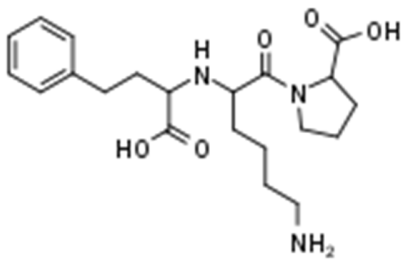
Supplementary File

Development and Validation of an HPLC-FLD Method for the Determination of NDMA and NDEA Nitrosamines in Lisinopril Using Pre-Column Denitrosation and Derivatization Procedure

Eleni Tsanaktsidou, Lamprini Kanata, Sofia Almpani, Constantinos K. Zacharis and Catherine K. Markopoulou *

Laboratory of Pharmaceutical Analysis, Department of Pharmacy, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
* Correspondence: amarkopo@pharm.auth.gr; Tel.: +30-2310-997-665



Lisinopril

Figure S1. Structural formula of lisinopril.

Table S1. Differences between the present approach and Boczar et al. [1] methodology.

Proposed approach	**Boczar's method** [1]
Extraction: Use of liquid-liquid microextraction technique for the extraction of NAs from Lisinopril. Low volumes of DCM and water (600 μL of each solvent) were used. A very simple LLME technique is suggested, using only a glass sealed syringe and an eppendorf tube.	Extraction: Use of liquid-liquid extraction technique for the extraction of NAs from Enalapril maleate. 5 mL of DCM are used and a white homogeneous suspension was formed, then filtered through a paper filter into a 5-mL volumetric flask. The resulting solution was transferred to a separation funnel, and 5 mL of acetate buffer, pH 5.6 was added. Complex extraction technique including several steps and using high volume of extraction solvent.
Denitrosation procedure: 20 μL of the denitrosation reagent and 600 μL of the DCM phase were used. The vial was left capped for 30 minutes at room temperature in the dark and then heated for 2 hours at 70°C to completely evaporate the DCM solvent and acids. Procedure duration: 150 min Denitrosation reagent: 20 μL.	Denitrosation procedure: 50 μL of denitrosation reagent and 1 mL of DCM sample solution were used. The vials were left for 30 min at room temperature in the dark and then heated for 20 min at 60 °C. Afterwards, the vials were opened and left for 3 h at 60 °C in an oven, to completely evaporate the solvent and the acids. Procedure duration: 230 min Denitrosation reagent: 50 μL
Derivatization procedure: The concentration of dansyl chloride was equal to 10 μg/mL and the diluent was acetonitrile. In order to obtain the desired pH for derivatization, 250 μL of 25 mM borate buffer (pH 10.5) were used.	Derivatization procedure: The concentration of dansyl chloride was equal to 0.5 mg/mL and the diluent was acetone. In order to obtain the desired pH for derivatization 50 μL of 1-M NaOH and 200 μL of 0.5-M $NaHCO_3$ were used. In section 3.3, the authors concluded that dansyl chloride suffers from low sensitivity and it is contaminaded from DMA, so they decided to continue their studies with Fmoc-Cl as derivatisation agent.
Dansyl-Cl: Derivatization duration: 30 min at 40 °C. Inexpensive reagent.	F-moc: Derivatisation duration: 60 min at 40 °C. Expensive reagent.
Chromatographic conditions:	Chromatographic conditions:

Isocratic elution program. mobile phase: 20 mM phosphate buffer (pH 2.8 with H_3PO_4) and acetonitrile 55:45 v/v column: LC-C18 DB 250 × 4.6 mm, 5.0 μm, Supelco Runtime = 13 min.	Gradient elution program. mobile phase: acetonitrile/water column: NovaPak C18, 150 × 3.9 mm, 4.0 μm Runtime = 30 min.

Table S2. Conducted experiments and measured responses for "Crossed D-Optimal" (CDO) design.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	**A: V den. factor**	**B: deriv. time**	**C: deriv. temper**	**area NDMA**	**area NDEA**
1	25	30	40	60779	6837
2	25	30	40	57796	6998
3	25	30	40	56245	6044
4	25	45	40	57927	7993
5	25	30	40	46173	5734
6	40	45	50	56862	8485
7	40	30	40	58765	7978
8	25	30	40	54823	6252
9	25	30	30	58518	8795
10	10	45	50	58900	8467
11	25	30	40	54309	6691
12	40	15	50	57839	8257
13	25	15	40	57263	8586
14	10	30	40	56460	9057
15	25	30	50	58307	8529
16	40	15	30	52504	7321
17	40	45	30	60583	9150
18	10	45	30	61615	9151
19	10	15	50	58864	8259
20	10	15	30	60405	9402

Reference

1. Boczar, D.; Wyszomirska, E.; Zabrzewska, B.; Chyła, A.; Michalska, K. Development and validation of a method for the semi-quantitative determination of n-nitrosamines in active pharmaceutical ingredient enalapril maleate by means of derivatisation and detection by hplc with fluorimetric detector. *Appl. Sci.* **2021**, *11*, 7590. https://doi.org/10.3390/app11167590.