CLINICAL TRIAL

SINGLE-CENTER, OPEN, RANDOMISED, CROSS-OVER, WITH TWO PERIODS OF TREATMENT CLINICAL TRIAL TO COMPARE BIOAVAILABILITY AND ASSESS THE BIOEQUIVALENCE OF 5 grams CREATINE MONYDRATE AND 5 grams OF BUFFERED CREATINE MONOHYDRATE (KRE-ALKALYN), APPLIED AS A SINGLE DOSE TO 4 HEALTHY VOLUNTEERS UNDER FASTING CONDITIONS

Content

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FINAL REPORT

Date of study: September, 2007

1. Content

STUDY PARTICIPANTS:

Sponsor:	**All American Pharmaceutical and Natural Foods Corporation**
Clinical Centre:	**"Dr. I.S.Greenberg" Medical Centre**
Principle investigators:	**Dr. Kamen Stoychev, MD** **Dr. Stanislav Yanev, PhD, MD**
Analysis and Statistics:	**Dr. Stanislav Yanev** **Bozhidarka Pandova** **Dept.Drug Toxicology, BAS** **Acad.G.Bonchev Str., bl.23** **1113 Sofia, Bulgaria**
CRO:	**Dr. Kamen Stoychev** **All American Pharmaceutical and Natural Foods Corporation**

Date: August, 2007

Clinical study was performed under the principals of GCP and GPL.

2. STUDY SYNOPSIS

SPONSOR:	All American Pharmaceutical and Natural Foods Corporation
NAME OF THE ACTIVE SUBSTANCE:	Creatine 5 g
TITLE OF STUDY:	SINGLE-CENTER, OPEN, RANDOMISED, CROSS-OVER, WITH TWO PERIODS OF TREATMENT CLINICAL TRIAL TO COMPARE BIOAVAILABILITY AND ASSESS THE BIOEQUIVALENCE OF 5 grams CREATINE MONYDRATE AND 5 grams OF BUFFERED CREATINE MONOHYDRATE (KRE-ALKALYN), APPLIED AS A SINGLE DOSE TO 4 HEALTHY VOLUNTEERS UNDER FASTING CONDITIONS
PRINCIPAL INVESTIGATORS:	Dr. Kamen Stoychev, MD
STUDY SITE:	"Dr. I.S.Greenberg" Medical Centre
PHASE:	I - bioequivalence
PURPOSE OF TRIAL:	Investigation of the bioequivalence of two creatine formulations.
DESIGN:	Single-center, randomized, open, balanced, two-way cross-over study after single intake of CREATINE 5 g in healthy volunteers.
SUBJECTS:	Protocol foreseen: 4 volunteers Survey: 4 volunteers Drop-outs: 0 volunteer Completed the study: 4 volunteers
INCLUSION CRITERIA:	Healthy subjects aged 18 – 45, with height/weight ratio under MLIC (1983) rules.
STUDY MEDICATIONS:	Test formulation: **Kre-Alkalyn, 5 grams, powder** Reference formulation: **Creatine Monohydrate, 5 mg, powder**
ROUTE OF INTAKE:	Per oral
TREATMENT DURATION:	Single dose with 3 days wash out period
SAFETY CRITERIA:	Physical status; appearance of adverse reactions; changes in clinic-laboratory parameters.
CLINICAL PROCEDURE:	Each volunteer received in fasting condition under the randomization scheme single dose of 5 g CREATINE (as test or reference preparation) in two periods of treatment with 3 days wash out period between them. Blood samples for determination of CREATINE plasma concentrations were withdraw at 0, 0,5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, hours after medication.

BIOANALYTICAL REPORT

ANALYSIS OF CREATINE IN PLASMA

THIS ANALYTICAL REPORT CONSISTS
OF THE FOLLOWING SUB-REPORTS:

- PRE-STUDY VALIDATION
- STUDY RESULTS
- ADDENDUM – EXAMPLES OF CHROMATOGRAMS

The results reported apply to the testing of the samples as described.

The results in this report may not be reproduced in part.

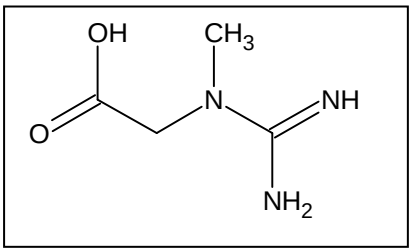
PRE-STUDY VALIDATION

ANALYSIS OF CREATINE IN PLASMA

METHOD DESCRIPTION

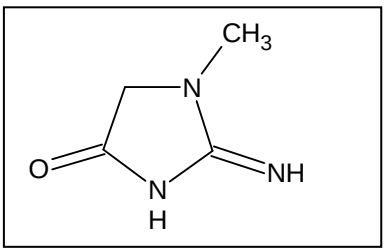
Analyte:

CREATINE
(Sigma)



Molecular Formula = $C_4 H_9 N_3 O_2$
Formula Weight = 131.133

CREATININE
(Sigma)



Molecular Formula = $C_4 H_7 N_3 O$
Formula Weight = 113.118

Summary:

The method used for CREATINE (CRE) and CREATININ (CRN)plasma determination was developed and validated in Department of Drug Toxicology, Inst. Neurobiology, BAS, Sofia based on significantly modified procedure described by Persky A. et al (2003).

0.25 ml of plasma was mixed after thawing with 0.5 ml buffer. Then samples were passed through SPE columns. The elutes was resuspended in 100 µl of 10% perchloric acid and were transferred to the autosampler vial. The vial was briefly shaken and 50 µl of the aqueous phase was injected to HPLC.

The plasma content of CRE and CRN in control and unknown samples is determinate using external standard method of calibration curves of chromatographic peaks area of known amounts of CRE and CRN.

Quality Assurance:

The study was conducted according to the principles of good laboratory practice. In-house standard operating procedures were applied during all phases of the study. Grades and batch numbers of all chemicals used were recorded and equipment used was checked and calibrated before use. All calculations and data transcriptions were checked by the analyst who performed the original calculation or transcription. In addition the internal auditor checked results on a daily basis. Final data was approved by the laboratory director before release. All raw data, validation data, summaries and reports are stored in the archives of Department of Drug Toxicology, Inst. Neurobiology, BAS, Sofia.

Equipment and materials

A. Chemicals

Reagent	Grade	Supplier	Batch. No.
Perchloric acid	Pure for analysis	Fluka	54998
Phosphoric acid	Pure for analysis	Fluka	44678
Potassium phosphate monobasic	Pure for analysis	Sigma	5379

Water was prepared in-house from Millipore water purifier.

B. Reference compounds

Name	Supplier	Lot/Batch No.
CREATINE MONOHYDRATE	Sigma	C3630
CREATININE	Sigma	C4255

Certificate of Analysis are kept on file.

B. Equipment

Glassware	Description	Supplier
Volumetric flasks	Grade A	Alkem
Graduated cylinders	Grade A	Alkem
Tubes (15 ml)	Plastic	Valerus

Dispensers	Make	Supplier
Variable volume	Pipetteman 1000	Eppendorff
	Pipetteman 200	Eppendorff
	Multipette	Eppendorff

Lab. Equipment	Type	Manufacturer
Analytical balance	KERN 770	Mettler
Top loading balance	KERNGJ	Mettler
Vortex mixer	Heidolph Reax	Heidolph
pH meter	WTW	Beckman
Centrifuge	MPW - 310	Poland
Shaker	Thyys10	Germany
Magnetic Stirrer	MR 3001 K	Thermolyne Corporation

PLASMA TREATMENT

0.25 ml of plasma was mixed after thawing with 0.5 ml buffer. Then samples were passed through SPE columns. Elutes was resuspended in 100 µl of 10% perchloric acid and were transferred to the autosampler vial. The vial was briefly shaken and 50 µl of the aqueous phase was injected to HPLC.

CHROMATOGRAPHIC CONDITIONS

The analytical method is developed on Waters reverse phase liquid chromatography system equipped with:

- Quaternary pump 600E
- PDA 996 set at multiplot regime.

Chromatographic conditions:

Pre-column:
AJO-6075 Polar-RP, 4.0x2.0 mm, Synergi

Analytical column
Polar-RP 80Å, 150x4,6 mm, 4µm, Synergi
Working temperature of the analytical column: 25^{o}C
Mobile phase:
- Buffer (0.05 M phosphate buffer (pH 4.0 with 70% perchloric acid);

Flow rate: 1 ml/min
Detection: UV= multiplot
Injection volume: 50 µl

Instrument control and integration is performed on Pentium 566 computer by Waters Empower software and the chromatograms are stored and reproduced by the same system.

CALCULATIONS

During the validation procedures all data was subjected to regression analysis using different regression equations. The equation that gave the best results, based on accuracy, for the entire validation range was selected for the calculation of concentrations of CRE and CRN in unknown samples. For this study a linear regression equation without weighting was found to be the most suitable to cover the dynamic range.

ANALYTICAL METHOD VALIDATION

Methods employed by Dept. Drug Toxicology undergo a three-phase validation prior to use. A pre-study validation is performed during the developmental stages of a new method. This is followed, if necessary, by a confirmatory revalidation performed immediately prior to commencement of trial sample analysis. During the actual analysis of the study samples the within-study validation is performed on a daily basis to monitor actual performance of the method over the analytical period of the particular study. Methods used for these validations are based on FDA Guidance for the Industry, Bioanalytical method validation (May 2001).

Validation Parameters

Definitions and Acceptance Criteria:

Specificity and selectivity: Selectivity of the analytical method will be determinate by the comparing the chromatograms of diluents, CRE standards, blank plasma and spiked plasma samples. In chromatogram of diluents and blank plasma sample there should not be any interfering peak at retention times of CRE and CRN, while retention times of CRE and CRN standards should correspond to retention times of the same component in spiked plasma samples.

The validation of specificity and selectivity of analytical method is done by **System suitability test**. Waters Empower software provides these data automatically under the criteria of European Pharmacopoeia:

- Precision of the detector response: RSD of the detector response for 6 injections of standard solution is <1%.
- Resolution factor (Rs) and Capacity Factor (k') between peaks of CRE and an unknown peak is >2.
- Tailing (symmetry) factor for CRE is <2.0.
- The number of plate count (N) as measure for column efficiency is for CRE >2000.

Linearity and range of determination: Linearity will be performed at 7 concentration points (excluding blank values) in the concentration range from 1 to 200.0 µg/ml for CRE and CRN, in 3 different days. Spiked plasma samples will be prepared and analysed as described in analytical procedure. Linear regression equation, slope and intercept, correlation coefficient and coefficient of determination will be calculated. The results will be also present graphically.
Criterion: $R^2 > 0.999$

Sensitivity: two parameters define the sensitivity of the method, the Limit of Quantification (LOQ) and Limit of Detection (LOD) The LOQ is that concentration of CRE which can be quantitatively determined with accuracy and precision better than RSD < 20%, and a signal-to-noise ratio better than 10:1. The LOQ will be determined using 6 replicate determinations. The LOD is the concentration of analyte that can be reliably differentiated from background levels but not quantitated with

sufficient accuracy or precision. A signal-to-noise ratio of between two and three is generally acceptable.

Recovery: indicates losses incurred during sample processing. Recoveries of more than 80% are normally required however reproducible recoveries lower than this is acceptable.
Criterion: minimum 80%

PRE-STUDY VALIDATION

PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROLS

Dynamic Range:

After a single oral dose of 5g of CREATINE, maximum plasma levels for CRE in some volunteers of approximately 200 µg/ml are to be expected. A validation range approximately from 1 to 200 µg/ml was therefore chosen to allow determination of CRE plasma levels in the largest possible interval.

RESULTS OF PRE-STUDY VALIDATION

1. Specificity and selectivity

Selectivity was performed by recording the chromatograms of diluents and standards of CRE and CRN. In the chromatogram of diluents samples there were not peaks matching that of **CRE** and **CRN** (Rt around **2.4** min and **2.7** min correspondingly**)**. In volunteers samples the peaks around **5.2 min** and **9.8** min which area is changing with the sampling time, probably are due to CRE metabolites (**?)**.

The method selectivity is demonstrated on **Figure 1**, where are demonstrated overlay chromatograms from samples with known concentrations of CRE and CRN injected directly or passed through the SPE columns.

The parameters of **System suitability** (**Fig. 1**) for CRE and CRN showed the following results:

Precision of the detector response: 2.5 – 3.3%
Resolution factor (Rs): 2.3 (SD 0.01)
Capacity factor (k'): 0.36 – 0.52 (SD 0.01)
Symmetry factor (**tailing**): 1.1 (SD 0.003)
The number of theoretical plates (N) for CRE is > 8000.

2. Linearity and range of determination

Linearity was performed at 7 concentration points for CRE and CRN (excluding blank values) in concentration range from 1 to 200 µg/ml, run in 3 different days. Spiked water samples were prepared and analysed as described in analytical procedure. Linear regression equation, coefficient of regression, slope of regression line and intercept were calculated. The results for CRE and CRN are presented in **Table 1 and Table 1a,** correspondingly. The graphical presentation of linearity is shown in the **Figs. 2 and 2a**.

Table 1. Intercept, slope and r^2 for CRE calibration curve (mean from 3 days)

	intercept	slope	r^2
mean	**0.00329**	**0.0002687**	**0.999833**
SD	0.000815	0.0000102	
C.V. (%)	24.77506	3.7960	

Table 1a. Intercept, slope and r^2 for CRN calibration curve (mean from 3 days)

	intercept	slope	r^2
mean	**0.0001017**	**0.00033936**	**0.99876**
SD	0.0000215	0.0000102	
C.V. (%)	21.14506	3.005657	

In the concentration range of **1 to 200 µg/ml** the dependency of peak area of CRE and CRN to concentration of CRE and CRN is linear with regression line presented as **y = bx + a** where **y** is the average peak area while **x** represents concentration of CRE or CRN, **b** is the slope, **a** the intercept and $\mathbf{r^2}$ is the coefficient of determination.

3. Sensitivity:

LOQ (limit of quantification): The lowest concentrations of CRE which corresponded of criteria of precision, accuracy and signal-to-noise ratio 10:1 (RSD of the lowest concentration of CRE and CRN from the calibration curve to be < 20%) were found out to be **1 µg/ml (Fig. 2 and 2a).**

LOD (limit of detection): Upon the accepted criterion **(**signal-to-noise ratio 3:1) the LOD for CRE and CRN was determined to be around **0.2 µg/ml**.

6. Recovery

The recovery of the analyte from plasma was quantified at tree different concentrations over the calibration range used (10, 100 and 200 µg/ml). This was performed using five individually spiked plasma samples at each concentration assayed, and comparing CRE and CRN peak so obtained with those of aqueous solutions of similar concentrations after dividing the endogenous CRE and CRN concentrations. The results are presented in **table 2** and **fig. 1**.

Table 2. Recovery of CRN spiked to plasma samples

Concentration (ng/ml)	**Area for InSt (n=5)**	**Area for ExSt (n=5)**	**Analytical yield (%)**
200	5229360	5414495	**96.49**

100	2515333	2555104	**98.44**
10	**264311**	**269733**	**97.98**
Mean yield			97.63

The mean analytical yield of CRE and CRN spiked to plasma samples as compared with CRE and CRN injected directly to HPLC system is around **97.6%** and **95.4%** correspondingly**.**

8. Conclusion:

The HPLC analytical method for CRE and CRN determination in spiked human plasma showed parameters of precision, accuracy, linearity, limits of quantification and detection and specificity in the limits of validation criteria. The results of this validation show that CRE and CRN can be analyzed in human plasma in the concentration range 1 to 200 ng/ml according to the method described with sufficient reliability for pharmacokinetic studies.

9. Signatures:

BIOANALYTICAL REPORT FOR HPLC DETERMINATION OF CREATINE IN HUMAN PLASMA

Validation date: 10th – 15th August 2007

Analyst: .....(B.Pandova)

Head of the Analytical lab:.....(S.Yanev)

STUDY RESULTS

ANALYSIS OF CREATINE IN PLASMA

ANALYTICAL REPORT

1. Conduct of Study:

A total of 104 frozen plasma samples were received by Dept.Drug Toxicology, Inst.Neurobiology, BAS on the 15th of August 2007 for the CREATINE study. These samples were stored in a freezer set at -28°C and were analyzed in batches by the method described in validation report between the 25th and 27th of August 2007. Each batch consisted of six calibration standards and three double quality control samples, together with study samples from both phases of two volunteers.

2. Protocol realization in the time of the CREATINE determination in plasma of healthy volunteers:

The results of plasma analysis of samples from 4 volunteers after the intake of test and reference preparations from 13 time intervals (104 samples all together) showed a greater concentration of creatine from the buffered creatine group. Plasma levels below the limit of quantification were denoted with LOQ and missing samples with "M".

7. Conclusion:

The data presented in this report indicate that the method used for the determination of CREATINE and CREATININE in plasma samples generated during the clinical phase of the study was suitably validated prior to analysis and remained consistent throughout the period of analysis. The results are therefore suitable for pharmacokinetic analysis. The buffered creatine (Kre-Alkalyn) group had better absorption then the regular creatine group with less creatinine levels.

8. Signatures:

BIOANALYTICAL REPORT FOR HPLC DETERMINATION OF CREATINE IN HUMAN PLASMA

Date of analysis: 25th - 28th August, 2007

Analyst: **(B.Pandova)**

Head of the Analytical lab:.. . (S.Yanev)

CONFIDENTIAL/PROPRIETARY STATEMENT