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Simultaneous determination of amitriptyline, nortriptyline and four hydroxylated metabolites in serum by capillary gas–liquid chromatography with nitrogen–phosphorus-selective detection

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Abstract

A method for the simultaneous quantification of the antidepressant drug amitriptyline, its demethylated metabolite nortriptyline and four hydroxy metabolites (*E*-10-hydroxyamitriptyline, *Z*-10-hydroxyamitriptyline, *E*-10-hydroxynortriptyline, *Z*-10-hydroxynortriptyline) in human serum or plasma has been developed. The method is based on a three-step liquid–liquid extraction followed by gas–liquid chromatography (split–splitless injection, HP-5, 25 m×0.2 mm I.D., 0.33 μm capillary) with nitrogen–phosphorus-selective detection (GLC–NPD). The limits of detection are 1.5 ng/ml for amitriptyline, nortriptyline, *E*-10-hydroxyamitriptyline and *Z*-10-hydroxyamitriptyline and 3 ng/ml for *E*-10-hydroxynortriptyline and *Z*-10-hydroxynortriptyline. The within-day and between-day precision is between 6 and 15% at three concentrations (low, moderate and high) for amitriptyline, nortriptyline and *E*-10-hydroxy metabolites. At low concentrations of 10 ng/ml, the precision of the assay of the *Z*-10-hydroxy metabolites has been found to be up to 19%. Accuracy is between 91 and 115% for all analytes. The performance of the assay of the hydroxy metabolites is mainly determined by the cleanness and the deactivation of the quartz insert of the injector port. Therefore, every day a freshly cleaned and deactivated insert was used.

Keywords: Amitriptyline; Nortriptyline; Hydroxyamitriptyline; Hydroxynortriptyline

1. Introduction

Therapeutic drug monitoring (TDM) of tricyclic antidepressant drugs, in most laboratories, is still limited to the parent compound plus the demethylated metabolite, e.g. amitriptyline (At) plus nortriptyline (Nt). It is expected, that the analysis of the hydroxy metabolites (Fig. 1) becomes more meaningful in future, because their contribution to the antidepressant effect and to side-effects is well-known and becomes more and more documented in clinical investigations [1,2].

Some methods for the simultaneous assay of parent drug+demethylated metabolite+hydroxy metabolites in clinical samples have already been described in literature [3–13] (Table 1).

An early method used thin-layer chromatography [11]. Most methods are based on HPLC with liquid–

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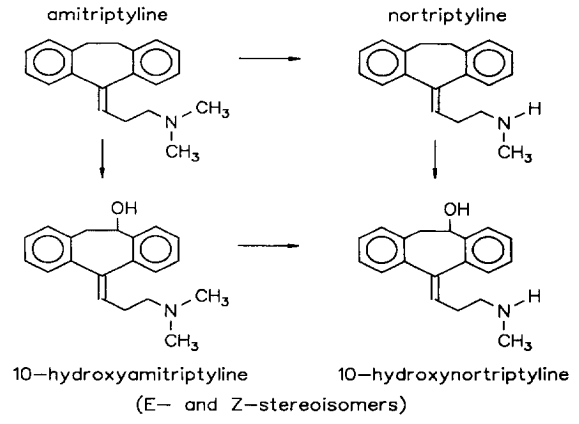

Fig. 1. Metabolism scheme of amitriptyline.

liquid extraction for sample preparation. Column-switching techniques, which allow the direct injection of serum or plasma, are now the most recent and most promising development [12]. However, some disadvantages are evident when using HPLC. The chromatographic separation is lower than with capillary GLC. For instance, in the case of amitriptyline hydroxy metabolites, as visible in chromatograms in e.g. [12], sometimes there is a poor separation of the *Z*- and *E*-stereoisomers or no separation at all. In contrast to GLC, to purchase a constant quality of column material remains a problem in the case of deactivated RP–HPLC phases. Such phases are necessary for the analysis of lipophilic compounds with one substructure, which is

Table 1
Methods for the analysis of antidepressant hydroxy metabolites in serum or plasma

Analytes	Sample preparation	Method	Limit of detection (ng/ml)	Ref.
Tr, DTr, 2HyTr	LLE	GC–NPD	Tr, DTr: 3 2HyTr: 4	[3]
Tr, DTr, 2HyTr, 2HyDTr	LLE deriv.	GC–MS	Tr, 2HyTr, 2HyDTr: 4[a] DTr: 2[a]	[6]
I, DI, 10HyI, 10HyDI, 2HyI, 2HyDI	LLE	HPLC–UV	Each: 1.5	[4]
Cl, DCl, 8HyCl, 8HyDCl	LLE	HPLC–ED	Cl, DCl: 0.3[a] 8HyCl, 8HyDCl: 0.2[a]	[5]
At, Nt, total *E*,*Z*10HyAt, total *E*,*Z*10HyNt	Direct inj.	HPLC	Each: 5–10	[7]
At, Nt, *E*10HyAt, *E*10HyNt, *Z*10HyAt, *Z*10HyNt	LLE	HPLC–UV	Each: 1	[8]
At, Nt, *E*10HyAt, *E*10HyNt, *Z*10HyNt	LLE	GC–NPD	Each: 0.5	[9]
At, Nt, total *E*,*Z*10HyAt, *E*10HyNt, *Z*10HyNt	LLE	HPLC–UV	At, 10HyAt: 5 Nt, *E*10HyNt, *Z*10HyNt: 10	[10]
At, Nt, *E*10HyNt	LLE	TLC–UV	Each: 5–10	[11]
At, Nt, *E*10HyAt, *E*10HyNt, *Z*10HyAt, *Z*10HyNt	Direct inj.	HPLC–UV	Each: 5 (1[b])	[12]
At, Nt, total *E*,*Z*10HyAt, Total *E*,*Z*10HyNt	LLE deriv.	GC–MS	At, Nt, *E*,*Z*10HyAt: 0.5 *E*,*Z*10HyNt: 1	[13]

At: amitriptyline, Nt: nortriptyline, Tr: trimipramine, DTr: desmethyltrimipramine, I: imipramine, DI: desipramine, Cl: clomipramine, DCl: desmethylclomipramine, Hy: hydroxy metabolites, LLE: liquid–liquid extraction.

[a] Limit of quantification.

[b] 'Enrichment modus', therefore, multiple injections to precolumn.

likely to form hydrogen bonds, such as hydroxy metabolites. As stated in [12], to provide a constant performance of the analysis, every batch has to be tested anew. By means of HPLC the enantioselective assay of amitriptyline hydroxy metabolites in urine [14], serum and cerebrospinal fluid [15] of patients was reported.

The disadvantage of GC–MS for the analysis of amitriptyline hydroxy metabolites is the loss of the stereoisomer substructure, due to derivatization. Therefore, only total *E,Z*-hydroxyamitriptyline and *E,Z*-hydroxynortriptyline levels have been measured [13]. It seems, that a good alternative, with respect to sensitivity, specificity and economy remains capillary GLC with nitrogen–phosphorus sensitive detection [3,9]. The method [9] is limited to only three amitriptyline hydroxy metabolites, because no reference substance of *Z*-10-hydroxyamitriptyline was available. However, from the chromatogram shown, separation of *Z*-10-hydroxyamitriptyline cannot be expected.

Because of the increased interest in the hydroxy metabolites of antidepressant drugs, the aim of the present work was to develop a sensitive and reproducible GLC method for the simultaneous quantification of amitriptyline, nortriptyline and four hydroxy metabolites in human serum or plasma. Two injection techniques, split–splitless and cold on column injection, have been tested.

2. Experimental

2.1. Chemicals

E-10-Hydroxyamitriptyline (*E*10HyAt), *Z*-10-hydroxyamitriptyline (*Z*10HyAt), *E*-10-hydroxynortriptyline (*E*10HyNt) and *Z*-10-hydroxynortriptyline (*Z*10HyNt) were kindly donated by H. Lundbeck Laboratory (Copenhagen, Denmark). The internal standard (I.S.) clomipramine hydrochloride was kindly donated by Ciba–Geigy (Basel, Switzerland). Amitriptyline hydrochloride was from Rzeszowa Zhimizeska Fabrika, RZF (Rzeszow, Poland), nortriptyline hydrochloride was from Troponwerke (Cologne, Germany). The organic solvents *n*-hexane p.a., methanol p.a., isoamyl alcohol p.a. (isomeric mixture) and toluene were from Merck (Darmstadt, Germany). For the preparation of aqueous NaOH and HCl, water Pestanal from Riedel-de Haën (Seelze, Germany) was used. NaOH p.a. and concentrated HCl p.a. were obtained from Merck. NaCl, concentrated H_2SO_4, H_2O_2 (30%) and potassium dichromate were obtained from Laborchemie Apolda (Apolda, Germany). Dichlorodimethylsilane was purchased from Chemiewerk Nünchritz (Nünchritz, Germany).

2.2. Solutions

Aqueous NaOH (1 *M*) with 6% NaCl was prepared by dissolution of 20 g NaOH and 30 g NaCl in 500 ml water. HCl (0.1 *M*) was prepared by diluting 4.2 ml of concentrated HCl in 500 ml of water. The extraction solvent was prepared by mixing 97 volumes of *n*-hexane with 3 volumes of isoamyl alcohol. For the silanization of glassware, a solution of 5% dichlorodimethylsilane in toluene was used. Chromic-sulfuric acid was prepared by moistening 25 g potassium dichromate with water and dissolution in 500 ml H_2SO_4. Caro's acid was prepared by mixing 250 ml H_2O_2 (30%) with 250 ml H_2SO_4. Drug-free plasma and serum of healthy individuals was used for the preparation of calibration samples.

2.3. Reference and standard solutions

A 11.36-mg amount of amitriptyline·HCl, 11.36 mg of nortriptyline·HCl and 11.24 mg of clomipramine·HCl were dissolved each in 100 ml of water to obtain stock solutions of 100 μg/ml of the base. A 50-μl volume of clomipramine stock solution was diluted by adding to 4950 μl of water. This solution was used as the I.S. (1 μg/ml clomipramine base). About 2 mg of each hydroxy metabolite was weighed exactly and dissoluted in a volume of 0.01 *M* HCl, calculated to obtain a concentration of 100 μg/ml hydroxy metabolite (e.g. 2.05 mg in 20.5 ml). Volumes (50 μl) of amitriptyline, nortriptyline and each hydroxy metabolite stock solution were diluted together with 4700 μl of water. This reference solution (each analyte 1 μg/ml) was used for the preparation of serum standards of 5 to 40 ng/ml. For the preparation of serum standards of 25 to 250 ng/ml a ten-fold more concentrated reference solution was used.

2.4. Glassware

Glasswares were silanized with 5% dichlorodimethylsilane in toluene monthly. A special cleaning procedure, including 30 min sonification in 0.001 *M* HCl, was applied.

2.5. Sample preparation

A three-step liquid–liquid extraction procedure plus one washing step was applied. Serum (2 ml) was mixed in a 10-ml glass tube with 100 μl of standard solution containing 1 μg/ml clomipramine. A 0.5-ml volume of aqueous NaOH with 6% NaCl and 4 ml *n*-hexane–isoamyl alcohol (97:3, v/v) was added. The first extraction step was carried out by shaking for 30 min. After 5 min centrifugation at 1500 *g*, 3.5 ml of the organic layer were transferred to 1.25 ml 0.1 *M* HCl in a 10-ml glass tube and shaken for 30 min. After 2 min of centrifugation, the organic layer was discarded. A 1-ml volume of *n*-hexane–isoamyl alcohol was added, the two phases were vortex-mixed for 30 s and separated again by 2 to 5 min centrifugation. From the lower phase (0.1 *M* HCl) 1.0 ml was taken away carefully and placed in a 4-ml glass tube. To enable the handling of a small volume such as 100 μl (see below), the inner diameter of these tubes was 8 mm instead of 15 mm for the tubes used in the first and second extraction steps. A 150-μl volume of the aqueous NaOH and 100 μl of *n*-hexane–isoamyl alcohol were added and vortex-mixed for 30 s. After 2 min of centrifugation, as much as possible of the organic layer (ca. 80 μl) was separated to a tapered 4-ml glass tube. The solution was evaporated to dryness for 3 min in a vacuum evaporator and reconstituted in 20 μl of *n*-hexane–isoamyl alcohol. By standing for 12 min at 40°C in a heating block (Tesochne DP-2P), the solution was evaporated to approximately 5 μl. A 2-μl aliquot was injected onto the GC system.

2.6. Apparatus

A Hewlett–Packard 5890 series II+ gas chromatograph equipped with a nitrogen–phosphorus detector (NPD) and a split–spitless injector port was used for the analysis. Separation was obtained with a 25 m×0.2 mm I.D., 0.33 μm HP-5 capillary and nitrogen (0.7 ml/min, automatic pressure control) as the carrier gas. Flow-rates of the detector gases were: air 100 ml/min (275 kPa), hydrogen 2.5 ml/min (100 kPa) and the auxiliary gas, nitrogen, 27 ml/min (220 kPa). The injector port and the detector port were maintained at 300°C. The injector was operated in the split–splitless mode. The split (30 ml/min) was opened 0.05 min after injection. The oven was operated isothermally at 240°C. An integrator HP 3398 series II was used for the calculation of retention times, peak widths and peak areas.

In the cold on-column modus the following temperature program was used: oven: T_1=120°C, T_2=240°C, r_1=30°C/min, 20 min at T_2; injector port: T_1=125°C, T_2=245°C, r_1=30°C/min, 20 min at T_2. A special cold on-column equipment (HP) with fused-silica needle and a duck-bill septum was used for the injection.

2.7. Preparation of the inserts of the injector port

For the assay of hydroxy metabolites a new insert was used every day. The inserts were cleaned by standing in chromic-sulfuric acid for 30 min, carefully soaked with water and treated with Caro's acid (see above) for 30 min. After again carefully washing with water and methanol, the dry inserts were silanized by standing 24 h in 5% dichlorodimethylsilane in toluene, then washed with methanol, soaked for 24 h in methanol and dried at 80°C.

2.8. Quantification

Calibration curves were constructed by plotting the peak-area ratios ($A_{analyte}/A_{I.S.}$) of eight concentrations between 5 and 40 ng/ml for At, Nt, *E*10HyAt, *Z*10HyAt and *Z*10HyNt, between 20 and 160 ng/ml for *E*10HyNt and of ten concentrations between 25 and 250 ng/ml for At and Nt.

2.9. Accuracy and precision

Accuracy was assessed by determining the concentrations of drug measured in samples (10 ng/ml, 25 ng/ml and 50 ng/ml for *E*10HyAt, *Z*10HyAt and *Z*10HyNt, 25 ng/ml, 75 ng/ml and 150 ng/ml for *E*10HyNt and 25 ng/ml, 100 ng/ml and 200 ng/ml

for At and Nt) relative to the known concentration added. Precision was determined utilising the coefficient of variation (%) of the within-day ($n=6$) and between-day ($n=12$, during two months) variations.

3. Results and discussion

In Fig. 2, typical chromatograms obtained from extracts of drug-free serum, of calibration serums (low and high level) and serum of a depressive patient, who received a dose of 150 mg amitriptyline per day, are shown. In drug-free serum, no peaks of endogenous material interfering with the analysis appeared. Symmetrical peaks and baseline separation of, not only, At and Nt, but also of the stereoisomers of hydroxy metabolites and the I.S. clomipramine, indicate a sufficient performance for the identification and quantification of all analytes. Moreover, the superiority to HPLC seems to be evident when these chromatograms are compared with some HPLC methods.

The shape of the peaks of the hydroxy metabolites was considerably dependent on the injector insert. The chromatographic separation of the hydroxy metabolites is dependent on the time of use of one cleaned and deactivated insert, and is illustrated in Fig. 3 by means of the peak width of *E*10HyAt. When the peak width exceeded 0.16 min, the separation and quantification was found to become insufficient. This repeatedly happened after a time of use of approximately two days or after about twenty injections of serum extracts. Thus, for the aim of a safe and qualitatively consistent analysis, every

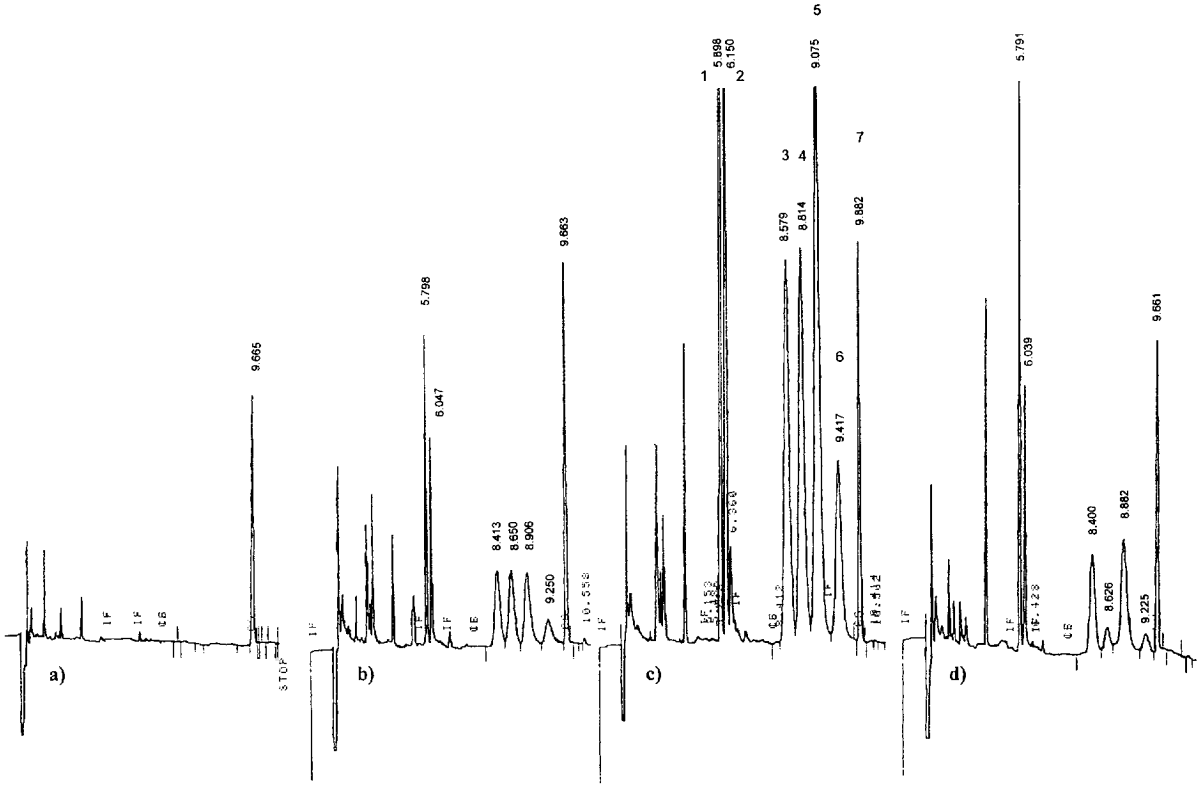

Fig. 2. Chromatograms of (a) blank plasma, (b) plasma spiked with 25 ng/ml At, Nt and *E*10HyNt and 10 ng/ml *E*10HyAt, *Z*10HyAt and *Z*10HyNt, (c) plasma spiked with 200 ng/ml At and Nt, 150 ng/ml *E*10HyNt and 50 ng/ml *E*10HyAt, *Z*10HyAt and *Z*10HyNt and (d) plasma of a depressive patient, who received a dose of 150 mg of amitriptyline per day (At 58 ng/ml, Nt 40 ng/ml, *E*10HyAt 18 ng/ml, *Z*10HyAt 4.7 ng/ml, *E*10HyNt 49 ng/ml, *Z*10HyNt 8.8 ng/ml), chart speed changed from 0.5 to 1.5 cm/min in the region of hydroxy metabolites. Peaks: 1=At; 2=Nt; 3=*E*10HyAt; 4=*Z*10HyAt; 5=*E*10HyNt; 6=*Z*10HyNt; 7=I.S.

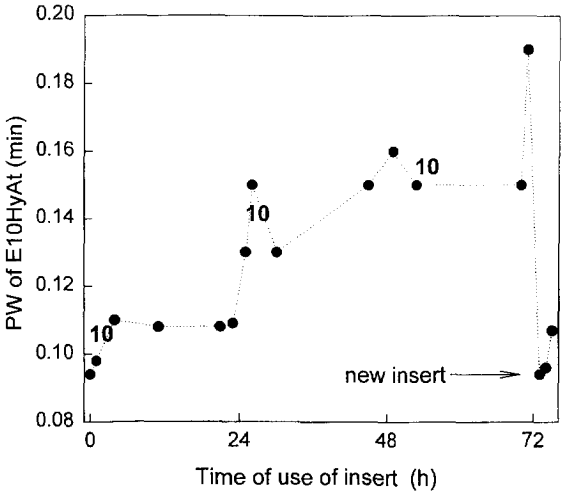

Fig. 3. Dependence of the peak width of *E*10HyAt on the time of use of one insert, the number indicates the number of injection of serum extracts in the course of this test.

morning a new insert was used. It may be speculated that moisture of the extraction solvent, some endogenous material, which is available in variant concentrations in individual samples, or contaminations from stoppers can affect the application time of one insert. However, this was not investigated in detail. It should be emphasized, that the time of use of one insert was considerably longer if only pure solvent but no serum extracts have been injected. Therefore, the mechanism of loss of performance of the inserts is not due to thermal destruction of the deactivation surface (silanization). The mechanism is as follows: non-evaporable contamination of the serum accumulates in the insert. During the injection, the hydroxy metabolites are adsorpted to this active surface and the transfer to the capillary is delayed. Two phases of the contamination have to be distinguished. First, there occurs just broadening of peaks and a minimal lowering of peak areas due to decomposition. After about twenty injections and up to several weeks of use of one insert, At and Nt alone could be analysed without problems. However, then the peaks of the hydroxy metabolites appeared just as one wide band and finally completely vanished, because of a higher degree of contamination.

The sample preparation was optimised with regard to a high extraction yield of the analytes and a very low co-extraction of endogenous, not evaporable material, which is mainly responsible for the contamination of the inner surface of the inserts and the loss of performance during long-term application. The polarity of the extraction solvent, therefore, the portion of isoamyl alcohol or a more polar alcohol in mixture with hexane, is the most important variable influencing the extraction. A mixture of 97 volumes of *n*-hexane and 3 volumes of isoamyl alcohol was found to yield pure enough extracts not contaminating the insert after just a few injections, but at a cost of a lowered extraction yield of the analytes. When testing *n*-hexane–isoamyl alcohol (98.5:1.5, v/v), the extraction yield of the hydroxy metabolites was too low. With the present method the overall analytical recoveries were calculated as follows: At 7.8%, Nt 6.7%, *E*,*Z*10HyAt 11% and *E*,*Z*10HyNt 8.3%. Taking into account the loss of volumes in course of the sample preparation process, the theoretical maximal recovery is RV^{th}_{max} (%)=(0.875×0.8×0.8×0.4)×100=22.4%. Thus, the extraction yields were: At 35%, Nt 30%, *E*,*Z*10HyAt 49% and *E*,*Z*10HyNt 37%. Increasing the time of extraction did not increase the extraction yield.

In strong acid solution, the hydroxy metabolites are decomposed by dehydration. Therefore, it has to be ascertained, that during the sample preparation, which contains a 30-min extraction with 0.1 *M* HCl, no dehydrated 10-hydroxyamitriptyline and 10-hydroxynortriptyline were formed. The mentioned products, which were prepared by simple standing of the hydroxy metabolites in 12 *M* HCl at room temperature for 2 h, gave a distinct doublet, signals at 6.50 and 6.68 min, in a test chromatogram. In the chromatograms of extracts of the sample preparation such signals have never been found, because of this it is concluded that the method is free of this complication.

Calibration curves of the six analytes were described by the formula $y=bx+a$, where y is the peak-area ratio of the analyte $A_{analyte}/A_{I.S.}$ and x is the concentration of the analyte in ng/ml. The slopes and the intercepts of each analyte are summarised in Table 2. For At and Nt, the negative intercepts of the calibration between 25 and 250 ng/ml and the slightly decreased slope of the calibration between 5 and 40 ng/ml indicate a very slight deviation from linearity in the region <20 ng/ml. This obviously does not influence the performance of the method,

Table 2
Data of calibration curves

Analyte	Range (ng/ml)	n	Slope (b)	Intercept (a)	r	Range (ng/ml)	n	Slope (b)	Intercept (a)	r
At	25–250	10	0.0233	−0.1027	0.998	5 to 40	8	0.0201	0.0154	0.998
Nt	25–250	10	0.0176	−0.0964	0.996	5 to 40	8	0.0147	0.0138	0.994
E10HyAt	5–40	8	0.0162	0.00367	0.993					
Z10HyAt	5–40	8	0.0167	0.00457	0.993					
E10HyNt	20–160	8	0.00888	−0.0156	0.992					
Z10HyNt	5–40	8	0.0085	−0.0106	0.994					

because concentrations at normal doses are far above 20 ng/ml and the calibration between 25 and 250 ng/ml will cover the majority of analytical problems (therapeutic window: At+Nt=80 to 250 ng/ml). However, in investigations of single-dose kinetics the calibration from 5 to 40 ng/ml can be used.

The determination of the analytes was accurate and precise as summarised in Table 3. Precision, as determined by the coefficient of variation of the within-day and between-day variations of samples of 25, 100 and 200 ng/ml (At and Nt), 25, 75 and 150 ng/ml (*E*10HyNt) and 10, 25 and 50 ng/ml (*E*10HyAt, *Z*10HyAt and *Z*10HyNt) was consistently between 6 and 19%. Accuracy, indicated as the concentration of drug measured relative to the known concentrations, was in a range of 91–115%. The lower limit of detection was 1.5 ng/ml for At, Nt, *E*10HyAt and *Z*10HyAt and 3 ng/ml for *E*10HyNt and *Z*10HyNt under the described conditions at a signal-to-noise ratio of 3. The quantification of the *Z*-stereoisomers in patient samples will sometimes be beset with problems because in man the formation of *E*-stereoisomers is favoured and the *Z*-isomers are known to occur at considerably lower levels (<15

Table 3
Accuracy and precision of the assay

Analyte	Concentration added (ng/ml)	Measured concentration (mean±S.D.) (ng/ml)		Accuracy (%)		Precision (R.S.D., %)	
		Within-day (n=6)	Between-day (n=12)	Within-day (n=6)	Between-day (n=12)	Within-day (n=6)	Between-day (n=12)
At	25	26.8±3.9	27.6±2.8	107	110	14.6	10.2
	100	97.1±6.0	95.9±6.6	97	96	6.2	6.9
	200	191.0±15.1	188.8±14.2	96	94	7.9	7.5
Nt	25	27.6±4.2	28.4±3.3	110	114	15.2	11.6
	100	100.0±6.8	103.1±8.0	100	103	6.8	7.8
	200	202.5±24.3	209.7±17.6	101	105	12.0	8.4
*E*10HyAt	10	9.2±0.7	10.5±1.3	92	105	7.2	12.4
	25	27.8±1.9	26.9±2.7	111	108	6.8	10.0
	50	56.4±5.2	54.9±4.1	113	110	9.2	7.5
*Z*10HyAt	10	9.7±1.7	10.5±1.2	97	105	17.3	11.4
	25	28.6±2.9	27.9±3.3	114	112	10.1	11.8
	50	57.6±5.6	57.1±3.5	115	104	9.7	6.1
*E*10HyNt	25	23.4±3.1	24.5±1.5	94	98	13.2	6.1
	75	68.5±10.3	75.2±6.2	91	100	15.0	8.2
	150	156.9±21.6	155.5±9.8	105	104	13.8	6.3
*Z*10HyNt	10	10.2±1.5	10.4±1.9	102	104	14.7	18.3
	25	24.0±3.3	24.5±4.6	96	98	13.7	18.8
	50	53.9±7.8	52.5±4.9	108	105	14.5	9.3

ng/ml). However, due to their higher levels, the *E*-stereoisomers are expected to be clinically more important and their concentrations are easily quantifiable with the present method.

In clinical practice, antipsychotics and antianxiety agents are sometimes prescribed concomitantly with amitriptyline. From Table 4 it is found that none of the drugs likely to be co-administered interfered with the method. Although antidepressants such as trimipramine, mianserine and *cis*-doxepine were not separated from Nt, this will not impair the performance of the method because usually two antidepressants are not co-administered. This argument also applies to the use of the antidepressant clomipramine as the internal standard. Furthermore, clomipramine in serum, not originating from the addition as the internal standard, will be clearly identified by the simultaneous appearance of desmethylclomipramine.

Table 4
Retention times and relative retention times of potential interfering drugs

Drug or metabolite	t_r (min)	Relative t_r
At	5.855	1.000
Nt	6.101	1.042
*E*10HyAt	8.509	1.453
Z10HyAt	8.726	1.490
*E*10HyNt	8.995	1.536
Z10HyNt	9.340	1.595
Clomipramine (I.S.)	9.810	1.675
Desmethylclomipramine	10.509	1.795
Imipramine	6.259	1.069[a]
Desipramine	6.577	1.123
Trimipramine	6.180	1.055[b]
Desmethyltrimipramine	6.637	1.134
Mianserine	6.192	1.058[b]
Maprotiline	8.276	1.413
Opipramol	No signal	
cis-Doxepin	6.059	1.035[b]
trans-Doxepin	6.326	1.080
Trazodone	No signal	
Fluoxetine	2.728	0.466
Clopenthixol	No signal	
Chlorprothixene	12.194	2.083
Chlorpromazin	12.254	2.092
Fluphenazine	No signal	
Haloperidol	No signal	
Levomepromazine	13.067	2.232
Perazine	No signal	
Promethazine	7.255	1.239
Thioridazine	No signal	
Alprazolam	No signal	
Diazepam	10.722	1.813
Oxazepam	8.238	1.407
Nitrazepam	No signal	
Zopiclon	No signal	
Zolpidem	No signal	

[a] $R_S = 1.19$.
[b] Not separated from Nt.

The method was tested in therapeutic drug monitoring of a depressive patient over three weeks of therapy. At a dose of 150 mg per day, the amitriptyline concentration and the nortriptyline concentration were 58 and 40 ng/ml, respectively. Only *E*10HyNt, as known from literature, reached serum levels in the same range (49 ng/ml). *E*10HyAt occurred at a medium concentration (18 ng/ml) and for the *Z*-stereoisomers considerable lower concentrations were calculated, Z10HyAt=4.7 ng/ml and Z10HyNt=8.8 ng/ml. In general, this example reflected the average metabolization pattern of amitriptyline in man. However, in some patients considerable deviations from this pattern have been reported. The clinical implications of such deviations from normal metabolization have to be investigated more.

To improve the sensitivity, a cold on-column injection technique with fused-silica needle and duck-bill septum (Hewlett–Packard) was tested. A three- to five-fold increase of sensitivity was found. Therefore, the limits of detection decreased to 0.5 ng/ml for At, Nt, *E*10HyAt and Z10HyAt and 1 ng/ml for *E*10HyNt and Z10HyNt. However, after this method was applied for two days, the separation of the hydroxy metabolites considerably deteriorated. As a first sign, the separation of Z10HyNt and I.S. disappeared. The reason for this behaviour is the contamination of the inner surface of the capillary. To achieve original performance, no simple manipulation such as the change of inserts is possible when using on-column injection, therefore, further investigation and validation was stopped. It is not possible to sacrifice, every two days, 3 to 5 meters of the column (or a retention gap). Besides, a retention gap was found to be insufficiently deactivated for the analysis of hydroxy metabolites.

4. Conclusions

The main limitation of gas–liquid chromatography for the analysis of antidepressant hydroxy metabolites is the injection process, therefore, the contamination of the inner surface of the injector insert. More care has to be spent on this problem, than in the assay of the parent drug and demethylated metabolite alone. This crucial problem was not recognized enough in the hitherto published methods, thus, an easy reproduction may be difficult. If a sufficient modus of the injection was found, as shown in the present method, the application of GLC provides some advantages over HPLC, e.g. the improved separation of stereoisomers. However, an injector for GLC with an integrated system for the self-cleaning or self-substitution of the injector insert is desirable.

The present method can be easily reproduced and applied in the research and therapeutic drug monitoring of antidepressant drugs. In contrast to Ref. [9], the separation of four instead of only three amitriptyline hydroxy metabolites, by means of capillary GLC, is described.

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