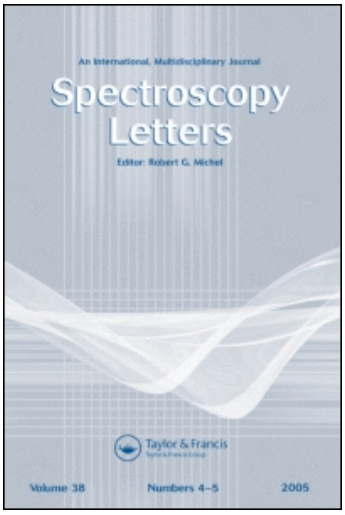

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ABSTRACT

To develop a method whereby polar biological metabolites can be analyzed using readily available GC instrumentation and enhance detection limits of small water-soluble amines as a potential clinical

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diagnostic method for analyzing both biologically produced amines as well as synthetic pharmaceutical compounds, we have investigated two derivatization techniques for biogenic amines employing fluorescent compounds, namely 9-fluorenylmethyl chloroformate (FMOC) and 1-pyrenyldiazomethane (PDAM). Both compounds provide a stable, non-polar element to the derivatized amines that facilitates the use of GC-MS methods for analysis as well as allow for the use of fluorescence detection, which increases overall sensitivity. The study presented herein is an application of these derivatization techniques to the simple biogenic amines creatine and creatinine, followed by analysis using GC-MS with ECD detection and HPLC with both UV and fluorescence detection. Results will show that the coupling of creatine to PDAM and creatinine to FMOC allow both compounds to enter the gas phase to a sufficient extent for gas chromatographic separation and mass spectrometric identification. In terms of fluorescence detection, 0.5 μg creatinine that is derivatized with FMOC is at least seven times more sensitive using fluorescence detection when compared to UV detection of the same compound.

Key Words: NMR, nuclear magnetic resonance; GC, gas chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; UV, ultraviolet-visible; HP, Hewlett-Packard©.

INTRODUCTION

Creatine, a small biogenic amine produced naturally by the body, is stored in a phosphorylated form (phosphocreatine) in skeletal muscle and is responsible for aiding in the regeneration of depleted adenosine triphosphate (ATP) by helping the body to rephosphorylate adenosine diphosphate (ADP), particularly in situations of high physical exertion.[1,2] Due to its importance in assisting the body in regenerating readily available energy stores via ATP synthesis, creatine has become popular in the nutritional supplement industry, where it exists primarily in the form of creatine monohydrate.[3,4] In addition, meat that is consumed in the diet provides a significant amount of creatine to the body as well (approximately 5 g creatine/1 kg uncooked meat).[5]

Excess creatine that is not used in energy production is removed from the body in the urine where it may exist in one of two forms, either as creatine or in a cyclic form known as creatinine. Conversion of creatine to creatinine is directly related to pH and temperature; therefore, depending on an individual's urinary pH, the potential for detecting either one or both of the compounds exists.[2] In general, the human body excretes creatine at a relatively constant rate, which makes the measurement of creatine levels in urine a

useful tool for clinical analysis. For example, renal function is often assessed by quantitating levels of both creatine and creatinine in the urine.[6] Forensic urine drug-testing laboratories, on the other hand, can determine the suitability of a urine sample for analysis based on the creatine level, which serves as an indicator of whether or not a sample has been diluted.[7] Therefore, the ability to accurately detect both creatine and creatinine in a biological fluid such as urine at sub-microgram levels could be highly advantageous to clinicians, exercise researchers, forensic investigators, and so forth.

Based upon their polar nature, the quantitative analysis of many biological metabolites such as creatine and creatinine by techniques such as GC-MS is an arduous task. Unfortunately, alternative techniques such as LC-MS, which is highly efficient in such analyses, are not always available. More recently, fluorescence detection coupled to HPLC has become a useful method for detecting trace levels of such compounds without the need for expensive LC-MS apparatus.[8]

In an effort to enhance the detection limits for both of these amines as well as to provide a stable mechanism by which GC-MS may be used, the use of 9-fluorenylmethyl chloroformate (FMOC) and 1-pyrenyldiazomethane (PDAM) derivatization techniques have been explored. Results will show that not only does the presence of such compounds enhance detection by both UV as well as fluorescence, but the introduction of such large non-polar elements also increases thermal stability, which in turn increases the robustness of using GC-MS when analyzing such compounds as well.

EXPERIMENTAL

Reagents

Creatine monohydrate, creatinine, FMOC, methanol, acetonitrile, and acetone were purchased from Aldrich Chemical Company (Milwaukee, WI). PDAM was purchased from Molecular Probes (Eugene, OR). Solid-phase microextraction (SPME) fibers were purchased from Supelco (Bellefonte, PA). All chemicals were used without further purification.

Derivatization Procedure

Derivatization of creatinine with FMOC involved adding 500 μL of sample (1 mM creatinine) to 500 μL of 0.4 M borate buffer (pH 8.0), followed by the addition of 250 μL of 8 mM FMOC (in acetone). Derivatization of creatine with PDAM involved adding 500 μL of sample

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(1 mM creatine) to 500 μL of 0.4 M acetate buffer (pH 4.2), followed by the addition of 250 μL of 8 mM PDAM (in acetone). Samples were each vortexed for 30 sec at room temperature to ensure that the reaction was complete (Fig. 1).

Instrumental Methods

NMR Spectroscopy: Brüker Avance 200 MHz

For confirmation of successful derivatization, 600 μL samples of 100 mM creatine, creatinine, PDAM-creatine, and FMOC-creatinine were prepared using either a 90% acetate buffer (pH 4.2) with 10% D_2O or a 90% borate buffer (pH 8.0) with 10% D_2O. Proton-decoupled carbon and DEPT-135 spectra were collected using 256 scans on a 5 mm BBO probe ($T = 298$ K).

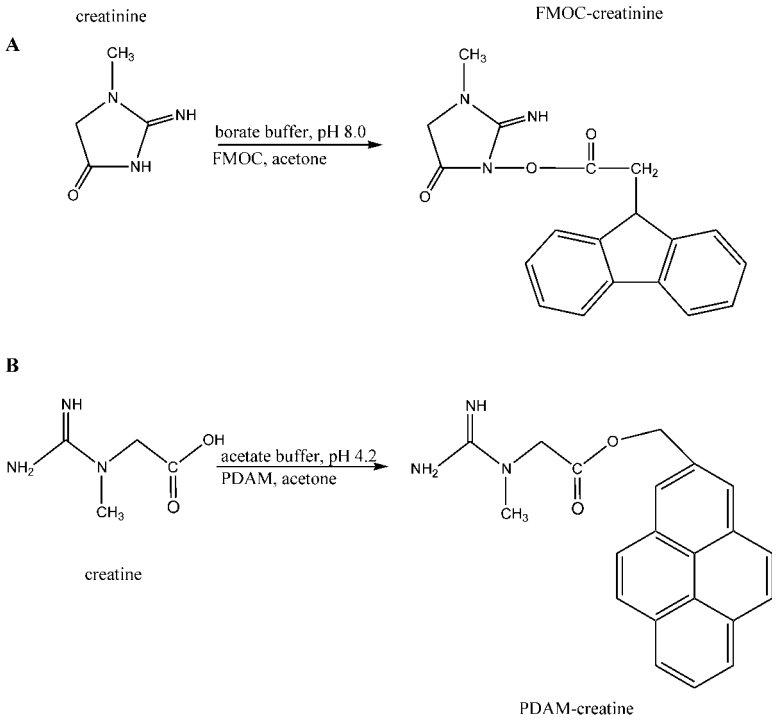

Figure 1. Procedure for derivatizing (a) creatinine using FMOC and (b) creatine using PDAM.

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GC-MS: HP GCD Plus

Samples were extracted by immersing an 85 mm polyacrylate SPME fiber in NaCl saturated solutions of (i) PDAM-creatine and (ii) FMOC-creatinine for 1 hr. The fiber was desorbed for each sample for 30 sec at 230°C. The initial oven temperature was 40°C for 5 min, followed by ramping at 10°C/min until reaching a final temperature of 300°C. Chromatographic separation was achieved using a 30 m × 0.25 mm × 0.2 mm dimethylsiloxane column at a flow rate of 1.0 mL/min.

HPLC-UV-MS: Varian ProStar HPLC and VG Quatro MS

Samples were injected and separated using a Varian ProStar HPLC coupled to a VG Quatro mass spectrometer for detection. Separation was achieved using a Phenomenex C_{18} 2 mL column that was held at 40°C. Following derivatization, samples were presented to the autosampler for injection. MS was scanned from m/z 120 to 500 and utilized APCI for sample introduction into the MS system. HPLC conditions were 90 : 10 H_2O : MeOH for 3 min, followed by a gradient to 5 : 95 H_2O : MeOH over 22 min and finally held at 5 : 95 for 20 min.

HPLC-Fluorescence: Hewlett Packard Series 1050

Samples of FMOC-creatinine were analyzed using a Hewlett Packard quaternary gradient HPLC with internal UV detector coupled in series to a Waters 470 scanning fluorescence detector. Samples were introduced via 10 μL injections, and separation was achieved using a Phenomenex C_{18} column (flow rate: 1.0 mL/min). HPLC conditions: isocratic elution using a 60 : 40 mixture of two buffers: Buffer A (0.05 M acetate buffer, pH 4.2/methanol/acetonitrile 50 : 40 : 10) and Buffer B (0.05 M acetate buffer, pH 4.2/acetonitrile 50 : 50). UV detection was set at 254 nm. Fluorescence detector conditions were: λ_{ex}, 280 nm and λ_{em}, 305 nm.

RESULTS AND DISCUSSION

NMR Spectroscopy

^{1}H-decoupled ^{13}C spectra were collected to verify that derivatization of both creatine and creatinine had occurred as desired. In both FMOC-creatinine and PDAM-creatine, coupling occurred as expected, with FMOC coupling

through the amine moiety of creatinine and PDAM coupling through the carboxylic acid moiety of creatine (spectra not shown).

GC-MS

Based on their polar nature, GC analyses of both creatine and creatinine are difficult. Following derivatization with either the non-polar PDAM or FMOC groups, thermal stability of both compounds increased significantly. As a result, both creatine and creatinine were more effectively introduced into the gas phase for subsequent MS analysis. Unstabilized creatine has difficulty entering the gas phase, with the resultant mass spectrum indicating poor relative abundance and fragment stability. In contrast, the PDAM-stabilized creatine is carried much more readily into the gas phase as evidenced by more intense fragment peaks as well as a stable fragmentation pattern. Similar results were obtained for creatinine, with the FMOC-stabilized creatinine showing a much more stable fragmentation pattern and higher intensity of peaks. In both compounds, the M^+ peak m/z 113 is readily identifiable in the derivatized compounds, whereas only M-2 is identifiable (very weak) in the underivatized forms (spectra not shown). Thus, derivatization using FMOC or PDAM allows for the identification of the molecular ion peak for both biogenic amines.

HPLC-UV-MS

Preliminary studies were conducted to determine the feasibility of using FMOC and PDAM derivatization methods as potential techniques for detecting creatine and creatinine levels in a biological matrix such as urine, focusing on FMOC-creatinine in this particular investigation. As can be seen in Table 1, the coupling of FMOC to creatinine alters the LC retention time significantly, improving separation that should enhance analyses, particularly in complex

Table 1. Retention times for creatinine and FMOC-creatinine.

Compound	Retention time (min)
Creatinine	3.853
Creatinine-FMOC	31.932
FMOC	29.409

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mixtures of analytes such as urine. However, at high concentrations, it appears that spontaneous dimerization of creatinine occurs as evidenced by the peak at m/z 227 taken from extracted ion chromatograms (not shown). An unfortunate feature of this particular derivatization method that requires basic pH conditions for FMOC coupling involves creatine spontaneously cyclizing to form creatinine, making a distinction between the two impossible. Therefore, if one was to use this technique to derivatize a mixture of both creatine and creatinine, one would have to couple a protecting group to creatine (such as PDAM) prior to FMOC derivatization to prevent spontaneous cyclization. Such an investigation of dual-derivatization is currently underway.

HPLC-Fluorescence

Preliminary studies were also conducted to compare the detection limits of FMOC-creatinine qualitatively using both UV and fluorescence detection. Based on initial findings, it appears that the use of fluorescence detection increases the detection of FMOC-creatinine by at least a factor of seven (Fig. 2) due to the fluorescent properties of FMOC. Likewise, one would expect similar increases for PDAM-creatine due to the nature of the PDAM group as well. Current investigations using both of these compounds to quantify detection limits are ongoing.

In recent years, the search for better analytical methods for helping clinicians to quantify the levels of creatine and creatinine found in bodily fluids

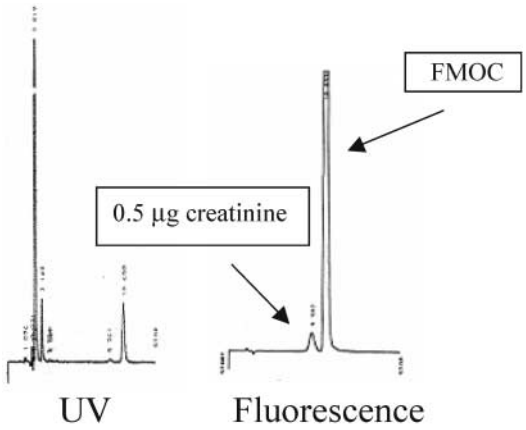

Figure 2. Comparison of HPLC chromatogram for 0.5 µg FMOC-creatinine using UV detection (left) and fluorescence detection (right).

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such as urine has regained impetus. To date, most techniques for such analyses are based on enzymatic reactions which are often inaccurate due to the interference of other chemical compounds that may be present.[2] More recently, the use of methods such as HPLC and CE has been explored to ascertain their ability to circumvent such problems.[8]

The study described herein is a description of how one might apply GC-MS to an analysis of this nature, as well as improve upon HPLC techniques that are already being investigated. By introducing a non-polar functional group to either creatine or creatinine, one can increase the probability that these polar compounds will remain stable enough to enter the gas phase, thus allowing for subsequent analysis on a standard GC or GC-MS instrument. Similarly, the introduction of these bulky fluorescent "tags" also affects detection limits and retention time when using LC apparatus as well, as evidenced by the HPLC-MS data described earlier. While UV detection is not enhanced tremendously, the change in retention time does improve chromatographic separation. However, if fluorescence detection is available, such derivatives could potentially be detected at much lower concentrations, which may be of importance to clinical research.

An added advantage to using non-polar derivatization techniques such as the ones described in this study for clinical applications is that sample preparation can be greatly reduced, particularly if one uses SPME methods. Theoretically, by introducing a small amount of the derivatization agent directly into a biological sample such as urine (which has been adjusted to the proper pH) followed by brief vortexing at room temperature, one can quickly derivatize any creatine or creatinine present. Following standard SPME extraction techniques, either through headspace analysis or by direct immersion of the fiber into the solution, one can quickly extract the sample directly from urine without the need for subsequent sample handling. Thus, relatively small sample volumes could yield a large amount of information with minimal sample workup.

CONCLUSION

The use of FMOC and PDAM as derivatization agents for studying biogenic amines appears to have a great deal of potential as a clinical alternative to enzymatic analyses. As has been shown, FMOC and PDAM derivatization both appear to assist in the introduction of such thermally unstable molecules as creatine and creatinine into the gas phase to allow for GC-MS analysis. This allows for the detection of both compounds using a technique which has previously seen little use in this area. Finally, if fluorescence detection if available, derivatization in this manner may enhance sensitivity and detection significantly. As shown in the case of FMOC-derivatized creatinine, sensitivity

increased by at least a factor of seven. The ease with which derivatization occurs and the sensitivity of such a method may lead to the use of such techniques for clinical analyses in the near future.

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