Simultaneous Analysis of Creatine, Creatinine, and Several Breakdown Products of Creatine in Dietary Supplements using High Pressure Liquid Chromatography with Photodiode Array Detection

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Abstract

ROBERT C. THOMPSON Albion Advanced Nutrition, Clearfield, Utah: Simultaneous Analysis of Creatine, Creatinine , and Several Breakdown Products of Creatine in Dietary Supplements using High Pressure Liquid Chromatography with Photodiode Array Detection. Creatine is one of the over the counter substances that has experienced increased popularity in strength and fitness training. The safety of creatine and creatinine has been established for many years, but the potential of toxic substances from both synthesis and breakdown does exist. Cyanamide, dicyanodiamide, and 6-Azauracil are all known contaminants of creatine which all have been shown to be toxic to humans. Creatinine could also be classified as a creatine contaminant, but is much less toxic than the other compounds tested. When present in a creatine supplement, creatinine has no usefulness. An HPLC method for the simultaneous detection of cyanamide, dicyanodiamide, 6-azouracil, creatinine, and creatine was developed. Cyanamide when put into solution dime rizes to dicyanodiamide. This makes it difficult to test for cyanamide, but the presence of any dicyan odiamide may be an indication that cyamamide is present as well. This method uses reverse phase C18 HPLC separation with a paired ion mobile phase , and photodiode array detection. Very good ligearity for all compounds tested over a broad analytical range was achieved. (R^2 = 0.999 with a range of 2.00 to .02 µg on column) Recoveries in the range of 99 to 101% were achieved for all compounds tested using stan dard curves of separate masses for reference. Detection limits of 20ng on column for the compounds tested was achieved, and we believe that based on the current data ppm detection levels are possible. In conclusion, we believe that this method is a good quality control tool for the screening of toxic compounds and metabolites known to be present in creatine dietary supplements.

Introduction

The dietary supplement creatine has experienced an increase in sales due to its popularity in the world of bodybuilding and other physical fitness activities. With the passage of DSHEA (Dietary Supplement Health and Education Act) in 1994, supplements like creatine are regulated as food requiring little raw material testing. The compound creatinine has been identified as one of several known contaminants that can be found mixed in with the creatine raw material. Creatinine is a relatively non-toxic compound, and has no use as a dietary supplement. Other identified contaminants found in creatine are cyanamide, dicyanodiamide (dicyandiamide), and 6-azauracil. With the use of HPLC (High Performance Liquid Chromatography) we have developed an analytical method to test the purity of creatine. This method can easily identify contaminants such as creatinine, cyanamide, dicyanodiamide, and 6-azauracil. The technique requires a reverse phase paired ion mobile phase and photo diode array detector. Cyanamide itself presents a unique challenge with this method because it dimerizes to dicyanodiamide when dissolved in the extraction solvent (10mM KH_2PO_4). When dissolved into a solution at a specific concentration, cyanamide will produce a linear standard curve with approximately 79% percent conversion to dicyanodiamide. (see Table 1) Based on current data it is not clear whether this conversion is pH dependent, or if a different extraction solvent will prevent this conversion. We can only say that under current conditions, the presence of dicyanodiamide is and indication that cyanamide is possibly present as well.

Standard	Initial Cyamamide [Conc]	HPLC Data Peak Area Cyamamide	Peak Area Dicyanodiamide	Percent * Dicyanodiamide
1	1.003	615781	2381142	79.5%
2	0.502	298979	1165796	79.6%
3	0.251	151691	608598	80.0%
4	0.125	76900	300387	79.6%
5	0.063	39015	150383	79.4%
6	0.031	23675	75848	76.2%
7	0.016	11273	39282	77.7%
8	0.008	7133	25777	78.3%
	Slope	608832	2362652	
	Intercept	924	-460	
	R^2	0.9997	0.9998	

Table 1 * Percent dicyanodiamide is based on peak area

Procedure

The fact that creatine and creatinine differ in structure only by a single water molecule lost during the cyclization of creatine to creatinine, presents a difficult challenge separating these two compounds by HPLC. With the use of our paired ion HPLC separation method, standard curves were generated by serial dilutions which allowed us to determine range concentrations, and the respective correlation coefficients. (Table 2) Our initial concentrations were determined based on the response factor of each individual compound, as well as, taking into account that creatine is the main analyte present with the other four compounds being analyzed as trace level contaminants.

Analyte	Supplier	CAS #	Standard Curve Range in µg/ml	Correlation Coefficient
Creatine • H_2O	Acros	6020-87-7	8.4 - 1084	0.9996
Creatinine	Sigma	60-27-5	1.9 - 243	0.9999
Cyanamide *	Sigma	420-04-2	7.8 - 1003	0.9997 *
Dicyanodiamide	Acros	461-58-5	1.2 - 159	0.9982
6-Azauracil	Fluka	461-89-2	1.6 - 209	0.9999

Table 2

* The dimerization of cyanamide to dicyanodiamide restricts the direct analysis of this compound in this assay. Cyanamide must be analyzed with its own separate standard curve.

With good linearity of all analytes in the range tested, we randomly chose five commercially available creatine products to use in a test trial for our newly developed method. From a choice of many products, we decided on using two raw materials and three finished products for this trial. We purchased three finished products from a local retailer and the raw materials from a chemical supplier. Each of the five test products were sampled three times to allow an average, standard deviation and relative standard deviation to be calculated and recorded. Samples were labeled A, B, C, D and E for record keeping. Separate weighings were denoted by using a number with the letter. (example A1,A2 A3 etc.) External controls were also added so that the targeted analyte concentration could be checked for accuracy. All calculations are reported on Table 3.

Control Data

	Creatine Measured [Conc.]	% Recovery	Creatinine Measured [Conc.]	% Recovery	Citric Acid Measured [Conc.]	% Recovery	Dicyanodiamide Measured [Conc.]	% Recovery	6-Azauracil Measured [Conc.]	% Recovery
1	0.497	99.74%	0.116	96.58%	0.405	99.70%	0.127	99.53%	0.120	101.5%
2	0.494	99.11%	0.120	99.67%	0.408	100.5%	0.127	99.34%	0.120	101.4%
3	0.491	98.68%	0.124	103.3%	0.410	101.1%	0.127	99.41%	0.120	101.9%
4	0.487	97.71%	0.126	105.2%	0.409	100.8%	0.127	99.49%	0.121	102.2%
	Ave.	**98.81%**	Ave.	**101.2%**	Ave.	**100.5%**	Ave.	**99.44%**	Ave.	**101.7%**

Creatinine Data

Sample	Mass in mg	HPLC Result	Percent Creatine	Statistical Data	Label Claim
A1	118.3	2.31	1.96%	Ave = 1.96%	n.a.
A2	119.2	2.33	1.97%	Std = 0.0080%	
A3	120.0	2.37	1.97%	Rsd(ppt) = 4.08	
B1	51.00	0.0605	0.119%	Ave = 0.139%	n.a.
B2	50.60	0.0696	0.138%	Std = 0.0235%	
B3	51.70	0.0835	0.160%	Rsd(ppt) = 149.0	
C1	51.40	0.115	0.223%	Ave = 0.224%	n.a.
C2	51.40	0.113	0.219%	Std = 0.0053%	
C3	50.40	0.117	0.231%	Rsd(ppt) = 28.0	
D1	501.5	0.0440	0.00877%	Ave = 0.0104%	n.a.
D2	500.8	0.0590	0.0118%	Std = 0.0015%	
D3	500.8	0.0570	0.0138%	Rsd(ppt) = 139.0	
E1	499.3	0.0990	0.0183%	Ave = 0.0168%	n.a.
E2	500.1	0.0418	0.0163%	Std = 0.0011%	
E3	500.8	0.0695	0.0167%	Rsd(ppt) = 63.9	

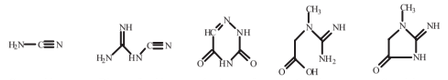
Creatine Data

Sample	Mass in mg	HPLC Result	Percent Creatine	Statistical Data	Label Claim
A1	118.3	50.8	42.9%	Ave = 43.0%	n.a.
A2	119.2	51.5	43.2%	Std = 0.12%	
A3	120.0	51.8	43.0%	Rsd(ppt) = 2.71	
B1	51.00	52.0	101.9%	Ave = 101.6%	n.a.
B2	50.60	51.3	101.4%	Std = 0.36%	
B3	51.70	52.5	101.5%	Rsd(ppt) = 2.56	
C1	51.40	51.9	101.0%	Ave = 101.0%	101.0%
C2	51.40	51.8	100.8%	Std = 0.31%	
C3	50.40	51.1	101.4%	Rsd(ppt) = 3.09	
D1	501.5	56.1	11.2%	Ave = 11.1%	111.1%
D2	500.8	55.3	11.0%	Std = 0.097%	
D3	500.8	55.6	11.1%	Rsd(ppt) = 8.05	
E1	499.3	52.3	10.5%	Ave = 10.5%	104.8%
E2	500.1	51.4	10.3%	Std = 0.21%	
E3	500.8	53.6	10.7%	Rsd(ppt) = 19.80	

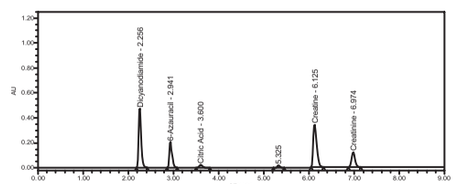
Citric Acid Data

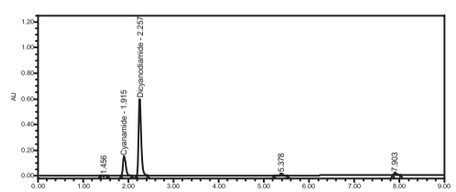
Sample	Mass in mg	HPLC Result	Percent Creatine	Statistical Data	Label Claim
A1	118.3	36.9	31.2%	Ave = 31.2%	n.a.
A2	119.2	37.4	31.4%	Std = 0.12%	
A3	120.0	37.5	31.7%	Rsd(ppt) = 3.91	
B1	51.00	0.0	0.0%	Ave = 0.0%	n.a.
B2	50.60	0.0	0.0%	Std = 0.00%	
B3	51.70	0.0	0.0%	Rsd(ppt) = 0.00	
C1	51.40	0.0	0.0%	Ave = 0.0%	n.a.
C2	51.40	0.0	0.0%	Std = 0.00%	
C3	50.40	0.0	0.0%	Rsd(ppt) = 0.00	
D1	501.5	1.0	0.2%	Ave = 0.2%	n.a.
D2	500.8	1.7	0.3%	Std = 0.09%	
D3	500.8	0.8	0.2%	Rsd(ppt) = 363.01	
E1	499.3	3.4	0.7%	Ave = 0.6%	n.a.
E2	500.1	3.5	0.7%	Std = 0.08%	
E3	500.8	2.8	0.6%	Rsd(ppt) = 119.27	

Table 3



Creatine – Creatinine HPLC Method





Instrument: Waters 2695 Seperations Module
Column: Phenomenex LUNA 3µ C18(2) 4.6 x 150mm Part# OOF-4251-E0
Injection Volume : 10µl
Mobile Phase : %C Acetonitrile
%D 25mM Ammonium Phosphate mono-basic , 5mM Na Hexanesulfonate pH 2.5
Flow rate: 1.00 ml/min

Detector: Waters 2996 Photodiode Array
Detection: PDA 200nm - 400nm
Analytical Wavelength
205nm

W2690/5 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		1.0	0.0	0.0	1.0	99.0	
2	1.00	1.0	0.0	0.0	1.0	99.0	1
3	7.00	1.0	0.0	0.0	14.0	86.0	6
4	8.00	1.0	0.0	0.0	25.0	75.0	1
5	9.00	1.0	0.0	0.0	1.0	99.0	3
6	19.00	1.0	0.0	0.0	1.0	99.0	-1

All samples, standards, and controls were run inter-dispersed throughout the sample set over a 24 hour period. (Table 4)

Empower software — Sample Set Data

Reported by User: R Charlie Thompson (Charlie) Project Name: Creatine_Creatinine

	SampleName	Sample Type	Vial	Run Time (Minutes)	Injection Volume (µl)	Acquisition Method Set	Label	Level
1	Control #1	Control	25	19.00	10.00	Creatine Creatinine Grad	Control	C
2	Standard Stock	Standard	26	19.00	10.00	Creatine Creatinine Grad	Stock	1
3	Sample A1	Unknown	27	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
4	Sample A2	Unknown	28	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
5	Sample A3	Unknown	29	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
6	Standard S1	Standard	30	19.00	10.00	Creatine Creatinine Grad	Std_1	2
7	Sample B1	Unknown	31	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
8	Sample B2	Unknown	32	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
9	Sample B3	Unknown	33	19.00	10.00	Creatine Creatinine Grad	Raw_Material	S
10	Standard S2	Standard	34	19.00	10.00	Creatine Creatinine Grad	Std_2	3
11	Control #2	Control	25	19.00	10.00	Creatine Creatinine Grad	Control	C
12	Sample C1	Unknown	35	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
13	Sample C2	Unknown	36	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
14	Sample C3	Unknown	37	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
15	Standard S3	Standard	38	19.00	10.00	Creatine Creatinine Grad	Std_3	4
16	Sample D1	Unknown	39	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
17	Sample D2	Unknown	40	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
18	Sample D3	Unknown	41	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
19	Control #3	Control	25	19.00	10.00	Creatine Creatinine Grad	Control	C
20	Standard S4	Standard	42	19.00	10.00	Creatine Creatinine Grad	Std_4	5
21	SampleE1	Unknown	43	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
22	SampleE2	Unknown	44	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
23	SampleE3	Unknown	45	19.00	10.00	Creatine Creatinine Grad	Finished_Product	S
24	Standard S5	Standard	46	19.00	10.00	Creatine Creatinine Grad	Std_5	6
25	Control #4	Control	25	19.00	10.00	Creatine Creatinine Grad	Control	C
26	Standard S6	Standard	47	19.00	10.00	Creatine Creatinine Grad	Std_6	7
27	Standard S7	Standard	48	19.00	10.00	Creatine Creatinine Grad	Std_7	8

Table 4

Conclusion

This HPLC technique for detecting and measuring the amounts of creatine, creatinine, cyanamide, dicyanodiamide, and 6-azauracil will be useful in testing the purity of raw materials and finished products. The challenge with this type of assay is the ability to measure a very large amount of the analyte (creatine) and simultaneously be able to measure trace amounts of contaminates that may be present. Controls were placed randomly throughout the assay to provide confidence in the linearity of the standard curves during the analytical run. Our controls show acceptable recovery which gives us confidence towards accuracy and precision. We also were able to calculate from the standard curves the linear range and estimate limits of detection for each analyte tested. The calculated recovery percentage of the creatine and creatinine in the controls demonstrated that a small amount of creatine was converted to creatinine during the 24 hr. run time. Each sample tested ended up having low relative standard deviation. The test results also show all of the finished products either met or exceeded the manufacturer's label claim. We found insignificant levels of creatinine in all of the raw materials and the finished products. As previously mentioned cyanamide and dicyanodiamide presented a problem due to the fact that when cyanamide is added to a solution, it converts to dicyanodiamide until both have reached equilibrium. All commercial samples tested negative for cyanamide and 6-azauracil. One raw material sample did test positive for dicyanodiamide but at a very low amount of less than 0.05%. This was below the minimum concentration of 19 nanograms on column. All other samples tested negative for dicyanodiamide. We also noted that citric acid was present in two of the finished products and one of the raw materials. We adjusted the method and were able to add citric acid to the standards. We found that citric acid did not interfere with any other standards present in the chromatographic separation. This allowed us to quantify citric acid in any raw material or finished product.

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