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Journal of Chromatography B, 716 (1998) 382–386

JOURNAL OF CHROMATOGRAPHY B

Short communication

High-performance liquid chromatography–mass spectrometry for the quantification of nortriptyline and 10-hydroxynortriptyline in plasma

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Received 2 March 1998; received in revised form 4 June 1998; accepted 16 June 1998

Abstract

A highly sensitive and selective method for the quantification of nortriptyline and its major 10-hydroxy metabolite in plasma is described. The method is based on liquid–liquid extraction in combination with acid dehydration of the 10-hydroxy metabolite to the less polar 10,11-dehydronortriptyline. Deuterium labelled internal standards ($[^2H_4]$NT and $[^2H_3]$10-OH-NT) were used and the compounds were separated by reversed-phase HPLC and detected using atmospheric pressure chemical ionisation and mass spectrometry. The limit of quantification was 0.8 ng/ml for both compounds. A 1-ml volume of plasma was used for analysis in the concentration range 0.8–32 ng/ml. The within- and between-day coefficients of variation were 11% in the low, 1.6 ng/ml range, and 7% at 8 ng ml/ml. Using this method it was possible to quantify plasma concentrations for 168 h following a single oral dose of 25 mg of nortriptyline with good accuracy and precision.

Keywords: Nortriptyline; 10-Hydroxynortriptyline

1. Introduction

The tricyclic antidepressant drug nortriptyline (NT), has been in clinical use for about 40 years. For NT, as well as several other tricyclic antidepressants (TCAs), the clinical response is dependent on the plasma concentrations. As early as the 1960s, Hammer and Sjöqvist reported a 30–40 fold variation in the steady-state plasma concentration of TCAs in patients treated with the same dose [1]. The utility of therapeutic drug monitoring for TCAs is nowadays well established [2,3].

NT is mainly metabolised to 10-OH-NT, which is excreted in the urine as *E*- and *Z*-isomers. Formation of the major metabolite, *E*-10-OH-NT is catalysed by the polymorphic cytochrome *P*450 isozyme, CYP2D6 [4,5], whose activity is under monogenetic control with about 7% of Caucasians but only 1% of Orientals being classified as poor metabolisers (PM) [6–8]. These individuals show longer half-lives of NT, and a lower rate of formation of 10-OH-NT than extensive metabolisers (EM) [4,5].

Various methods for therapeutic drug monitoring (TDM) have been published for NT as well as other TCAs like amitriptyline (AT). For this purpose, the methods are limited to the parent compound and steady-state plasma concentrations. Some of these methods include simultaneous quantification of NT and 10-OH-NT. These are based on gas chromatography (GC) with nitrogen phosphorous detection

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0378-4347/98/$19.00
PII: S0378-4347(98)00299-0

[9,10], or mass spectrometric (MS) detection [11–13], and HPLC with UV detection [14,15].

GC often results in a better chromatographic separation with higher selectivity and sensitivity than HPLC separation and was earlier used in single dose studies in our laboratory [13].

Kiel et al. [15], describe a sensitive HPLC method for NT and 10-OH-NT using UV detection at a wavelength of 214 nm. LC–MS allows the use of internal standards (I.S.s) labelled with stabile isotopes, and this combination gives a better accuracy, precision and good sensitivity, similar to GC–MS procedures. However, an extensive sample work-up procedure is needed for quantification of plasma concentrations as low as 1 ng/ml.

In this paper, we describe a LC–MS method developed for the quantification of NT and 10-OH-NT for 168 h in the range 0.8–32 ng/ml following a single oral dose of 25 mg NT. This low dose is suitable for pharmacogenetic studies in healthy volunteers. The method involves several liquid–liquid extraction steps and dehydration of 10-OH-NT to the less polar 10,11-dehydroNT (Fig. 1) which yields the sum of the two *E*- and *Z*-isomers. Since the *Z*-isomer is a minor metabolite [5], separation of the two isomers does not further contribute to the result. Deuterium labelled I.S.s were used and the compounds were separated on a reversed-phase C_{18} column, followed by atmospheric pressure chemical ionisation (APCI) and MS detection.

This method was later applied in a pharmacogenetic study where the plasma concentrations of NT and 10-OH-NT were measured in relation to the CYP2D6 genotype in Chinese healthy subjects. This study is reported separately [16]

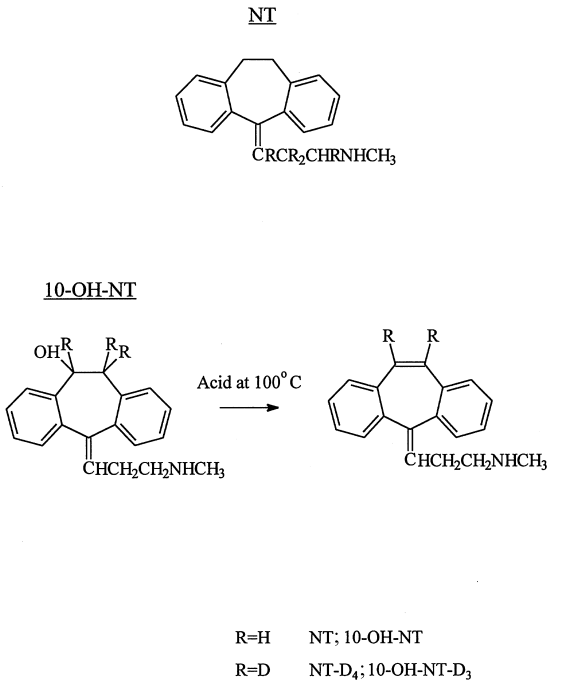

Fig. 1. Chemical structures of nortriptyline, E-10-hydroxy-nortriptyline and dehydration of 10-OH-NT to 10,11-dehydro-NT.

2. Experimental

2.1. Chemicals

Nortriptyline and 10-hydroxynortriptyline were donated by Lundbeck (Copenhagen, Denmark). Deuterium labelled I.S.s, NT with four deuterium atoms (a personal gift, synthesised 1974) and 10-OH-NT labelled with three deuterium atoms was synthesised as described by Bertilsson et al. [13]. The chemical structures are shown in Fig. 1. Stock solutions were prepared in 0.01 *M* HCl and stored in a refrigerator. All other chemical reagents were of analytical grade (Merck).

2.2. Apparatus

The HPLC system consisted of a Waters 600-MS silk tertiary system (Waters Associates, Milford, MA, USA) with a CMA 200 autosampler (CMA Microdialysis, Solna, Sweden). The compounds were separated on a Zorbax SB C_{18} reversed-phase column (75×4.6 mm, 3.5 μm particle size, Rockland Technologies) protected by Zorbax SB C_{18} precolumn (12.5×4 mm). The mobile phase was acetonitrile–25 m*M* ammonium acetate buffer (40:60, v/v) adjusted to pH 4.2 with acetic acid. The flow rate was 1.0 ml/min at room temperature and was continuously degassed with helium.

The HPLC was connected to a Finnigan TSQ tandem mass spectrometer (Finnigan, San Jose, CA, USA) via an APCI interface. The vaporiser temperature was 500°C, and the heat capillary was held at 200°C. The corona current and voltage were 5 μA

and 4.5 kV, respectively. The MS was run in the selective ion monitoring (SIM) mode focused on the positive ions 264 and 268 (M+1) for NT and the deuterium labelled I.S. [$^2H_4$]NT at a retention time of 5.1 min. The 10-hydroxy metabolite and the corresponding I.S. [$^2H_3$]10-OH-NT were detected as dehydro compounds (Fig. 1) and monitored at *m/z* 262 and 264 at a retention time of 4.1 min.

2.3. Preparation of calibration standards and quality control samples

The standards and quality control samples (QC) were prepared by spiking blank plasma with different concentrations of NT and *E*-10-OH-NT. The calibration curve ranged from 0.8–32 ng/ml plus a zero sample (0, 0.8, 1.7, 4, 8, 16 and 32 ng/ml). The QC samples were made from separate weighings and two concentrations were used, 1.8 and 8 ng/ml. QC plasma samples were stored at −20°C, while the standards were prepared freshly prior to the analysis.

2.4. Extraction procedure

Plasma samples, 1 ml in duplicates, I.S.s (30 μl of a solution containing 9 μg/ml [$^2H_4$]NT and 0.7 μg/ml [$^2H_3$]10-OH-NT, and 50 μl of 2.5 *M* NaOH were mixed and extracted with 5 ml diisopropyl ether for 30 min. After centrifugation for 10 min at 2000 *g*, the samples were placed in a freezing bath at −33°C (Lauda RC 20 CS, Königshofen, Germany) for about 5 min. The organic phase was poured to a new test tube containing 1 ml of 0.25 *M* sulphuric acid and extracted for 10 min. After centrifugation the tubes were replaced in the freezing bath and after 5 min, the organic phase was discarded. The tubes, containing the frozen acid were placed in a water bath at 100°C. After 1 h, 0.3 ml of 2.5 *M* NaOH and 3 ml of diisopropylether was added and the mixture was extracted for 10 min and centrifuged. The organic phase was transferred to a new test tube containing 300 μl of 50 m*M* acetic acid and extracted for 10 min and centrifuged. The organic phase was aspirated off and the tubes were placed under a stream of nitrogen to evaporate the ether residue. A 150-μl volume of the final acid phase was injected onto the HPLC system.

2.5. Extraction recovery

The extraction recovery was checked by spiking NT at a concentration of 8 ng/ml to blank plasma and following the extraction procedure described above. The peak area of NT was compared with a reference sample where blank plasma was extracted and the same amount of NT added to the final 300 μl 50 m*M* acetic acid. Unfortunately, there was no reference substance available for recovery determination of the 10,11-dehydro-NT.

3. Results and discussion

3.1. Selectivity

NT and the 10-de-hydroxy metabolite eluted closely but separately after dehydration of 10-OH-NT to the less polar 10,11-dehydro-NT compound. The retention times of 10,11-dehydro-NT and NT were 4.1 and 4.5 min, respectively. Without dehydration, the 10-hydroxy metabolite was not completely separated from the solvent front and the unstable baseline at a retention time of less than 1.5 min resulted in unsatisfactory variations in the peak area determination. Moreover, a prolonged retention time which was suitable for 10-OH-NT, resulted in a decreased limit of quantification for NT.

Fig. 2a shows the ion chromatogram from one subject at time zero before intake of a single oral dose of 25 mg NT. The ions *m/z* 264 and 268 correspond to the deuterium labelled I.S.s, [$^2H_3$]10-OH-NT and [$^2H_4$]NT, respectively (one deuterium atom of [$^2H_3$]10-OH-NT is lost during the dehydration to [$^2H_2$]10,11-dehydro-NT, Fig. 1). Fig. 2b shows the ion chromatogram of the two compounds and the respective I.S.s in a plasma sample obtained from the same subject 48 h after drug intake. The additional ions *m/z* 262 and *m/z* 264 correspond to 10-OH-NT measured as 10,11-dehydro-NT and NT, respectively.

3.2. Accuracy and precision

The calibration curves were calculated by means of least-square regression of the peak area ratio of analytes/I.S.s versus the nominal concentrations of

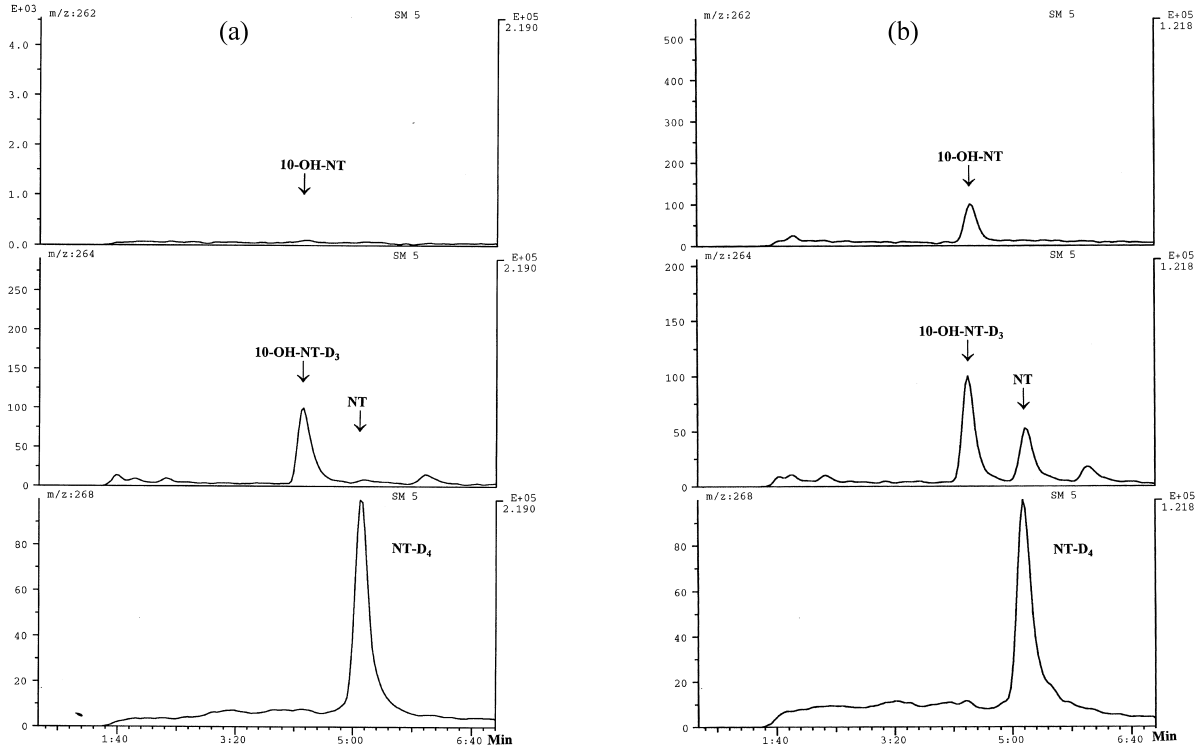

Fig. 2. Chromatogram of the determination of NT and 10-OH-NT by HPLC separation on a C_{18} reversed-phase column and mass spectrometric detection in one healthy subject after intake of 25 mg nortriptyline. (a) Ion chromatogram of a plasma sample drawn before drug intake where the positive ions (M+1) 264 and 268 correspond to [$^2H_2$]10,11-dehydro-NT and [$^2H_4$]NT respectively. (b) Ion chromatogram from the same subject in a plasma sample drawn 48 h after drug intake. The additional ions, 262 and 264, correspond to 10-OH-NT (measured as 10,11-dehydro-NT) and NT. The concentrations were 3.6 and 8.8 ng/ml of the respective compound.

the analyte. The calibration curves of the two compounds were linear within the analysed range 0.8–32 ng/ml ($r^2>0.99$). The limit of detection (LOD) was 0.2 ng/ml and the limit of quantification (LOQ) was estimated to be 0.8 ng/ml for both compounds. The extraction recovery was 80% for NT. Although we were unable to measure the extraction recovery for 10-OH-NT, the recovery appears to be equal for NT and 10,11-OH-NT, since the LODs and LOQs are similar for the two compounds.

The within-run ($n=7$) and day-to-day ($n=21$) coefficients of variation (C.V.) were 11% and 6.8% at the concentrations of 1.8 and 8 ng/ml, respectively, for both compounds.

The described chromatographic system in combination with the highly selective mass spectrometric detection results in a very robust and reproducible quantification. However, a relatively time consuming and complex analytical procedure was used. In the future, experiments will be done with the intention to improve and simplify this procedure. Fig. 3 shows the plasma concentrations versus time curves for NT and 10-OH-NT in a Chinese subject who is an extensive metaboliser of CYP2D6. Using this method, it was possible to follow the plasma concentrations of both the parent compound and the formed hydroxymetabolite for 168 h following a low single oral dose.

Acknowledgements

This study was supported by the Swedish Medical Research Council (03902), EU Biomed 2 (BMH4-CT-0291) and the Karolinska Institute.

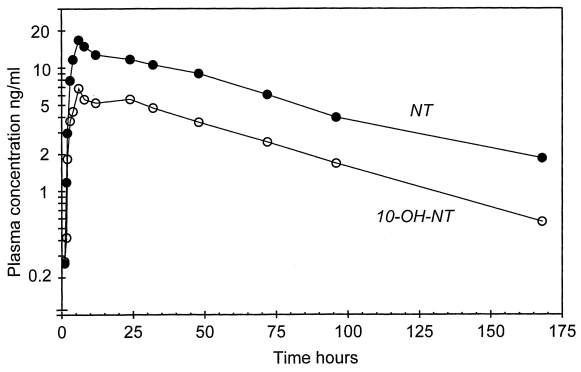

Fig. 3. Plasma concentration–time profile of NT (●) and 10-OH-NT (○) in one volunteer receiving a single oral dose of 25 mg nortriptyline.

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