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Servicebereich Alzserv

Method-SOP: 107-138/1

2-ANA/BL-Dr.Ru/Eg
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Assay of Creatine and Determination of the Impurities Dicyandiamide, Creatinine and Dihydrotriazine in Creatine monohydrate by HPLC

1 Principle

Substances are separated by HPLC using an ion exchange column and determined at a wavelength of 225 nm. The calculation is performed with external standards.

2 Reagents

Water, deionised
Creatine monohydrate, standard of known purity, Fluka 27900
Creatinine standard of known purity, Fluka 27910
Dicyandiamide standard of known purity, Degussa AG
Dihydrotriazine standard of known purity, Degussa AG
Ammoniumdihydrogen phosphate, p.a., Riedel de Haen 30401
Phosphoric acid, 85 %, Fluka 79621

3 Equipment

High performance liquid chromatograph consisting of:
- Autoinjector 234, Abimed
- Pump 307, Abimed
- UV-VIS detector 115, Abimed (sensitivity 0.005)

Calculation software Gilson Unipoint 1.90
Column, Nucleosil 5 SA, EI 250/4, Macherey-Nagel
Analytical balance, readability 0.1 mg
Analytical balance, readability 0.001 mg
Ultrasonic bath
pH-meter
Standard laboratory equipment

4 Procedure

4.1 Preparation of Standard Solutions

Weigh approx. 250 mg creatine monohydrate into a 100 ml volumetric flask, dissolve in water with the help of an ultrasonic bath and make up to volume with water. The solution is stable for 3 days.

Weigh approx. 0.5 mg creatinine into a 100 ml volumetric flask, dissolve in water with the help of an ultrasonic bath and make up to volume with water. Pipett 10 ml of this solution into a 100 ml volumetric flask and make up to the volume with water. The solution is stable for 3 days.

Weigh approx. 1 mg dicyandiamide into a 100 ml volumetric flask, dissolve in water with the help of an ultrasonic bath and make up to volume with water. Pipett 2 ml of this solution into a 100 ml volumetric flask and make up to the volume with water.

Weigh approx 0.5 mg dihydrotriazine into a 100 ml volumetric flask, dissolve in water with the help of an ultrasonic bath and make up to volume with water. Pipett 2 ml of this solution into a 100 ml volumetric flask and make up to the volume with water.

4.2 Preparation of Sample

Weigh approx. 250 mg of sample into a 100 ml volumetric flask, dissolve in water with the help of an ultrasonic bath and make up to volume with water.

Caution: Prepare the solution fresh for every determination, because creatinine is formed in the solution. The samples should be in the ultrasonic bath only for a short period, because heat promotes the forming of creatine.

5 System Suitability Test

The suitability of the system must be verified every day: Inject the standard solution for creatine monohydrate six times. Calculate the coefficient of variation of the peak areas of the last five in-jections; it should not exceed 2 %. Otherwise the equipment should not be used. Calculation of the coefficient of variation (C_v):

$$C_v = \frac{S_{FI} * 100}{F_m}$$

C_v = coefficient of variation [%]
S_{FI} = standard deviation of peak areas
F_m = mean of peak areas

For the calculation of S_{Fl} and F_m see SOP 706.1.

6 HPLC Determination

Standard and sample solutions should be injected in the following sequence:
Standard solution creatine monohydrate, 6 sample solutions, standard solution creatine monohydrate, 6 sample solutions, etc. Inject the standard solutions of creatinine and dicyandiamide at least once a day.
If necessary (creatinine- and dicyandiamide peaks are present) inject the standard solutions frequently. Usually there is no dihydrotriazine detectable in the sample. In case a peak appears a dihydrotriazine standard solution has to be prepared and injected in order to determine the dihydrotriazine content.
Each sample must be analysed twice.

7 Chromatographic Operating Conditions

Wavelength	225 nm	
Flow	1 ml/min	
Mobile phase	dissolve 23 g ammonium dihydrogen phosphate in 1 l water and adjust the solution to a pH-value of 4.0 with phosphoric acid	
Injection volume	20 µl	
Column temperature	ambient	
Retention times	creatine	approx. 3.5 min
	dicyandiamide	approx. 2.8 min
	dihydrotriazine	approx. 5.4 min
	creatinine	approx. 8.6 min

8 Calculation

Calculation is made by the software or with the following formula:

$$Geh = \frac{F_{Pr} * E_{St} * G_{St} * U}{F_{St} * E}$$

Geh = Content [%]
F_{Pr} = peak area or peak height of sample signal
F_{St} = peak area or peak height of standard signal
E_{St} = weight of standard [mg]
G_{St} = content of standard [%]
E = weight of sample [mg]
U = conversion factor (creatine monohydrate to creatine = 0.8792)

For the standard use the mean of at least two injections.

For creatinine, dicyandiamide and dihydrotriazine the content must be multiplied by 10000, because the residues of these substances are stated in mg/kg. These substances are usually calculated using the peak heights.

The result must be the mean of two determinations. The results are reported with the same precision as stated in the specifications; the raw data are documented with one more significant figure than stated in the testing protocol.

For the calculation it must be considered, whether the results will be reported as creatine (usually) or creatine monohydrate. The molecular masses are:

Creatine: 131.14 g/mol Creatine monohydrate: 149.15 g/mol

9 Characteristic Statistical Data

9.1 Assay of Creatine

Precision: sixfold determination of a typical sample
mean: 88.8 % C_v=0.95 %

Linearity: A calibration plot is linear for sample concentrations between 200 – 300 mg/100 ml.
(r = 0.9998)

Specifity: Due to the chromatographic separation of creatine, creatinine, dicyandiamide and dihydrotriazine the analytical method is specific for the determination of creatine.

Accuracy: (Trueness): Because of the existing data about precision, linearity and specifity as well as the analysis of the reference substance the analytical method is considered to be true.

9.2 Determination of Dicyandiamide, Creatinine and Dihydrotriazine

Precision: (calculated from the data of the determination of recovery)
sixfold determination in each case

	mean	C_V
DCD	42.9 mg/kg	2.9 %
Creatinine	95.4 mg/kg	7.0 %

Recovery rate (accuracy):

Approx. 100 mg/kg creatinine and approx. 40 mg/kg dicyandiamide are added to a creatine monohydrate sample (no dicyandiamide detectable, 44 mg/kg creatinine).

mean recovery rate	- for creatine:	93.4 %
(six replicates)	- for dicyandiamide:	101.7 %

Limit of detection and limit of quantitation:

The following LODs and LOQs are calculated from the deviation of the data for the recovery rates:

	LOD [mg/kg]	LOQ [mg/kg]
creatinine	20	67
dicyandiamide	4	13
dihydrotriazine	4.5	15

(estimated from the signal to noise ratio of a dihydrotriazine standard solution with 0.00445 mg/100 ml)

Linearity:

DCD: 7 calibration points between 0.001055 – 0.1055 mg/100 ml (approx. 4 – 400 mg/kg)
r = 0.9998

Creatinine: 4 calibration points between 0.00293 – 0.0585 mg/100 ml (approx. 12 – 230 mg/kg)
r = 0.9999

Dihydrotriazine: 4 calibration points between 0.00445 and 0.445 mg/100 ml (approx. 18 – 1780 mg/kg)
r = 0.9999

Specifity: Due to the chromatographic separation of creatine, creatinine, dicyandiamide and dihydrotriazine the analytical method is specific for the determination of creatine, dicyandiamide and dihydrotriazine.

9 Remark

This method is suitable for the determination of creatine in mixtures containing creatine, which are soluble in water. This method may also be used for the determination of creatine in creatine pyruvate or creatine citrate.

(Creatine pyruvate $C_7H_{13}N_3O_5$ 219.19 g/mol)
(Creatine citrate $C_{18}H_{35}N_9O_{13}$ 585.51 g/mol)

The mentioned suppliers serve only as examples. If chemicals of other suppliers are used they must have at least the same quality.

10. Enforcement

Step	**Org. – Unit / site**	**Name**	**Date**	**Signature** *[on the original only]*
Prepared by	B-CS P (QKL) / Essen	Käseborn	14.05.03	X X X X X
Checked by	B-CS P (QKL) / Essen	Käseborn	14.05.03	X X X X X
Approved by	B-CS P (QKL) / Essen	Käseborn	14.05.03	

Reviewed regarding validity *[on the original only]*

Date	*Signature*	*Date*	*Signature*	*Date*	*Signature*