrate contains multiple target arginine residues, thereby increasing the combinations of potential methylated products. Finally, some PRMTs have been shown to methylate themselves, making the enzyme itself a substrate; such automethylation adds an additional level of complication [9,14]. Previous studies on the bisubstrate mechanism of PRMTs concur that substrate binding and product dissociation are sequential [15–17]. With respect to PRMT6 we have already demonstrated that the bisubstrate mechanism is sequential and ordered such that AdoMet is the first substrate to bind and AdoHcy the last product to dissociate [15]. Our findings are supported by structural investigations of PRMT4, which show that AdoMet binding induces the normally disordered N-terminus of PRMT4 to form a helix that serves as a binding ridge for the protein substrate [18]. In another study using PRMT1, it was proposed that substrate binding to PRMTs is random but still sequential [17]. We suggest that this disparity in mechanism may arise from the different methods used to quantitate methyl transfer [6].

Despite the importance of PRMTs in cellular processes, methods for measuring PRMT activity have not been comprehensively examined. Here, we focus on methods that we routinely use and methods developed by other groups, highlighting the advantages and disadvantages of each. Other methods, such as the use of commercially available antibodies directed against methylated arginine residues will not be examined in detail. The techniques for

measuring PRMT activities are divided into three categories based on the minimal enzymatic reaction scheme (Fig. 1): analysis of the intact methylated protein, quantification of the product AdoHcy, and measurement of the methylated arginine residues MMA, aDMA, and sDMA after acid hydrolysis.² We find that intact protein separation by gel electrophoresis followed by detection (using radioactivity from an isotopic label on the transferred methyl group) can demonstrate the methylation of multiple substrates, including automethylation. However, this method can be difficult for inhibitor studies, poor methyl-acceptors, and low activity methyltransferases. Measuring PRMT activity by quantification of the coproduct AdoHcy is useful for product inhibitor studies, but can suffer from high background caused by automethylation and the spontaneous formation of AdoHcy from AdoMet. Methods that detect methylated arginines derived from acid hydrolysis of PRMT reaction samples can determine the relative amounts of MMA, aDMA, and sDMA, in addition to quantification of PRMT activity. We describe methods of separation of these methylated arginine derivatives by thin layer, reverse phase, or cation exchange chromatography, and mass spectrometry. Of the several methods described herein, we point out the advantages of a UPLC–MS/MS approach because of its unambiguous differentiation of MMA and aDMA, its very low limit of quantitation, and its general applicability to any PRMT and substrate combination without the need for methylated polypeptide standards or radioactivity.

Data analysis considerations

Densitometric analysis of phosphor images with radioactive TLC and SDS–PAGE data

For quantification of radioactive signals, gels or TLC plates are best exposed to storage phosphor screens that have a wide linear dynamic range, form images from ionizing radiation faster, and are generally considered to be more quantitative than film [19]. We use a Typhoon 9210 phosphor imager (GE Healthcare) to develop the screens. Relative band intensity (in the case of SDS–PAGE gels) or spot intensity (in the case of TLC) can be measured by densitometry. For this purpose we use ImageQuant 5.2 (Molecular Dynamics), which also calculates an error for the signal relative to background. Signals can also be quantified with standards; for example, 0.045 to 9 pmol *S*-adenosyl-L-[methyl-¹⁴C]methionine ([methyl-¹⁴C]AdoMet) spotted onto filter paper for gels and onto silica plates for TLC can be used to generate standard curves. Ideally, standards should be radioactive isotopes of the product being analyzed, but this is often not feasible.

Calculation of enzyme kinetic parameters

The accurate derivation of bisubstrate steady-state enzyme kinetic parameters requires initial velocity measurements where both substrates are varied simultaneously. The resulting data can be analyzed via Lineweaver–Burk, Hanes–Wolf, or Eadie–Hofstee plots of initial velocities for each concentration of a substrate that is varied in the presence of multiple fixed concentrations of the other substrate. Subsequent replot of apparent V_{max} for each fixed concentration against the varied substrate allows calculation of the kinetic parameters [20]. Apparent V_{max} values can also be calculated in a similar way using nonlinear least squares fitting to the Michaelis–Menten equation for a Uni–Uni reaction

$$v = \frac{V_{max}[A]}{K_m + [A]} \quad (1)$$

² Hydrolysis is carried out on dried samples in a Pico-Tag WorkStation (Waters) under vacuum at 110 °C in the presence of 6 N HCl in the vapor phase for 24 h.

However replots of the apparent V_{\max} are still needed to derive kinetic parameters as above. PRMTs follow a sequential bisubstrate enzyme mechanism [6,15,17]. The initial velocity for such a mechanism can be described by

$$v = \frac{V_{\max}[A][B]}{K_S^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B]}, \quad (2)$$

where K_S^A is the dissociation constant for the first substrate to bind, K_m^A and K_m^B are the Michaelis constants, and [A] and [B] are the concentrations for the first substrate and second substrate to bind, respectively. Ideally, these enzymatic parameters can be derived by plotting a range of initial velocities against variation in both substrates and using nonlinear least squares fitting to Eq. (2) with available software [21–25]. It is also possible to calculate bisubstrate enzyme kinetic parameters by nonlinear least squares fitting of the initial velocities with a varied substrate at a single fixed concentration of the other substrate to Eq. (2). This analysis method requires software that can simultaneously fit multiple enzymatic parameters and requires user-defined initial estimates for those kinetic parameters. The final results may be affected by the choice of such initial estimates [22,23].

Analytical performance statistics

Deciding which PRMT assay to use depends on the availability of resources and equipment, and more importantly, whether the objective is to determine substrate specificity or quantification of enzyme activity. The choice of an appropriate method may be influenced by its analytical performance statistics, some of which include limit of detection (LOD), limit of quantitation (LOQ), and signal-to-noise (S/N) ratio [26]. The S/N ratio is the ratio of the magnitude of the signal such as radioactivity in cpm or UV absorbance peak area over the signal in the absence of analyte/reaction product detected (i.e., random noise). There are various ways to calculate S/N depending on the technique being used; however, most data analysis software will generate these data. For reliable quantitation, data should have a S/N greater than 2 or 3. The LOD is the minimum concentration of analyte that can be detected with confidence, and it is usually defined as the mean of several blanks and 1.6–3 times the standard deviation of those blanks. The LOQ is the minimum concentration of analyte that can be reliably quantified, and it is usually defined as the mean of several blanks and 3–10 times the standard deviation of those blanks [26]. These parameters are a guide to the suitability of an assay, but the true reliability of any quantification is dependent on the linearity of the standard curve and the S/N of the experimental samples.

Methods of measuring the activities of PRMTs

Approaches to measuring arginine methylation can be grouped into three categories: those that measure the amount of methylation on the intact protein, those that measure the appearance of the coproduct AdoHcy, and those that measure the amount of MMA, and aDMA or sDMA in hydrolysates of the methylated protein product. All of these methods allow for detection of methyl transfer. However, some techniques are better suited for qualitative analysis while others for quantitative purposes.

Intact protein analysis via gel electrophoresis

Intact protein analysis is perhaps the most direct method for measuring arginine methylation, as it requires little or no processing of the methylated protein product. The simplest approach is to use methyl-labeled radioactive [*methyl*- ^{14}C]AdoMet or [*methyl*- ^3H]Ado-

Met as the cosubstrate for the reaction and separate radioactively methylated products by gel electrophoresis. An example of such

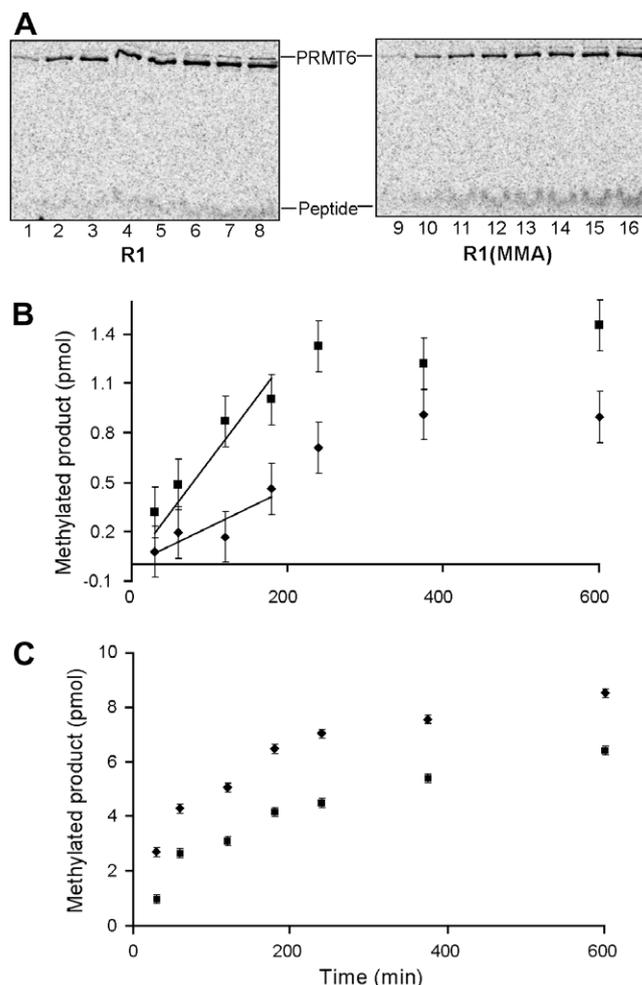


Fig. 2. Assaying the activity of $\text{H}_6\text{PRMT6}$ with the R1 and R1(MMA) peptides using SDS-PAGE. (A) Storage phosphor scans exposed to 16.5% tricine SDS-PAGE gels [27] for 48 h from progress reactions with $\text{H}_6\text{PRMT6}$ (0.77 μM) at 0, 30, 60, 120, 180, 240, 375, and 600 min at 37 $^\circ\text{C}$ with the R1 peptide (lanes 1–8, respectively) and the R1(MMA) peptide (200 μM) (lanes 9–16, respectively) and 112.5 μM [*methyl*- ^{14}C]AdoMet (0.23 kBq/ μL) in a final 20- μL volume. The positions of automethylated $\text{H}_6\text{PRMT6}$ and the peptides are indicated, and the phosphor images have been uniformly adjusted for brightness and contrast to aid visualization. (B) Progress curves derived by densitometry from (A) for R1 (\blacklozenge) and R1(MMA) (\blacksquare). The lines drawn were used to derive the slopes to estimate the initial rates listed in Table 2. The linear range is indicated with straight lines from 30 to 180 min. (C) The progress curves derived by densitometry from PRMT6 automethylation in the presence of R1 (\blacklozenge) and R1(MMA) (\blacksquare). Error bars in (B) and (C) represent standard deviations for the associated band densities as calculated by ImageQuant 5.2 (Molecular Dynamics).

Table 1
Amino acid sequences for peptide PRMT substrates.

Name	Sequence	Reference
Histone H4 tail ^a	SGRGKGGKGLGKGGAKRHRKVV	[6,31]
Histone H3 tail ^a	ARTKQTARKSTGGKAPRQLATKAAW	[53]
SmB'	WPPGMRPPPPGMRPPPPGMRPPRP	[57]
Tat	YGRKKRRQRRRAPQDSQ	[54]
R1	WGGYSRGGYGGW	[15]
R1(MMA)	WGGYSR _(MMA) GGYGGW	[15]
R1(aDMA)	WGGYSR _(aDMA) GGYGGW	[15]
R1(sDMA)	WGGYSR _(sDMA) GGYGGW	[15]

^a Tail refers to the N-terminal tail of the respective histone.

Table 2
Initial rate of activity of H₆PRMT6 with R1 or R1(MMA) using various methods.

Substrate	Analyte	Method	Initial rate (pmol/min mg)
R1	R1(MMA)	SDS–PAGE	117
R1(MMA)	R1(aDMA)	SDS–PAGE	235
R1	MMA	TLC	354 ^a
R1(MMA)	aDMA	TLC	1055 ^a
R1	R1(MMA)	MS	423 ^a
R1(MMA)	R1(aDMA)	MS	1032 ^a

^a These values are derived from [15].

an experiment is depicted in Fig. 2A. H₆PRMT6³ was incubated with the R1 or R1(MMA) substrates (Table 1) (described previously [15]). Reactions⁴ were incubated for 0, 30, 60, 120, 180, 240, 375, and 600 min and loaded onto 16.5% tricine SDS–PAGE gels [27]. The dried gels were exposed to storage phosphor screens along with 0.045–9 pmol [*methyl*-¹⁴C]AdoMet as a standard, and the activity quantified via densitometry using ImageQuant 5.2 (Molecular Dynamics). In agreement with our previous results, the monomethylated R1(MMA) peptide is the preferred substrate for H₆PRMT6. However, the initial rates of methylation of the R1 and R1(MMA) peptides, as measured by densitometry of the phosphor image signal, (Fig. 2B), are about 3-fold lower than what we have found for similar enzymatic reactions previously quantified using MS and TLC (Table 2) [15]. This is likely caused by the diffuse nature of the methylated peptide bands resulting in low S/N. As a consequence, most of the time points for methylation of R1 and one time point for R1(MMA) are below the LOQ. Regardless, these data are presented in Fig. 2B for comparison to other methods. The initial apparent rates of methylation derived from these gel data are, not surprisingly, inconsistent with those values derived previously by TLC and MS under similar conditions (Table 2) [15]. In contrast, the error associated with the more narrow-focused automethylated PRMT6 bands (Fig. 2C) is much lower. These results suggest that electrophoretic separation of peptides that migrate as diffuse bands can confound densitometric analysis of radioactivity detected after exposure to storage phosphor screens.

The inherent problems associated with radioactivity detection in gels with low activity enzymes are particularly apparent when performing inhibition studies. While low signal may be mitigated to some extent by increasing reaction volume, there is a limit to the gel-loading volume, and methylated protein products concentrated by precipitation may not necessarily redissolve for gel loading and may not have been quantitatively precipitated in the first place. Using radioactive protein standards on each gel might reduce the batch-to-batch variability between gels, but this requires in-gel radiolabeled standards and reduces the number of available wells for analytes. For the present study we used dilutions of [*methyl*-¹⁴C]AdoMet to construct the standard curve for indirect quantification. Compared to other techniques available, we found that quantification of methylation using SDS–PAGE is less reliable and generally less suitable for enzymatic studies to determine kinetic parameters than the procedures described below.

The diffuse mobility of small (i.e., 1–3 kDa) PRMT peptide substrates on SDS–PAGE is caused by the relatively small size of the peptides and their comigration with the SDS front [27]. In addition, the high positive charge conferred by the relatively high ratio of arginine to total amino acid residues tends to retard the mobility of the normally uniform, negatively charged SDS-associated pro-

teins during SDS–PAGE [28,29]. A simple established technique to obtain high-resolution bands for positively charged peptides and proteins is Triton X-100 acid urea PAGE [30]. This method has been applied to substrates of PRMTs and may help to improve the LOQ of gel-based assays [31]. Another method to improve the LOQ and selectivity is the use of antibodies that are specific to MMA, aDMA, or sDMA residues within protein products. This approach provides the improved S/N garnered by Western blotting, but still requires methylated standards for quantification [32–34].

Regardless of the method, a key strength of the gel electrophoresis technique is the potential for simultaneous detection of multiple methylated products, provided that proteins separate in the gel. We detected both automethylated H₆PRMT6 in addition to the methylated R1 and R1(MMA) substrates (Fig. 2). A comparison of intensity of automethylated PRMT6 in the presence of R1 or R1(MMA) reveals that less automethylation occurs in the presence of the better methyl-acceptor, R1(MMA) (Fig. 2).

Sequencing intact proteins by MS

Recent studies have focused on the application of MS to the qualitative and quantitative measurement of arginine methylation using top-down or bottom-up sequencing techniques. The bottom-up strategy requires proteolytic digestion of the methylated product and subsequent collision-induced dissociation (CID) MS/MS fragmentation to sequence the cleaved peptides, whereas the

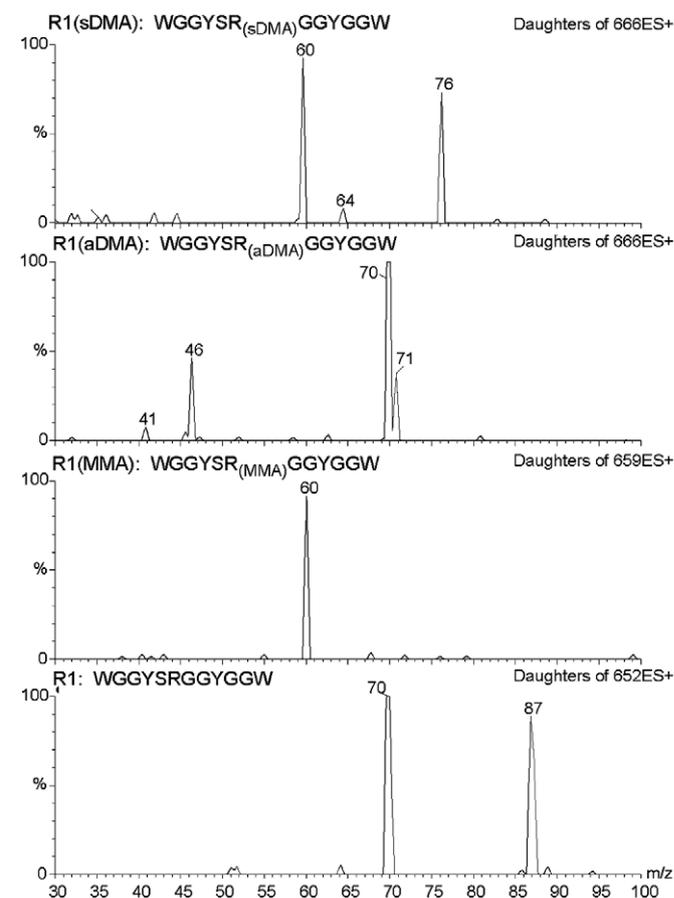


Fig. 3. Fragmentation of arginine-containing peptides. The fragmentation patterns of the +2 charged species of the R1, R1(MMA), R1(aDMA), and R1(sDMA) peptides using ESI ionization with a cone voltage of 20 V and CID fragmentation with a collision energy of 40 eV similar to a previously described protocol [39]. The amino acid sequence, methylation state, and the +2 *m/z* are listed above the corresponding spectra.

³ Two forms of PRMT6 are used in reactions throughout this work, one with an N-terminal GST tag (GST-PRMT6), and the other with an N-terminal hexa-histidine tag (H₆PRMT6).

⁴ In all original experiments described in this work methylation reactions were carried out at 37 °C in a methylation buffer, which is composed of 50 mM HEPES, pH 8, 10 mM NaCl, 1 mM DTT.

top-down technique of electron-transfer dissociation (ETD) fragmentation is used to specifically cleave peptide bonds and thus sequence an intact polypeptide [35]. Both techniques can, in principle, be used to sequence proteins, but ETD is generally considered superior because it results in more reliable peptide backbone cleavage, leading to greater sequence coverage [36]. ETD is also superior with respect to the detection of posttranslational modifications, as it does not result in cleavage of modifications observed with CID [37]. The ETD approach represents the most powerful tool for identification and sequencing of methylated arginine products (approximately 50 kDa maximum) because it can determine both the position and the number of methyl groups on an arginine residue, and unlike CID, it is not adversely affected by sequences with multiple arginine residues. With respect to arginine methylation, the chief disadvantage of ETD is that it cannot distinguish between aDMA and sDMA; however, this can be mitigated by simultaneous CID and ETD fragmentation as reported previously [35].

Despite the obvious advantages to the top-down approach in measuring arginine methylation, generation of diagnostic fragment ions using CID has proven useful for enzyme classification and sequence position of methylation. CID fragmentation of the previously described R1 series of peptides (Table 1), which differ by a single methyl group, yielded fragments that were unique to each peptide (Fig. 3). As noted previously, the fragment ion 46 m/z corresponding to dimethylammonium is diagnostic for peptides with aDMA and is not observed in the fragmentation pattern of peptides bearing arginine, sDMA, or MMA [38,39]. In contrast to earlier studies we do not find evidence that the fragment ion 73 m/z is diagnostic for MMA-containing peptides [35]; however, this ion is observed in abundance for the free MMA amino acid (discussed below). The fragment ion 70 m/z is not diagnostic for peptides with sDMA as has been reported [35]. In fact, we find that for the R1 series of peptides this fragment is present in R1(aDMA) and the arginine-containing R1 peptide. In addition, the fragment ion 70 m/z can be produced from arginine, MMA, aDMA, and sDMA amino acids, suggesting that it is not diagnostic for any specific type of arginine methylation [6].

Direct quantification of methylated peptides by MS

It is possible to use MS to directly detect methylation of peptide substrates for PRMTs without the need for fragmentation. Using UPLC–MS, we have already shown that it is possible to achieve

both a baseline separation of the R1 (Table 1) series of peptides that differ by a single methyl group and to quantify total methylation [15]. Using ESI without fragmentation generates several species: the molecular ion and several multiply charged species (one of which is usually the most abundant). We have found that for the R1 series of peptides the +2 mass was the most abundant so that one and two methylations produced masses +7 and +14 m/z greater than the substrate m/z . Using this technique we were able to determine the kinetic mechanism and calculate kinetic parameters for H₆PRMT6 [15]. This approach does not require any sample processing and multiple samples can be analyzed if the chromatographic run time is sufficiently short. Unfortunately, this method requires methylated peptides for generation of standard curves. Such standards must be made by chemical synthetic means, limiting the scope of substrates that can be investigated due to the synthetic boundary on peptide length (35–65 residues) using conventional techniques.

Measuring AdoHcy

The reaction scheme in Fig. 1 suggests that a simple and direct way to measure the activity of PRMTs is quantification of the coproduct AdoHcy that results from the transfer of the methyl group of AdoMet to an arginine residue in the substrate protein. We have developed two similar methods for the measurement of AdoHcy using the MS [15] and MS/MS [40] techniques, both of which have limitations and advantages.

The rate of production of AdoHcy from H₆PRMT6 with constant R1(MMA) and increasing AdoMet as measured using a UPLC–MS assay detecting AdoHcy⁺ (385 m/z) (similar to a previously described assay [15]) is shown in Fig. 4A as a Hanes–Woolf plot. Normally determination of bisubstrate enzymatic parameters requires variation of both substrates (in this case R1(MMA) and AdoMet); yet if the K_m of one substrate has been determined, then the other can be estimated by varying its concentration in the presence of saturating concentrations of the substrate with the known K_m . At 1.24 mM, the concentration of R1(MMA) is 6.7-fold higher than its previously estimated K_m of 184 μ M [15], producing an 88% saturation of H₆PRMT6 with R1(MMA). The estimated K_m for AdoMet of H₆PRMT6 is 17.4 μ M (Table 3), which is similar to 18.6 μ M that was previously determined by measuring accumulation of R1(aDMA) using an MS assay under the same conditions [15]. The similarity of the results suggests that this technique is valid

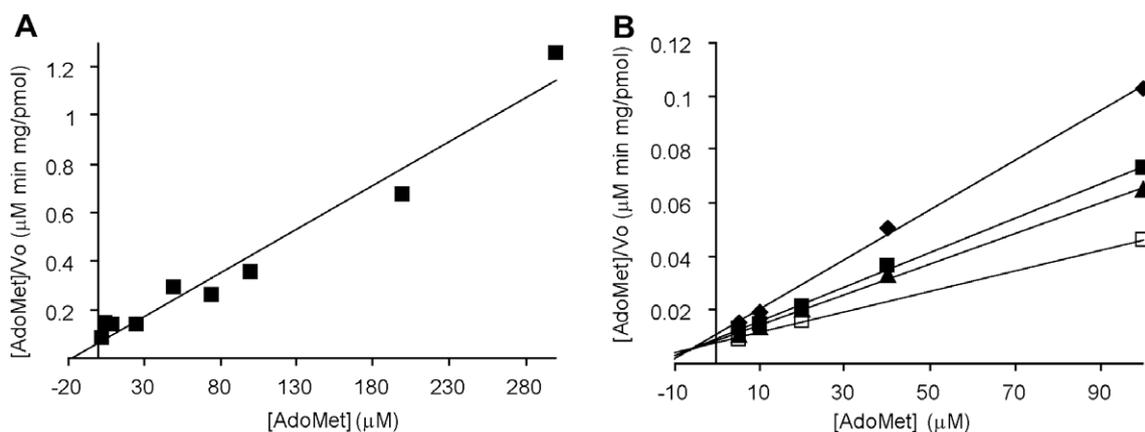


Fig. 4. Estimating kinetic parameters by measuring AdoHcy production. (A) The Hanes–Woolf plot of the rate of AdoHcy produced by H₆PRMT6. Saturating R1(MMA) peptide (1.24 mM) with (0.77 μ M) enzyme and variable AdoMet (2.5–300 μ M) in a 40- μ L volume was incubated for 90 min at 37 °C. AdoHcy was measured by UPLC–MS: selected ion recording (SIR) for 385 m/z , corresponding to AdoHcy⁺ with a cone voltage of 20 V similar to a previously described assay [15]. (B) PRMT1 (58 nM) incubated with AdoMet varied from 5 to 100 μ M at fixed GST-GAR concentrations of 1.1 μ M (◆), 2.1 μ M (■), 4.3 μ M (▲), and 25.8 μ M (□) in a total volume of 40 μ L incubated for 30 min at 37 °C. Reactions were stopped by flash freezing and samples were thawed immediately prior to analysis. Total AdoHcy was measured using a previously described UPLC–MS/MS assay [40].

Table 3
Kinetic parameters derived for PRMT1 with GST-GAR using various MS assays to detect AdoHcy and methylated arginines.

Enzyme	Substrate	Analyte	Method	X	Linear fit		Nonlinear fit	
					$K_m X$ (μM)	V_{max} (nmol/min mg)	$K_m X$ (μM)	V_{max} (nmol/min mg)
H ₆ PRMT6	R1(MMA)	AdoHcy	MS	AdoMet	17.4 ^a	0.280 ^a	19.5 ^a	0.34 ^a
GST-PRMT1	GST-GAR	AdoHcy	MS/MS	GST-GAR	1.5 ^b	2.60 ^b	1.7 ^b	2.60 ^b
GST-PRMT1	GST-GAR	AdoHcy	MS/MS	AdoMet	10.5 ^b	2.14 ^b	13.3 ^b	2.10 ^b
GST-PRMT1	GST-GAR	MMA/aDMA	MS/MS	GST-GAR	1.7 ^b	2.05 ^b	1.2 ^b	1.90 ^b
GST-PRMT1	GST-GAR	MMA/aDMA	MS/MS	AdoMet	2.7 ^b	1.88 ^b	2.0 ^b	1.70 ^b

^a Apparent value.

^b GST-GAR does not represent a single substrate but rather a family of related substrates; therefore, the constants derived are apparent values.

for estimation of K_m , but the estimated V_{max} is more than 10-fold smaller after background subtraction than the previously measured V_{max} using methylated product detection [15]. In the case of H₆PRMT6 a high background that is at least partly caused by high automethylation confounds the estimation of V_{max} , whereas the automethylation background does not affect the estimation of K_m because the low enzyme concentration remains constant as substrate concentrations are varied.

We have also developed a more sensitive UPLC–MS/MS assay for the measurement of AdoHcy, which detects the parent ion AdoHcy⁺ (385 *m/z*) and associated fragment ions (134 and 136 *m/z*) [40]. This alternate method of detecting AdoHcy provides an increase in the S/N and a lower LOQ (Supplemental Table 1) than the UPLC–MS method described above. We tested this assay with PRMT1 and GST-GAR by incubation with increasing concentrations of AdoMet and increasing fixed concentrations of GST-GAR (Fig. 4B). As expected, the intersecting lines correspond to a sequential bisubstrate enzymatic mechanism [6,15,17]. Table 3 lists the resulting enzymatic parameters derived assuming an ordered sequential Bi–Bi enzymatic mechanism (Eq. (2)).

Tandem MS assays similar to the one presented above have been described for the detection of AdoHcy in plasma [41] or enzymatic AdoHcy formation [42]. Both assays detect AdoHcy via the fragment ion 250 *m/z* corresponding to AdoHcy lacking adenine (*S*-ribosyl-homocysteine). However, we were able to achieve a low LOQ by utilizing the fragment ions described above. It should be noted that GST-GAR migrates as a series of bands on SDS–PAGE because of partial degradation during purification, and therefore does not represent a single substrate but a family of related substrates [7]. Nevertheless, GST-GAR is an established general methyl-accepting substrate for testing PRMT activity.

Despite the differences in sensitivity between the assays for detecting AdoHcy, both methods exhibit a low LOQ, high S/N, and no required sample processing. In addition, the standard for quantification, AdoHcy, is commercially available and inexpensive. Measuring PRMT activity by quantification of AdoHcy can be useful in enzyme kinetic studies where methylated protein products are used as product inhibitors. This strategy has been used successfully to determine the bisubstrate enzyme mechanism of H₆PRMT6 [15].

The chief disadvantage of detection of AdoHcy for PRMT activity measurements is high background AdoHcy signal [40]. There are at least three sources of this background including AdoHcy contamination in commercially available AdoMet, automethylation, and the nonenzymatic pseudo-first-order AdoHcy formation from AdoMet. Once AdoMet is purified according to established methods [6], the most important source of background AdoHcy signal is from automethylating enzymes such as PRMT1, PRMT4, PRMT6, and PRMT8 [9,14]. We have previously demonstrated that the nonenzymatic pseudo-first-order formation of AdoHcy from AdoMet is promoted by high pH and temperature, and is likely caused by the transfer of methyl groups from AdoMet to water forming methanol [40]. While disconcerting, this source of AdoHcy represents less than 15% of the total background for reactions with 58 nM PRMT1. Whatever its source, high background AdoHcy can mask

the activity of PRMTs; therefore, in order to successfully implement a technique that detects AdoHcy, no-substrate controls are required for each concentration of AdoMet used and their subsequent subtraction from experimental samples.

The strategy of AdoHcy detection for the measurement of PRMT activity has been used successfully, albeit indirectly, by means of a coupled photometric assay [43]. This assay is capable of determining the K_m values for PRMT enzymes, and the results are generally in the same range for similar length peptide substrates as we have observed with PRMTs 1, 2, and 6 [6,15]. The authors experienced similar difficulty with high background signal as we did, and recommended against the use of AdoMet concentrations higher than 250 μM [44]. Thus, the assay was deemed unable to accurately measure V_{max} where the K_m for AdoMet was high such that saturation could not be achieved with less than 250 μM AdoMet. We have not found this restriction necessary when controls lacking protein substrate for each concentration of AdoMet are employed and the AdoMet is purified. Nevertheless, the subtraction of background signal results in an increased error at each measurement that must be accounted for.

Other assays have been developed for the detection of AdoHcy, but not for the expressed purpose of measuring *in vitro* PRMT activity [45,46]. Adaptation of these assays to the study of PRMT activities should be relatively straightforward. For example, the activity of protein isoaspartyl methyltransferase has been measured by quantification of AdoHcy using HPLC [45,46]. As with all assays used for AdoHcy detection that we have discussed, persistent background signals can be problematic and must be addressed accordingly [46].

Detection of MMA, aDMA, and sDMA from PRMT catalysis

The products of PRMT-catalyzed reactions are methylated arginine residues (Fig. 1); therefore, qualitative and quantitative information can be garnered by measuring the accumulation of these methylated amino acids. The easiest way to affect quantitative recovery of these amino acids from the protein product is complete acid hydrolysis of all peptide bonds using 6 N HCl *in vacuo* for 24 h at 110 °C. Direct measurements of all methylated arginine species allows for determination of the initial rates of reactions for enzymes, kinetic analysis, and determination of the type of PRMT (i.e., type I or II), as well as providing insight into the mechanism of multiple methylations. Following acid hydrolysis, methods of separation and detection are required for analysis of recovered methylated arginine amino acids. This usually entails a form of chromatography such as TLC, FPLC, HPLC, or UPLC for which the method of detection can include radioactivity, fluorescence, or MS.

Detection of derivatized methylated arginines

Separation of derivatized amino acids via HPLC and detection using fluorescence is an established technique that has been applied to measurement of methylated arginines in plasma since they are markers for cardiovascular disease [47–49]. We have adapted

one of these techniques utilizing fluorescent OPA derivatization, except here we derivatize hydrolysates of PRMT methylated protein substrates. Depicted in Fig. 5A is a chromatogram of acid-hydrolyzed reactions of PRMT2 with [methyl- ^{14}C]AdoMet and GST-GAR and controls without enzyme or substrate that have been derivatized with OPA and separated by analytical RP-HPLC similar to a previously described method [49]. OPA derivatization is used primarily as a means to increase retention time of the highly polar methylated amino acids, which would otherwise elute in the void

volume. However, radioactivity was used as the method of detection, rather than fluorescence since it does not require an additional solid-phase extraction step to remove other amino acids [49]. The presence of these other amino acids, which are also derivatized with OPA, could confound the interpretation of the fluorescence chromatograms. The sample included MMA, aDMA, and sDMA standards, and UV absorbance was used for detection of the standards rather than fluorescence. Under these conditions it is possible to achieve a baseline separation among MMA, aDMA, and sDMA [49]. Methylation activity was detected using radioactivity and the trace showing the radioactivity of fractions collected from this sample reveal the presence of peaks with retention times consistent with MMA and aDMA, confirming that PRMT2 is a type I enzyme (Fig. 5A). In addition, we find that approximately 3-fold more MMA is produced than aDMA, consistent with previous results using MS [6]. The controls with either PRMT2 or GST-GAR failed to show significant radioactivity, confirming that MMA, aDMA, or sDMA cannot be made spontaneously from [methyl- ^{14}C]AdoMet and substrate (GST-GAR) alone (Fig. 5A). Derivatization with OPA has the advantage of being rapid and complete, and the chromatographic separation times using this method are relatively short (9 min), making it possible to readily process multiple samples.

A potential obstacle with derivatization using OPA is the relatively short half-life of OPA amino acids formed in the presence of mercaptoethanol, necessitating the refrigeration of samples and rapid analysis for accurate quantitation [47]. This difficulty can be mitigated by the use of mercaptopropionic acid with OPA [48] or the derivatizing agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor) [47]. Both techniques produce more stable amino acid derivatives [47]. For the experiments detailed in Fig. 5A derivatization with mercaptoethanol was initiated 1 min before injection. The qualitative nature of this experiment together with the short interval between reaction and injection limits the importance of the decay of OPA-derivatized amino acid.

High-resolution cation exchange separation of methylated arginines

The additional step of derivatization of methylated arginines can introduce variability that would not be present if one could directly measure the amino acids. A near baseline separation of MMA, aDMA, and sDMA has been achieved with high-resolution cation exchange chromatography using cross-linked sulfonated polystyrene resin [5,11,50]. This technique is exemplified by Fig. 5B, which demonstrates the separation of an acid-hydrolyzed reaction of PRMT2 with [methyl- ^3H]AdoMet and GST-GAR as well as two similar controls lacking either enzyme or substrate. Two peaks of radioactivity can be observed and the retention times are consistent with aDMA and MMA, confirming type I activity for PRMT2 using an independent technique [6]. However, unlike the assay with derivatized methylated amino acids described above and previous results [6], we find that the aDMA and MMA are formed in approximately equal proportion (the MMA:aDMA ratio is ~ 1.15 , taking into account that aDMA produces 2-fold more radioactivity than MMA). This may be explained by the shorter incubation time and/or the much lower concentration of AdoMet used ($0.66\ \mu\text{M}$ as opposed to $112.5\ \mu\text{M}$). This method has the advantage of being an established technique utilized to determine the many types of arginine methylation [50]. However, the separations of aDMA and sDMA are not at baseline and the chromatographic run time is relatively long (greater than 1 h), making the processing of multiple samples more labor-intensive. As with the OPA method described above, it is important to perform controls of enzyme alone or substrate alone.

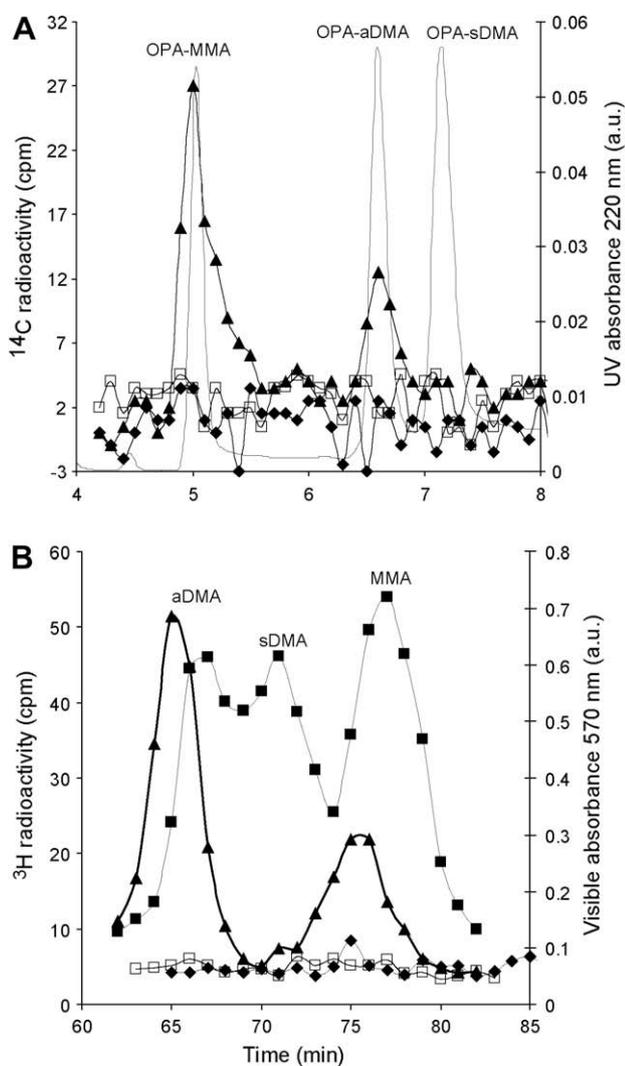


Fig. 5. Comparison of reverse-phase HPLC and ion exchange methods of separation of methylated arginines derived from hydrolyzed reactions of PRMT2 with GST-GAR. (A) PRMT2 ($0.19\ \mu\text{M}$) with GST-GAR ($0.9\ \text{mg/mL}$ total protein) and $112.5\ \mu\text{M}$ ($0.23\ \text{kBq}/\mu\text{L}$) [methyl- ^{14}C]AdoMet in a $100\text{-}\mu\text{L}$ volume incubated for 16 h at $37\ ^\circ\text{C}$ was acid-hydrolyzed, derivatized with OPA and mercaptoethanol, and injected immediately onto a SunFire $4.6 \times 150\ \text{mm}$ C18 HPLC column. Shown are radioactivity from ^{14}C methyl groups transferred in counts per minute (cpm) for PRMT2 with GST-GAR (\blacktriangle), PRMT2 alone (\square), and GST-GAR alone (\blacklozenge), as well as the UV absorbance at $240\ \text{nm}$ from OPA-derivatized standards MMA, aDMA, and sDMA (continuous gray line). (B) PRMT2 ($1.0\ \mu\text{g}$ total protein) with GST-GAR ($1.5\ \mu\text{g}$ total protein) and $0.66\ \mu\text{M}$ ($1.97\ \text{kBq}/\mu\text{L}$) [methyl- ^3H]AdoMet (Perkin Elmer) in a final volume of $308\ \mu\text{L}$ was incubated at $37\ ^\circ\text{C}$ for 1 h, TCA-precipitated, acid-hydrolyzed and separated on a sulfonated polystyrene cation exchange column. Fractions were collected and mixed with scintillation fluid and radioactivity was measured on a scintillation counter. Shown is the radioactivity from ^3H methyl groups transferred in cpm for PRMT2 with GST-GAR (\blacktriangle), PRMT2 alone (\square), and GST-GAR alone (\blacklozenge). The standards MMA, aDMA, and sDMA were spiked into the samples and postcolumn-derivatized with ninhydrin [59] and Ruhemann's purple is detected at $570\ \text{nm}$ (\blacksquare , gray line).

Separation of methylated arginines using TLC

Radioactive methylated arginine amino acids derived from acid hydrolysis of reactions with PRMTs have also been separated using silica gel and cellulose TLC in one and two dimensions [11,15,51,52]. Depending on the choice of stationary and mobile phases a baseline separation among arginine, MMA, aDMA, and sDMA can be achieved. As with other techniques that measure radiolabeled methylated arginines the signal intensity is higher than from the intact protein product because the signal is concentrated in two analytes (MMA and either aDMA or sDMA) rather than a series of discrete, multiply methylated products. This effect is dramatic when comparing the radioactive signal from methylated amino acids separated by TLC to methylated protein bands separated by gel electrophoresis, as illustrated in Fig. 6. A comparison of methylation reactions with PRMT4 using various substrates separated by tricine SDS–PAGE gel [27] (Fig. 6A, top) or acid-hydrolyzed and separated by TLC using a recently described assay that includes a sample cleanup step [6,15] (Fig. 6A, bottom) reveals that activity that can be difficult to detect by gel can be more readily observed as hydrolysates separated via TLC. For the substrate peptides H3 tail [53], H4 tail [6,31], and Tat [54] corresponding to lanes 2, 3, and 5, respectively, the radioactive signals are 3.0-, 1.4-, and 1.7-fold higher than background for a gel phosphor image, whereas the signals are 27-, 12-, and 13-fold higher than background for MMA and 69-, 6.0-, and 4.5-fold higher for aDMA, respectively, on a TLC plate phosphor image (Fig. 6A). This disparity in signal is even more dramatic when one considers that only 10% of the hydrolyzed reaction was applied to the TLC, whereas the entire reaction mixture was loaded onto the gel. Similar results are observed with GST-PRMT6 and the same substrates (Fig. 6B). As described above these results are at least partly explained by the

fact that the radioactive signals from small peptides are concentrated into two spots on TLC (MMA and either aDMA or sDMA) rather than a diffuse band on SDS–PAGE gels. On average, the radioactivity from a diffuse band of a small peptide (1.3 kDa) on an SDS–PAGE gel is spread out over approximately 6 times more area than on TLC. In addition, it is possible that small peptides diffuse out of the gel matrix during fixing, staining, and destaining, giving the appearance of low total methylation. However, it has also been suggested that small peptides do not bind Coomassie blue dye well giving the appearance of less peptide in the gel while also being consistent with low total methylation [27,55,56]. Furthermore, we cannot rule out the possibility that differences in image transfer via ionizing radiation onto storage phosphor screens exist between gels and TLC plates due to differences in their matrices, and that this may account for the improved signal detection for TLC plate phosphor images.

As with other methods that separate methylated amino acids from acid-hydrolyzed PRMT reactions the TLC technique can allow for quantification of MMA, aDMA, and sDMA [15]. Independent measurement of these methylated amino acids provides important information that is not revealed by electrophoretic analysis. In this case we observe that, similar to PRMTs 1 and 2, the relative production of MMA to aDMA is determined by the substrate [6]. For PRMT4 more aDMA is produced than MMA with the H3 tail substrate, but the opposite ratio of aDMA to MMA is observed for histone H4, H4 tail peptide, SmB peptide [57], and Tat peptide by densitometry. With GST-PRMT6 more aDMA is produced than MMA with histone H4, but the opposite ratio of aDMA to MMA is observed for H4 tail, H3 tail, SmB, and Tat peptides.

Despite the qualitative and quantitative information garnered using TLC as a method of separation for MMA, aDMA, and sDMA amino acids, some limitations to this technique exist. For example, detection of methylated arginines requires the use of radioactive AdoMet since generalized visualization methods such as ninhydrin detect nearly all amino acids in a protein hydrolysate, and the signal from these species can obscure the signal from the relatively small number of methylated arginine residues. In addition, elaborate cleanup steps are required to remove the hydrolysis products of radioactive AdoMet that can confound interpretation of TLC data [6,15] (see Supplemental Fig. 1. for the whole TLC plate). Although multiple samples and TLC plates can be developed and exposed to storage phosphor screens simultaneously, the protracted TLC development times, hydrolysis, and cleanup steps make this technique time consuming. Finally, quantification must be indirect. For example, we have used radioactive AdoMet as a standard for densitometry since radiolabeled MMA, sDMA, and aDMA are expensive and not widely available.

Quantification of methylated arginines using mass spectrometry

We have described a UPLC–MS/MS assay that identifies the parent mass for MMA⁺ and diagnostic daughter ions, as well as the parent mass for DMA⁺ and a daughter ion diagnostic for aDMA. This technique is, therefore, capable of unambiguous detection and quantification of MMA, aDMA, and sDMA [6]. Furthermore, the relatively short chromatographic separation time (less than 5 min) of this technique and relative stability of methylated amino acids allows for the processing of multiple samples, making it ideal for kinetic characterization of PRMT enzymes. We tested this assay with PRMT1 incubated with increasing concentrations of AdoMet and fixed concentrations of GST-GAR (Fig. 7A). This allowed for a direct comparison of this assay with the UPLC–MS/MS method for AdoHcy detection in the experiment described in Fig. 4B. Accordingly, Fig. 7A shows a family of intersecting lines indicative of a sequential bisubstrate enzymatic mechanism that is similar to that depicted in Fig. 4B. In addition, the enzymatic parameters de-

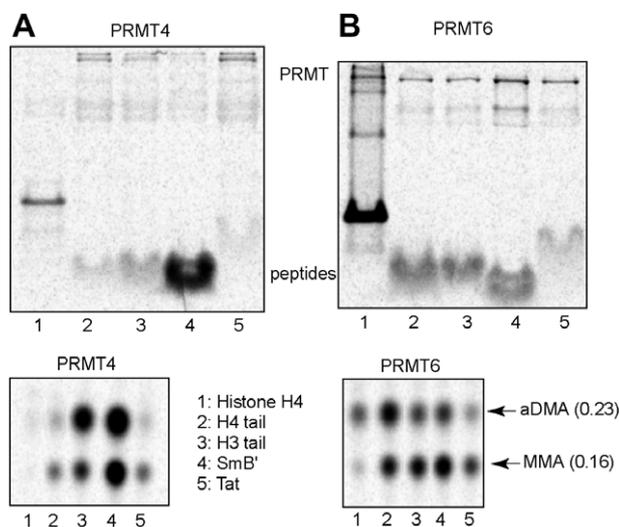


Fig. 6. Comparison of tricine SDS–PAGE and TLC methods for measuring methyltransferase activity using PRMTs 4 and 6. Storage phosphor screens exposed to 16.5% tricine SDS–PAGE gels [27] (top) from half of the reactions with 9.8 μ M histone H4, or 100 μ M H4 tail, H3 tail, SmB', or Tat synthetic peptides (lanes 1 through 5, respectively) (Table 1) with 112.5 μ M (0.23 kBq/ μ L) [*methyl*-¹⁴C]AdoMet in a final 40- μ L volume for 16 h with (A) PRMT4 (0.3 mg/mL total protein) or (B) PRMT6 (0.75 mg/mL total protein). The other half of the corresponding reactions were acid-hydrolyzed into amino acids, 10% of the hydrolyzed material was separated via TLC, and the resulting TLC plates were exposed to storage phosphor screens (lower panels). The mobile phase was 75% ethanol and 25% concentrated ammonium hydroxide. The retardation factor (R_f) values are shown in parentheses and are consistent with aDMA and MMA as shown previously [15]. The lanes in the TLC have been rearranged to make the order consistent with the corresponding gel (the entire TLC plate is shown in Supplemental Fig. 1.). Both the gel and TLC images have been adjusted for brightness and contrast to aid in visualization.

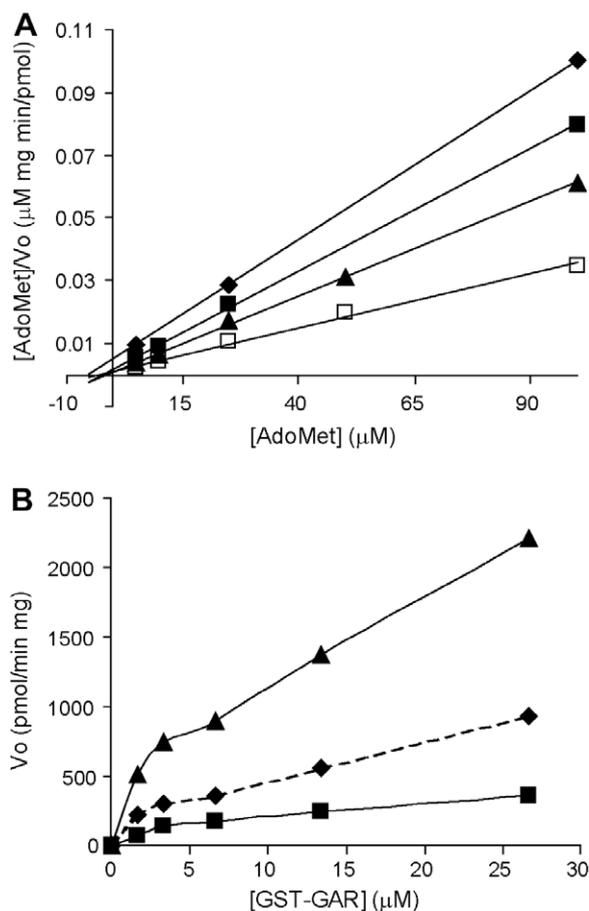


Fig. 7. Detection of MMA and aDMA from acid hydrolysis of reactions with PRMT1 and GST-GAR using UPLC-MS/MS. (A) Hanes-Woolf plots of the initial velocity derived by detection of MMA and aDMA and total methyl groups transferred are calculated as $2(\text{aDMA}) + \text{MMA}$. PRMT1 (58 nM) with AdoMet varied from 5 to 100 μM at fixed GST-GAR concentrations of 1.7 μM (◆), 6.7 μM (■), 13.3 μM (▲), and 26.7 μM (□) in a total volume of 40 μL was incubated for 30 min at 37 °C. Reactions were stopped by flash freezing followed by lyophilization, acid hydrolysis, and reconstitution in initial mobile phase conditions 0.1% aqueous formic acid and 0.05% TFA. (B) The production of MMA (■), aDMA (◆), and total methylation (▲) with varying concentrations of (GST-GAR) and 5.0 μM AdoMet was measured using the assay in (A) with PRMT1 (58 nM). The concentration of GST-GAR was determined by amino acid analysis, and is given in micromoles per liter of arginine residues derived from the entire GST-GAR sequence, even though not all of these residues are known to be methylated.

rived by the detection of MMA and aDMA are largely congruent with those derived by the UPLC-MS/MS detection of AdoHcy (Table 3). The exception is the AdoMet K_m value, which appears to be 3-fold higher where AdoHcy is detected; however, the AdoMet K_m value derived using detection of MMA and aDMA is similar to that derived for PRMT1 using the same assay where histone H4 is the substrate [6]. The higher AdoMet K_m value derived from the assay for AdoHcy detection may be caused by error introduced from background subtraction, or by the minimum AdoMet substrate concentration being above the expected K_m . We also note that the enzyme kinetic parameters derived using linearized Hanes-Woolf plots or nonlinear least squares fitting to the Michaelis-Menten equation for a Uni-Uni reaction for each fixed substrate and varied substrate are similar (Table 3).

As with other methods that can detect methylated amino acids derived from acid hydrolysis of PRMT methylated protein products, this assay can independently measure MMA and aDMA. We have already shown that this additional information is useful (Figs. 5 and 6). Initial production of MMA, aDMA, and total methylation produced by PRMT1 with varying concentrations of GST-GAR and

5.0 μM AdoMet are shown in Fig. 7B. As reported previously, aDMA is produced at a greater rate than MMA [6], and this is true at all concentrations of GST-GAR used (data not shown). These results along with the TLC data (Fig. 6) are congruent with our earlier work showing that the substrate and not the enzyme determine the relative amounts of aDMA and MMA for type I PRMTs [6].

The UPLC-MS/MS assay for detection of MMA, aDMA, and sDMA was further validated by quantification of the recovery of MMA and aDMA from the acid hydrolysis of the previously described methylated peptides R1(MMA) and R1(aDMA) (Supplemental Table 2). The results show quantitative recovery of MMA and aDMA from 0.005 to 0.1 μM methylated peptide with a minimum S/N of 23, indicating that little loss of detectable methylated arginine occurs during hydrolysis, reconstitution, and analysis. Furthermore, we have already shown that the LOQ of standards MMA and aDMA are 0.7 and 0.47 nM, respectively, but that experimental samples often exhibited noise associated with them that necessitated rejection of data with a S/N of less than 3 [6]. The background noise associated with experimental samples can be reduced by a cleanup step prior to hydrolysis. Matched samples of PRMT1 incubated with GST-GAR and AdoMet were precipitated with 15% TCA, or the dried samples washed with 95% acetone, and we found that the chromatogram peak areas for control and TCA-precipitated samples were similar, but a wash step with 95% acetone increased peak area and S/N slightly (Supplemental Table 3). However, we have found that the increase in peak area and S/N afforded by a 95% acetone wash cannot always justify the increased time for processing the samples, which could be as much as an additional 16 h, depending on the substrate.

We have already observed that some fragment ions detected with the UPLC-MS/MS detection of MMA and aDMA are diagnostic for each methylated amino acid. Accordingly we, along with others, have observed that the fragment ion 46 m/z always appears to be present in aDMA-containing peptides and proteins, as well as the aDMA amino acid [6,35,38,39]. Despite the discrepancies in the predictive validity of other fragment ions from peptides and proteins, it is possible to differentiate all methylated arginine amino acids by the UPLC-MS/MS method described above because MMA and DMA are easily distinguished by the molecular weight of the parent compound (difference of 14 Da), and the fragment ion 46 m/z differentiates aDMA and sDMA. In addition, the baseline chromatographic separation of aDMA and MMA (0.5 min) provides an added level of certainty in differentiation between MMA and DMA [6].

As noted above, the fragment ion 70 m/z is not diagnostic for peptides with any particular methylated arginine, and when methylated amino acids are fragmented under similar conditions this fragment ion is observed with arginine, MMA, aDMA, and sDMA, indicating that it represents a structure common to all four amino acids [6]. These findings are in agreement with previous studies on methylated peptides [39]. However, it should be noted that fragment ions do not need to be diagnostic to be chosen for quantification in MS/MS assays. Their presence in the fragmentation patterns from the parent mass justifies their use for quantification.

Since this UPLC-MS/MS method detects methylated arginine amino acids, quantification is readily achieved with commercially available MMA, aDMA, or sDMA standards regardless of substrate and without the need for radioactive AdoMet. Any other method of quantification would require a unique set of methylated peptide standards for each substrate of interest for a truly robust quantification. Moreover, such methylated peptide standards must be made by chemical synthetic techniques, which are only capable of making short peptides, thus limiting the scope of substrates that can be investigated.

As with other methods that detect methylated arginine amino acids the key disadvantages of this technique are the time (24 h)

required for acid hydrolysis and the irreversible conversion of all proteins in the reaction mixture to component amino acids. These techniques also cannot differentiate between automethylation and substrate methylation. For the measurement of total methylation activity an additional error is introduced by summing the initial rates of formation of MMA and aDMA; nevertheless, this error can be accounted for by the appropriate propagation of errors.

Comparison of PRMT activity and substrate specificity

When comparing PRMT substrate specificity and activity, the methyl-accepting peptide or protein is usually the focus of interest. The methyl donor AdoMet is often overlooked in these situations, yet its concentration is critical to the interpretation of experimental data. As we have suggested above, one can estimate the apparent K_m values for a given methyl-accepting substrate by varying its concentration in the presence of a fixed saturating AdoMet concentration. However, if insufficient AdoMet is included in the reaction, V_{max} values are not reached and the apparent K_m values for methyl-accepting substrates may differ from the actual value obtained in the presence of saturating AdoMet. We have identified variation in the K_m values of AdoMet for PRMTs 1, 2, and 6 that range from 1.0 to 18.6 μM [6,15]. Whereas an AdoMet concentration of 8.0 μM would be sufficient to saturate PRMT1 to 90%, nearly 150 μM AdoMet would be needed to saturate PRMT6 to 90%. The issue of K_m for AdoMet can also be problematic when using [*methyl*- ^3H]AdoMet, which is commercially available at a concentration of 6.6 μM (Perkin Elmer; NET155H001MC) or 12–17 μM (GE Healthcare; TRK581). AdoMet at the undiluted concentration of 6.6 μM would produce 87% PRMT1 enzyme saturation, but only 26% PRMT6 enzyme saturation. This could lead to an underestimation of the activity of enzymes with low affinity for AdoMet and potentially erroneous conclusions about substrate specificity. For example, it has been recently reported that unlike PRMT1, recombinant forms of PRMT4 were unable to methylate GST-GAR using either 0.8 or 4.5 μM [*methyl*- ^3H]AdoMet [58]. However, we have shown that PRMT4 can readily methylate GST-GAR when 112.5 μM [^{14}C]AdoMet is used [6]. To remedy this issue, we suggest combining a fixed concentration of unlabeled AdoMet with [*methyl*- ^3H]AdoMet to achieve a higher overall AdoMet concentration to saturate the enzyme [14]. This is not required for [*methyl*- ^{14}C]AdoMet, which despite its much lower specific activity is available as a concentrated solution of ~ 450 μM (GE Healthcare; product number CFA360).

Conclusion

We have outlined a number of methods that can be used for quantitative and qualitative analysis of PRMT activity. Each method has unique advantages and disadvantages, which can help determine the choice of assay. The method of gel electrophoresis separation of radioactively methylated samples and subsequent exposure to storage phosphor screens has the advantage of being easy to perform without processing, but the radioactivity is difficult to directly quantify and this method is not suited for enzymes with low activity or substrates that are poor methyl-acceptors. Mass spectrometry of intact peptide samples can be used for quantitation, and the use of ETD-MS sequencing can determine the type and the position of methylation within the substrate sequence. Theoretically, with ETD-MS it is possible to simultaneously detect the position and type of many posttranslational modifications in addition to arginine methylation. We have, along with others, cautiously used methods that detect AdoHcy to quantify PRMT activity. We find that parallel, no-substrate controls are required for accurate quantitation of PRMT activity. This approach is useful for the determination of enzymatic mechanism using product inhi-

bition assays. For quantitation of PRMT activity we generally prefer methods that use acid hydrolysis followed by independent detection of MMA, aDMA, and sDMA despite the increase in processing time. We have shown that some of these methods are less time consuming and more selective than others. In general, we prefer to use UPLC-MS/MS because of its unambiguous differentiation of MMA and aDMA, its very low LOQ, and its general applicability to any PRMT and substrate combination without the need for methylated standards or radioactivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.09.021.

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