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Quantitative analysis of amitriptyline and nortriptyline in human plasma and liver microsomal preparations by high-performance liquid chromatography

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Abstract

A simple, rapid, highly selective and sensitive method for the analysis of amitriptyline and nortriptyline in plasma and human liver microsomes is described. It is suitable for the routine analysis of large numbers of samples using readily available instrumentation and low cost consumables. The detection limit was 2 ng/ml for both compounds and calibration curves were linear over a wide range of concentrations and passed through the origin. The within-batch and between-batch coefficients of variation for amitriptyline and nortriptyline were less than 7.4% and 12.8%, respectively. A series of compounds, including inhibitors used for probing cytochrome P450 activity in vitro, were tested for interference in the assay. Only ketoconazole caused interference and the assay was modified to allow samples containing ketoconazole to be analysed.

Keywords: Amitriptyline; Nortriptyline

1. Introduction

Amitriptyline is an effective tricyclic antidepressant drug which has been in wide clinical use for 40 years. It is metabolised by hepatic cytochrome P450 mainly to nortriptyline and hydroxyamitriptyline [1]. Further oxidative metabolism of these products also occurs. Therapeutic dosing with amitriptyline results in steady-state plasma concentrations of drug and its N-demethylated metabolite nortriptyline in the range 0.05–0.30 μg/ml [2]. To monitor amitriptyline and its metabolite nortriptyline for 24 h following single doses, a method with much higher sensitivity is needed to measure concentrations in the range 0.005–0.010 μg/ml [3]. High sensitivity is also required for studies of the in vitro N-demethylation of amitriptyline. In addition such an assay requires a high degree of selectivity to exclude the possibility of co-elution of peaks from a range of inhibitors (and/or their metabolites) used to define cytochrome P450 specificity.

Various analytical techniques have been employed for the determination of amitriptyline and nortriptyline in plasma. These include ultraviolet spectroscopy (UV) [4], radioimmunoassay [5], radioisotope derivative analysis [6]; gas-liquid chromatography with nitrogen-specific [7] or electron capture detection [8,9], gas chromatography–mass spectrometry (GC–MS) [10] with chemical-ionisation [11] or electron-impact [12,13] detection and high-

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performance liquid chromatography (HPLC) [14–21]. These methods have rather low sensitivity.

Several newer methods with high sensitivity have been published. For example, Dolezalova et al. [22] developed an HPLC method using on-line solid-phase extraction with a lower limit of quantitation about 40 ng/ml. Although these methods are suitable for routine use, the cost of consumables such as solid-phase extraction kits, may be unacceptably high if large numbers of samples are to be analysed.

Furthermore, those methods that are capable of detecting very low concentrations of amitriptyline and nortriptyline in plasma require sophisticated and expensive instrumentation [10,23,24], or are associated with long retention times, poor peak resolution, or low selectivity [25,26]. Moreover, none of the above methods have been applied to in vitro metabolism studies where an additional problem can result from the co-elution with metabolites of interest, of inhibitor compounds used for probing specific enzyme activities.

In this paper we describe a rapid, sensitive and selective assay suitable for amitriptyline and nortriptyline in plasma and for studies of the human in vitro N-demethylation of amitriptyline to nortriptyline. The method involves high recovery solvent extraction of these compounds followed by HPLC analysis with UV detection.

2. Experimental

2.1. Chemicals and reagents

Amitriptyline hydrochloride, nortriptyline hydrochloride, desipramine hydrochloride, diazepam, ketoconazole, 7-ethoxycoumarin, quinidine sulphate and triacetyloleandomycin were purchased from Sigma (Poole, UK). Furafylline was a gift from Professor W. Pfleiderer (University of Konstanz, Konstanz, Germany) and *E*-10-OH-amitriptyline, *Z*-10-OH-amitriptyline, *E*-10-OH-nortriptyline and *Z*-10-OH-nortriptyline were donated by Lundbeck Pharmaceuticals (Copenhagen, Denmark). Sulfaphenazole was purchased from Ultrafine Chemicals (Manchester, UK). All other reagents were obtained commercially and were of the highest grade of purity.

2.2. HPLC instrumentation

The chromatograph comprised a Model 501 pump (Waters, Watford, UK), a Guard-Pak pre-column module (Waters), a Z Module column system containing Nova-Pak C_{18} reversed-phase material (5 μm particle size), a Spectroflow Model 773 UV detector (HPLC Technology, Macclesfield, UK) and a Model 3390A integrator (Hewlett-Packard, UK). The mobile phase was water–acetonitrile (70:30) containing triethylamine (1%, v/v) adjusted to pH 3 with orthophosphoric acid. In experiments where ketoconazole was used, the mobile phase was changed to water–acetonitrile (65:35) containing triethylamine (1%, v/v) adjusted to pH 3 with orthophosphoric acid. Chromatography was carried out using a flow-rate of 2 ml/min and at room temperature. Detection was at 240 nm.

2.3. Source of liver, preparation of microsomes and method of incubation

Liver tissue from human donors was used with the approval of the Hospital Ethics Committee. Microsomes were prepared and incubated as described by Otton et al. [27].

2.4. Sample preparation

Plasma or incubation samples (1 ml), internal standard (200 μl of desipramine, 1 μg/ml or, in ketoconazole experiments, 200 μl diazepam, 5 μg/ml) and sodium hydroxide (5 *M*, 0.1 ml) were vortex-mixed for 10 s. Butan-1-ol in hexane (5 ml, 2% v/v) was added and the mixture vortex-mixed for 1 min and then centrifuged at 2000 *g* for 5 min at 4°C. The organic phase was evaporated to dryness at 40°C using a vacuum-vortex evaporator. The residue was reconstituted in the mobile phase (200 μl) and 50 μl was injected onto the HPLC system. Calibration standards were prepared by adding known amounts of amitriptyline and nortriptyline to pooled drug-free human plasma or heat-inactivated, diluted (20 fold) rat liver microsomes.

3. Results

3.1. Selectivity

The chromatographic conditions were optimised to obtain baseline separation of desipramine (internal standard), nortriptyline and amitriptyline with retention times of 6.3, 8.5 and 10.5 min, respectively (Fig. 1). Chromatograms from drug-free plasma or incubation samples contained no peaks co-eluting with these compounds. The following compounds, which are either metabolites of amitriptyline and nortriptyline, or inhibitors of cytochrome P450, were tested for chromatographic interference: *E*-10-hydroxyamitriptyline, *Z*-10-hydroxyamitriptyline, *E*-10-hydroxynortriptyline and *Z*-10-hydroxynortriptyline, quinidine, triacetyloleandomycin, mephenytoin, furafylline and diazepam. They were tested both as authentic standards and after incubation for 15 min at a concentration of 50 μM with microsomes from a human liver possessing high activity for a range of cytochromes P450. Only authentic ketoconazole and incubate containing ketoconazole produced an interfering peak which had a retention time close to nortriptyline. Ketoconazole and nortriptyline were separated as described above and the modified assay was used in all experiments involving ketoconazole. Representative chromatograms at amitriptyline and nortriptyline concentrations near lower limit of determination are shown in Figs. 1 and 2.

3.2. Extraction recovery

The recoveries of amitriptyline, nortriptyline and desipramine at a concentration of 0.050 μg/ml were 90%, 87% and 76%, respectively.

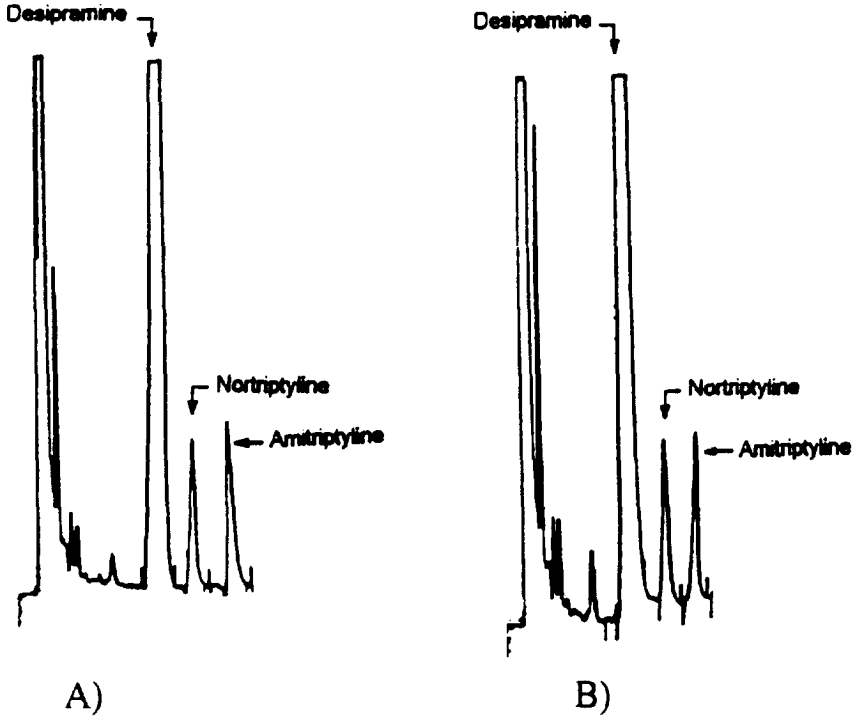

Fig. 1. Representative chromatograms following analysis of: (A) a plasma sample containing amitriptyline and nortriptyline (2 ng/ml each); 50 μl of the reconstituted residue (200 μl) was injected onto the HPLC system. (B) An incubation sample containing amitriptyline and nortriptyline (2 ng/ml each); 50 μl of the reconstituted residue (200 μl) was injected onto the HPLC system.

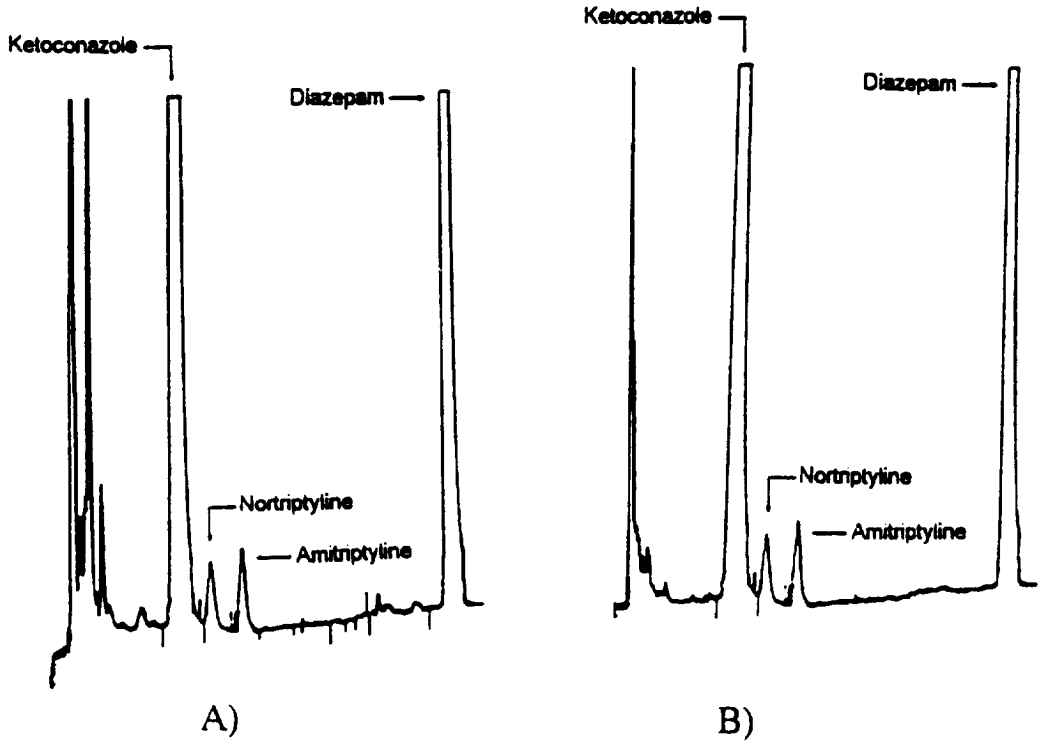

Fig. 2. Representative chromatograms using a modified assay to avoid ketoconazole interference: (A) a plasma sample containing amitriptyline and nortriptyline (2 ng/ml each) and ketoconazole (5 μM); 50 μl of the reconstituted residue (200 μl) was injected onto the HPLC system. (B) An incubation sample containing amitriptyline and nortriptyline (2 ng/ml each) and ketoconazole (5 μM); 50 μl of the reconstituted residue (200 μl) was injected onto the HPLC system.

3.3. Linearity

Calibration curves for amitriptyline (0.002–5 μg/ml, r=0.9969) and for nortriptyline (0.002–1.5 μg/ml, r=0.9991) were linear and passed through the origin. The linearity of the modified assay is stated in Tables 3 and 4.

3.4. Precision and sensitivity

The limit of determination of amitriptyline and nortriptyline in both plasma and incubation samples was 2 ng/ml. Lower concentrations can be determined by injecting a larger volume of reconstituted residue onto the HPLC system but at the expense of a lower assay precision. Data on assay precision are shown in Table 1, Table 2, Table 3, and Table 4.

3.5. Application of the assay

Chromatograms following the analysis of plasma from a healthy volunteer who had taken a single

Table 1
Coefficients of variations for the analysis of amitriptyline and nortriptyline in plasma

Concentration (μg/ml)	Coefficient of variation (%)	
	Within-batch	Between-batch
Amitriptyline		
0.002	7.4	12.8
0.2	2.7	8.9
5	2.1	7.8
Nortriptyline		
0.002	7.2	12.2
0.15	2.5	7.6
1.5	3.6	4.8

Table 2
Coefficients of variations for the analysis of amitriptyline and nortriptyline in human liver microsomal incubations

Concentration (μg/ml)	Coefficient of variation (%)	
	Within-batch	Between-batch
Amitriptyline		
0.002	6.3	12.4
0.2	4.2	10.3
5	2.4	8.9
Nortriptyline		
0.002	5.7	12.5
0.15	5.2	10.5
1.5	4.9	6.3

50-mg dose of amitriptyline, and a human liver microsomal sample incubated (60 min) with amitriptyline are shown in Figs. 1 and 2. Fig. 3 shows the plasma profiles of amitriptyline and nortriptyline over 24 h in two representative patients after a single dose of amitriptyline (50 mg). Fig. 4 shows a plot of nortriptyline formation against time following the incubation of amitriptyline with liver microsomes from two human donors.

4. Discussion

We describe a simple, rapid, highly selective and sensitive method for the analysis of amitriptyline and nortriptyline in plasma and human liver microsomes. Using this assay (a) plasma concentrations can be detected for 24 h following single dose administration of amitriptyline and (b) the generation of nortriptyline from amitriptyline by human liver

Table 3
The specifications of the modifed assay to avoid ketoconazole interference in plasma samples

Concentration (μg/ml)	Coefficient of variation (%)		Linearity range (μg/ml)	r^a	Determination limit (μg/ml)
	Within-batch	Between-batch			
Amitriptyline					
0.002	7.1	10.3	0.002–5	0.998	0.002
0.5	5.2	8.2			
5	3.6	5.5			
Nortriptyline					
0.002	6.7	12.0	0.002–1.5	0.999	0.002
0.2	4.1	8.1			
1.5	3.2	4.9			

[a] Correlation coefficient of calibration curve.

Table 4
The specifications of the modified assay to avoid ketoconazole interference in human liver microsomal incubations

Concentration (μg/ml)	Coefficient of variation (%)		Linearity range (μg/ml)	r^a	Determination limit (μg/ml)
	Within-batch	Between-batch			
Amitriptyline					
0.002	7.2	11.2	0.002–5	0.999	0.002
0.5	5.5	9.6			
5	3.1	9.1			
Nortriptyline					
0.002	6.5	12.9	0.002–1.5	0.991	0.002
0.2	4.3	11.1			
1.5	4.1	6.7			

[a] Correlation coefficient of calibration curve.

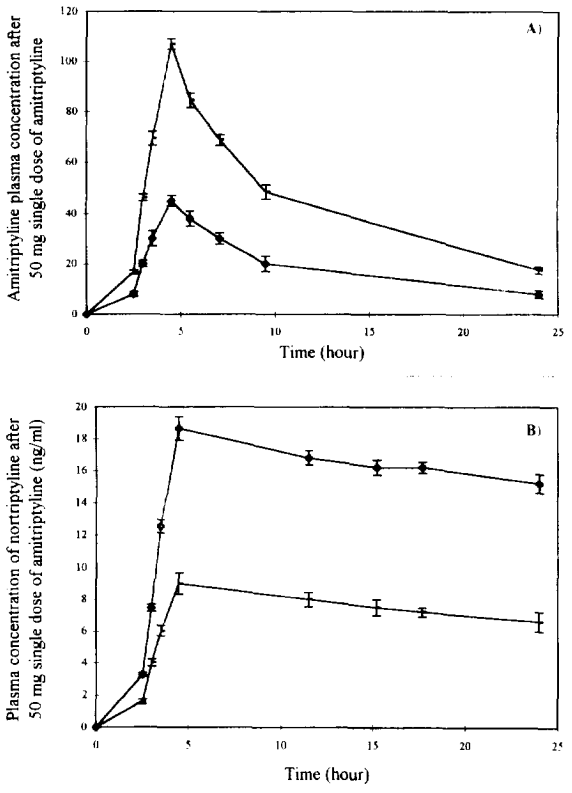

Fig. 3. Representative plasma concentration–time profiles of (A) amitriptyline and (B) nortriptyline formed from amitriptyline, in two volunteers after taking a 50-mg single oral dose of amitriptyline. Each point is the mean ± S.D. of five replicates.

microsomes can be monitored over a wide range of substrate concentrations. Out of a series of compounds, including inhibitors used for probing cytochrome P450 activity in vitro, only ketoconazole produced an interfering peak with the same retention time as nortriptyline. A modified assay overcoming this problem, was used in studies involving ketoconazole.

Acknowledgments

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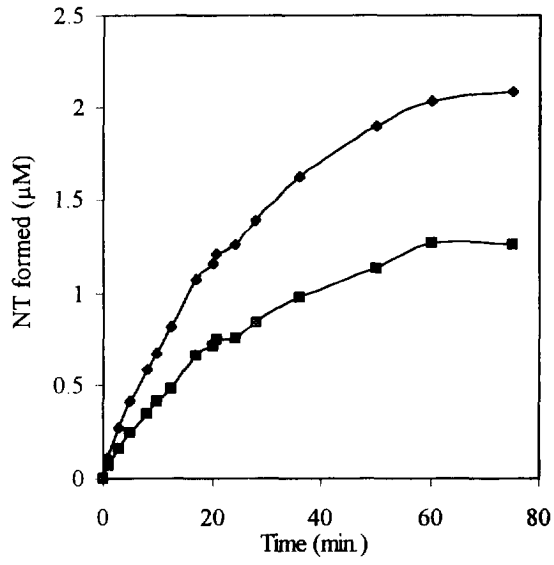

Fig. 4. Representative plot for the rate of nortriptyline formation from amitriptyline in microsomes from two human livers. Each point is the mean of five replicates.

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