EAGLE BIOSCIENCES

Hydroxypyridinium Crosslinks HPLC Assay

Catalog Number: CSL34-H100
100 Tests
For Research Use Only. Not for use in diagnostic procedures.
v. 1.0

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1. Intended purpose

The Hydroxypyridinium Crosslinks HPLC Assay kit is intended for the quantitative determination of the hydroxy-pyridinium crosslinks in urine. The Hydroxypyridinium Crosslinks HPLC Assay kit is for research use only and not to be used in diagnostic procedures.

2. Introduction

Pyridinium and deoxypyridinium are built as intramolecular crosslinkers during the maturation of collagen. During bone resorption collagen is degraded and the pyridinium-crosslinks are excreted. They are not reutilized by the body and excreted in the urine. The crosslinks are not released during the de novo synthesis of collagen. They are specific for resorptive processes. Neither PYD nor DPD are taken up by nutrition. Therefore the determination of the crosslinks is independent from the diet. PYD is found in cartilage, ligaments and bone, whereas DPD is rather specific for bone.

The crosslink excretion shows a strong circadian rhythm with a maximum in the early morning hours. Reliable data can be obtained with a 24h or a creatinine corrected morning urine.
An elevated crosslink excretion is shown in osteoporotic women. Crosslink levels decrease during treatment with estrogen (Seibel et al. 1994). Therefore they are suited for therapy monitoring (Ganero et al. 1994). The measurement of pyridinium crosslinks is an independent predictor for future bone fractures (Ganero et al. 1995). Increased crosslink concentrations are also described in patients with Paget's disease, primary hyperparathyroidism and bone metastasis.

Hydroxypyridinium Crosslinks HPLC Assay kit application for the pyridinium crosslinks makes it possible to determine pyridinium and deoxypyridinium in an easy, fast and precise method. The Hydroxypyridinium Crosslinks HPLC Assay kit includes all reagents in a ready to use form for preparation and separation of the samples with exception of the columns (IC3201rp) and the controls (IC3201ko). Both can be supplied separately by Eagle Biosciences. Beside the complete test kits it is possible to order all components separately. Please request our single component pricelist.

3. Warnings and precautions

- All reagents of the Hydroxypyridinium Crosslinks HPLC Assay kit are strictly intended for Research Use Only.
- Test kit and column are concerted. Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange the Hydroxypyridinium Crosslinks HPLC Assay kit components from different lots.

- The hydrolysis reagent contains acid and has to be handled carefully. It is corrosive and causes burns. It should be handled with gloves, eye protection and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition immediately contact a physician.
- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Material Supplied

Article no.	Component	Designation	Amount
IC3201lm	ELU	Mobile phase	1000 ml
IC3201ka	CAL	Calibrator, lyophilized	3 vials
IC3201hy	HYDRO	Hydrolysis reagent	100 ml
IC3201rb	REAC	Reaction buffer	
IC3201rba		Reagent I	2 x 35 ml
IC3201rbb		Reagent II	2 x 140 ml
IC3201w1	WASH1	Washing solution I	
IC3201waa		Reagent I	2 x 170 ml
IC3201wab		Reagent II	2 x 340 ml
IC3201w2	WASH2	Washing solution II	1 x 100 ml
IC3201el	ELUSO	Elution solution	1 x 55 ml

5. Additional special equipment

- Glass tubes for hydrolysis
- Solid phase extraction cartridges and equipment
- Various pipettes
- HPLC with Fluorescence-detector
- HPLC column Crosslinks (IC3201rp)
- Cryostat or heating block

6. Reagent preparation

Preparation of the calibrator

- Reconstitute the **calibrator (CAL)** in **5.5 ml** deionized water. Aliquot and store them at -20°C. Avoid repeated freeze-thaw cycles. The concentration of the crosslinks might have minor changes from lot to lot.
- All other test reagents are stable at 20-25 °C up to the date of expiry stated on the label.

Preparation of the reaction buffer

- Add the total content of REAC reagent I to the bottle of REAC reagent II and leave the mixture 12 hours at room temperature. The prepared reaction buffer (REAC) is stable for 3 month.

Preparation of the washing solution I

- Add the total content of WASH I reagent I to the bottle of WASH I reagent II and leave the mixture 12 hours at room temperature. The prepared washing solution I (WASH1) is stable for 3 month.

7. Specimen

- Urine could be used in this test system. A two hour urine sampling between 07:00 and 10:00 am correlates well with a 24h urine.
- Samples are stable for 24h at room temperature and up to one week at 2-8 °C. For longer storage the samples should be kept at -20°C.

8. Procedure

Principle of the method

For the determination of the pyridinium crosslinks, the samples are hydrolyzed and extracted on solid phase extraction cartridges. The probe is centrifuged and 100 µl of the supernatant are injected into the HPLC system. The isocratic separation is via HPLC at 30°C using a "reversed phase" column. One run lasts 15 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered plasma calibrator; the concentration is calculated via integration of the peak areas or heights.

Sample preparation

1. Pipette into glass tubes:

   1 ml sample, CAL or CTRL

   +

   1 ml HYDRO (**caution:** Hydrochloric acid)

2. Mix well. Leave the tubes for **4 hours at 120°C**
3. Soak 1 ml WASH1 through the SPE cartridge by vacuum.
4. Mix

   0.5 ml WASH1

   +

   0.5 ml hydrolyzed cold urine

   +

   3 ml REAC

5. Shake very well, pipette in the SPE cartridge and let it soak through by mild vacuum.
6. Rinse the cartridge with **6 ml** WASH1 under mild vacuum.
7. Dry the cartridge by vacuum for 10 min.
8. Rinse the cartridge with 1 ml WASH2 under mild vacuum.
9. Dry the cartridge by vacuum for 10 min.
10. Elute the sample with **0.5 ml** ELUSO under vacuum
11. Centrifuge and inject **100 µl** of the supernatant for chromatography into the HPLC-system

Chromatographic settings

Column material:	Merck Superspher, 4 µm	
Column dimension:	125 mm x 4 mm	
Flow rate:	1-1.5 ml/min	
Fluorescence detection:	Excitation	290 nm
	Emission	400 nm
Injection volume:	100 µl	
Running time:	20 min	
Temperature:	30 °C	

Treatment of the HPLC column

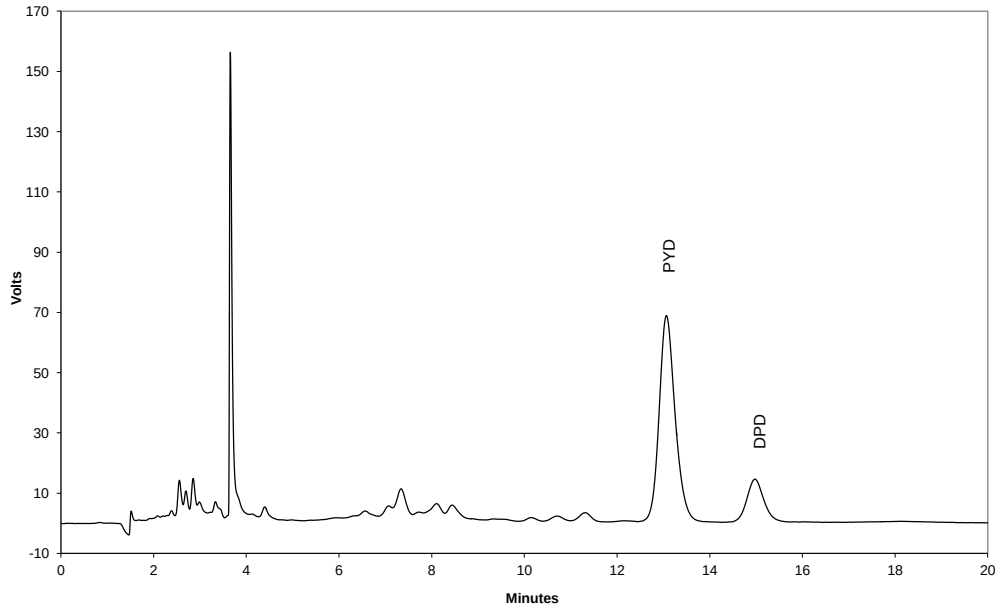
After the analysis the column should be flushed with 15 ml deionized water (1 ml/min) and stored in 50% methanol in deionized water (ca. 15 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with approx. 30 ml eluent.

9. Calculation of analytical results

Calculation

$$\text{Conc. sample} = \frac{\text{peak area patient} * \text{conc. calibrator}}{\text{peak area calibrator}}$$

Typical chromatogram



10. **Internal** Quality Control

Reference intervals

Mean ± 1 SD	31.1 ± 10.4 nmol PYD/mmol creatinin
	8.8 ± 3.8 nmol DPD/mmol creatinin
Range	14.8 – 59.2 nmol PYD/mmol creatinin
	3.8 – 26.2 nmol DPD/mmol creatinin

We recommend that each laboratory should develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data. (Source: Seibel et al. (1992). J. of. Clin. Endocrin. and Metabol., Vol. 74, No. 3, 481-486.)

11. Validation data

Precision and reproducibility

Intra-Assay:	PYD	3.5 % (335 pmol/ml)	[n = 6]
	PYD	2.7 % (856 pmol/ml)	[n = 6]
	DPD	3.6 % (74 pmol/ml)	[n = 6]
	DPD	2.3 % (190 pmol/ml)	[n = 6]
Inter-Assay:	PYD	5.5 % (332 pmol/ml)	[n = 6]
	PYD	1.9 % (792 pmol/ml)	[n = 6]
	DPD	4.0 % (75 pmol/ml)	[n = 6]
	DPD	3.6 % (178 pmol/ml)	[n = 6]

Linearity

PYD	up to 4500 pmol/ml
DPD	up to 1000 pmol/ml

Detection limit

PYD	2.4 pmol/ml
DPD	3.2 pmol/ml

Recovery

PYD	97.2 %
DPD	96.5 %

12. Limitations of the method

Serum, plasma or whole blood should not be used.

13. Disposal

The mobile phase (ELU), washing solution I (WASH1) and II (WASH2), reaction buffer (REAC) and elution solution (ELUSO) must be disposed as non-halogenated solvent. The hydrolysis solution (HYDRO) could be neutralized with NaOH and if the pH value is neutral it can be disposed as salt solution. (**Important:** Reaction will produce extreme heat, be careful) Please refer to the appropriate national guidelines.

14. Troubleshooting

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
Continue baseline is drifting	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flowcell is dirty	Clean flow cell

17. Literature references

- Seibel et al. (1992). TEM, Vol. 3, No. 7, 263-270.
- Robbins et al. (1991). Europ. J. of Clin. Invest., Vol. 21, 310-315.
- Seibel et al. (1992). J. of. Clin. Endocrin. and Metabol., Vol. 74, No. 3, 481-486.

For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.