Metabolic Considerations in the death of Autumn Klein

And conviction of Robert Ferrante.

Robert Ferrante, inmate # LW6933

From Carol Gebert, PhD.

For relevantce and background see FDA guidances Safety Testing of Drug Metabolites at http://www.fda.gov/OHRMS/DOCKETS/98fr/FDA-2008-D-0065-GDL.pdf and exploratory IND studies: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm078933.pdf, and drug-drug interactions: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf

This document summarizes the academic literature that supports the proposition that (a) Autumn Klein suffered toxicity from creatine metabolites in potential combination with retinoic acid, and (b) Quest Diagnostics measured a creatine metabolite. See six main points below, with academic support of these propositions.

1. (Importance of specific transporters) Creatinine is widely known to be the first metabolite of creatine. Its clearance by kidney filtration is established medicine. The article by Optivia and Gilead, abstract below, explicitly names the drug transporter OAT2 as the most efficient pump for creatinine, followed by OCT2 and OCT3. http://www.ncbi.nlm.nih.gov/pubmed/24646860

Kidney Int. 2014 Aug;86(2):350-7. doi: 10.1038/ki.2014.66. Epub 2014 Mar 19.

Contribution of the organic anion transporter OAT2 to the renal active tubular secretion of creatinine and mechanism for serum creatinine elevations caused by cobicistat.

Lepist EI[1], Zhang X[2], Hao J[1], Huang J[2], Kosaka A[2], Birkus G[1], Murray BP[1], Bannister R[1], Cihlar T[1], Huang Y[2], Ray AS[1].

Author information

[1]Gilead Sciences, Foster City, California, USA.
[2]Optivia Biotechnology, Menlo Park, California, USA.

Abstract

Many xenobiotics including the pharmacoenhancer cobicistat increase serum creatinine by inhibiting its renal active tubular secretion without affecting the glomerular filtration rate. This study aimed to define the transporters involved in creatinine secretion, applying that knowledge to establish the mechanism for xenobiotic-induced effects. The basolateral uptake transporters organic anion transporter OAT2 and organic cation transporters OCT2 and OCT3 were found to transport creatinine. At physiologic creatinine concentrations, the specific activity of OAT2 transport was over twofold higher than OCT2 or OCT3, establishing OAT2 as a likely relevant creatinine transporter and further challenging the traditional view that creatinine is solely transported by a cationic pathway. The apical multidrug and toxin extrusion transporters MATE1 and MATE2-K demonstrated low-affinity and high-capacity transport. All drugs known to affect creatinine inhibited OCT2 and MATE1. Similar to cimetidine and ritonavir, cobicistat had the greatest effect on MATE1 with a 50% inhibition constant of 0.99 μM for creatinine transport. Trimethoprim potently inhibited MATE2-K, whereas dolutegravir preferentially inhibited OCT2. Cimetidine was unique, inhibiting all transporters that interact with creatinine. Thus, the clinical observation of elevated serum creatinine in patients taking cobicistat is likely a result of OCT2 transport, facilitating intracellular accumulation, and MATE1 inhibition.

2. (Additional clearance path by metabolism) When the kidney transporters do not function properly, then creatinine has enzymatic pathways that result in metabolites such as formaldehyde, methylamine, sarcosine and urea. See abstract below and *in vivo* data from mice. http://www.ncbi.nlm.nih.gov/pubmed/10859677 Note also that Autumn Klein did NOT have elevated urine creatinine. So where did it go?

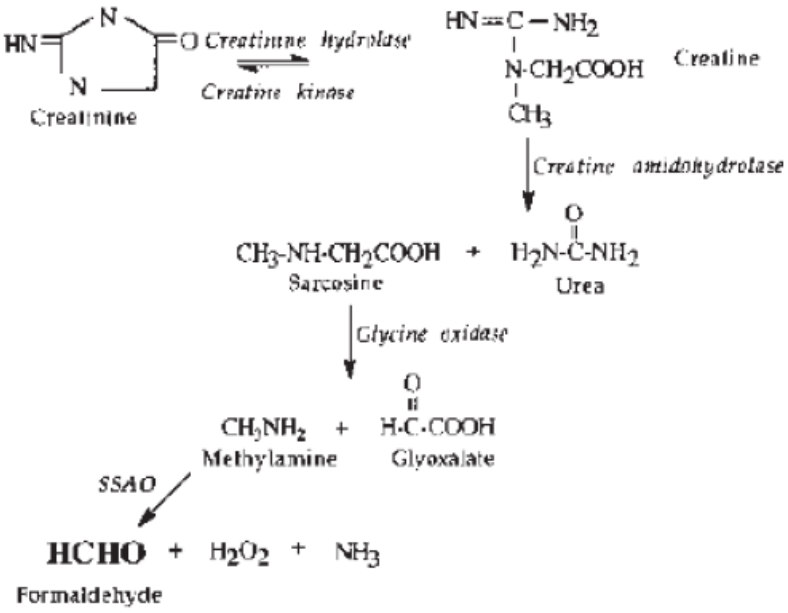
Potential cytotoxic effect of chronic administration of creatine, a nutrition supplement to augment athletic performance

Medical Hypotheses (2000) 54(5), 726–728

P. H. Yu, Y. Deng

Neuropsychiatry Research Unit, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Summary Creatine is alleged to be an ergogenic aid to enhance sports performance and recently became a popular sports nutrition supplement. Although short-term supplementation of creatine has not been associated with major health risks, the safety of prolonged use has caused some concern. The present study demonstrates that creatine is metabolized to methylamine, which is further converted to formaldehyde by semicarbazide-sensitive amine oxidase (SSAO). Formaldehyde is well known to cross-link proteins and DNAs, and known to be a major environmental risk factor. SSAO-mediated production of toxic aldehydes has been recently proposed to be related to pathological conditions such as vascular damage, diabetic complications, nephropathy, etc. Chronic administration of a large quantity of creatine can increase the production of formaldehyde, which may potentially cause serious unwanted side-effects. © 2000 Harcourt Publishers Ltd



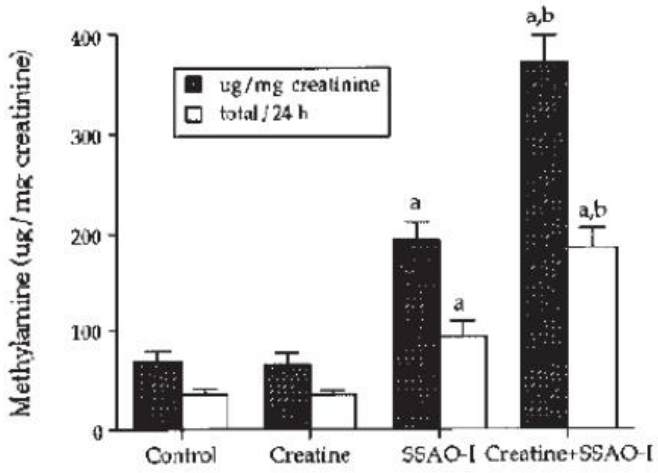


Fig. 2 Effect of creatine and SSAO inhibitor on urinary excretion of methylamine in CD1 Swiss white mice. A single dose of creatine (50 mg/kg, p.o.) and/or SSAO inhibitor (SSAO-I), (E)-4-fluoro-β-fluoroethylene-benzenebutamine (2 mg/kg, i.p.) were administered to the mice, and 24 h urines were collected in a vessel imbedded in dry ice. Methylamine was estimated with a fluorometric HPLC procedure as recently described (15). Both total excretion of methylamine in 24 h (open column) and amount of methylamine based on creatinine (filled column) were indicated. a = significantly different ($P < 0.01$) from the untreated controls; b = significantly different ($P < 0.01$) between SSAO-I vs creatine+SSAO-I groups ($n = 6$ for each group). The animal treatments were in strict accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee.

The enzymatic predictions are verified by human *in vivo* experiments by Jacques Poortmans, Belgium. (see below for two examples)

Med Sci Sports Exerc. 2005 Oct;37(10):1717-20.
http://www.ncbi.nlm.nih.gov/pubmed/16260971

Effect of oral creatine supplementation on urinary methylamine, formaldehyde, and formate.

Poortmans JR1, Kumps A, Duez P, Fofonka A, Carpentier A, Francaux M.

Higher Institute of Physical Education and Physical Therapy, Free University of Brussels, Brussels, Belgium. jrpoortm@ulb.ac.be

Abstract PURPOSE: It has been claimed that oral creatine supplementation might have potential cytotoxic effects on healthy consumers by increasing the production of methylamine and formaldehyde. Despite this allegation, there has been no scientific evidence obtained in humans to sustain or disprove such a detrimental effect of this widely used ergogenic substance.

METHODS: Twenty young healthy men ingested 21 g of creatine monohydrate daily for 14 consecutive days. Venous blood samples and 24-h urine were collected before and after the 14th day of supplementation. Creatine and creatinine were analyzed in plasma and urine, and methylamine, formaldehyde, and formate were determined in 24-h urine samples.

RESULTS: Oral creatine supplementation increased plasma creatine content 7.2-fold ($P < 0.001$) and urine output 141-fold ($P < 0.001$) with no effect on creatinine levels. **Twenty-four-hour urine excretion of methylamine and formaldehyde increased, respectively, 9.2-fold (P = 0.001) and 4.5-fold (P = 0.002) after creatine feeding**, with no increase in urinary albumin output (9.78 +/- 1.93 mg x 24 h(-1) before, 6.97 +/- 1.15 mg x 24 h(-1) creatine feeding).

CONCLUSION: This investigation shows that short-term, high-dose oral creatine supplementation enhances the excretion of potential cytotoxic compounds, but does not have any detrimental effects on kidney permeability. This provides indirect evidence of the absence of microangiopathy in renal glomeruli.

Also:

J Sports Sci. 2009 May;27(7):759-66. Doi: 10.1080/02640410902838237.
http://www.ncbi.nlm.nih.gov/pubmed/19437189

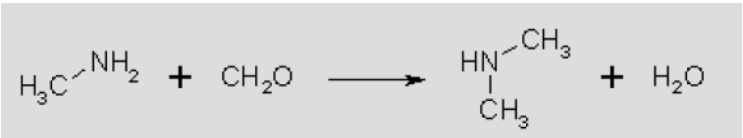
Urinary creatine and methylamine excretion following 4 x 5 g x day(-1) or 20 x 1 g x day(-1) of creatinemonohydrate for 5 days.

Sale C[1], Harris RC, Florance J, Kumps A, Sanvura R, Poortmans JR.

[1]School of Science and Technology, Nottingham Trent University, Nottingham, UK.
Craig.sale@ntu.ac.uk

Abstract: In this study, we examined the effect of two creatine monohydrate supplementation regimes on 24-h urinary creatine and methylamine excretion. Nine male participants completed two trials, separated by 6 weeks. Participants ingested 4 x 5 g x day(-1) creatine monohydrate for 5 days in one trial and 20 x 1 g x day(-1) for 5 days in the other. We collected 24-h urine samples on 2 baseline days (days 1-2), during 5 days of supplementation (days 3-7), and for 2 days post-supplementation (days 8-9). Urine was assayed for creatine using high-performance liquid chromatography and methylamine using gas chromatography. Less creatine was excreted following the 20 x 1 g x day(-1) regime (49.25 +/- 10.53 g) than the 4 x 5 g x day(-1) regime (62.32 +/- 9.36 g) (mean +/- s; $P < 0.05$). Mean total excretion of **methylamine** (n = 6) over days 3-7 was 8.61 +/- 7.58 mg and 24.81 +/- 25.76 mg on the 20 x 1 g x day(-1) and 4 x 5 g x day(-1) regimes, respectively ($P < 0.05$). The lower excretion of creatine using 20 x 1 g x day(-1) doses suggests a greater retention in the body and most probably in the muscle. Lower and more frequent doses of creatine monohydrate appear to further attenuate formation of methylamine.

Note also that metabolites can react with each other to form yet more chemicals, depending on the distribution of key enzymes. Dimethylamine (DMA) and paraformaldehyde are chief among these.



(CG: I highly suspect **dimethylamine** is the culprit analyte. Else, formic acid would build up.)

3. (Co-medications) Autumn Klein is on-record as having taken Accutane for some time. Note that Accutane is isotretinoin, a retinoid. Retinoic acid is known to down-regulate drug transporters in hepatocytes, with OAT2 being measured in this study, abstract below. http://www.ncbi.nlm.nih.gov/pubmed/23352986

Eur J Pharm Sci. 2013 Mar 12;48(4-5):767-74. doi: 10.1016/j.ejps.2013.01.005. Epub 2013 Jan 23.

Differential regulation of drug transporter expression by all-trans retinoic acid in hepatoma HepaRG cells and human hepatocytes.

Le Vee M[1], Jouan E, Stieger B, Fardel O.

Author information

Abstract

All-trans retinoic acid (atRA) is the active form of vitamin A, known to activate retinoid receptors, especially the heterodimer retinoid X receptor (RXR):retinoic acid receptor (RAR) that otherwise may play a role in regulation of some drug transporters. The present study was designed to characterize the nature of human hepatic transporters that may be targeted by atRA and the heterodimer RXR:RAR. Exposure of human hepatoma HepaRG cells and primary human hepatocytes to 5 μM atRA down-regulated mRNA levels of various sinusoidal solute carrier (SLC) influx transporters, including organic anion transporting polypeptide (OATP) 2B1, OATP1B1, organic cation transporter (OCT) 1 and organic anion transporter (OAT) 2, and induced those of the canalicular breast cancer resistance protein (BCRP). The retinoid concomitantly reduced protein expression of OATP2B1 and OATP1B1 and activity of OATPs and OCT1 and induced BCRP protein expression in HepaRG cells. Some transporters such as OATP1B3 and the bile salt export pump (BSEP) were however down-regulated by atRA in primary human hepatocytes, but induced in HepaRG cells, thus pointing out discrepancies between these two liver cell models in terms of detoxifying protein regulation. atRA-mediated repressions of OATP2B1, OATP1B1, OAT2 and OCT1 mRNA expression were finally shown to be counteracted by knocking-down expression of RARα and RXRα through siRNA transfection in HepaRG cells. atRA thus differentially regulated human hepatic drug transporters, mainly in a RXR:RAR-dependent manner, therefore establishing retinoids and retinoid receptors as modulators of liver drug transporter expression.

More investigation of the role of retinoic acid is suggested. E.g. a review of xenobiotic-retinoid interactions here: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4526766/

Note that Autumn Klein was diagnosed with a mitochondrial dysfunction. This abstract (full text here: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4452429/) suggested a link between retinoids and mitochondrial toxicity.

Oxid Med Cell Longev. 2015; 2015: 140267.
Published online 2015 May 19. doi: 10.1155/2015/140267
PMCID: PMC4452429

Vitamin A and Retinoids as Mitochondrial Toxicants

Marcos Roberto de Oliveira *

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Abstract Go to:

Vitamin A and its derivatives, the retinoids, are micronutrient necessary for the human diet in order to maintain several cellular functions from human development to adulthood and also through aging. Furthermore, vitamin A and retinoids are utilized pharmacologically in the treatment of some diseases, as, for instance, dermatological disturbances and some types of cancer. In spite of being an essential micronutrient with clinical application, vitamin A exerts several toxic effects regarding redox environment and mitochondrial function. Moreover, decreased life quality and increased mortality rates among vitamin A supplements users have been reported. However, the exact mechanism by which vitamin A elicits its deleterious effects is not clear yet. In this review, the role of mitochondrial dysfunction in the mechanism of vitamin A-induced toxicity is discussed.

Note also the drug has been linked to headaches, a severe symptom experiences by Autumn Klein in the months before her death.

J Dermatolog Treat. 2015 Apr 17:1-5. [Epub ahead of print]

Safety of alitretinoin for severe refractory chronic hand eczema: Clinical studies and postmarketing surveillance.

Morris M[1], Schifano L, Fong R, Graff O.

⊕ Author information

Abstract

BACKGROUND: Alitretinoin is approved for the treatment of adults with severe chronic hand eczema (CHE) refractory to potent topical steroids. In the 6 years since launch, approximately 250 000 patients have been treated with alitretinoin.

OBJECTIVE: To compare the postmarketing safety surveillance experience of alitretinoin with data from clinical trials and key safety issues with other retinoids.

METHODS: An integrated safety analysis of the pivotal studies of alitretinoin and postmarketing adverse event (AE) reports received since approval for alitretinoin were analyzed.

RESULTS: In the pivotal trials, headache, erythema, nausea, increased blood triglycerides and increased blood creatinine phosphokinase were the most frequently reported AEs. Headache, hyperlipidemia and nausea were also frequently reported postmarketing AEs, but depression was relatively more frequently reported than in the pivotal trials. Inflammatory bowel disease and benign intracranial hypertension were rare, and very few cases have been reported in postmarketing surveillance. There have been no reports of teratogenicity in humans consequent to fetal exposure.

CONCLUSIONS: Safety data collected in pivotal trials and postmarketing surveillance suggest that alitretinoin is well tolerated by patients with CHE with a relatively low incidence of serious reactions. The adverse reaction profile is congruent with reported effects of other marketed oral retinoids.

4. (Dosage) Autumn Klein was prescribed 10g/day of creatine. The European Sports Medicine Society recommends just 2g/day of supplementation. Note the dosages studies in this review article:

LEADING ARTICLE

Sports Med 2000 Sep; 30 (3): 155-170
0112-1642/00/0009-0155/$20.00/0

Adverse Effects of Creatine Supplementation

Fact or Fiction?

Jacques R. Poortmans[1] and *Marc Francaux*[2]

1 Physiological Chemistry, Higher Institute of Physical Education and Readaptation, Free University of Brussels, Bruxelles, Belgium
2 Institute of Physical Education and Readaptation, Catholic University of Louvain, Louvain-la-Neuve, Belgium

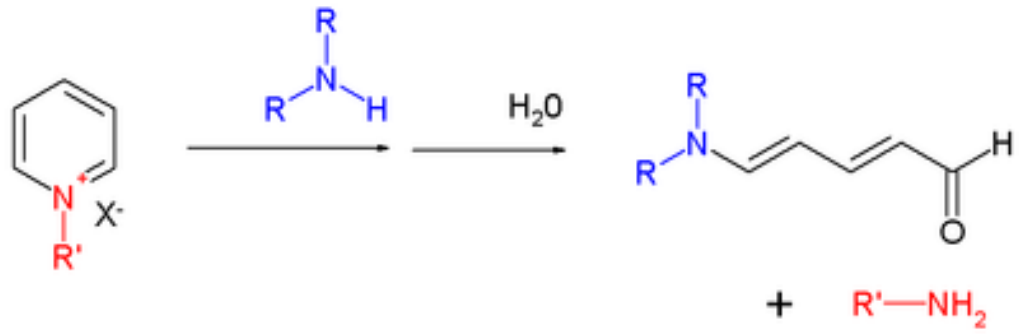
Exogenous creatine supplements are often consumed by athletes in amounts of up to 20 g/day for a few days, followed by 1 to 10 g/day for weeks, months and even years. Usually, consumers do not report any adverse effects, but body mass increases. There are few reports that creatine supplementation has protective effects in heart, muscle and neurological diseases. Gastrointestinal disturbances and muscle cramps have been reported occasionally in healthy individuals, but the effects are anecdotal. Liver and kidney dysfunction have also been suggested on the basis of small changes in markers of organ function and of occasional case reports, but well controlled studies on the adverse effects of exogenous creatine supplementation are almost nonexistent.

Table I. Body mass changes induced by creatine supplementation

Study	Year	Gender	Population	Dosage (g/day)	Number of days	Effect on body mass (%)
Short term (<10 days)						
Balsom et al.[23]	1993	M	Active	24	6	+1.5
Balsom et al.[24]	1993	M	Trained	20	6	+1.2
Greenhaff et al.[25]	1994	M	Active	20	5	+1.0
Stroud et al.[26]	1994	M	Active	20	5	+1.3
Balsom et al.[27]	1995	M	Active	20	6	+1.4
Dawson et al.[28]	1995	M	Active	20	5	+1.0
Green et al.[29]	1996	M	Sedentary	20	5	+1.1
Mujika et al.[30]	1996	M, F	Swimmers	20	5	+1.0
Vandenberghe et al.[31]	1996	F	Active	0.5 g/kg	6	Stable
Becque et al.[32]	1997	M	Weight lifters	20	5	+2.3
Godly & Yates[33]	1997	M, F	Cyclists	20	5	Stable
Grindstaff et al.[34]	1997	M, F	Swimmers	21	9	Stable
Hamilton-Ward et al.[35]	1997	F	Athletes	25	7	Stable
Prevost et al.[36]	1997	M, F	Students	18.8	5	Stable
Stout et al.[37]	1997	M	Football	21	5	Stable
Terrillion et al.[38]	1997	F	Runners	20	5	Stable
Vandenberghe et al.[39]	1997	F	Students	20	4	Stable
Bermon et al.[40]	1998	M, F	Active	20	5	Stable
Maganaris & Maughan[41]	1998	M	Active	11.4	5	+2.3
Oöpik et al.[42]	1998	M	Karatekas	20	5	+1.3
Snow et al.[43]	1998	M	Active	30	5	+1.3
Robinson et al.[44]	1999	M	Active	20	5	+1.4
Volek et al.[45]	1999	M	Trained	25	7	+2.1
Oöpik et al.[46]	1999	M	Wrestlers	20	5	+1.3
Urbanski et al.[47]	1999	M	Active	20	5	+1.0
Medium term (>10 days)						
Earnest et al.[48]	1995	M	Weight lifters	20	14	+1.9
Thompson et al.[49]	1996	F	Swimmers	2	42	Stable
Goldberg & Bechtel[50]	1997	M	Football	3	14	+0.8
Kirksey et al.[51]	1997	M, F	Athletes	0.3 g/kg	42	+2.0
Stout et al.[37]	1997	M	Football	10.5	51	Stable
Vandenberghe et al.[39]	1997	F	Students	5	60	Stable
Volek et al.[52]	1997	M	Trained	3	47	+1.8
Bermon et al.[40]	1998	M, F	Active	3	47	Stable
Kreider et al.[53]	1998	M	Football	16	28	+1.0
Francaux & Poortmans[54]	1999	M	Active	3	63	+2.9
Leenders et al.[55]	1999	M, F	Swimmers	10	14	Stable
Stone et al.[56]	1999	M	Football	8	35	+1.4
Volek et al.[45]	1999	M	Trained	5	77	+6.3
Rawson et al.[57]	1999	M	Old	20	30	+0.6
Francaux et al.[58]	2000	M	Active	21	14	Stable

Note that the highest dosages are 20g/day, and that only one study had duration of longer than a few weeks at this dose. Note the article mentions athletes. A reasonable assumption of body mass for a male athlete is ~210lb, or 2x that of Autumn Klein. Thus, her effective dose was actually the equivalent of 20g/day for a male athlete. Autumn took this dose for 50 days, far longer than any study cited in this table. Furthermore, if her OAT2 and OCT transporters were down-regulated by retinoids, then her effective dose was some multiple again. Yet her urine creatinine was not elevated. The only conclusion to reach is that Autumn Klein likely had high levels of *secondary* creatine metabolites in her blood.

5. (Interference of pyridine assays by creatine metabolites.) Of the expected metabolites, dimethylamine is already well characterized as a pyridine ring opener, in a reaction class called Zincke aldehydes.



This hypothesis was informally confirmed by LabChem, manufacturer of the reagent used by Quest Diagnostics, in an email exchange with the Operations Manager in August, 2015. Copied here:

> **Subject:** Pyridine-Barbituric Acid Reagent - interferences?
>
> Hi there,
> I am seeking information about your Pyridine-Barbituric Acid Reagent:
> http://www.labchem.com/tools/msds/msds/LC22190.pdf
>
> I understand it is a chromophore for cyanide assays. Do you know if the reagent also reacts with **organic amines**? I am especially interested to ask if dimethylamine is a known ring-altering substance likely to cause the pyridine reagent to change color. We have a sponsor with a metabolite that we fear is cyanide-mimetic, and need to know which diagnostic tests might give false positives.
>
> Carol Gebert Ph.D.
> Director of Business Development
> Woodland Biosciences
> cgebert@woodlandpharma.com
> office: 617 513 5280
>
> --------- Original Message ---------
> Subject: RE: Pyridine-Barbituric Acid Reagent - interferences?
> From: "Matt Petrina" <mattp@labchem.com>
> Date: 8/19/15 9:44 am
> To: "'cgebert@woodlandpharma.com'" <cgebert@woodlandpharma.com>
> Carol,
> My guess is that organic amines would react similarly (vs cyanide) with the pyridine-barbituric acid method. Note that APHA method 4500-CN uses Chloramine-T as an indicator. I would suggest reviewing the APHA method and potentially adapting it to your situation, but the only way to really know is to try an experiment. I hope this is helpful.
> Regards,
> Matt
>
> **Matthew V. Petrina**
> Operations Manager
>
> Jackson's Pointe Commerce Park – Building 1000
> 1010 Jackson's Pointe Court

Zelienople, PA 16063
www.labchem.com
Office: 412-826-5230 X116
Fax: 724-473-0647

From: cgebert@woodlandpharma.com [mailto:cgebert@woodlandpharma.com]
Sent: Wednesday, August 19, 2015 3:51 PM
To: Matt Petrina
Subject: RE: Pyridine-Barbituric Acid Reagent - interferences?

Matt - Thanks for your reply, and your advice. I guess anything that opens the ring into an aldehyde will test positive.
I don't suppose you know of any gov or academic lists of known interferences, do you?
Carol Gebert Ph.D.
Director of Business Development
Woodland Biosciences
cgebert@woodlandpharma.com
office: 617 513 5280

Carol,
Unfortunately I'm not aware of any such lists of known interferences. Good luck in your research.
Regards,
Matt

Matthew V. Petrina
Operations Manager

In support of these academic studies I suggest the following controls to collect data specific to the case of Robert Ferrante and Autumn Klein:

(a) To spike human blood samples with creatinine, mono-methylamine (MMA), dimethylamine (DMA), paraformaldehyde, urea and other known creatine metabolites and perform a Conway-method cyanide assay using the pyridine reagent.

(b) To incubate aqueous creatine /creatinine at various pH (2-5) at various temperatures (4deg to 60) for 48hrs, and then perform GC/MS to verify degradation products include DMA. This control tests whether the Quest protocol step of acid addition converts creatinine to DMA.

(c) To dose rats with creatine, and confirm literature results identifying metabolites. Or just perform an LD50 in mice to find the lethal dose.

(d) To retest any frozen blood samples still remaining from Autumn Klein, by GC/MS and LC/MS to identify known creatine metabolites, and to perform comparative mass balances of these findings to those from Quest Diagnostics' first assay results.