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Additional Information

Quantification of nortriptyline in plasma by HPLC and fluorescence detection

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A simple, sensitive and specific high-performance liquid chromatography method has been developed for the determination of nortriptyline (NT) in plasma samples. The assay involved derivatization with 9H-fluoren-9-ylmethyl chloroformate (Fmoc-Cl) and isocratic reversed-phase (C_{18}) chromatography with fluorescence detection. The developed method required only 100 μl of plasma sample, deproteinized and derivatized in one step. Calibration curves were lineal over the concentration range of 5–5000 ng/ml. The derivatization reaction was performed at room temperature in 20 min and the obtained NT derivative was stable for at least 48 h at room temperature. The within-day and between-day relative standard deviation was below 8%. The limit of detection (LOD) was 2 ng/ml, and the lower limit of quantification (LLOQ) was established at 10 ng/ml. The method was applied on plasma collected from rats, at different time intervals, after intravenous administration of 0.5 mg of NT.

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1. Introduction

Nortriptyline, 3-(10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5-ylidene)-N-methyl-1-propanamine (NT), is a tricyclic antidepressant drug widely used in the treatment of unipolar depression, since it is a non-selective serotonine uptake inhibitor [1,2]. Also, there is growing evidence of its efficacy for pharmacological smoking cessation therapy [3,4].

Transdermal drug delivery systems are used to release drugs through the skin for therapeutic use as an alternative to oral, intravascular, subcutaneous and transmucosal routes. Several transdermal products and applications include hormone replacement therapy, pain management, angina pectoris, neurological disorders such as Parkinson's disease, and smoking cessation.

In order to develop a transdermal delivery system of NT for smoking cessation therapy and to evaluate its ability to provide sustained plasma levels of the drug during preclinical studies in rats, a sensitive analytical method using a small volume of the sample is needed. The method must be sensitive enough to determine the low plasma concentrations required for smoking cessation therapy (about 40 ng/ml) [5].

Several HPLC analytical methods for NT analysis in plasma samples have been published, but, in general, these methods involve excessively large plasma samples (0.5–1 ml) for their application to small laboratory animals such as rats [6–16], use long concentration processes or expensive equipment (liquid chromatography–tandem mass spectrometry) [17–20].

The aim of this study was to develop a HPLC analytical method for NT in plasma samples having a low detection limit, using a small volume of the sample and a straightforward treatment.

2. Materials and methods

2.1. Reagents

Nortriptyline (NT) and Fmoc-Cl (9H-fluoren-9-ylmethyl chloroformate) were purchased from Sigma Chemical (Madrid, Spain) and Fluka (Buchs, SG, Switzerland), respectively. Acetonitrile was of HPLC grade and all other analytical reagents were of analytical grade.

2.2. Standard solutions

A stock solution of NT (10 μg/ml) was prepared in water. Appropriate dilutions in water and plasma were prepared from this stock solution to obtain calibration standards in the range of 5–5000 ng/ml. Although rat plasma was used in the experiments, blank human plasma was also subjected to the derivatization and chromatographic processes to check the absence of interference peaks from endogenous human plasma components.

Abbreviations: Fmoc-Cl, 9H-fluoren-9-ylmethyl chloroformate; LOD, limit of detection; LLOQ, lower limit of quantification; NT, nortriptyline; RSD, relative standard deviation.

Dilutions for calibration standards were prepared daily, while quality control samples were prepared in plasma, divided in small aliquots and stored at −20 °C until use.

2.3. Derivatization procedure

The following solutions were used for derivatization of NT: Fmoc-Cl in acetonitrile (0.25 mg/ml) prepared daily, 0.2 M borate buffer (pH 9.5) and 1 M citric acid. A sample volume of 100 μl, 100 μl of borate buffer, 300 μl of acetonitrile and 100 μl of Fmoc-Cl solution were mixed in a 1.5 ml Eppendorf tube. After shaking, the mixture was kept at room temperature for 20 min to allow the derivatization of NT. Then, 20 μl of 1 M citric acid were added and the mixture was centrifuged at 5000 × *g* for 5 min. The supernatant was separated and transferred to a glass vial for HPLC analysis. A volume of 50 μl was injected into the HPLC system.

2.4. Chromatographic conditions

The HPLC system consisted of a quaternary pump HP series 1050, an Agilent Technology 1100 series autoinjector, and an HP 1046A programmable fluorescence detector. HPLC system control and data processing were performed by Chromeleon Chromatography Management System (Chromeleon 6.8, Dionex Corporation, Sunnyvale, USA). Chromatographic separation was performed on a Waters Spherisorb S5 ODS2 (4.6 mm × 250 mm) column at room temperature. The mobile phase consisted of a mixture of acetonitrile/water (85/15, v/v), delivered at a flow rate of 1 ml/min. The fluorescence detector was set at 260 nm (excitation) and 310 nm (emission).

2.5. Method validation

The calibration curve was divided into two concentration ranges, from 5 to 300 ng/ml and from 300 to 5000 ng/ml, respectively. The calibration curves (peak area versus nominal concentration) were constructed using a least squares linear regression analysis for the calculation of the slope, intercept and correlation coefficient. The accuracy (bias) and precision (relative standard deviation; RSD) of the assay were determined from the analysis of plasma samples spiked with NT at four concentrations (10, 100, 300 and 2000 ng/ml). Within-day accuracy and precision were calculated from six replicate analyses on the same day. Between-day accuracy and precision were calculated from results obtained in 6 different analyses carried out within 2 weeks. The limit of detection (LOD) was estimated as the concentration of NT in plasma giving rise to a peak whose height is three times the noise. The lower limit of quantification (LLOQ) was determined as the concentration of the lower standard with accuracy within 80–120% and relative standard deviation within 20%. The recovery of NT from plasma was calculated by comparing the peak areas obtained with standards prepared in plasma (10, 100, 300 and 2000 ng/ml) to the peak areas obtained with standards prepared in water at the same NT concentrations.

In order to evaluate the application of the method pharmacokinetically, NT was administered to five male Wistar rats (280–310 g). Rats were subjected to jugular vein cannulation on the day before drug administration [21] to facilitate intravenous drug administration (0.5 mg of NT dissolved in saline) and blood sampling with heparinised syringes. Blood samples (0.25 ml) were collected immediately prior to the administration of NT (0 min) and at the following post-dosing times: 2, 5, 10, 20, 40, 60, 120, 180 and 240 min. After centrifugation (2000 × *g* for 5 min), plasma was separated and frozen at −20 °C until analysis of NT.

3. Results and discussion

Fmoc-Cl is a reagent used as a UV/fluorescence tag for derivatizing amino acids for HPLC analysis and for preparing N-Fmoc amino acids for solid-phase peptide synthesis. It is also used as a N-protecting reagent for peptide and oligonucleotide syntheses. Due to its high reactivity with amino groups it is also used for the analysis of drugs containing primary or secondary amino groups. However, the practical uses of Fmoc-Cl are limited by the requirement that excess Fmoc-Cl must be removed before analysis, since the reagent itself is fluorescent.

The proposed reaction pathway between the secondary amine in NT and Fmoc-Cl is shown in Fig. 1. To avoid the extractive step separating the derivatized NT from the excess of Fmoc-Cl reagent, the concentration of reagent and chromatographic conditions were selected to obtain an adequate separation of corresponding peaks in the chromatograms. On the other hand, to simplify the treatment of plasma samples, the deproteinization and derivatization reaction were performed in the same step.

3.1. Chromatographic variables

To achieve the most effective chromatographic separation and analysis, the mobile phase was optimized by examining the effect of pH (in the range of pH 3–7 in steps of one pH unit) and varying the percentage of methanol and acetonitrile content (form 60% to 90%). Other chromatographic variables were investigated including flow rate and column type. Three C_{18} columns were examined: Kromasil C-18 (4.6 mm i.d. × 150 mm length, 5 μm particle diameter), Waters Spherisorb ODS-2 (4.6 mm × 250 mm, 5 μm) and Waters Nova-Pak C-18 (3.9 mm × 150 mm, 4 μm). The best chromatographic separation was obtained using Waters Spherisorb ODS-2 and the chromatographic conditions described in Section 2.4.

3.2. Optimization of the derivatization procedure

To study the time course of derivatization, several reaction times were tested: 2, 5, 10, 20, and 30 min. As can be seen in Fig. 2, the derivatization reaction of NT with Fmoc-Cl is very fast at room temperature in the presence of borate buffer (pH 9.5). From the data in Fig. 2, a reaction time of 20 min was selected for analyzing plasma samples containing NT.

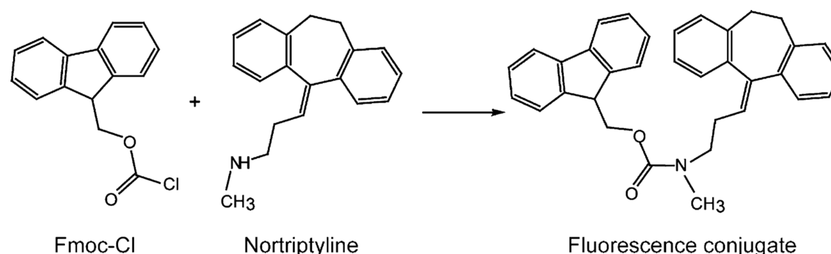

Fig. 1. Proposed reaction pathway of Fmoc-Cl with nortriptyline.

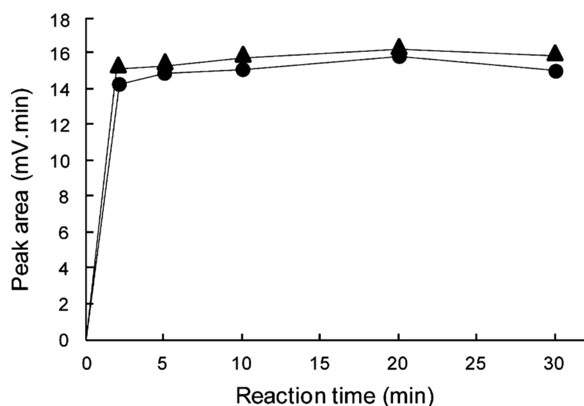

Fig. 2. Peak area of NT derivative as a function of reaction time at room temperature. (•) NT standard prepared in water, (�) NT standard prepared in plasma.

3.3. Assay validation

Fig. 3 shows two chromatograms corresponding to blank rat plasma and rat plasma spiked with NT. Peaks corresponding to endogenous components extracted from blank plasma and excess of reagent eluted before the peak of the NT derivative. Only two small peaks eluted after the peak of the NT derivative, but sufficiently apart to avoid interference with the analyte peak. Human blank plasma was also assayed and no interfering peaks were observed at, or near, the retention time of the NT derivative.

The stability of the NT derivative was checked at room temperature by comparing the peak areas obtained immediately after finishing the reaction and, again, after 48 h at room temperature. Practically, the same peak area value was obtained, which indicates that the derivative is stable for at least 48 h at room temperature.

Due to the wide calibration range (5–5000 ng/ml) and saturation of detector response, typically observed with fluorescence detection, the calibration curve was divided into two concentration ranges (5–300 ng/ml and 300–5000 ng/ml) and each concentration range was used with a different detector amplification factor. For both calibration ranges, a linear relationship between the peak area and the nominal concentration of NT was obtained, with values of correlation coefficient higher than 0.998.

The LOD of the method was approximately 2 ng/ml and the LLOQ was established at 10 ng/ml. The bias of the method was lower than 4%, and both the within-day and between-day precision were lower than 8% (Table 1).

When standards of NT prepared at the same concentration in plasma and water were compared, slightly smaller peaks of the NT derivative were observed for the standards prepared in plasma (as can be seen in Fig. 2). The mean recovery of NT from plasma was between 85% and 92% for the four concentrations assayed (10, 100, 300 and 2000 ng/ml). Since NT binds to plasma proteins in a high proportion (92%) [22], the incomplete recovery of NT from plasma could be attributed to an incomplete deproteinization by acetonitrile. Nevertheless, this fact should not affect the analytical determination of NT concentrations in plasma samples if calibration curves are prepared in blank plasma.

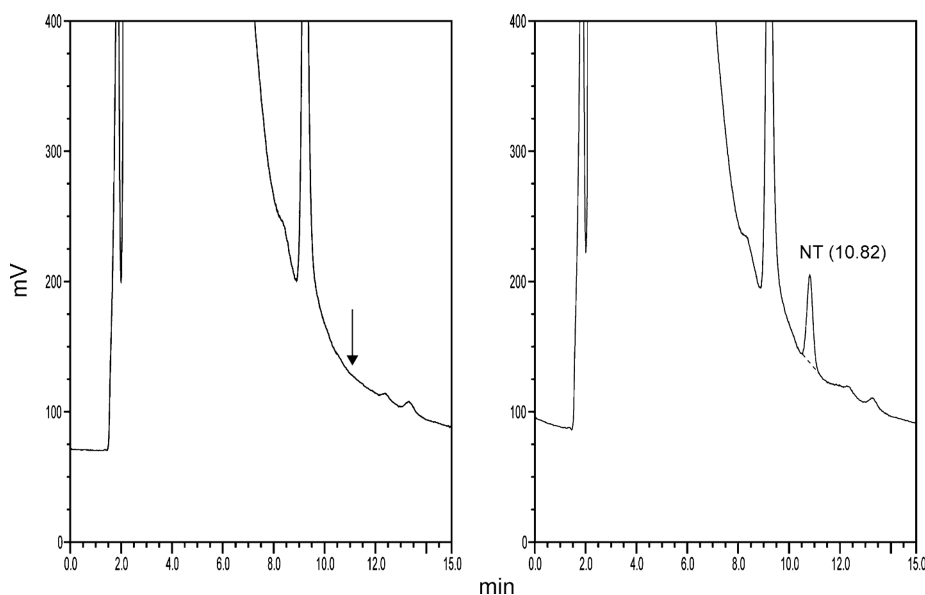

Fig. 3. Chromatograms corresponding to blank plasma and plasma spiked with NT (50 ng/ml).

Table 1
Within- and between-day precision and accuracy for determination of NT in spiked rat plasma.

Nominal concentration (ng/ml)	Within-day			Between-day		
	Observed concentration[a] (ng/ml)	RSD[b] (%)	Bias (%)	Observed concentration (ng/ml)	RSD (%)	Bias (%)
10	9.7 ± 0.6	6.2	−3.0	10.3 ± 0.8	7.7	3.0
100	103.0 ± 5.0	4.8	3.0	97.7 ± 6.5	6.6	−2.3
300	298.9 ± 2.1	0.7	−0.4	302.5 ± 3.8	1.3	0.8
2000	1982 ± 39	1.9	−0.9	1999 ± 70	3.5	−0.05

[a] Mean ± standard deviation, $n = 6$.
[b] Relative standard deviation.

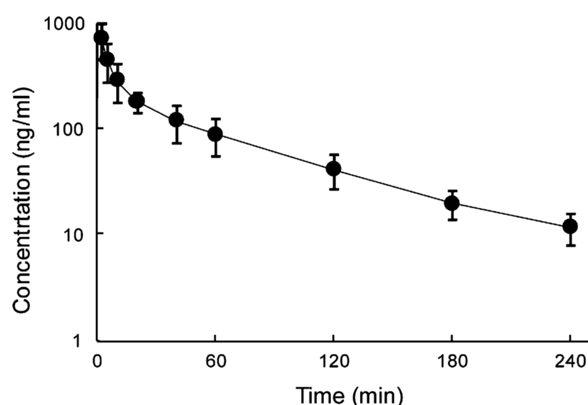

Fig. 4. Mean plasma concentrations of NT versus time after intravenous administration of 0.5 mg of NT to rats (n = 5).

The literature available on HPLC methods for the analysis of NT in plasma samples using conventional detectors shows that practically all of these methods are based on UV detection [6–8,10–12,14–16]. Due to the relatively low therapeutic window of NT concentrations for the treatment of depression (50–150 ng/ml) [23] or for smoking cessation (≥40 ng/ml) [22], plasma or serum samples containing NT have to be concentrate before if an HPLC equipped with a UV detector is used for the quan- tification of this drug at the indicated concentration values. Thus, a sample volume of 1 ml is typically concentrated to 50–250 μl by means of liquid–liquid or solid-phase (SPE) extraction, solvent evaporation and reconstitution with mobile phase or another adequate solvent. These steps are time consuming. On the other hand, when a pharmacokinetic study is conducted in rats, a limited volume of plasma is available for each sampling time if several samples are taken from the same animal (about 100–150 μl when 10 samples are withdrawn in a period of several hours), which avoids the possibility of performing a concentration process in order to determine low drug concentrations. However, the described method, based on fluorescence detection of the reaction product of Fmoc-Cl with NT, allows for the quantification of NT concentrations as low as 10 ng/ml using only 100 μl of sample. The only treatment of samples consisted in the addition of reagents and subsequent centrifugation, the supernatant being ready for direct injection onto the chromatograph.

3.4. *Application of the method*

The method was used to analyze samples obtained form rats intravenously dosed with 0.5 mg of NT. The analyzed samples showed no interference peaks from the endogenous plasma constituents. Mean plasma concentrations of NT were from 725 ng/ml, at 2 min post-dose, to 12 ng/ml at 4 h post-dose, and the plasma concentration–time profile showed a typical two exponential decay (Fig. 4).

4. Conclusion

In the present study, a simple, specific and sensitive HPLC method based on Fmoc-Cl derivatization for the quantification of NT in plasma was developed. The method required only 100 μl of plasma sample, which was deproteinized and derivatized in only one step, moreover, the obtained derivative was stable at room temperature. The LLOQ of the method was 10 ng/ml, and no interference with endogenous plasma peaks was observed.

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References

[1] P.K. Gillman, Br. J. Pharmacol. 151 (2007) 737.
[2] M.E. Thase, Dialogues Clin. Neurosci. 8 (2006) 217.
[3] J.D. Anczak, R.A. Nogler, Clin. Med. Res. 1 (2003) 201.
[4] J.R. Hughes, L.F. Stead, T. Lancaster, Cochrane Database Syst. Rev. 1 (2007), Art. No.: CD00003.
[5] M.E. Mooney, V.I. Reus, J. Gorecki, S.M. Hall, G.L. Humfleet, R.F. Mun˜oz, K. Delucchi, Clin. Pharmacol. Ther. 83 (2008) 436.
[6] R.H. Queiroz, V.L. Lanchote, P.S. Bonato, D. de Carvalho, Pharm. Acta Helv. 70 (1995) 181.
[7] P. Ghahramani, M.S. Lennard, J. Chromatogr. B: Biomed. Appl. 685 (1996) 307.
[8] R. Theurillat, W. Thormann, J. Pharm. Biomed. Anal. 18 (1998) 751.
[9] G. Tybring, J. Nordin, J. Widén, J. Chromatogr. B: Biomed. Sci. Appl. 716 (1998) 382.
[10] O.V. Olesen, P. Plougmann, K.J. Linnet, J. Chromatogr. B: Biomed. Sci. Appl. 746 (2000) 233.
[11] A. Oztunc, A. Onal, S. Erturk, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 774 (2002) 149.
[12] C. Frahnert, M.L. Rao, K. Grasmäder, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 794 (2003) 35.
[13] D. Bose, A. Durgbanshi, A. Martinavarro-Domínguez, M.E. Capella-Peiró, S. Carda-Broch, J. Esteve-Romero, M. Gil-Agustí, J. Pharmacol. Toxicol. Methods 52 (2005) 323.
[14] C. Alves, C. Fernandes, A.J. Dos Santos Neto, J.C. Rodrigues, M.E. Costa Queiroz, F.M. Lanc¸as, J. Chromatogr. Sci. 44 (2006) 340.
[15] T. Shinozuka, M. Terada, E. Tanaka, Forensic Sci. Int. 162 (2006) 108.
[16] M. Woz´niakiewicz, R. Wietecha-Posłuszny, A. Garbacik, P. Kos´cielniak, J. Chromatogr. A 1190 (2008) 52.
[17] H. Kirchherr, W.N. Kühn-Velten, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 843 (2006) 100.
[18] K. Titier, N. Castaing, M. Le-Déodic, D. Le-Bars, N. Moore, M. Molimard, J. Anal. Toxicol. 31 (2007) 200.
[19] A.J. Santos-Neto, J. Bergquist, F.M. Lanc¸as, P.J. Sjöberg, J. Chromatogr. A 1189 (2008) 514.
[20] A.R. Breaud, R. Harlan, M. Kozak, W. Clarke, Clin. Biochem. 42 (2009) 1300.
[21] F. Torres-Molina, J.C. Aristorena, C. Garcia-Carbonell, L. Granero, J. Chesa-Jiménez, J. Pla-Delfina, J.E. Peris-Ribera, Pharm. Res. 9 (1992) 1587.
[22] A. Gjelstad, K.E. Rasmussen, S. Pedersen-Bjergaard, Anal. Bioanal. Chem. 393 (2009) 921.
[23] E. Richelson, American Psychiatric Association Task Force on Treatments of Psychiatric Disorders. Treatments of Psychiatric Disorders, vol. 3, American Psychiatric Association, Washington, DC, 1989, pp. 1773–1786.