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Physicochemical Characterization of Creatine *N*-Methylguanidinium Salts

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ABSTRACT. Creatine is widely used as a dietary supplement for body builders to enhance athletic performance. As the monohydrate, its low solubility in water and high dose lead to water retention and gastrointestinal discomfort. Hence, alternative creatine derivatives with enhanced water solubility and potential therapeutic advantages have been synthesized. As a zwitterionic compound, creatine can form salts at the *N*-methyl guanidinium or carboxylic acid functional groups. In this study, we determined the aqueous solubilities and partition coefficients of six *N*-methyl guanidinium salts of creatine compared to those of creatine monohydrate; two of these were new salts, namely, creatine mesylate and creatine hydrogen maleate. The aqueous solubilities of the salts were significantly more than that of creatine monohydrate with the hydrochloride and mesylate being 38 and 30 times more soluble, respectively. The partition coefficients of the creatine salts were very low indicating their relatively high polarity. Permeabilities of creatine pyruvate, citrate, and hydrochloride in Caco-2 monolayers were compared to that of creatine monohydrate. Aside from the creatine citrate salt form that had reduced permeability, there were no significant differences in permeability characteristics in Caco-2 monolayers. Typical of an amphoteric compound, creatine is least soluble in the pH region near the isoelectric point.

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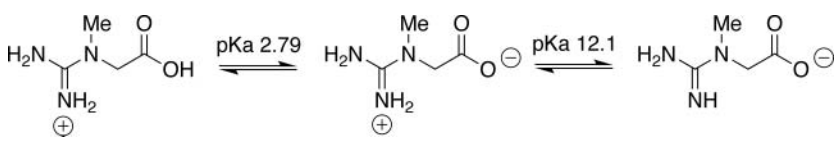
INTRODUCTION

Creatine, most commonly in the form of the monohydrate, is widely used as a dietary supplement for bodybuilding and for its potential to improve exercise and athletic performance (Persky & Brazeau, 2001). Creatine may also be useful in the treatment of certain diseases, especially those involving muscular atrophy or fatigue associated with impaired energy production (Persky & Brazeau, 2001; Wyss & Kaddurah-Daouk, 2000). As creatine monohydrate is typically used at relatively high doses (5–25 g/day), its water solubility of 16.6 mg/ml (Dash & Sawhney, 2002) dictates that it is administered as an oral suspension, leading to water retention and possible gastrointestinal discomfort (Persky & Brazeau, 2001).

It is possible to make salts of the zwitterionic creatine at either the carboxylic acid or *N*-methyl guanidinium functional groups (Figure 1). Due to relatively low aqueous solubility of creatine monohydrate, there have been several creatine *N*-methylguanidinium salts introduced in the market as dietary supplements that are claimed to be more water-soluble than creatine monohydrate. These include the pyruvate, hydrogen citrate, and hydrochloride salt forms. Other creatine *N*-methylguanidinium salts have been described in patents and/or patent applications. These salt forms include the hydrobromide (Rudakova, Pospelova, & Yurkevich, 1967); nitrate (Dhar & Ghosh, 1961); mesylate (Blatt et al., 2006); dihydrogen phosphate and hydrogen oxalate (Dhar & Ghosh, 1961); malate (Boldt, 2004; Cornelius & Haynes, 2004; Qian, Ye, & Huang, 2005); maleate, fumarate, and tartarate (Boldt, 2004); lipoate (Buononato & Festuccia, 2003; Gardiner, 2000); hydrogen maleate, hydrogen fumarate, hydrogen tartarate, and hydrogen malate (Negrisoli & Del Corona, 1996); ascorbate (Pischel, Weiss, Gloxhuber, & Mertschenk, 1998); and bicarbonate (Kneller, 2008).

Many of these creatine *N*-methylguanidinium salts are formed with acids, which are insufficiently acidic to directly form a salt with creatine (Figure 1). Thus, it is not clear if these "salts" are merely physical mixtures, weak complexes, or "addition salts" (Pischel et al., 1998) of creatine monohydrate and the acid in question. The only way to form such salts with weak acids is using an ion-exchange between a creatine *N*-methylguanidinium salt and a salt of the acid as described by Qian et al. (2005) in the synthesis of creatine maleate using creatine hydrochloride or creatine sulfate with sodium or calcium maleate; by Arnold (2001) in the synthesis of creatine pyruvate from creatine hydrochloride and sodium pyruvate; and by Gardiner, Heuer, and Molino (2006) in the synthesis of creatine citrate from tripotassium citrate and creatine hydrochloride.

FIGURE 1. Creatine acid–base equilibria.



The purpose of this study was firstly, to synthesize additional salts, namely creatine mesylate and creatine hydrogen maleate, and secondly, to determine and compare the physicochemical properties of these and other commercially available salts, namely creatine hemisulfate, creatine hydrochloride, creatine pyruvate, and "creatine citrus" (citrate complex). The aim was to identify a water soluble salt that may have the following potential advantages over creatine monohydrate: (a) increase solubility promoting increased bioavailability (b) lower oral dose (c) decrease side effects (d) be able to be formulated into a more diverse range of formulations e.g., capsules or a topical product for (e) a wider range of therapeutic applications e.g., anti-inflammatory effects.

MATERIALS AND METHODS

Materials

Creatine monohydrate (Creapure^{TM}) and creatine pyruvate were obtained from Degussa, creatine citrate, i.e., "creatine citrus" was obtained from Peak Nutrition, and creatine hydrochloride (Miller, Vennerstrom, & Faulkner, 2009) was obtained from Vireo Systems. With the exception of the 1-octanesulfonic acid sodium salt that was obtained from Fluka, all other reagent solvents were obtained from Sigma-Aldrich and used as received.

Nuclear Magnetic Resonance (NMR), Elemental Analysis, and Melting Point Determination

Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra of each new salt in DMSO-d_6 were recorded on a 500-MHz spectrometer. All chemical shifts are reported in parts per million (ppm) and are relative to internal TMS (0 ppm). Elemental analyses were determined by M-H-W Laboratories. Melting points were obtained using differential scanning calorimetry and were uncorrected.

Creatine Mesylate

A suspension of creatine monohydrate (0.08 mol, 11.93 g) in deionized water (120 ml) warmed to 59°C was added to a stirred solution of methane sulfonic acid (0.08 mol, 7.69 g) in ethanol (EtOH) (200 ml) at 59°C. The reaction mixture was stirred for 10 min at 59°C and then allowed to cool to room temperature. The solvents were removed in vacuo affording creatine mesylate (11.19 g, 58%) as a white crystalline solid that was filtered and washed with cold EtOH: melting point, 179.8°C; ^{1}H NMR δ 2.40 (s, 3H), 2.95 (s, 3H), 4.16 (s, 2H), 7.44 (s, 4H). Anal. calculated for $C_5H_{13}N_3O_5S$: C, 26.43; H, 5.77; N, 18.49; found: C, 26.60; H, 5.70; N, 18.39.

Creatine Hydrogen Maleate

To a stirred solution of maleic acid (0.04 mol, 10.40 g) in EtOH was added a suspension of creatine monohydrate (0.04 mol, 5.98 g) in water (80 ml) at 59°C. The solution was stirred for 10 min at 59°C before cooling to room temperature. The reaction

solution was then cooled to 5°C for 24 hr after which a creatine monohydrate precipitate that got formed was collected using suction filtration. Cold acetonitrile (300 ml) was added to the filtrate and cooled to 0°C to precipitate additional creatine monohydrate that was then removed using suction filtration. This process was repeated thrice until no additional precipitate was observed. After removing the solvents in vacuo, crude creatine hydrogen maleate (6.18 g, 59%) was filtered and washed with cold acetonitrile. Three crystallizations from 80% aqueous EtOH afforded analytically pure creatine hydrogen maleate: melting point 157.8°C; ^{1}H NMR δ 2.95 (s, 3H), 4.14 (s, 2H), 6.03 (s, 2H), 7.33 (s, 4H). Anal. calculated for $C_8H_{13}N_3O_6$: C, 38.87; H, 5.21; N, 17.00; found: C, 38.71; H, 5.49; N, 16.85.

HPLC Analysis of Creatine Salts

The HPLC system consisted of a Shimadzu SCL-10A controller, an SIL-10AF autosampler, dual LC-10AT pumps, an SPD-10A UV-VIS detector set to monitor absorbance at both 210 and 235 nm, and a CTO-10AS column oven set to 30°C, with a Waters Atlantis® T3 column (4.6 × 100 mm, 3 μm, C18). The isocratic mobile phase consisted of 20% v/v acetonitrile, 5-mM formic acid, and 5-mM 1-octanesulfonic acid sodium salt (apparent pH 2.8) at a flow rate of 1.5 ml/min. Simultaneous quantitative determination of creatine and creatinine content in test solutions was based on the UV absorbance at 210 nm (creatine and creatinine) and 235 nm (creatinine only). The UV absorbance of creatinine at 235 nm allows for confirmation of creatinine concentration at both wavelengths. Calibration curves were generated using stock solutions (500 μg/ml) of creatine monohydrate and creatinine diluted in the mobile phase to concentrations of 3, 10, 30, and 50 μg/ml. For lower concentrations, calibration curves were prepared in mobile phase at concentrations of 0.1, 0.3, 1, and 3 μg/ml. Linear regressions gave excellent agreement between concentrations and detector response within the experimental concentration ranges ($R^2 = 0.999$).

Saturation Solubility Determination

Using preliminary measurements as a guide, the saturated solubility of each creatine salt in deionized water was determined in triplicate by adding increasing amounts to 5 ml of solvent in screw-capped glass bottles placed in a shaking water bath at 25°C. After 1.5 hr, the saturated solutions were vortexed and 2-ml aliquots removed and centrifuged in microcentrifuge tubes at 11,000 rpm for 5 min. Creatine concentrations were analyzed by HPLC by diluting 500 μL of supernatant with mobile phase (500 μL). The mean ± standard deviation of the saturation solubility of each salt were calculated from the corresponding standard curves. Extended equilibration times for saturation were performed with no observable change in creatine concentration. Extending the equilibration time of creatine in these acidic saturated solutions did result in cyclization of creatine to creatinine as expected. Using the equilibration times outlined above, no significant creatinine concentration was observed in any of the saturation solubility experiments.

Determination of the pH-Dependent Saturation Solubility of Creatine Monohydrate

The pH-dependent solubility of creatine monohydrate was determined at 25°C in triplicate. Excess creatine monohydrate was added to 20-ml glass vials containing

1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.4, and 8.5 were measured; pH was adjusted by adding 0.1-M HCl or 0.1-M NaOH. The suspensions were equilibrated for 2 hr in a shaking water bath at 25°C and the final pH recorded. Aliquots were then removed and after centrifuging at 3,900 rpm for 10 min, the supernatant was collected, filtered through a 0.22-μm hydrophilic Aladn^{TM} syringe filter, and diluted in mobile phase and the creatine monohydrate concentration determined by HPLC analysis.

Thermal Analysis

Melting points of each creatine salt were obtained by differential scanning calorimetry (DSC) using a Shimadzu model DSC-50 with a TA-50WS controller. Samples (~5 mg) were placed in flat aluminum pans, crimped, and heated from 30 to 330°C at a rate of 10°C/min in an atmosphere of nitrogen (20 ml/min). To ensure that the true saturation solubility, rather than the apparent solubility, i.e., of free creatine, was being measured at each pH (Pudipeddi, Serajuddin, Grant, & Stahl, 2002), 3 hr after equilibration, 1-ml aliquot was removed, lyophilized for 72 hr, and the thermogram of the remaining compound obtained and compared to that of the original salt.

Water–Octanol Partition Coefficient Determination

To assay the concentrations of each salt in both the aqueous and octanol phase after equilibrium, the creatine salt in the octanol phase was extracted in water as described below. Standard solutions of creatine monohydrate in mobile phase were prepared in the range of 0.1 to 50 μg/ml by serial dilution of a 500-μg/ml stock solution. Two series of standards (0.1, 0.3, 1.0, 3.0 μg/ml and 3.0, 10, 30, 50 μg/ml) were prepared. For the partitioning studies, 25 ml of each creatine salt was prepared in octanol–saturated water at a nominal creatine concentration of 6.0 mg/ml. Due to very low partition coefficients, this relatively high concentration was used to ensure that each salt could be quantified in the octanol phase. Triplicate aliquots (5 ml) of each creatine monohydrate or creatine salt solution were placed in glass scintillation vials and then an equal volume (5 ml) of water-saturated octanol was added. Triplicate 5-ml samples of aqueous solutions of each creatine derivative served as controls to test for decomposition. The samples were allowed to equilibrate in a slowly shaking 25°C water bath for 2 hr and then the aqueous phases were assayed after appropriate dilutions with mobile phase. To assay the concentration of the creatine salt in the octanol phase, 4 ml of the water-saturated octanol phase was removed and placed in scintillation vials and 4 ml of water was added to extract the water-soluble salt. The samples were equilibrated for an additional 2 hr in a slowly shaking water bath at 25°C to extract the creatine from the octanol into the aqueous phase. The concentration of each derivative so extracted was then quantified after diluting 500-μL aliquots in 500-μL mobile phase. Due to the very small amount of creatine partitioning into the octanol phase, larger quantities of creatine (100, 200, 300, 400 μL of a 10-μg/mL stock solution) were also employed to more accurately assay the aqueous phase. The mean of the partition coefficient for each creatine compound was calculated and recorded as a log P value.

Caco-2 Monolayer Permeability

The permeability of various creatine salt forms was evaluated using the established human intestinal epithelial cell line derived from a human colon carcinoma (Caco-

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