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Analysis of phase II metabolites of methamphetamine by solid-phase extraction and liquid chromatography with tandem mass spectrometric detection

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1. Introduction

(+)-Methamphetamine (METH) abuse is one of the most serious health problems in the United States and Europe due to its addictive properties and potential neurotoxic effects [1, 2, 3]. METH abuse appears to have stabilized or decreased slightly in the general populations, however abuse in certain populations is increasing [4, 5]. Treatments for drug abuse are often tested in animal behavioral pharmacology models. One of the first *in vivo* tests conducted in animals is to follow the concentration of the drug and its metabolites as a function of time. In this way, one can determine how long a potential medication must be active and one can decide if specific tissues are potentially better targets for medication.

Glucuronidation and sulfation are important phase II reactions in the biotransformation of xenobiotics for proper excretion from the body [6]. These phase II compounds are generally biologically less reactive than the parent molecules; however, some studies suggest that when conjugated, the compounds are more active than the parent molecules [7]. Compounds such as morphine-6β-glucuronide, a metabolite of morphine, and minoxidil sulfate, a metabolite of minoxidil, more commonly known as Rogaine, are examples where the phase II metabolites of these compounds are

bioactive [6, 7]. These studies show that in some cases, difference in potency between the parent and conjugated molecules can be up to 100-fold [6]. The biological activity of the phase II metabolites of methamphetamine is unknown.

For further research on the disposition of these compounds in the human body, standards must be available. Rats have been shown to undergo similar pathways as humans when metabolizing methamphetamine, only differing in the concentrations of the glucuronide and sulfate conjugates they produce [8]. Therefore, rats can be used to produce the standards. Isolation of the standards from rat urine can be performed by solid phase extraction. Further purification can be performed by a higher resolution liquid chromatography. Four different methods for solid phase extraction were evaluated for their ability to isolate glucuronide and sulfate metabolites of METH. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to evaluate the effectiveness of each isolation procedure.

Figure 1: METH Metabolism in Humans and Rats

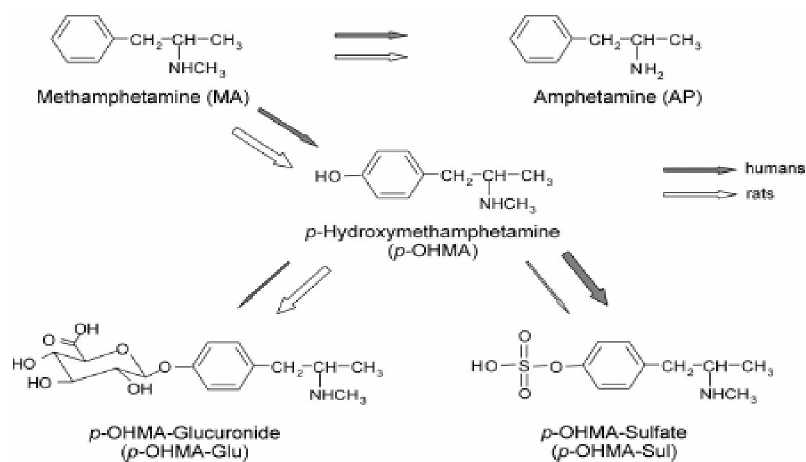


Figure 1. Proposed main metabolic pathways for MA in humans (➔) and rats (⇨).

Proposed pathway for the metabolism of METH in rats and humans [8]

2. Methods

2.1 Chemicals and reagents

The internal standard, Methamphetamine-D5, was purchased from Sigma (Saint Louis, MO). LC-MS grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburg, PA). Water was purified before use with a Millipore Milli-Q Synthesis A10 system (Millipore, Bedford, MA). All other reagents used were purchased from Fisher Scientific (Pittsburg, PA).

2.2 Urine collection

Male Sprague Dawley rats were administered 10 mg/kg-day METH using subcutaneous osmotic minipumps. Rats were placed in metabolism cages for 4 days with free access to food and water. Urine samples were collected twice a day and stored at -20˚C. METH was obtained from the National Institute on Drug Abuse (Bethesda, MD).

2.2 Extraction method 1

This extraction method was adapted from the Chen et al. method for sample preparation [9]. Sep-Pak C_{18} cartridges (1ml x 100mg) were pretreated with 10ml methanol, 5ml 25% of acetonitrile in 10mM phosphate buffer (pH 2.1), and 10ml distilled water. Urine (0.5ml) was mixed with 50µl of 4µg/ml of the internal standard methamphetamine-D5 and 3ml of 0.5M ammonium sulfate buffer (pH 9.3). The urine was then passed through a pretreated cartridge. The cartridge was washed with 5ml of 5mM ammonium sulfate buffer (pH 9.3) and 0.5ml of distilled water. The compounds

were then eluted with 2ml of 25% acetonitrile in 10mM phosphate buffer (pH 2.1). The eluate was mixed with 3ml of 0.5M ammonium sulfate buffer and passed through a new pretreated cartridge. The cartridge was washed with 5ml of 5mM ammonium sulfate buffer and 0.5ml of distilled water. The compounds were then eluted with 2ml of methanol and concentrated to about ~300μl under nitrogen stream at 40˚C. The sample was then injected into a small centrifuge tube and methanol was used to balance the volumes of the samples. After centrifuging for 5 min at 14000 rpm, the samples were analyzed using LC-MS/MS.

2.3 Extraction method 2

Urine (0.5ml) was mixed with $Ba(OH)_2$ starting at 100μl in the first sample then increased by 50μl in each sample up to 450μl in the last sample. The samples were centrifuged for five minutes at 14000 rpm. Sep-Pak C_{18} (1ml x 100mg) cartridges were pretreated with 10ml of methanol, 5ml of 25% acetonitrile in 10mM phosphate buffer (pH 2.1), and 10ml of distilled water. The samples were subjected to method 1 extraction steps and analyzed using LC-MS/MS.

2.4 Extraction method 3

Six Sep-Pak C18 cartridges were pretreated with 1.0ml of methanol and 2ml of 50mM sodium dihydrogenphosphate adjusted to pH 7.0 with sodium hydroxide. Urine (0.5ml) was mixed with 0.1ml of 2.5mM sodium 1-heptanesulfonate and applied to a pretreated cartridge. The cartridges were rinsed twice with 0.5ml of 8% methanol in 0.2M carbonate buffer (pH 11) and once with 0.5ml of 5% methanol in 50mM carbonate buffer (pH10). The compounds were then eluted with 300μl of 85% methanol in 2M

phosphoric acid. The samples were centrifuged for 5 minutes at 14000 rpm then analyzed using LC-MS/MS.

2.4 Extraction method 4

This extraction method was adapted from the method described by Strahm et al. [10]. SPE Oasis WAX mixed-mode polymeric anion-exchange cartridges (6ml) were pretreated with 2ml of 2M acetate buffer (pH 5.2). Urine (4ml) was mixed with 2ml of 2M acetate buffer (pH 5.2) and passed through a pretreated cartridge. The cartridge was then washed with 2ml of distilled water. For the glucuronide fraction, the compounds were eluted with 8ml of methanol/formic acid 10% in water (95:5). The cartridge was then washed with 2ml of methanol/ammonium hydroxide 5% in water (20:80). The sulfates were then eluted using 8ml of methanol/ammonium hydroxide 5% in water (90:10). Both fractions were evaporated under a nitrogen stream to dryness. The glucuronide fraction was re-dissolved in 200µl of methanol/acetic acid 1% in water (50:50) and the sulfate fraction was re-dissolved in 200µl of methanol/acetic acid 1% in water (20:80). The samples were analyzed by LC-MS/MS.

2.5 Acid Hydrolysis

This experiment was adapted from the Kazuaki Shimosato et al. method for hydrolysis of the conjugates [11]. Urine (1ml) was mixed with 1ml of 12M hydrochloric acid and incubated at 60˚C for 4 hours while sonicating. The samples were then mixed with 2ml of 2M acetate buffer (pH 5.2) and the pH of the sample was adjusted to approximately 5 using sodium hydroxide. Controls were then prepared using 1ml of

water and 2ml of 2M acetate buffer (pH5.2). Method 4 extraction was then performed on the samples and the fractions were analyzed using LC-MS/MS.

2.6 Blank Urine Samples

In order to determine whether or not the compounds detected were endogenous or were the result of dosage with METH, a method 4 extraction was performed on rat urine from rats that were not administered METH. The samples were then analyzed using LC-MS/MS.

2.7 Instrumentation

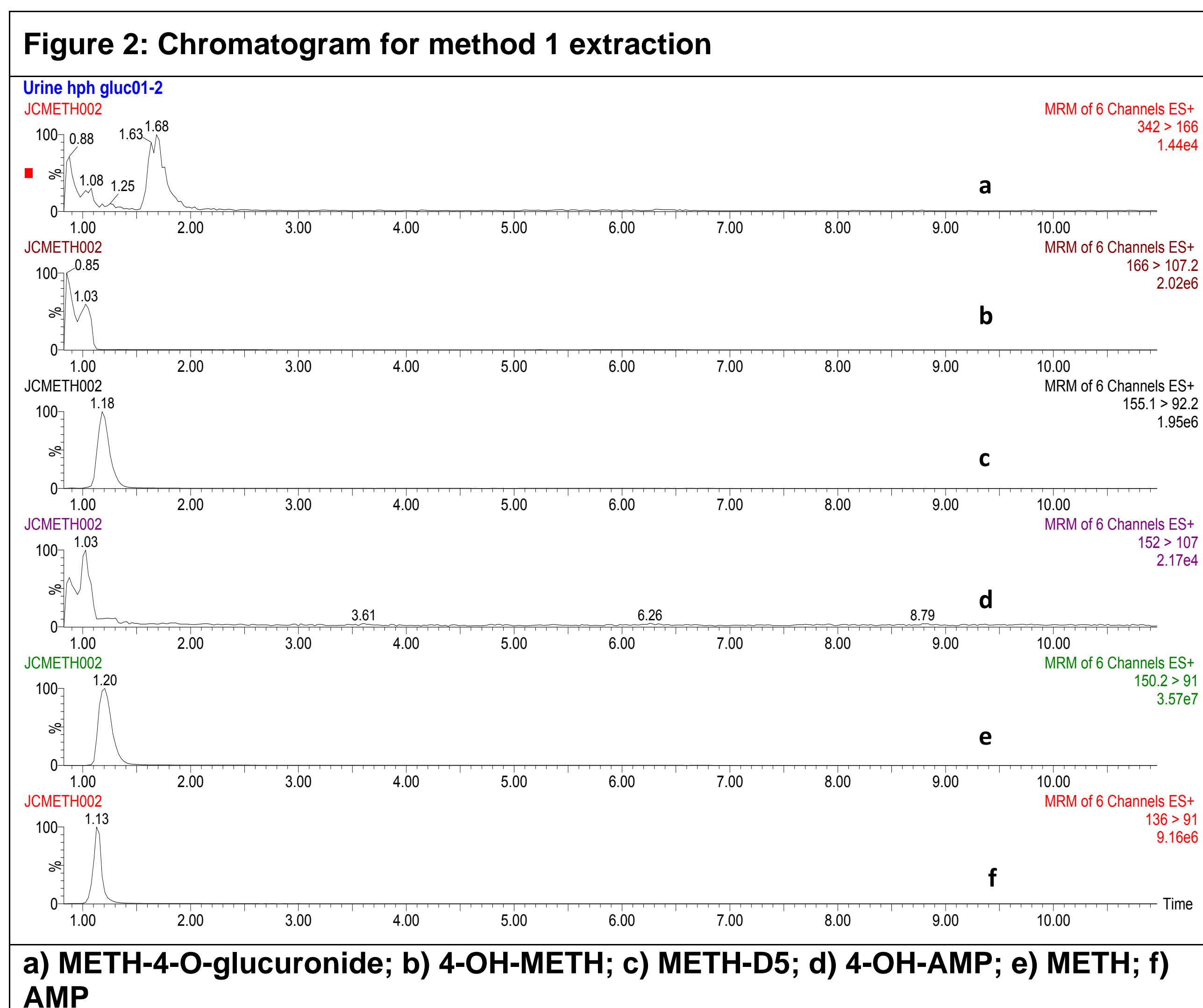
The LC system used was a Shimadzu LC 10AD paired to a Shimadzu SIL-HTA autosampler. The mass spectrometer used was a Quattro Premier triple-quadrupole fitted with a Z-spray interface with an ESI source operating in positive ion mode. A phenyl-hexyl-column maintained at 35˚C was used for separation. Mobile phase A was 20mM ammonium formate (pH 2.7) with 28% acetonitrile and mobile phase B was 20mM ammonium formate (pH2.7) with 95% acetonitrile. The flow rate was 0.4 ml/min. The linear gradient was as follows: 0 - 3 min, 0% B; 3 – 5 min: 0% B; 5 – 8 min: 100% B; 8 – 11 min: 100% B. The MS/MS experiments were performed by collision-induced dissociation with argon as the target gas (2×10^{-3} torr). METH-4-O-Glucuronide, METH-4-O-Sulfate, METH-D5, METH, AMP, 4-OH-METH, and 4-OH-AMP were quantitated using the following precursor → product m/z values: 342 → 166, 245.1 → 165.1, 155.1 → 92.2, 150.2 → 91, 136 → 91, 166 → 107.2, and 152 → 107 respectively.

3. Results

3.1 Method 1 results

For method 1, the mass spectrometer was set to detect peaks of METH-4-O-glucuronide, 4-OH-METH, METH-D5, 4-OH-AMP, METH, and AMP. The chromatogram of a method 1 extracted sample is shown in Figure 2.

Figure 2: Chromatogram for method 1 extraction



a) METH-4-O-glucuronide; b) 4-OH-METH; c) METH-D5; d) 4-OH-AMP; e) METH; f) AMP

The peaks of six samples from extraction method 1 were integrated and the average retention times and peak areas of the samples are provided in Table 1.

Table 1: Method 1 LC-MS/MS Results

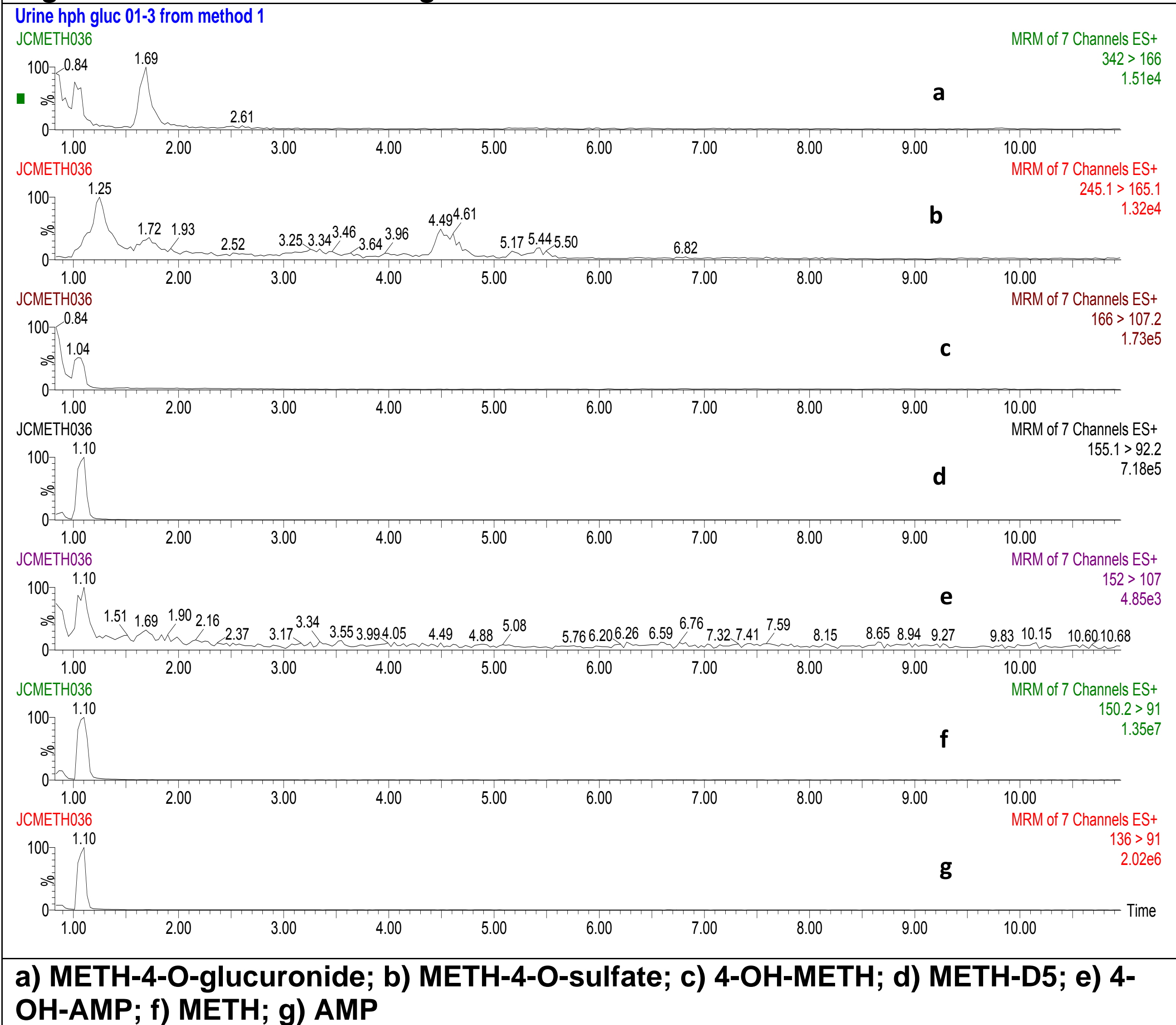
	Compound											
	342 > 166 4-O-METH-G		166 > 107 4-OH-METH		155 > 92 METH-D5		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
Average of 8 Samples	1.65 ± 0.02	1400 ± 679	0.97 ± 0.12	103208 ± 5291	1.20 ± 0.05	234608 ± 74436	1.04 ± 0.02	1532 ± 503	1.22 ± 0.06	40777211 ± 1249912	1.15 ± 0.04	628277 ± 293376

t_R is the retention time; PA is the peak area

3.2 Method 2 results

The mass spectrometer was set to detect the same compounds as method 1. The chromatogram of a sample from method 2 is shown in Figure 3.

Figure 3: Method 2 Chromatogram



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) METH-D5; e) 4-OH-AMP; f) METH; g) AMP

The peaks for 8 samples extracted using method 2 were integrated and the retention times and peak areas are provided in Table 2.

Table 2: Method 2 LC-MS/MS Results

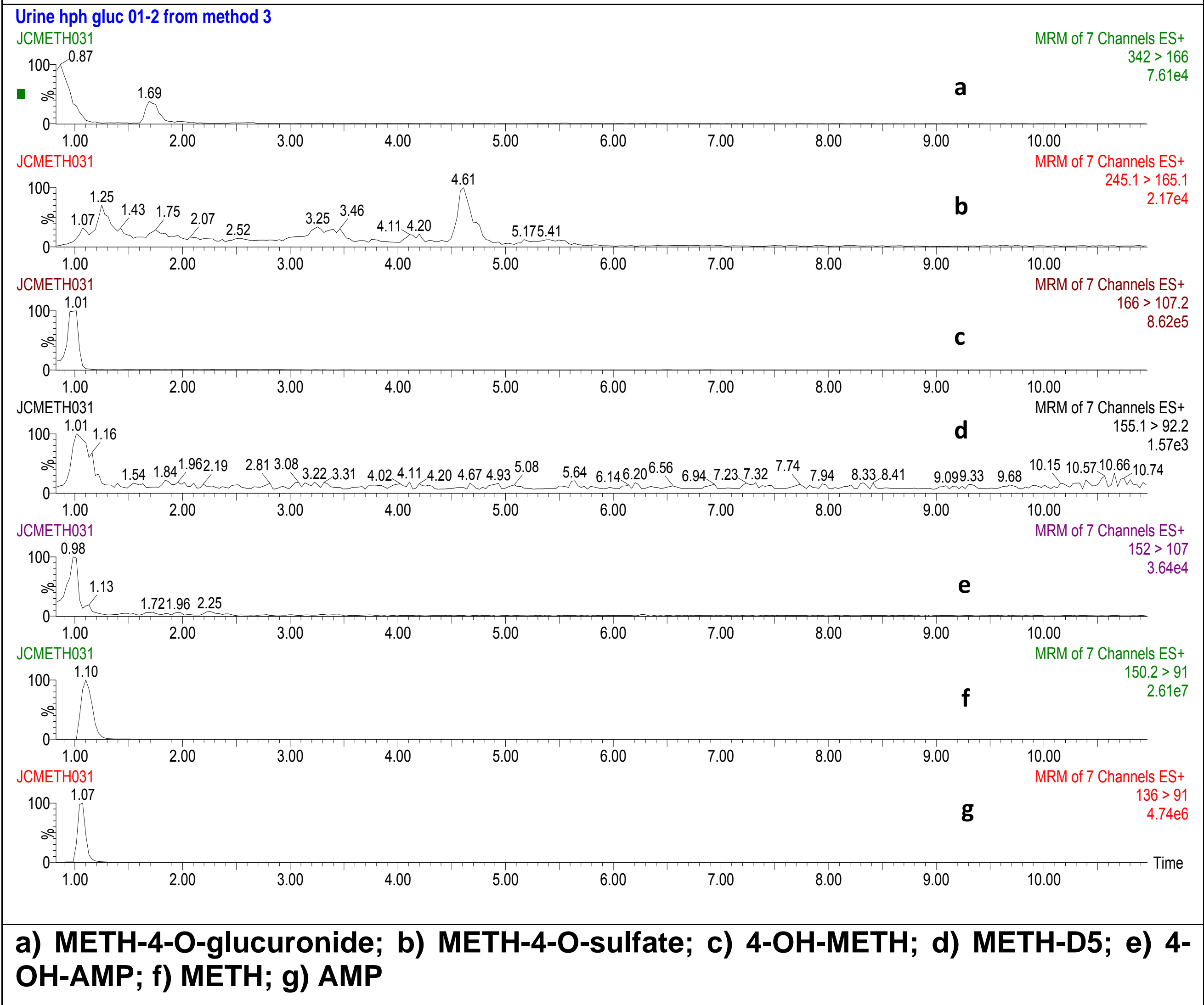
	Compound													
	342 > 166 4-O-METH-G		245 > 165 4-O-METH-S		166 > 107 4-OH-METH		155 > 92 METH-D5		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
Average of 8 Samples	1.66 ± 0.02	1551 ± 402	4.48 ± 0.04	957 ± 287	0.83 ± 0.02	30585 ± 15057.14	1.07 ± 0.00	57203 ± 30537	1.08 ± 0.015	755 ± 298	1.08 ± 0.016	1099692 ± 359672	1.07 ± 0.00	154939 ± 32474

t_R is retention time; PA is peak area

3.3 Method 3 results

For method 3, the mass spectrometer was set to detect peaks of METH-4-O-glucuronide, METH-4-O-sulfate, 4-OH-METH, 4-OH-AMP, METH, and AMP. The chromatogram of a method 3 extraction sample is shown in Figure 4.

Figure 4: Method 3 Chromatogram



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) METH-D5; e) 4-OH-AMP; f) METH; g) AMP

The peaks for 6 samples from a method 3 extraction were integrated and the retention times and peak areas are provided in Table 3.

Table 3: Method 3 LC-MS/MS Results

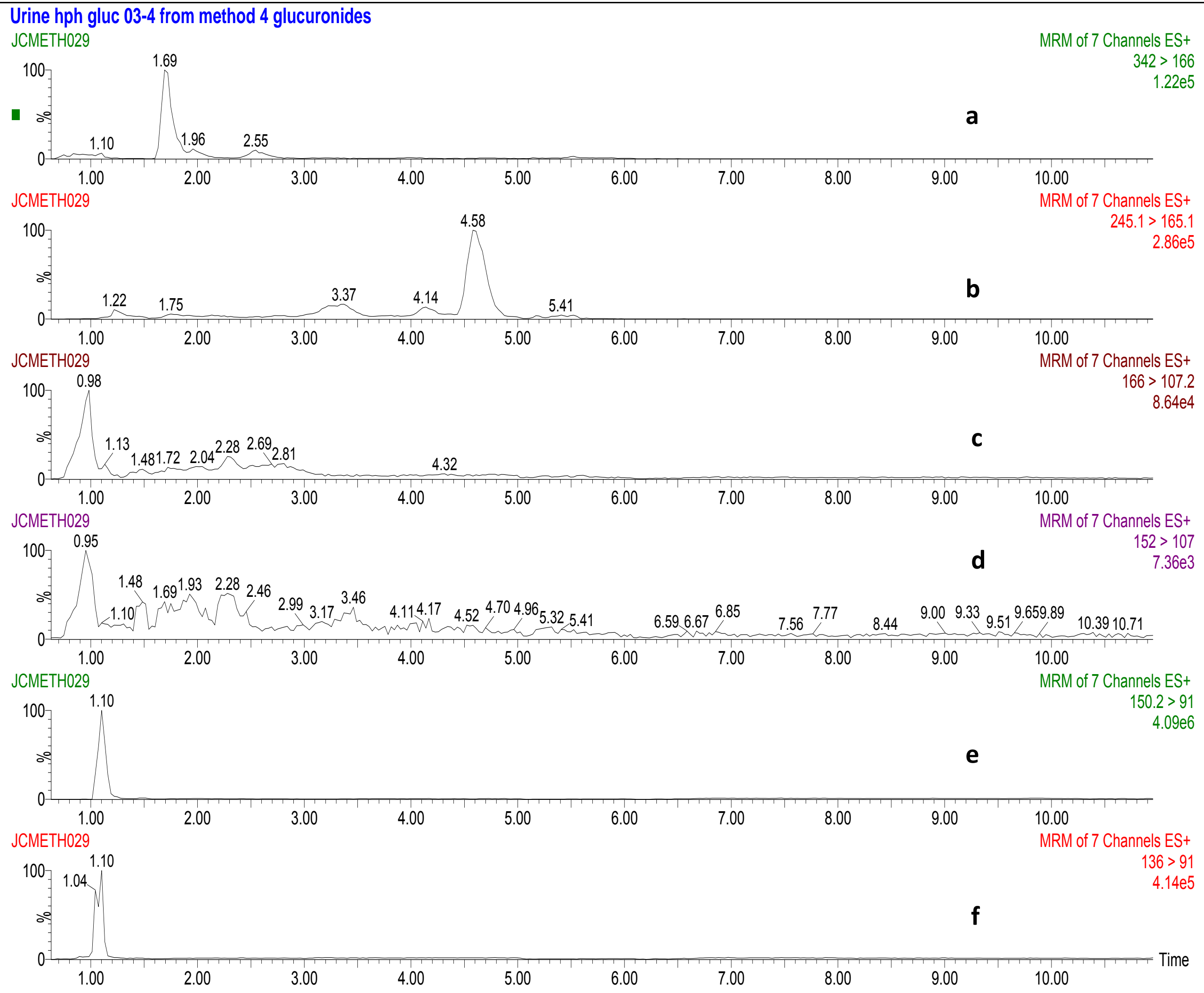
	Compound											
	342 > 166 4-O-METH-G		245 > 165 4-0-METH-S		166 > 107 4-OH-METH		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
Average of 6 Samples	1.76 ± 0.14	3508 ± 1056	4.66 ± 0.13	2311 ± 981	1.00 ± 0.02	122941 ± 30285	1.00 ± 0.025	4268 ± 783	1.12 ± 0.037	3123531 ± 792737	1.08 ± 0.02	368018 ± 177039

t_R is retention time; PA is peak area

3.4 Method 4 results

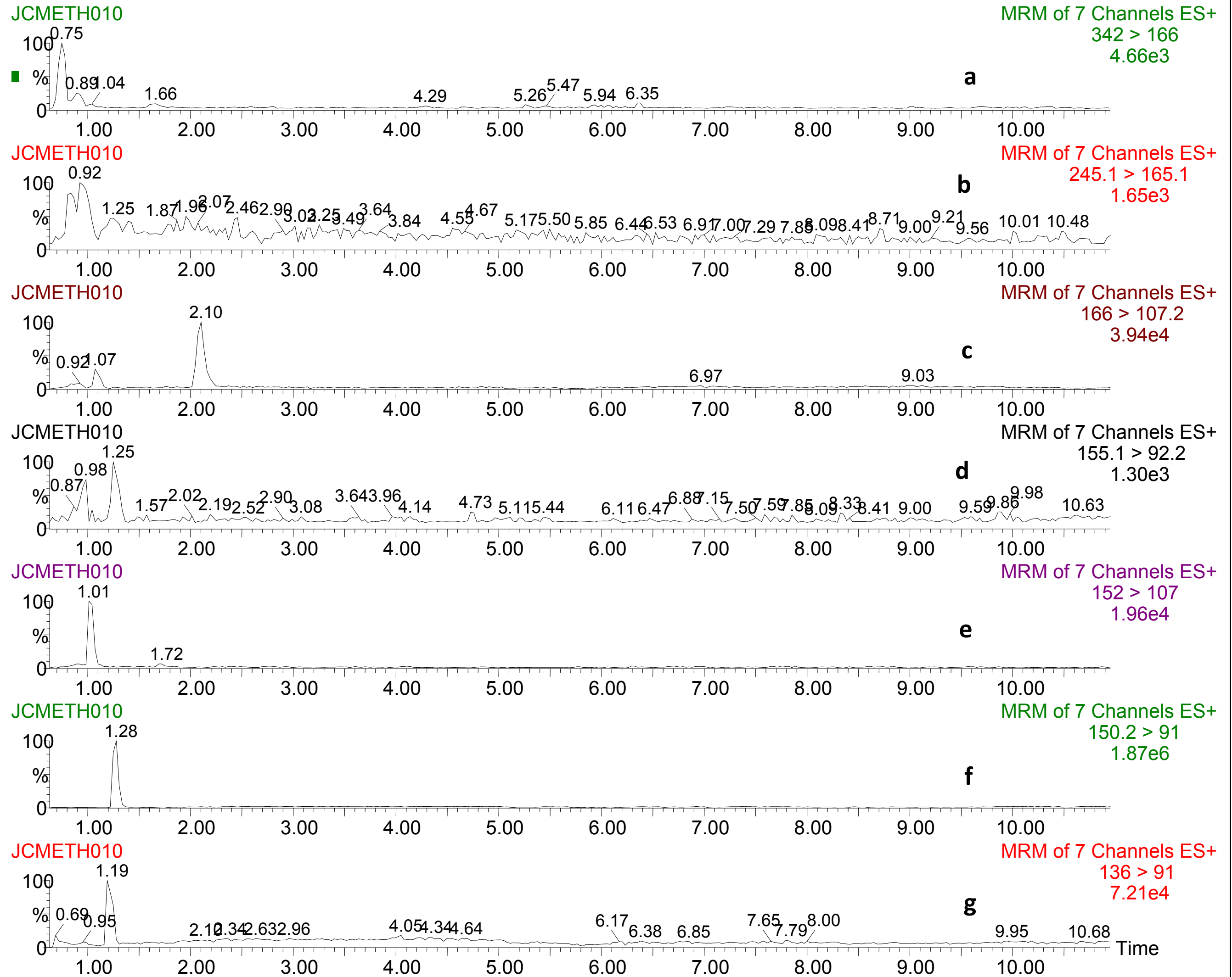
For method 4, the mass spectrometer was set to detect peaks of METH-4-O-glucuronide, METH-4-O-sulfate, 4-OH-METH, 4-OH-AMP, METH, and AMP. For each sample of urine, the glucuronide and sulfate were eluted separately. A chromatogram of a glucuronide fraction and a sulfate fraction are shown in Figure 5 and Figure 6 respectively.

Figure 5: Method 4 Chromatogram Glucuronide Fraction



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) 4-OH-AMP; e) METH; f) AMP

Figure 6: Method 4 Chromatogram Sulfate Fraction



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) 4-OH-AMP; e) METH; f) AMP

Peaks for 6 samples extracted using method 4 were integrated and the retention times and peak areas are provided in Table 4.

Table 4: Method 4 LC-MS/MS Results

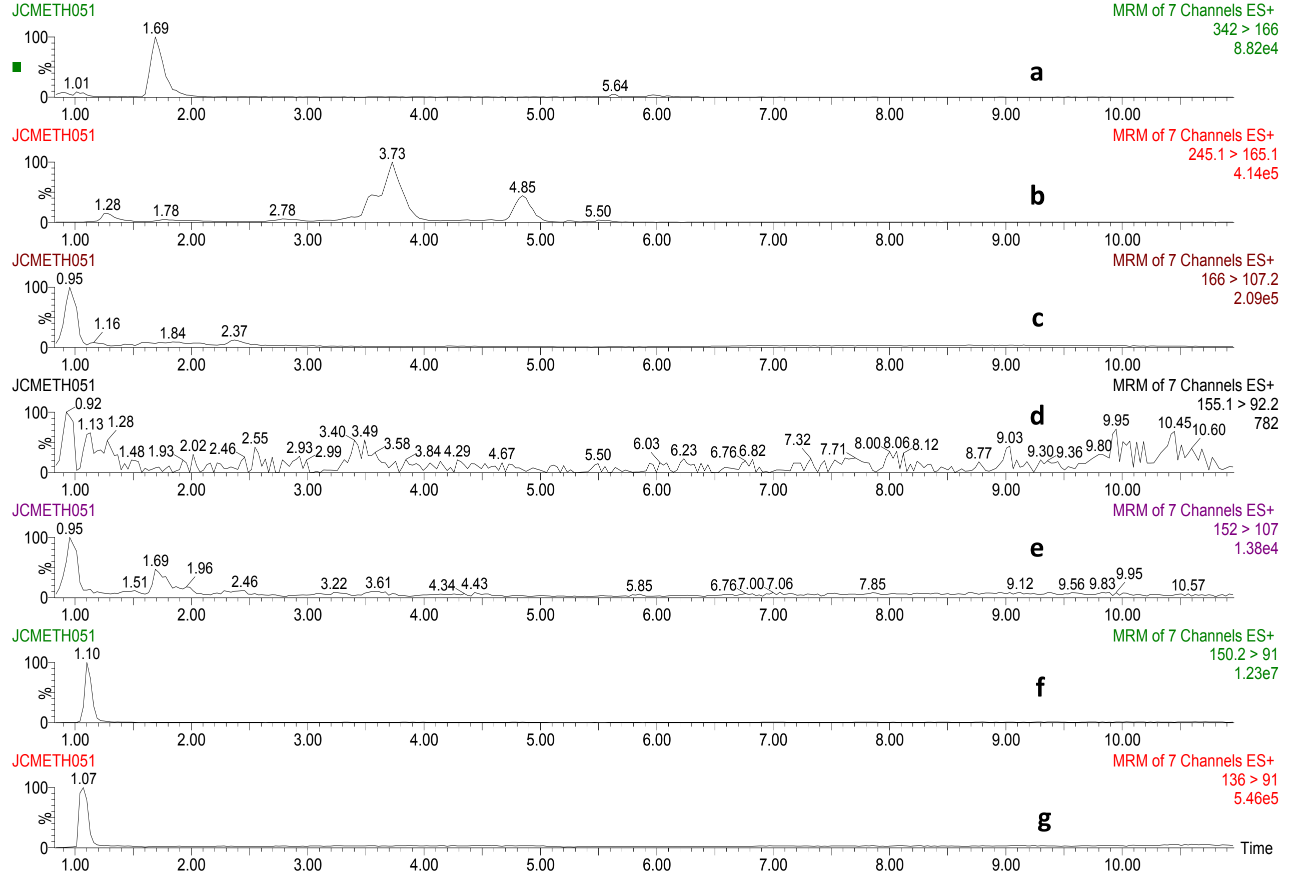
	Compound											
	342 > 166 4-O-METH-G		245 > 165 4-O-METH-S		166 > 107 4-OH-METH		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
	Glucuronide Fraction											
Average of 6 Samples	1.705 ± 0.016	15104 ± 1038	4.61 ± 0.00	53408 ± 9507	0.95 ± 0.00	13310 ± 1030	0.97 ± 0.015	1080 ± 81	1.10 ± 0.00	414153 ± 46931	1.075 ± 0.012	42419 ± 16253
	Sulfate Fraction											
Average of 6 Samples	Not Detected	Not Detected	Not Detected	Not Detected	0.99 ± 0.10	1904 ± 1621	1.03 ± 0.01	2149 ± 821	1.26 ± 0.02	332740 ± 299683	1.18 ± 0.04	37077 ± 39408

t_R is retention time; PA is peak area

3.5 Acid Hydrolysis Results

For the acid hydrolysis method, the mass spectrometer was set to use the same method as method 4. The chromatogram of a sample treated with HCl before extraction is shown in Figure 7.

Figure 7: Acid Hydrolysis Chromatogram



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) METH-D5; e) 4-OH-AMP; f) METH; g) AMP

Peaks for 3 samples treated with HCl and 3 samples untreated with HCl have been integrated and the retention times and peak areas are provided in Table 5.

Table 5: Acid Hydrolysis LC-MS/MS Results

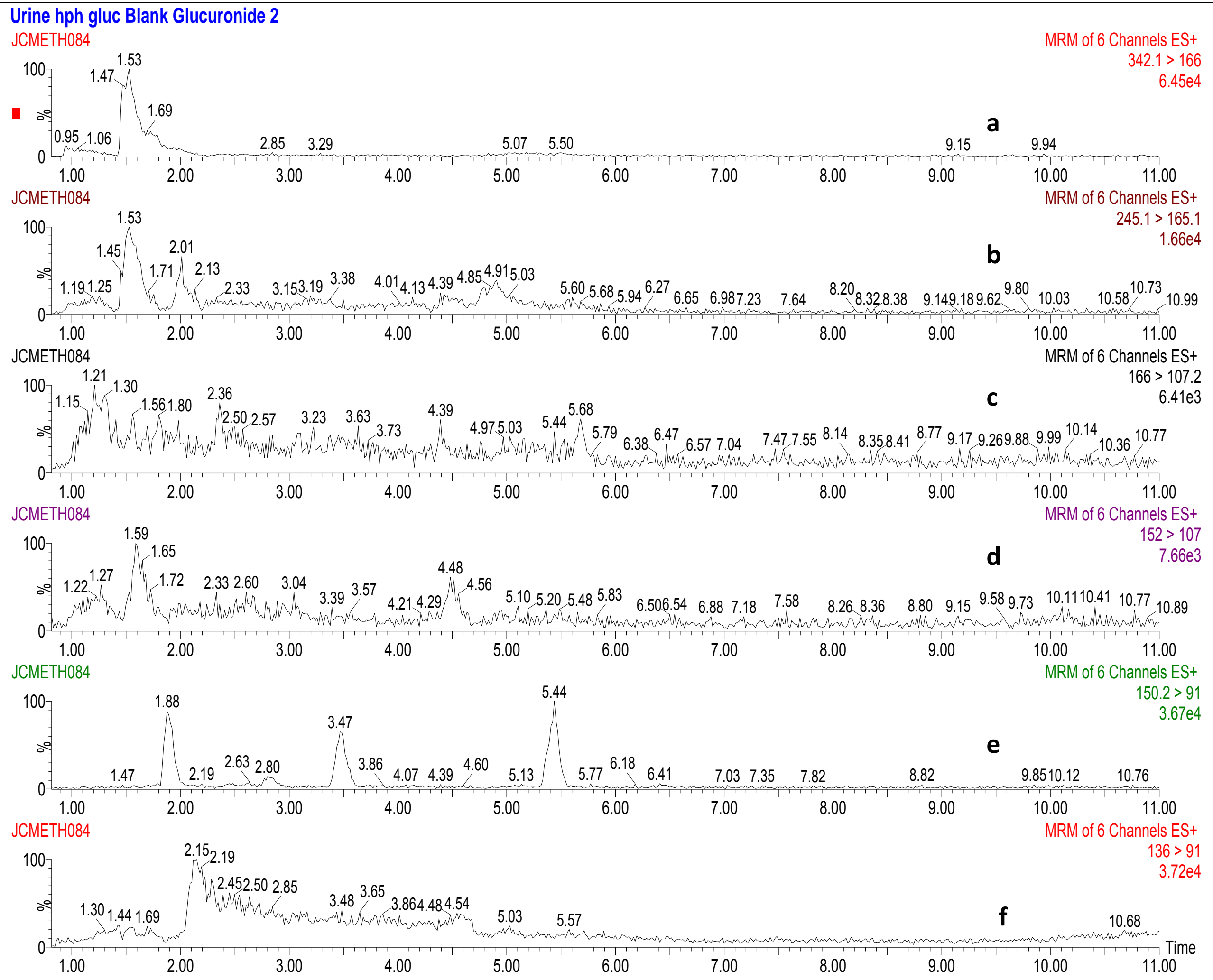
	342 > 166 4-O-METH-G		245 > 165 4-O-METH-S		166 > 107 4-OH-METH		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
	Glucuronide fraction treated with HCl											
Average of 3 Samples	1.69 ± 0.00	11698 ± 0.00	4.61 ±0.04	1764 ± 799	0.97 ± 0.02	37326 ± 16887	0.98 ± 0.04	2942 ± 1788	1.13 ± 0.04	1779815 ± 1244643	1.09 ± 0.02	91485 ± 55256
	Glucuronide fraction not treated with HCl											
Average of 3 Samples	1.70 ± 0.017	21276 ± 485	4.83 ± 0.017	39882 ± 2569	0.97 ± 0.017	16228 ± 2109	0.97 ± 0.017	1105 ± 74	1.11 ± 0.017	917000 ± 257691	1.07 ± 0.00	67341 ± 6387
	Sulfate fraction treated with HCl											
Average of 3 Samples	Not Detected	Not Detected	Not Detected	Not Detected	0.94 ± 0.017	17263 ± 4918	0.92 ± 0.00	930 ± 313	1.12 ± 0.017	458751 ± 238307	1.07 ± 0.00	32044 ± 18229
	Sulfate fraction not treated with HCl											
Average of 3 Samples	Not Detected	Not Detected	Not Detected	Not Detected	1.04 ± 0.00	1232 ± 468	1.04 ± 0.00	2410 ± 705	1.18 ± 0.017	362797 ± 19010	1.12 ± 0.017	20776 ± 2067

t_R is retention time; PA is peak area

3.6 Blank Urine

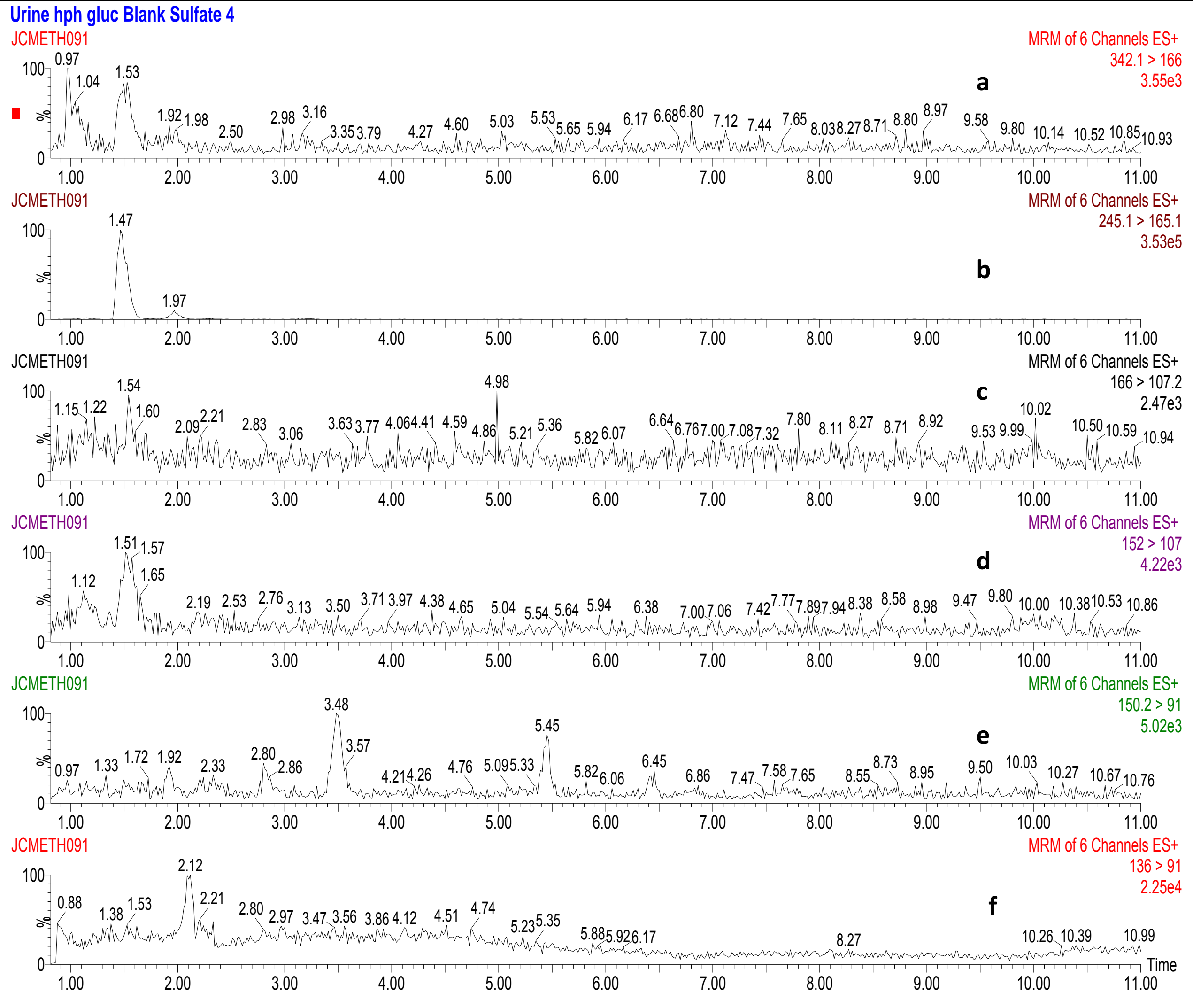
Urine from rats that were not administered METH was extracted using method 4. The chromatogram of a glucuronide faction and a sulfate fraction are shown in Figure 8 and Figure 9 respectively.

Figure 8 : Blank Sample Chromatogram Glucuronide Fraction



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) 4-OH-AMP; e) METH; f) AMP

Figure 9 : Blank Sample Chromatogram Sulfate Fraction



a) METH-4-O-glucuronide; b) METH-4-O-sulfate; c) 4-OH-METH; d) 4-OH-AMP; e) METH; f) AMP

The peaks for 6 extracted samples were integrated and the retention times and peak areas are provided in Table 6.

Table 6 : Blank Sample LC-MS/MS Results

	Compound											
	342 > 166 4-O-METH-G		245 > 165 4-O-METH-S		166 > 107 4-OH-METH		152 > 107 4-OH-AMP		150 > 91 METH		136 > 91 AMP	
Sample	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA	t_R	PA
	Glucuronide Fraction											
Average of 6 Samples	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
	Sulfate Fraction											
Average of 6 Samples	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected

No peaks were detected for the compounds of interest.

4. Discussion

4.1 Extraction Methods 1, 2, and 3

Methods 1 through 3 were able to extract glucuronide and sulfate, however the extraction recovery was poor. Samples extracted using Method 1 contained very large amounts of METH and AMP which could be reduced. Method 2 aided in the cleanup in the samples to some extent but as more barium hydroxide was added to the samples, the amount of glucuronide extracted decreased. This can be attributed to the fact that as more barium hydroxide was added, the pH increased to a high of 13. This occurred when 450 µl of 6 M barium hydroxide was added. At this pH, the integrity of the C18 stationary phase was compromised. In method 3, LC-MS/MS data showed similar recovery of the peak at 1.7 min (i.e. the glucuronide). The method used a surfactant which is often not appropriate or favorable for a mass spectrometer and therefore optimization of this method was not pursued. In fact the extraction of the glucuronide

might have been high with method 3, but the MS signal might have been compromised due to the presence of the surfactant.

4.2 Extraction method 4, acid hydrolysis and blank urine

Method 4 was able to produce large quantities of the phase II metabolites of METH and relatively clean samples. The amount of glucuronide detected in the samples extracted using method 4 were at a minimum increased by a factor of four compared to the other methods. It was designed to separate the glucuronide and sulfate into two fractions, however, LC-MS/MS data suggested that the sulfates came off the column in the glucuronide fraction in almost all cases. The sulfate fraction was of no use in extracting the phase II metabolites. Multiple peaks occurred in some cases, but after comparison with other method's data, it is evident that the glucuronide retention time is 1.69 min and the sulfate retention time is 4.61 min. The confirmation that these peaks are the glucuronide and sulfate metabolites comes from the acid hydrolysis results. If the glucuronide and sulfate were what was being detected by the mass spectrometer at the retention times that occurred, acid hydrolyzed samples would either not detect the corresponding peaks, or the peaks would have a lower peak area. Also, as the glucuronides and sulfates are hydrolyzed, the by-products are a glucuronic acid or sulfate and a 4-OH-METH. Because of this, it is expected that if the glucuronide and sulfate are being hydrolyzed, the amount of 4-OH-METH detected would increase. The samples that were subjected to acid hydrolysis showed peaks at the same retention time as the suspected phase II metabolites that were reduced by approximately half compared to untreated samples and the peak area for 4-OH-METH increased as expected. This supports the idea that the glucuronide fraction elutes from the column at

1.69 min and the sulfate fraction elutes from the column at 4.61 min and that the phase II metabolites are what is being detected. Also, urine used as blank samples provide more confirmation of the presence of the phase II metabolites. Blank urine should not contain glucuronide or sulfate of METH, therefore if the sample does not contain the peak thought of to be the glucuronide or sulfate, the data would suggest that the peaks detected in urine containing METH are indeed the glucuronide and sulfate. The LC-MS/MS data from the blank urine samples did not contain the peak at the suspected retention time of the glucuronide or sulfate and therefore further confirms the presence of the glucuronide and sulfate of METH.

4.3 Future studies

This study suggests that Method 4 was the most effective method for the purpose of sample preparation. In the future, this method will be used to isolate the glucuronide to be used as a standard. The isolated fractions will then be analyzed using nuclear magnetic resonance (NMR) spectroscopy for further confirmation of the structural details of 4-O-METH-Glucuronide and 4-O-METH-Sulfate. Isolation of these phase II metabolites will also provide a more complete description of the disposition of methamphetamine and it's potentially active metabolites.

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