Article

Determination of Amitriptyline and its Main Metabolites in Human Plasma Samples using HPLC-DAD: Application to the Determination of Metabolic Ratios after Single Oral Dose of Amitriptyline

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Foi desenvolvido e validado um método simples e rápido, empregando CLAE-DAD, para a determinação simultânea de amitriptilina, nortriptilina, E-10-hidroxiamitriptilina, Z-10-hidroxiamitriptilina, E-10-hidroxinortriptilina, Z-10-hidroxinortriptilina e desmetilnortriptilina em amostras de plasma humano. O método utiliza extração líquido-líquido em duas etapas e separação isocrática em fase reversa. A precisão apresentou D.P.R. % menor que 12.1% e a precisão esteve na faixa entre 93,1 e 102,5%. O limite de detecção foi 5 ng mL^{-1} para todos os analitos. Foram avaliadas razões metabólicas de desmetilação da amitriptilina em indivíduos genotipados para CYP2C19, com nítidas diferenças entre os valores obtidos nos voluntários com zero ou dois alelos ativos. O método é adequado para o monitoramento terapêutico de pacientes em terapia com amitriptilina, permitindo também a indicação da atividade de CYP2C19.

A simple and sensitive HPLC-DAD method for the simultaneous determination of amitriptyline, nortriptyline, E-10-hydroxyamitriptyline, Z-10-hydroxyamitriptyline, E-10-hydroxynortriptyline, Z-10-hydroxynortriptyline and desmethylnortriptyline in human plasma samples was developed and validated. The method employs a two step liquid-liquid extraction and a reversed phase separation with isocratic elution. Precision assays showed R.S.D % lower than 12.1% and accuracy was in the range of 93.1 to 102.5%. Lowest Limit of detection was 5 ng mL^{-1} for all analytes. Metabolic ratios of amitriptyline demethylation were evaluated in individuals genotyped for CYP2C19, with clear differences between volunteers with zero or two active alleles. The method is suitable for therapeutic drug monitoring of patients under amitriptyline treatment, also allowing the indication of CYP2C19 activity.

Keywords: amitriptyline, HPLC-DAD, CYP2C19, metabolic ratios

Introduction

Amitriptyline (AT), even after 40 years of its introduction still is a leading drug in the treatment of depression, specially in developing countries.[1,2] AT is metabolized mainly by demethylation forming nortriptyline (NT) (Figure 1), and by hydroxylation, leading to the formation of E-10-hydroxy (EHAT) and Z-10-hydroxyamitriptyline (ZHAT). NT is further demethylated to desmethylnortriptyline (NNT) and hydroxylated to E-10-hydroxy (EHNT) and Z-10-hydroxynortriptyline (ZHNT). The demethylation of AT and NT is mainly catalyzed by CYP2C19, with the participation of other CYP enzyme forms in higher drug concentrations.[3] The formation of the E-10-hydroxy metabolites is dependent on the activity of CYP2D6, with stereospecificity to the (-)-EHAT and (-)-EHNT metabolites.[3]

Therapeutic drug monitoring (TDM) in AT pharmacotherapy is usually based on the determination of plasma concentrations of AT and its main active

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metabolite, NT, once better clinical outcome in the treatment of depression is expected with plasma steady state concentrations of AT + NT in the range of 80 to 200 ng mL^{-1}, with an increased incidence of adverse effects when plasma levels are above 300 ng mL^{-1}.[4,5] Additional information can be obtained through the determination of the hydroxylated metabolites of AT and NT, once these metabolites are correlated with important clinical findings. A trend to better clinical outcome in the treatment of depression was observed with higher EHAT and EHNT plasma concentrations,[6] and some earlier reports associated the occurrence of severe cardiovascular toxicity to high plasma levels of EHNT and ZHNT after intake of therapeutic doses of NT.[7-10] Recently, some authors proposed AT individualized dose adjustments based on genotype-predicted phenotype of CYP2D6 and CYP2C19.[11] Another approach, to be investigated in this work, is the evaluation of the activity of CYP2C19 in AT pharmacotherapy through the determination of metabolic ratios for demethylation of AT after a single oral dose, as has been done with phenotyping probes as omeprazole.[12]

Several analytical methods have been developed for the determination of AT and some of its metabolites in human plasma using either gas,[13,14] or liquid chromatography.[15,16] To the best of our knowledge, there is only one report on simultaneous determination of AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT in biological samples, which was done by LC-MS/MS.[17] Therefore, there is no report of a low-cost HPLC-UV method to simultaneously determine AT and its 6 main metabolites. Additionally, the use of a diode array detector (DAD) detector coupled to the HPLC system permits the use of comprehensive databases of UV-spectra for DAD, allowing a reliable identification of the analytes and of unknown compounds eventually present on the sample.[18]

In this manuscript, we describe a simple and fast HPLC-DAD method for simultaneous determination of AT and its demethylated and hydroxylated metabolites in small samples of human plasma. The method was applied to a pilot study in the determination of demethylation metabolic ratios of AT after a single oral dose in individuals previously genotyped for CYP2C19.

Experimental

Chemicals

Amitriptyline (99%), nortriptyline (99%) and clomipramine (99%) hydrochlorides were obtained from Purifarma Química e Farmacêutica (São Paulo, Brazil). E-10-hydroxyamitriptyline (EHAT) (98%),
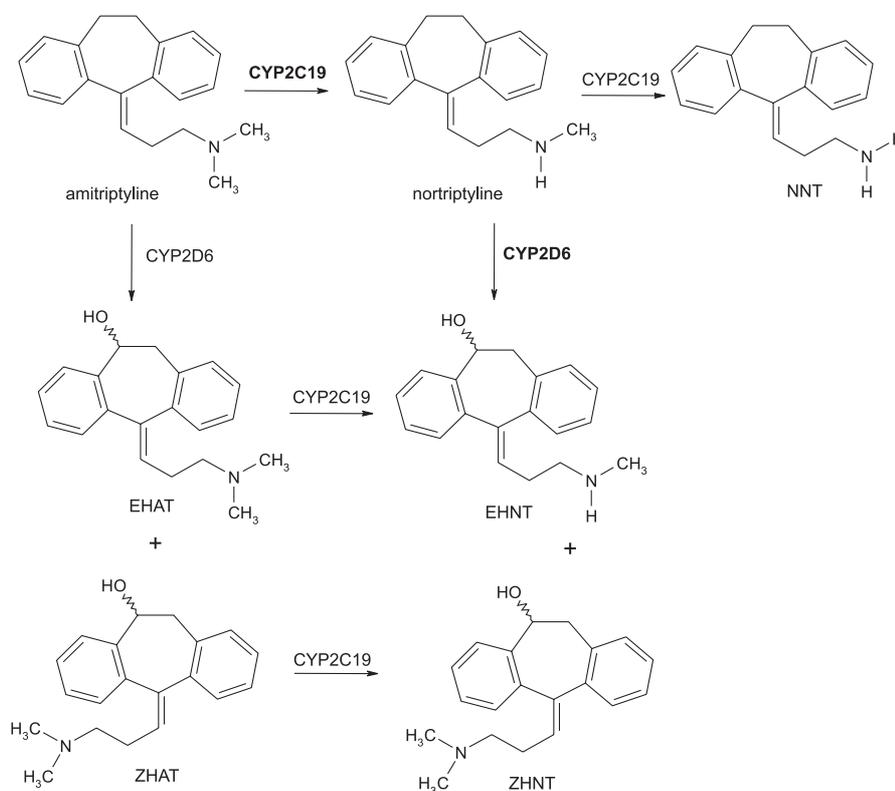
Figure 1. Main metabolic pathways of amitriptyline. Major routes are shown in bold.

Z-10-hydroxyamitriptyline (ZHAT) (99%), E-10-hydroxynortriptyline (EHNT) (99%), Z-10-hydroxy-nortriptyline (ZHNT) (98%) and desmethylnortriptyline (NNT) (97%) were kindly donated by Lundbeck A/S (Copenhagen, Denmark). HPLC grade acetonitrile, isopropylic alcohol, monobasic sodium phosphate, anhydrous dibasic sodium phosphate, hydrochloric and phosphoric acids were obtained from Merck (Darmstadt, Germany). HPLC grade *n*-hexane was obtained from Malinkrodt (Philisburg, USA). Ultrapure water was obtained by means of an Elga Purelab Ultra SC apparatus from Elga Labwater (High Wycombe, UK). Generic amitriptyline 25 mg tablets were manufactured by Eurofarma Laboratórios Ltda (São Paulo, Brazil).

Equipment

The chromatographic apparatus consisted of a binary pump LC-10AT, an on line degasser DGU-14, a column oven CTO 10AS, an automatic injector SIL-10AF and a diode array detector SPDM10A. The system was controlled by the software Class VP 6.13 SP2. The complete system was obtained from Shimadzu (Kyoto, Japan).

Chromatographic conditions

The separation was performed on a Lichrospher RP8ec (250 x 4.0 mm i.d., 5 μm) column with a guard column Lichrospher RP8e (10 × 4.0 mm i.d., 5 μm), both from Merck (Darmstadt, Germany). The integration wavelength was set at 210 nm, with diode array spectral acquisition in the range of 200 to 380 nm. The mobile phase consisted of phosphate buffer pH 2.3 and acetonitrile (63:37, v/v) at a flow rate of 1.0 mL min^{-1}. The column was kept at 30 °C during the analyses. The autosampler was maintained at room temperature.

Standard solutions

Stock solutions of the analytes were prepared in methanol at the concentration of 100 μg mL^{-1}, calculated as base. The stock solution of clomipramine (internal standard) was prepared in methanol at a concentration of 100 μg mL^{-1}. Stock solutions were stable for at least two months, when stored at -20 °C. Working solutions were prepared daily from stock solutions by dilution with methanol.

Biological samples

Pooled blank plasma used to obtain the calibration samples was obtained from healthy volunteers. EDTA blood samples (5 mL) were collected for genotyping and pharmacokinetic analysis. All samples were stored at –20 °C, being thawed just before use.

Sample preparation

To 500 μL of plasma in a glass-stoppered 10 mL centrifuge tube, 50 μL of internal standard working solution (2 μg mL^{-1}), 500 μL of Tris buffer pH 9.5 and 5 mL of hexane: isopropylic alcohol (95:5, v/v) were added. After vortex mixing (1 min), the mixture was centrifuged at 3500 g for 10 min and the organic phase was transferred to another glass stoppered tube to which 150 μL of phosphoric acid 0.1 % (v/v) was previously added. This mixture was vortex mixed for 1 min and then centrifuged at 3500 g for 10 min. The organic layer was discharged and 100 μL of the aqueous phase were transferred for a microvial for autosampler and 50 μL were injected.

Genotyping

The CYP2C19 genotyping assay was carried out using a Real-Time-PCR-based assay, (TaqMan® Assay # C__1329163_10, Applied Biosystems), following the manufacturer's instructions. The genotyping procedure screened for the CYP2C19 *1, *2 and *3 alleles.

Plasma calibration curves and Lower Limit of Quantitation

To 450 μL of blank plasma, 50 μL of the working standards containing all analytes were added, yielding final concentrations of AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT in the range of 5 to 500 ng mL^{-1}. To this mixture, 50 μL of internal standard working solution were added to yield a IS concentration of 200 ng mL^{-1}. Each calibration level was analyzed in sextuplicate. Calibration curves were constructed by means of the least-squares method, with a weighting factor of concentration^{-2}. The regression was evaluated for goodness-of-fit and lack-of-fit, with a confidence level of 5%. The Lower Limit of Quantitation (LLOQ) was established as the lowest concentration evaluated yielding acceptable precision (R.S.D. < 20 %) and accuracy (80-120 % of theoretical value), according do Shah *et al.*[19]

Selectivity, precision and accuracy

Control human blank plasma, obtained from 10 healthy volunteers, was assessed by the procedure described above to evaluate for the presence of inferring endogenous peaks.

In all runs, peaks related to AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT were evaluated with respect to their spectral purity and compared with reference spectra at DAD library. Precision and accuracy were examined by adding known amounts of analytes to blank plasma, yielding final concentrations of 15, 200 and 400 ng mL^{-1} of each analyte. Quality control samples were analyzed in triplicate on each of 6 days. The 18 measured concentrations *per* concentration level were subjected to analysis of variance (ANOVA), considering day as the grouping variable. Precision was expressed as percentage relative standard deviation (R.S.D. %). Accuracy was calculated by comparing the mean of the 18 measured concentrations per concentration level with the nominal concentration and expressed as percentage of the spiked concentration.

Extraction yield

The absolute recovery was determined at three different concentrations of analytes (15, 200 and 400 ng mL^{-1}). The plasma samples were analysed as in the *sample preparation* section. The absolute recovery was calculated by comparing the peak areas for extracted analytes from spiked plasma and a standard solution of the compounds in methanol containing internal standard with the same initial concentration (six samples for each concentration level).

Pharmacokinetic study design and sample collection

The pharmacokinetic pilot study protocol was approved by the Ethics Committees of Centro Universitário Feevale, and a written informed consent was obtained from the volunteers. The volunteers were previously genotyped for *CYP2C19*, according to the *Genotyping* section. Subjects were excluded of the study if they had any factor affecting AT pharmacokinetics or had a reported allergy to drugs. AT was administered in a single oral dose of 1 mg kg^{-1} body mass to the volunteers after a 8 hours fasting. Blood samples were collected pre-dose and at 1, 2, 3, 4, 5 and 7 hours after dosing. After collection, plasma was immediately separated, transferred to polypropylene tubes and frozen at –20 °C until assayed. Demethylation metabolic ratio was calculated as [AT]/[NT].

Results and Discussion

Chromatographic conditions

Under the chromatographic conditions described, AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT and the internal standard peaks were well resolved, without endogenous interfering peaks. Complete separation was achieved in less than 23 min. Fortunately, the chosen chromatographic conditions allowed the use of a comprehensive database of relative retention times and DAD spectra, acquired between 200 and 380 nm, as proposed by Pragst *et al.*[21] Fortunately, the combination of acidic mobile phase and fully endcapped reversed phase column provided adequate resolution and peak shapes, making the use of amine modifier unnecessary. The average retention times EHNT, EHAT, ZHNT, ZHAT, NNT, NT, AT and internal standard were 3.7, 4.1, 4.5, 5.1, 10.8, 13.4, 15.2 and 21.9 min, respectively (Figure 2).
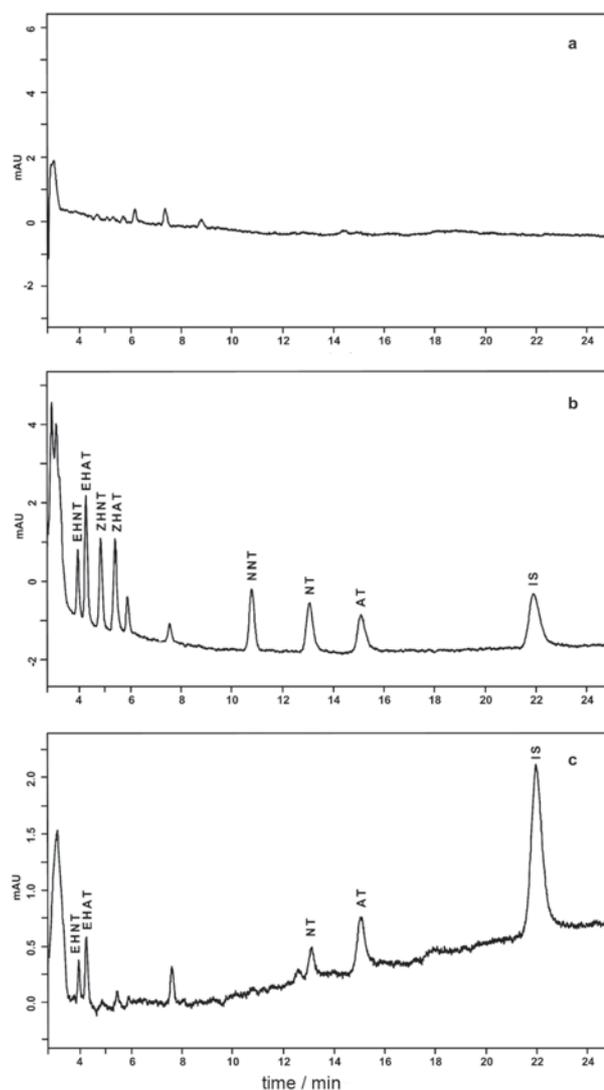
Figure 2. Chromatograms of: (a) blank plasma sample; (b) blank plasma sample spiked with 50 ng mL^{-1} of EHNT, EHAT, ZHNT, ZHAT, NNT, NT and AT, and 200 ng mL^{-1} of IS; and (c) plasma sample of a volunteer three hours after an oral intake of 1mg kg^{-1} AT. Concentrations in (c) are: EHNT 14.1 ng mL^{-1}, EHAT 16.7 ng mL^{-1}, NT 18.9 ng mL^{-1} and AT 46.1 ng mL^{-1}.

Sample preparation

Plasma and blood samples has been extracted for the determination of AT and metabolites mainly by solid-phase and liquid-liquid extraction procedures. The latter option, besides its favorable cost characteristics, can provide very clean extracts when submitted to a subsequent back-extraction to an acidic aqueous solution. The cleanness of these extracts allows the use of shorter wavelengths for analyte quantification, increasing sensitivity. Differently from other studies, we used small volumes of sample, allowing reanalysis if needed, even with typical amounts of collected plasma. The smallest extraction yield was obtained with EHNT (37.4%). All the other compounds presented recoveries above 70%, with maximum recovery of 83.7% for ZHAT (Table 2). Considering the lack of endogenous chromatographic interference with this two step liquid-liquid extraction, even the compounds with smaller yields presented adequate limits of detection. In addition, the fast preparation of samples due to the absence of a solvent evaporation step represents a considerable advantage. The whole sample preparation process does not take more than 25 minutes.

Validation method

Once area ratios of analytes to internal standard presented significant heteroscedasticity through the range of concentration of the calibration curve, weighted linear regression was used to fit the data, with a weighting factor of concentration^{-2}. Acceptable linearity's were obtained for all analytes, with values of r^2 between 0.992 for EHAT and 1.000 for NNT. The goodness of fit was highly significant and no significant lack-of-fit was observed in any of the calibration curves (Table 1). The LLOQ for all compounds evaluated was 5 ng mL^{-1}. Precision results were also satisfactory, with R.S.D. % values for intra-assay precision always lower than or equal to 10.5%, and R.S.D. % values for inter-assay precision lower than or equal to 12.1%. Accuracy was also acceptable, ranging from 93.1 to 102.5%. Complete precision, extraction yield and accuracy data are presented at Table 1. No interfering peaks were identified in blank samples. In all analysis, peaks were evaluated with respect to spectral purity by the DAD and we did not found any impurity at the peaks related to AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT and internal standard.

A group of five Brazilian volunteers received a single oral dose of 1mg kg^{-1} AT. In this group, 4 individuals presented two *CYP2C19* active alleles and one individual presented no active alleles. The maximal observed concentrations of AT, NT, EHAT, EHNT and ZHNT were 162.8, 32.4, 17.2, 65.4 and 43.0 ng mL^{-1}, respectively. Maximum detected ZHAT concentrations were below the LLOQ and were not quantified in any sample. Metabolic ratios for demethylation were calculated at the times where maximal AT and NT were achieved (Table 3). At 3 hours after drug administration, the volunteer without *CYP2C19* active alleles presented AT demethylation ratio ([AT]/[NT]) 10.6 times higher than the average of volunteers with two active alleles.

Our study shows that it is feasible to detect unusual activity of CYP2C19, at the start of the pharmacotherapy with AT, without the use of probe drugs or genotyping procedures. To achieve this purpose, a blood sample can be drawn 3 hours after oral AT intake and the demethylation ratio can be determined, with a simple and fast HPLC method.

Table 1. Assay linearity and regression analysis*

Substance	Parameter	Coefficient	Standard error	r^2	*F*-test for goodness of fit		*F*-test for lack-of-fit	
					F	p-value	F	p-value
AT	Slope	0.009	0.006	0.994	2722.45	0.0001**	0.0492	0.9951***
	Intercept	0.018	0.001					
NT	Slope	0.010	0.000	0.998	3562.9	0.0001**	0.0252	0.9987***
	Intercept	-0.001	0.004					
NNT	Slope	0.010	0.001	1.000	9188.2	0.0001**	0.2604	0.9004***
	Intercept	-0.002	0.000					
ZHAT	Slope	0.008	0.001	0.992	3201.3	0.0001**	0.0514	0.9517***
	Intercept	0.019	0.006					
EHAT	Slope	0.009	0.001	0.993	2998.7	0.0001**	0.0487	0.9814***
	Intercept	0.070	0.006					
ZHNT	Slope	0.004	0.000	0.994	3114.8	0.0001**	0.6297	0.9752***
	Intercept	0.020	0.002					
EHNT	Slope	0.006	0.000	0.993	2685.1	0.0001**	0.0341	0.9932***
	Intercept	0.029	0.004					

*Confidence level of 5 %, **significant, ***not significant.

Table 2. Precision, extraction yield and accuracy results (n = 18)

Spiked concentration / (ng mL^{-1})	Within-runR.S.D / (%)	Between-runR.S.D / (%)	Extraction yield / (%)	Accuracy / (%)
AT				
15	5.7	6.9	72.3	101.8
200	5.2	4.9	76.6	102.4
400	3.5	3.1	75.1	101.8
NT				
15	4.9	5.6	70.2	98.5
200	2.2	5.5	73.8	99.9
400	2.9	3.8	75.3	102.5
NNT				
15	3.8	4.3	69.5	102.7
200	3.1	4.9	69.5	100.9
400	2.2	4.3	72.6	101.1
EHAT				
15	7.9	9.1	68.5	95.5
200	7.2	8.8	68.4	98.3
400	6.9	7.4	71.8	97.6
ZHAT				
15	8.8	11.3	83.2	95.8
200	7.8	10.8	85.3	98.8
400	6.2	8.2	82.6	96.9
EHNT				
15	10.5	12.1	37.3	96.4
200	9.6	9.8	35.2	95.1
400	9.7	9.7	39.7	96.3
ZHNT				
15	10.5	11.1	63.4	93.1
200	10.3	9.8	67.4	96.8
400	8.8	9.3	65.1	95.3

Pharmacokinetic study.

Table 3. Metabolic ratios of AT demethylation 3 and 4 hours after a single oral dose and number of CYP2C19 active alleles

Volunteer	Number of CYP2C19 active alleles	[AT]/[NT] (t = 3 h)	[AT]/[NT] (t = 4 h)
1	2	2.44	2.00
2	2	2.32	2.11
3	2	1.64	2.29
4	2	0.87	0.49
5	0	19.24	12.52

Conclusions

A HPLC-DAD for the simultaneous determination of AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT in small human plasma samples was described. The sample preparation is based on a two-step liquid-liquid extraction, without a solvent evaporation step. The method showed to be precise, accurate, specific and sensitive. Currently, this is the first report on the simultaneous quantification of AT, NT, NNT, EHAT, ZHAT, EHNT and ZHNT by HPLC-DAD. With the presented method, it was possible to calculate demethylation ratios after a single oral dose of 1 mg kg^{-1} of AT. The demethylation ratios for AT, as determined in this work, correlated with CYP2C19 genotype.

Acknowledgments

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