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Enantiomer analysis of *E*- and *Z*-10-hydroxyamitriptyline in human urine

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SUMMARY

E- and *Z*-10-hydroxyamitriptyline (*E*- and *Z*-10-OH-AT) are racemic alcoholic metabolites of the antidepressant amitriptyline. Their enantiomers were separated by high-performance liquid chromatography as diastereomeric derivatives using *R*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent). Although *E*-10-hydroxyamitriptyline excreted in patient urine in free form or as the O-glucuronide consisted primarily of the (−)-enantiomer, the N-glucuronide contained similar amounts of the two enantiomers. *Z*-10-OH-AT was analysed in one patient and an excess of the (+)-isomer was found in the unconjugated, total conjugated and N-glucuronidated metabolite. The specific optical rotation of (−)-*E*-10-OH-AT was determined.

INTRODUCTION

Hydroxylation of the antidepressants amitriptyline (AT) and nortriptyline (NT) at position 10 is a major biotransformation pathway leading to lipophilic secondary alcohols. Since C-10 becomes chiral by introduction of an OH group, the geometric isomers *E*- and *Z*-10-hydroxyamitriptyline and -nortriptyline (*E*- and *Z*-10-OH-AT and -NT) consist of pairs of enantiomers. Synthetic racemic mixtures of the alcohols were shown to possess some pharmacological activity [1]; therefore it was of interest to analyse the enantiomeric composition of the 10-hydroxy compounds excreted as metabolites. Their diastereomeric glucuronides can be separated by high-performance liquid chromatography (HPLC) [2–4] and the enantiomers of *E*- and *Z*-10-OH-NT were separated by HPLC after derivatization of the secondary amino group with

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2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) [5]. This procedure could not be applied to the analysis of 10-OH-AT enantiomers; therefore an assay method was developed using the more electrophilic chiral reagent *R*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent) [6,7]. It was used to analyse the enantiomeric composition of *E*- and *Z*-10-OH-AT that was excreted in human urine in free form or as the O- or N-glucuronide.

EXPERIMENTAL

Chemicals

Synthetic racemates of *E*- and *Z*-10-OH-AT and -NT were kindly donated by Dr. A. Jørgensen (Lundbeck, Copenhagen, Denmark). 10,11-Dehydroamitriptyline was prepared by acid treatment of *E*-10-OH-AT [8]. Organic solvents used for HPLC were of analytical grade with high UV transmittance. β-Glucuronidase/arylsulphatase from *Helix pomatia* was purchased from Boehringer (Mannheim, F.R.G.), *R*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) from Roth (Karlsruhe, F.R.G.) and TAGIT from Polysciences (St. Goar, F.R.G.). Thin-layer chromatography (TLC) was performed on silica gel plates (Sil G/UV_{254}; Macherey-Nagel, Düren, F.R.G.).

Urine samples

Urine was collected during 24 h from three female in-patients receiving therapy of 100–200 mg AT per day.

Isolation of aglycones

Semipreparative procedure. Since the isomers of 10-OH-AT are more than 90% conjugated prior to excretion in urine [9], larger amounts can only be recovered from the glucuronide fraction. Urine (150 ml) was extracted on a 150 mm × 10 mm I.D. column of C_{18} silica gel (40–63 μm, Polygosil, Macherey-Nagel) that was washed with water and eluted with methanol. The eluate was evaporated, and the residue was dissolved in water, alkalinized with ammonia and extracted with two 10-ml portions of chloroform for removal of unconjugated metabolites. Traces of chloroform were removed by evaporation and, after neutralization with 2 *M* acetic acid, the conjugate mixture was purified by filtration over the anion-exchange gel SAX (acetate form, Sepralyte 40 μm, ICT, Frankfurt, F.R.G.) and adsorption on 6 ml of C_{18} Polygosil followed by elution with methanol. It was then applied to two TLC plates and run in *n*-butanol–acetic acid–water (4:1:1, v/v). The UV-absorbing band at R_F 0.35–0.5 containing the OH-AT O-glucuronides was eluted with methanol, and the residue was dissolved in 2 ml of 0.1 *M* sodium acetate buffer (pH 5.0). Conjugate hydrolysis was achieved with 2 ml of β-glucuronidase/arylsulphatase (11 U β-glucuronidase) within 24 h at 37°C. Aglycones were extracted from the

alkalinized incubate with chloroform and purified by TLC in 2-propanol–chloroform–water–2 *M* ammonia (13:8:0.8:0.3, v/v). The bands at R_F 0.76 (*E*-10-OH-AT) and 0.72 (*Z*-10-OH-AT) were removed after spraying the dried chromatogram with water, suspended in 2 ml of 2 *M* ammonia and extracted with two 2-ml portions of chloroform. Metabolites excreted in unconjugated form could be isolated by TLC from the chloroform extract, following the first purification on C_{18} Polygosil. For enantiomer analysis *E*- and *Z*-10-OH-AT were removed together, because their derivatives were well separated on HPLC.

Analytical procedure. Unconjugated *Z*-10-OH-AT had to be isolated from large volumes of urine as described above. *E*-10-OH-AT and conjugated *Z*-10-OH-AT (from patient 3) were obtained in amounts sufficient for chiral derivatization from 5 ml of urine that was alkalinized with ammonia and extracted with two 2-ml portions of chloroform. At 4-ml volume of the aqueous phase was incubated at pH 5.0 with 0.8 ml of *β*-glucuronidase/arylsulphatase (1.1 U *β*-glucuronidase per ml urine) for 24 h at 37 °C, liberated aglycones being extracted at alkaline pH with chloroform. Completeness of conjugate hydrolysis was proved by adding 3 *M* hydrochloric acid (0.2 ml) to the aqueous phase (0.3 ml) and heating to 90 °C for 30 min; the cooled sample was shown by HPLC [10] not to contain 10,11-dehydroamitriptyline, the decomposition product of 10-OH-AT O-glucuronide [8]. The chloroform extracts were purified by TLC, and 10-OH-AT was isolated by extraction as above.

For determination of the enantiomeric composition of *E*- and *Z*-10-OH-AT N-glucuronides, 50 ml of urine was extracted on C_{18} Polygosil, filtered over anion-exchange gel SAX, extracted on C_{18} Polygosil and separated by semi-preparative HPLC [10]. The methanol in the fractions eluted at 11–13 and 27–43 min was evaporated, and the residues were enzymically deconjugated using 0.3 ml of *β*-glucuronidase/arylsulphatase. *E*- and *Z*-10-OH-AT were extracted and purified by TLC. Complete hydrolysis was apparent from the failure to detect by HPLC 10,11-dehydroamitriptyline N-glucuronide in the aqueous phase heated with hydrochloric acid as above [10].

Enantiomer analysis of E- and Z-10-OH-AT

Prior to derivatization, traces of water in the extract residues were removed by adding 0.2 ml of dichloromethane and evaporating under nitrogen. MTPA-Cl–dichloromethane (0.25 g + 2 ml) was added in 1000–10 000 fold excess (5–100 *μ*l) followed by pyridine (3–50 *μ*l) that had been stored on potassium hydroxide pellets. After 12 h at room temperature, the samples were evaporated under nitrogen, diluted with 0.1 ml of dichloromethane and purified by TLC in diisopropyl ether–diethylamine–2-propanol (8:0.4:0.1, v/v). The MTPA derivatives of *E*- and *Z*-10-OH-AT showed R_F 0.53; no underivatized compounds were detected at R_F 0.35, and there was no side-reaction to 10,11-dehydroamitriptyline (R_F 0.60) by loss of water. The threshold for visual detection of the compounds is ca. 100 ng per spot; thus, reactions starting from 10

μg of 10-OH-AT were at least 99% complete. The derivatives were extracted with methanol, which was evaporated. The residue was dissolved in the mobile phase for HPLC.

Separation of diastereomeric MTPA derivatives was optimal on a 250 mm $\times$ 4.6 mm I.D. column with C_{18} silica gel (Nucleosil C_{18}, 5 μm, pore width 300 Å; Macherey-Nagel) with 50 m*M* perchloric acid (adjusted to pH 2.5)–acetonitrile (55:45), flow-rate 1.5 ml/min, with photometric detection at 254 nm. The overall recovery was 30%, and the minimum amount of enantiomeric mixture required for derivatization was 0.3 μg.

The method was calibrated by preparing mixtures of fixed amounts of racemic *E*-10-OH-AT and various amounts of the pure (–)-enantiomer obtained by enzymic hydrolysis of (–)-*E*-10-OH-AT O-glucuronide [4] and carrying them through the procedure. Analysis of the enantiomeric composition of *Z*-10-OH-AT was based on linear extrapolation of the peak-height ratio (first peak to second peak = 1.1) of the racemate; the area was the same for the two peaks of the racemate.

Photometry of the column effluent from 200 to 380 nm (Uvikon 720 LC, Kontron, Munich/F.R.G.) confirmed that no elimination reaction to 10,11-dehydroamitriptyline had taken place, as the absorption at 290 nm characteristic of the dehydro compounds was not detectable. The spectrum rather corresponded to that of 10-OH-AT. Moreover, MTPA-*E*-10-OH-AT purified by TLC could be hydrolysed to *E*-10-OH-AT by incubation with 6 *M* ammonia for 6 h at 30°C. No racemization occurred on storage of the MTPA derivatives for several days at −20°C, but traces of 10,11-dehydroamitriptyline were formed. Therefore, MTPA derivatives were always chromatographed immediately following their preparation.

Assignment of optical rotation to the enantiomers of Z-10-OH-AT and -NT

The *Z*-10-OH-NT enantiomers liberated from the O-glucuronide isomers I and II [4] were derivatized with TAGIT and assigned to dextro- and levorotation by their behaviour in HPLC [5].

Since *Z*-10-OH-AT could not be isolated from urine in amounts sufficient for measuring optical rotation, (+)- and (–)-*Z*-10-OH-NT were converted into the tertiary amines by reductive methylation [11]. The resulting enantiomers of *Z*-10-OH-AT with known optical rotation were derivatized with MTPA-Cl and subjected to HPLC. In parallel, enzymic deconjugation and derivatization were carried out on isomers I and II of *Z*-10-OH-AT O-glucuronide [4].

Specific rotation of E-10-OH-AT

Unconjugated *E*-10-OH-AT was prepared from 150 ml of urine from patients by isolation and hydrolysis of the glucuronide as described above. The amount was determined by UV photometry and its enantiomeric composition

by derivatization with MTPA-Cl. It was dissolved in 1.1 ml of methanol and the optical rotation (α_D) was measured in a Perkin-Elmer polarimeter 241.

Measurement of 10-OH-AT and glucuronides

Total 10-OH-AT excreted in conjugated form and as O-glucuronide was analysed as 10,11-dehydroamitriptyline after treatment with 1 *M* hydrochloric acid at 90 °C for 30 min; *E*- and *Z*-10-OH-AT, present in free form or liberated enzymically, were separated by HPLC [10]. 10-OH-AT N-glucuronide produced 10,11-dehydroamitriptyline N-glucuronide on heating with hydrochloric acid and was quantitated in this form by HPLC [10].

RESULTS

Assay method for E- and Z-10-OH-AT enantiomers

Diastereoisomers formed by derivatization with MTPA-Cl (Mosher's reagent, Fig. 1) were well separated by HPLC (Fig. 2). Derivatization of pure enantiomers of *E*-10-OH-AT prepared from the individual O-glucuronides led to the expected single HPLC peaks.

The procedure proved to be well reproducible: in ten duplicate determina-

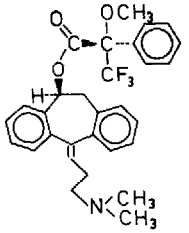

Fig. 1 Structure of the MTPA derivative of one enantiomer of *E*-10-OH-AT.

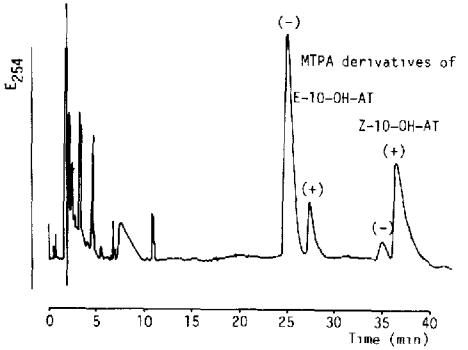

Fig. 2. HPLC of the MTPA derivatives of *E*- and *Z*-10-OH-AT from the unconjugated fraction in urine of patient 3.

tions the mean fraction of the (−)-isomer in *E*-10-OH-AT was 70% and the coefficient of variation was 1.4% of the mean. The peak-height ratio depended on the enantiomeric composition of *E*-10-OH-AT in a strictly linear fashion. A calibration curve constructed for mixtures containing 5–50% (+)-isomer had a correlation coefficient of $r=0.99$ ($n=9$) and went through the origin.

Interferences from other metabolites could be excluded by TLC prepurification of aglycones and MTPA derivatives. This resulted in low recoveries and consequently in a loss of sensitivity, but it did not lead to a change in the enantiomeric composition.

Assignment of optical rotation to the enantiomers of Z-10-OH-AT and -NT

On enzymic hydrolysis the slower-eluting *Z*-10-OH-NT O-glucuronide diastereomer II [4] gave *Z*-10-OH-NT, which reacted with TAGIT to give the faster-eluting derivative (Table I). Levorotation has been measured on the corresponding aglycone [5] such that the glucuronide isomers I and II can be designated as the (+)- and (−)-enantiomers, respectively.

N-Methylation of the enantiomerically pure aglycones led to the *Z*-10-OH-AT enantiomers, which on reaction with MTPA-Cl resulted in single derivatives with retention times in HPLC of 37 and 35 min for the (+)- and (−)-isomer, respectively. The same retention times were found when the aglycones of isomers I and II of *Z*-10-OH-AT O-glucuronide were derivatized with MTPA-Cl (Table I) which proves that as with the secondary amine the faster-eluting glucuronide contains as aglycone the (+)-enantiomer.

TABLE I

HPLC RETENTION TIMES OF DIASTEREOMERIC DERIVATIVES OF (+)- AND (−)-*Z*-10-OH-AT AND -NT

Retention times of O-glucuronides were measured in 0.02 *M* ammonium acetate buffer (pH 5)–methanol (63:37) [10].

Enantiomer	Retention time (min)		
	O-Glucuronide	TAGIT derivative	MTPA derivative
(+)-*Z*-10-OH-NT	27[a]	96	
(−)-*Z*-10-OH-NT	30.5[b]	91.5	
Methylated (+)-*Z*-10-OH-NT			37
Methylated (−)-*Z*-10-OH-NT			35
(+)-*Z*-10-OH-AT	21.5[a]		37
(−)-*Z*-10-OH-AT	24[b]		35

[a]Isomer I.
[b]Isomer II of Baier-Weber et al. [4].

Specific optical rotation of (−)-E-10-OH-AT

The optical rotation (α_D) of a sample of *E*-10-OH-AT isolated from a urine sample was −0.018° (concentration 0.064 g per 100 ml methanol) and it was found to contain 76% of the (−)-enantiomer. Thus, the specific rotation ($[\alpha]_D$) of pure (−)-*E*-10-OH-AT would be −54° which is similar to the value of −49° obtained on (−)-*E*-10-OH-NT [5].

Enantiomeric composition of urinary metabolites

Unconjugated as well as total conjugated *E*-10-OH-AT in urine from three patients contained an excess of the (−)-isomer. In contrast, separate analysis of the N-glucuronide revealed a slight excess of the (+)-enantiomer (Table II). The enantiomer composition of *E*-10-OH-AT O-glucuronide has not been determined directly. An estimate can be made taking the amounts and (−)/(+) ratios of total conjugates and N-glucuronide into consideration. However, the N-glucuronide amount represents the sum of *E*- and *Z*-10-OH-AT N-glucuronides, which could not be measured separately [10]. On the assumption that the *Z*-isomer contributed 10%, the (−)-isomer fraction in *E*-10-OH-AT O-glucuronide of the three patients was between 80 and 92%; with a contribution of 20% of the *Z*-form, the O-glucuronide would contain 77–87% (−)-*E*-10-OH-AT.

Unconjugated *Z*-10-OH-AT was analysed in two patients and found to con-

TABLE II

AMOUNTS AND ENANTIOMERIC COMPOSITION OF 10-OH-AT AND ITS CONJUGATES IN URINE FROM PATIENTS

Patients 1, 2 and 3 were treated with 100, 150 and 200 mg AT per day, respectively.

Compound	Patient 1		Patient 2		Patient 3	
	Percentage of dose	(−)/(+) ratio	Percentage of dose	(−)/(+) ratio	Percentage of dose	(−)/(+) ratio
E-10-OH-AT						
Unconjugated	0.38	82 18	0.14	80 20	0.23	70:30
Total conjugates	4.3	67:33	5.3	72 28	9.3	63 37
N-Glucuronide		46·54		48.52		40.60
Z-10-OH-AT						
Unconjugated	0.03	32.68			0.04	15 85
Total conjugates	0.22				2.6	33.67
N-Glucuronide						22.78
E- and *Z*-10-OH-AT						
N-Glucuronide	2.6		2.5		4.4	

tain more (+)- than (−)-isomer. The same applied to total conjugates and to purified *Z*-10-OH-AT N-glucuronide in one sample (Table II).

DISCUSSION

Attempts to achieve resolution of 10-OH-AT enantiomers on chiral columns containing α_1-acid glycoprotein [12], cellulose triacetate [13], TAGIT [14] or amino acid derivatives [15] were unsuccessful, as were experiments aimed at derivatization with *R*-(+)-phenylethyl isothiocyanate, N-trifluoroacetyl-*S*-(−)-prolyl chloride [15], D-camphorsulphonic chloride [16] or naproxen chloride [17]. The four alcohols could, however, be derivatized with the chiral reagent MTPA-Cl, which has been used for enantiomeric analysis of alcohols and amines [6] including a β-receptor blocker [18]. The reagent is sufficiently electrophilic for complete derivatization of the benzylic OH group, but under mild conditions elimination to 10,11-dehydroamitriptyline could be avoided. The diastereoisomeric esters were separated on C_{18} silica gel with 100 Å pore width, but quantitation was difficult owing to tailing. Better chromatograms were obtained from a stationary phase with larger pores.

The enantiomeric composition of the alcoholic metabolites in urine samples indicates product selectivity [19] in AT hydroxylation, with preferential formation of (−)-*E*- and (+)-*Z*-10-OH-AT. It must, however, be kept in mind that the major fraction of orally administered *E*-10-OH-AT does not appear in urine in free or conjugated form [10] and is possibly biotransformed by pathways exhibiting substrate enantioselectivity. Different fates of the two enantiomers on ingestion of the racemate have been demonstrated for *E*- and *Z*-10-OH-NT [5, 20].

Distinct differences were found with regard to the enantiomeric composition of the O- and N-glucuronides of *E*-10-OH-AT in urine samples from patients. This may be due to substrate enantioselectivity of the conjugating enzymes or to differences in the extent of in vivo re-hydrolysis of the four isomeric conjugates. Further, *E*-10-OH-AT N-glucuronide may in patients be preferentially produced via AT N-glucuronide, a pathway that was demonstrated to operate on AT N-glucuronide administered by intravenous infusion [10] and to lead to nearly equal amounts of (+)- and (−)-*E*-10-OH-AT N-glucuronide in urine (unpublished results).

Examples of other lipophilic aminoalcohols that exhibit substrate enantioselectivity in their disposition are oxaprotiline [21] and the β-receptor blockers. In particular, propranolol undergoes a complex metabolic fate in which all reactions tested, including glucuronidation, occur preferentially in one of the isomers [19].

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