

DOCUMENTATION OF LEAKAGE FROM A TRIAZINE BASED MIMETIC AFFINITY MATRIX DEVELOPED FOR PURIFICATION OF COAGULATION FACTOR VIIa

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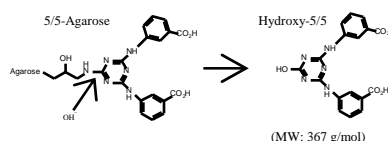


Abstract

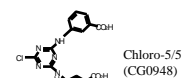
A synthetic ligand for purification of human recombinant Factor VIIa has previously been developed based on the rational design approach cited in ref. 1. The ligand is immobilised to an agarose matrix. Release of cleaved ligand into the product stream is a subject that has to be addressed when applying mimetic affinity ligands for purification of pharmaceuticals (ref. 2, 3). A reversed phase HPLC method with a limit of detection and limit of quantification in the same order of magnitude as obtained in ELISA based techniques (ref. 4) was developed to monitor the level of leakage. Leakage from the matrix was studied with batch incubation experiments by exposure to 1 M HCl, equilibration buffer, elution buffer and various concentrations of NaOH. No leakage was observed by treatment with the equilibration buffer or the elution buffer. Whereas 1 M HCl degrades the agarose backbone only, NaOH will release ligand into the liquid phase to an extent dependent on the concentration, exposure time and temperature. Elucidation of the structure of the released ligand was carried out by means of LC-MS and NMR spectroscopy. At process conditions including a Cleaning In Place procedure with 0.5 M NaOH, the content of released ligand in the eluate is below the limit of quantification.

Main leakage route – storage in NaOH

Formation of Hydroxy-5/5



Free ligand



RP-HPLC method

Column: Jupiter C4, 4.6 mmD x 250 mmL

Solvent A: 10 mM Na₂HPO₄, pH 7.0

Solvent B: 80 % Acetonitrile

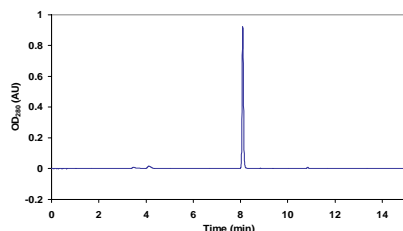
Regeneration: 80 % Acetonitrile + 0.09 % TFA

Detection: 232 nm

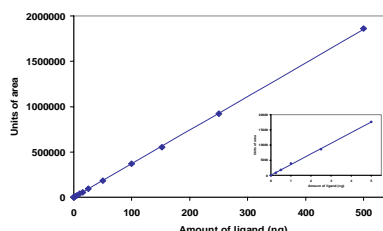
Temperature: 50 °C

Flow rate: 1.0 ml/min

Gradient: 0 – 50 % B, 17 min



RP-HPLC analysis of supernatant from 5/5-Agarose stored in NaOH

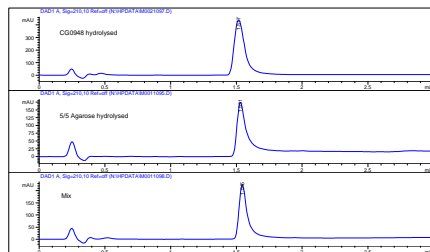


Linearity of method from 0 – 500 ng (insert: 0 – 5 ng)

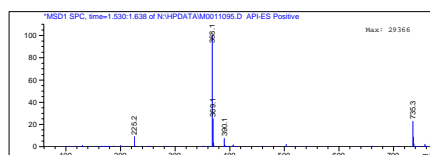
Characteristics

- Coefficient of Variance: 0.5 % (independent of ligand amount)
- Limit of Detection: 0.20 ng
- Limit of Quantification: 0.62 ng

Structure elucidation by LC-MS

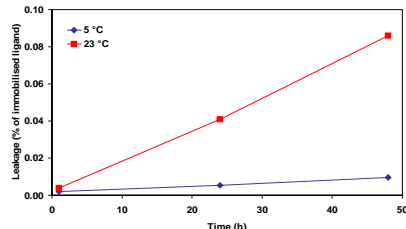


Chromatograms from LC-MS analysis of hydrolysed free ligand (top), supernatant from hydrolysed 5/5-Agarose (centre) and a mix (bottom)

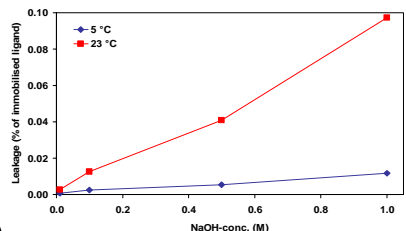


MS spectrum corresponding to peak at 1.59 min from LC-MS analysis of hydrolysed 5/5-Agarose

Batch incubation with NaOH



Leakage as a function of storage time in 0.5 M NaOH



Leakage as a function of the NaOH-concentration (24 h)

Conclusions

RP-HPLC method

- Very high sensitivity corresponding to a limit of quantification of 6.2 ng/ml if 100 µl sample is injected
- Linear from 0 – 500 ng ligand leachates both in the presence and absence of Factor VIIa

Verification of ligand leakage structure

- Formation of the main leakage component hydroxy-5/5 was verified by LC-MS
- MS-spectrum of the main peak showed a molecular weight of 367 g/mol identical with the molecular weight of hydroxy-5/5

Storage of the matrix in process buffers

- Leakage by storage in equilibration buffer, elution buffer or 20 % ethanol in 24 h at 23 °C is < 0.002 %.
- Both temperature, concentration of NaOH and storage time has an impact on leakage when 5/5-Agarose is incubated with a NaOH-solution
- The process is run at 5 °C i.e. leakage is significantly reduced compared to a temperature of 23 °C
- It was estimated that 3000 batch runs could be performed per production matrix if a 1 % -point decrease in ligand density, caused by regeneration with 0.5 M NaOH, is accepted

Wash-out of cleaved ligand

- Prior to a production run, the cleaved ligand, i.e. generated during the preceding regeneration phase, will be washed out of the column within 2.5 column volumes

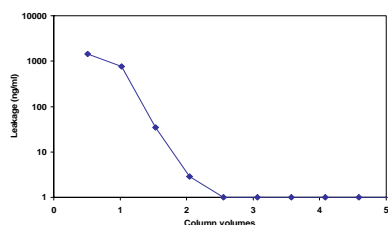
In-process analysis of leakage

- Research production has shown that the content of ligand leachates in the eluate from 5/5-Agarose normally is below the limit of quantification

Future work

- A long-term stability study of 5/5-Agarose in storage buffer and 0.5 M NaOH will be carried out

Wash-out of leached components



Wash-out of NaOH generated leakage components from 5/5-Agarose by equilibration buffer

References

- (1) Sproule, K. et al. New strategy for the design of ligands for the purification of pharmaceutical proteins by affinity chromatography. *J. Chromatogr. B* 740, 17-33 (2000).
- (2) Boyer, P.M. & Hsu, J.T. Protein purification by dye-ligand chromatography. *Adv. Biochem. Eng. Biotechnol.* 49, 1-44 (1993).
- (3) Johansson, B.L. Determination of Leakage Products from Chromatographic Media Aimed for Protein Purification. *BioPharm* 34-37 (1992).
- (4) Santambien, P., Hulak, I., Girot, P. & Boschetti, E. ELISA-based quantification of cibacron blue F3GA used as ligand in affinity chromatography. *Bioseparation*, 2, 327-334 (1992).

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