METABOLISM OF NOMIFENSINE (ALIVAL[®], MERITAL[®]): ISOLATION AND IDENTIFICATION OF THE CONJUGATES OF NOMIFENSINE-¹⁴C FROM HUMAN URINE

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- 1 It can be stated in regard to the metabolism of nomifensine that the metabolites and also the starting compound are present in alkaline urine almost completely in conjugated form.
- 2 The metabolites MI–M III represent about 30% of the radioactivity excreted with urine. Two-thirds are *N*-glucuronides and one-third *o*-glucuronides.
- 3 Nearly the entire remainder of the renally eliminated radioactivity is nomifensine which is present in conjugated form as *N*-glucuronide.
- 4 Thus over 90% of the metabolites formed in man and excreted via urine have been identified.

Introduction

Nomifensine, 8-amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline maleate (Hoe 984, Alival[®]) is a psychotropic drug with antidepressant properties. It does not belong to the conventional group of tricyclic antidepressant drugs, but to a new class of compounds. Its pharmacodynamic properties in man (Angst, Koukkou, Bleuler-Herzog & Martens, 1974; Rupp, 1974; Taeuber, 1974) and in animals (Hoffman, Ehrhart & Schmitt, 1971; Hoffmann, 1973) have been described earlier. Reports are also available on absorption, distribution in the organs, metabolism and excretion of ¹⁴C-labelled nomifensine (Hornke, Cavagna, Christ, Fehlhaber, Heptner, Kellner & Rupp, 1977; Kellner, Baeder, Christ, Heptner, Hornke & Ings, 1977; Kellner, Hornke & Volz, 1977; Heptner, Hornke, Cavagna, Fehlhaber, Rupp & Neubauer, 1978; Kellner & Hornke, 1978).

In the studies on the biotransformation of nomifensine in man the following main metabolites were detected: 4'-hydroxy-nomifensine, 4'-hydroxy-3'-methoxy-nomifensine and 3'-hydroxy-4'-methoxy-nomifensine, together account for approximately 20%.

In addition, the structures of four further metabolites, in all representing only 1%, could probably be identified. They are dihydroxy, dimethoxy, hydroxy-dimethoxy and desmethyl derivatives of nomifensine.

About 90% of the renally eliminated metabolites were excreted in conjugated form, over two-thirds of

Figure 1 The formula of nomifensine maleate, showing the position of the ¹⁴C label (★)

which can be converted to unchanged nomifensine after acid hydrolysis.

This study describes the isolation and identification of conjugates of [¹⁴C]-nomifensine as well as nomifensine metabolites which are unstable to acids and those that are stable, in human urine after oral administration.

Methods

1. Preparation and labelling

Nomifensine labelled with ¹⁴C in the 4 position of the tetrahydroisoquinoline ring was used for conducting the investigations (Figure 1). The compound was

synthesized in the Radiochemisches Labor of Hoechst AG (Gantz, Weimer & Schwarz, 1978), with a specific radioactivity of 0.20 mCi/g nomifensine maleate and was radiochemically pure, as tested by radio thin-layer chromatography (chloroform : cyclohexane : methanol : aqueous ammonia 40 : 40 : 15 : 2).

2. Procedure

In each case 25 mg radioactive ingredient was weighed out into hard gelatine capsules. Three male healthy volunteers each received 4 capsules containing a total of 100 mg radioactive ingredient

Figure 2 Extraction and purification scheme: conjugates

together with a breakfast. Emphasis in the experiments was put on the fact that volunteers had to excrete alkaline urine during the first 8 h, because adequate stability of the conjugates to be isolated can only be guaranteed at pH values above 7.2. For this reason, the volunteers were given sodium hydrogen carbonate in the form of Kaisernatron (up to 6 tablets of 960 mg NaHCO₃, Messrs. A. Holste Wwe, Bielefeld) every hour, starting 1 h before application, until 7 h after application, depending on the pH

values of the urine, which was checked hourly. The urine was collected hourly during the first 8 h, then two-hourly until 14 h after application, and the remainder in one portion until 24 h after application.

3. Radioactivity determination in the urine

Two parallel urine samples of 2 ml were mixed with 15 ml of an emulsifying scintillator and measured by means of the liquid scintillation technique, using an

external standard device for determining the efficiency. The scintillant consisted of xylene, a polyethoxyethanol, ethanol, 1.1% PPO and 0.1% dimethyl-POPOP.

4. Extraction of the urine samples (Figure 2)

Immediately after obtaining the urine samples they were adjusted to pH 8.5–9 with aqueous ammonia. The fractions 0–4 and respectively 4–8 h of the three volunteers were pooled and then freeze-dried. Two hundred ml urine lyophilisate in each case was extracted with two 150 ml portions of methanol to which had been 0.5% by volume of aqueous ammonia; the mixtures were then centrifuged and decanted. After the radioactivity measurement, the non-radioactive residue was discarded.

5. Processing the methanol extracts (Figure 2)

The methanol extracts were concentrated in a rotary evaporator under a current of NH_3 . The residues were then dissolved each in 100 ml aqueous ammonia (pH 8–9), added to an Amberlite XAD column and eluted consecutively with 1-litre portions of

water	(E I)
water/methanol 90/10	(E II)
water/methanol 70/30	(E III)
water/methanol 50/50	(E IV)
methanol	(E V)

Each eluant had been adjusted to pH 8–9 with a few drops of aqueous ammonia.

The eluates E III and E IV were retained for further processing, as they contained the main portion of the conjugates.

6. Thin layer chromatographic separation of the eluates E III and E IV

After concentrating these eluates under a current of NH_3 , further separation of the conjugates was carried out by thin layer chromatography.

For this purpose the evaporated residues of E III and E IV were each dissolved in 1.8 ml methanol/aqueous ammonia (1000/5), applied in the form of streaks to ready-made silica gel films Sil G UV₂₅₄, Macherey & Nagel, and developed in the solvent system 1-butylacetate/2-propanol/water/aqueous ammonia (30/50/15/5). Before use the plates had been given a preliminary run with methanol. The radioactive zone (R_F approx. 0.22) was cut out immediately after development and while still moist, eluted with ammoniacal methanol by the line-elution technique in a Desaga elutor. The eluates obtained were concentrated under a current of NH_3 and then purged with NH_3 gas. Samples pretreated in this way are stable for a prolonged time if stored in a freezer.

These samples were subsequently examined by mass spectrometry, as well as gas chromatography and thin layer chromatography.

7. Separation of the stable conjugates

In order to destroy the labile conjugates, a part of the substance isolated according to section 6 above was mixed with 0.1 ml 5 N HCl and with 5 ml methanol and heated at 50°C for 2 h. This mixture was concentrated and dissolved in a small quantity of methanol. The solution was separated by the TLC system described in (6) above.

Whilst the metabolites MI–MIII and the free nomifensine have an R_F -value of about 0.85, the stable conjugates sought have the R_F -value of approx. 0.22. This zone was cut out, separated by the line-elution technique with methanol and subjected to glucuronidase treatment.

8. Conjugate-splitting with β -glucuronidase and aryl sulphatase

The eluate obtained as described in section (7) above was concentrated and dissolved in 5 ml 0.1 M acetate buffer pH 5. After adding 0.5 ml β -glucuronidase (prep. from *Escherichia coli*, Boehringer, Mannheim) the mixture was incubated at 37°C for 22 h. This incubation had to be continued for a further 24 h after adding an additional 0.5 ml β -glucuronidase. The incubate was then evaporated to dryness in a rotary evaporator, extracted with two 4-ml portions of methanol, centrifuged and decanted.

The stable conjugates were not split during incubation carried out in the same way with aryl sulphatase (Boehringer, Mannheim).

9. Thin layer chromatography of the β -glucuronidase incubate

After extensively concentrating the methanol extract of the incubate, it was applied in the form of streaks to silica gel films Sil GUV₂₅₄, (Macherey & Nagel) and developed with the solvent system chloroform/1-butanol/aqueous ammonia (70/30/1). The radioactive zones were cut out, eluted by the line-elution technique with methanol, concentrated and examined by mass spectrometry.

10. Detection of D-glucuronic acid by thin layer chromatography

D-glucuronic acid and an acidified sample of the labile conjugate were developed in the solvent system ethyl acetate/formic acid/water (60/20/20) on cellulose films CEL 300 UV₂₅₄, (Macherey & Nagel). For visualizing the D-glucuronic acid the plates were

sprayed with a solution of 0.93 g aniline and 1.66 g *o*-phthalic acid in 100 ml water-saturated 1-butanol, and heated for 10 min at 105°C (Merck, 1970).

11. Silylation reactions

- (i) For gas chromatographic detection of *D*-glucuronic acid an acidified conjugate sample was concentrated in the rotary evaporator after adding 1 drop of aqueous ammonia. Then it was dried in vacuum over CaCl_2 in the desiccator until completely anhydrous. The sample was dissolved in pyridine and silylized with an excess of MSHFBA (*N*-methyl-, *N*-trimethylsilyl-heptafluoro-butyramide) at 70°C.
- (ii) For mass spectrometric measurement a conjugate sample was mixed with 50 μl of a 10% MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) solution in methylene chloride and left to stand for 90 min at room temperature.

12. Gas chromatographic detection of *D*-glucuronic acid

The gas chromatographic determinations were carried out directly with the 1–2% pyridine/MSHFBA solutions obtained during silylation (see section 11). For characterizing the components which had been obtained by the splitting of the labile conjugates, the following reference substances were available: *D*-glucuronic acid, *D*-glucuronic acid- γ -lactone, nomifensine and its metabolites MI, MII and MIII. Depending on the number of components per sample, 0.1–1.5 μl were injected.

Instrument: F 20 F, Perkin–Elmer
Column: length 2 m; internal diameter 2.7 mm
Stationary phase: 2.5% OV 17 on Chromosorb G AW-DMCS (30–100 mesh)
Temperature: 150–300°C; temperature program: 3 min at a constant temp. of 150°C, then increased by 25°C/min
Carrier gas: Helium (Messer Griesheim) inlet pressure 2.2 atm. gauge
Injection port: Temperature 330°C
Detector: FID; temperature 390°C

Under these conditions the retention times given in Table 1 were measured.

13. Structure determination by high-resolution mass spectrometry

The molecular weight of the persilylized unstable conjugate and its structure were determined by mass spectrometry (MS 902 S, AEI). The temperature of

Figure 3 Ratio of nomifensine conjugate to free base as a function of the pH value of freshly obtained human urine samples. ■ volunteer 1, ● volunteer 2, and ▲ volunteer 3

the ion source was 190°C, the electron energy 70 eV. The metabolites MI–M III were also identified in the same instrument after splitting the *o*-glucuronides with β -glucuronidase.

14. Evaluation of the radio thin layer chromatograms and estimation of the radioactivity amount

The site of the radioactive spots or streaks on the thin layer films was determined with the aid of a radio thin layer scanner (Berthold-Frieske). The radioactivity content in the extracts, eluates and residues was determined by liquid scintillation measurement (see section (4) above).

Results

Renal excretion

Experiments in humans had shown that nomifensine is excreted almost exclusively as a conjugate (Figure

Table 1 Retention times of the reference substances under the stated GC conditions

Substance	Retention time (min)
<i>D</i> -glucuronic acid	6.95/7.05 (double peak)
<i>D</i> -glucuronic acid- γ -lactone	6.8
Nomifensine	9.5
MI (4'-hydroxynomifensine)	11.0
II (4'-hydroxy-3'-methoxy-nomifensine)	11.9
M III (3'-hydroxy-4'-methoxy-nomifensine)	11.7

Figure 4 Radio thin layer chromatogram of natural human urine (pH 8.0) after administration of [^{14}C]-nomifensine. For thin layer chromatography conditions see Methods (9)

Figure 5 Radio thin layer chromatogram of a natural acid-hydrolysed human urine sample after administration of [^{14}C]-nomifensine. For thin layer chromatography conditions see Methods (9)

4); however, this is split relatively fast at pH values below 7.2 (Hornke *et al.*, 1977) (Figure 3).

In order to ensure that weakly alkaline values in the urine would be obtained during the trial, the volunteers were given sodium hydrogen carbonate in addition to nomifensine (Table 2).

During the first 8 h, on average 73% and within 24 h 90% of the applied radioactivity was renally excreted. As thin layer chromatographic tests of the natural urine samples revealed, virtually the entire radioactivity was present in conjugated form (Figure 4).

Table 2 pH values of the urine samples

Time	Volunteer 1	Volunteer 2	Volunteer 3
Before application			
until 2 h	5.1	5.4	5.2
until 1 h	5.1	5.3	5.3
1 to 0 h	7.2	6.7	7.1
After application			
0-1 h	8.0	7.7	7.8
1-2 h	8.0	7.8	7.9
2-3 h	7.9	7.7	7.8
3-4 h	7.9	7.8	7.8
4-5 h	7.9	7.8	7.9
5-6 h	7.9	7.6	7.8
6-7 h	7.8	7.5	7.8
7-8 h	8.0	8.0	8.2
8-10 h	8.0	8.0	8.2
10-12 h	7.1	6.7	7.2
12-14 h	6.5	7.2	7.1
14-24 h	6.3	6.7	7.2

Figure 6 Radio thin layer chromatogram of an acid-hydrolysed conjugate sample of [^{14}C]-nomifensine isolated from human urine. For thin layer chromatography conditions see Methods (6)

After acid hydrolysis (Figure 5) it was found that the metabolites M I–M III, are also excreted in labile conjugated form.

Identification of the structure of the unstable nomifensine conjugate

The radioactive conjugate samples obtained after column and thin layer chromatographic isolation and cleaning, according to TLC criteria, contained unstable, as well as an approximately 10% portion of acid-stable conjugates which could not be separated by means of the chromatographic partition system used (Figure 6).

The mass spectrometric examination of a sample did not produce any spectra, neither by electron impact ionization nor by chemical ionization. Due to extensive thermal cleavage in all spectra only molar peaks of free nomifensine (m/e 238) and its metabolites M I–M III were exhibited.

In consequence, the samples were silylated with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA). The TMS derivative exhibited a distinct molecular ion at m/e 702 (Figure 7). The empirical molecular formula of which was determined by high resolution mass spectrometry: $\text{C}_{34}\text{H}_{58}\text{N}_2\text{O}_6\text{Si}_4$.

Evaluation of the high resolution mass spectrum led to the identification of the key fragments shown in the following scheme (Figure 8). Among these fragments there are a number of ions, especially m/e 465, that prove the glycosidic linkage of glucuronic acid to the aglycone moiety (Fenselau, 1973). With this result the conjugate unambiguously could be identified as a TMS-derivative of nomifensine-*N*-glucuronide.

After hydrolysis of a conjugate sample with

Figure 7 Mass spectrum of the silylated *N*-glucuronide of nomifensine

Figure 8 Identity of key fragments after evaluation of the high resolution mass spectrum (Figure 7)

methanolic HCl, it was possible to detect glucuronic acid by thin layer chromatography and after silylation with *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA) by gas chromatography (Figure 9).

In accordance with the retention times, the main peaks of the gas chromatogram represent nomifensine (9.5 min) and *D*-glucuronic acid (6.95/7.05 min).

The peaks occurring after 10.5 min can be assigned to the silylized metabolites MI and MIII. These findings indicate that the metabolites, too, are excreted in the form of unstable conjugates. Owing to the low concentrations, no MS examination was carried out.

Clarification of the stable conjugates

As the unstable and stable conjugates exhibit the same chromatographic characteristics, the *N*-glucuronides of nomifensine and its metabolites MI–MIII were first destroyed by acid hydrolysis, in order to isolate the stable conjugates. It was then possible to separate and clean the stable conjugates chromatographically. After splitting with β -glucuronidase, the substances were isolated by thin layer chromatography from the sample obtained. The R_F -values of these substances correspond to those of the metabolites MI–MIII, for which the respective molecular ions m/e 254 and 284 could be detected by

Figure 9 Gas chromatogram of the conjugate of [^{14}C]-nomifensine after acid hydrolysis and silylation with MSHFBA (a) and of a glucuronic acid sample after silylation with MSHFBA (b). TMS derivatives of ■ glucuronic acid, ● nomifensine, ▼ metabolite M I and ▽ metabolites M II and M III. For gas chromatographic conditions see Methods (11)

high-resolution mass spectrometry. This means that the metabolites M I–M III are also excreted as *o*-glucuronides (Figure 10).

Small quantities of a further substance of molecular

Figure 10 The *o*-glucuronides of Metabolites (a) M I, (b) M II and (c) M III

weight 314 were also detected. High-resolution mass spectrometry revealed the empirical molecular formula $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_3$, which indicates that one hydroxy and two methoxy groups have been introduced into the nomifensine molecule.

Because of the small proportion of this compound, it was not possible to assign the position of the hydroxy and methoxy groups precisely.

References

- ANGST, I., KOUKKOU, M., BLEULER-HERZOG, M. & MARTENS, H. (1974). Ergebnisse eines offenen und eines Doppelblindversuches von Nomifendrosin im Vergleich zu Imipramin. *Arch. Psychiat. Nervenkr.*, **219**, 265–276.
- FENSELAU, C. (1973). Mass spectral analysis of Glucuronides. *J. med. Chem.*, **16**, 30.
- GANTZ, D., WEIMER, K. & SCHWARZ, A. (1978). Synthesen von radioaktiv markiertem Nomifensin. *Internal report*, Hoechst AG.
- HEPTNER, W., HORNKE, I., CAVAGNA, F., FEHLHABER, H.-W., RUPP, W. & NEUBAUER, H.-P. (1978). Metabolism of Nomifensine in man and animal species. *Arzneimittel-Forsch./Drug Res.*, **23**, 58–64.
- HOFFMANN, I. (1973). 8-Amino-4-phenyl-1,2,3,4-tetrahydroisoquinoline, a new antidepressant. *Arzneimittel-Forsch./Drug Res.*, **23**, 45–50.
- HOFFMANN, I., EHRHART, G. & SCHMITT, K. (1971). 8-Amino-4-phenyl-1,2,3,4-tetrahydroisoquinolin, eine neue Gruppe antidepressiver Psychopharmaka. *Arzneimittel-Forsch./Drug Res.*, **21**, 1045.
- HORNKE, I., CAVAGNA, F., CHRIST, O., FEHLHABER, H.-W., HEPTNER, W., KELLNER, H.-M. & RUPP, W. (1977). Alival-(Nomifensin)-Symposium über Ergebnisse der Experimentellen und Klinischen Prüfung (Symposium on results of experimental and clinical testing). *Pharmakokinetik und Metabolismus von Nomifensin*. (1976, Berlin, West, 99 104). Stuttgart, New York: F.K. Schattauer Verlag.
- KELLNER, H.-M., BAEDER, C., CHRIST, O., HEPTNER, W., HORNKE, I. & INGS, R.M.J. (1977). Kinetics and Metabolism of Nomifensine in Animals. *Br. J. clin. Pharmacol.*, **4**, 109S–116S.
- KELLNER, H.-M., HORNKE, I. & VOLZ, M. (1977). Pharmakokinetik und Metabolismus am ausgewählten Beispiel. *Der Kassenarzt*, **17**, 2402–2413.

- KELLNER, H.-M. & HORNKE, I. (1978). Pharmakokinetik und Metabolismus (Serie Nuklearmedizin VIII). *Ärztliche Praxis*, **30**, 234.
- MERCK, DARMSTADT (1970). Anfärbereagenzien für Dünnschicht- und Papierchromatographie (Staining reagents for thin layer and paper chromatography).
- RUPP, (1974). Relevants of screening test in human psychopharmacology; the clinical psychopharmacologist's point of view. In *Assessment of Pharmacodynamic Effects in Human Pharmacology*, ed. Hippus, H. Part I, pp. 43-49. Stuttgart: Schattauer Verlag.
- TAEUBER, K. (1974). Relevants of screening tests in human psychopharmacology; the psychological point of view. In *Assessment of Pharmacodynamic Effects in Human Pharmacology*, ed. Hippus, H. Part II, pp. 51-57. Stuttgart: Schattauer Verlag.

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