Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Chromatography B, 802 (2004) 339–345
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of picogram levels of midazolam, and 1- and 4-hydroxymidazolam in human plasma by gas chromatography–negative chemical ionization–mass spectrometry

C.B. Eap [a,*], G. Bouchoux [b], K. Powell Golay [a], P. Baumann [a]

[a] *Unité de Biochimie et Psychopharmacologie Clinique, Département Universitaire, de Psychiatrie Adulte, Hôpital de Cery, CH-1008 Prilly-Lausanne, Switzerland*
[b] *Département de Chimie, Laboratoire des mécanismes réactionnels, Ecole Polytechnique, F-91128 Palaiseau Cedex, France*

Received 12 June 2003; received in revised form 27 November 2003; accepted 16 December 2003

Abstract

Midazolam is a widely accepted probe for phenotyping cytochrome P4503A. A gas chromatography–mass spectrometry (GC–MS)–negative chemical ionization method is presented which allows measuring very low levels of midazolam (MID), 1-OH midazolam (1OHMID) and 4-OH midazolam (4OHMID), in plasma, after derivatization with the reagent *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide. The standard curves were linear over a working range of 20 pg/ml to 5 ng/ml for the three compounds, with the mean coefficients of correlation of the calibration curves ($n = 6$) being 0.999 for MID and 1OHMID, and 1.0 for 4OHMID. The mean recoveries measured at 100 pg/ml, 500 pg/ml, and 2 ng/ml, ranged from 76 to 87% for MID, from 76 to 99% for 1OHMID, from 68 to 84% for 4OHMID, and from 82 to 109% for *N*-ethyloxazepam (internal standard). Intra- ($n = 7$) and inter-day ($n = 8$) coefficients of variation determined at three concentrations ranged from 1 to 8% for MID, from 2 to 13% for 1OHMID and from 1 to 14% for 4OHMID. The percent theoretical concentrations (accuracy) were within ±8% for MID and 1OHMID, within ±9% for 4OHMID at 500 pg/ml and 2 ng/ml, and within ±28% for 4OHMID at 100 pg/ml. The limits of quantitation were found to be 10 pg/ml for the three compounds. This method can be used for phenotyping cytochrome P4503A in humans following the administration of a very low oral dose of midazolam (75 μg), without central nervous system side-effects.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Midazolam; Hydroxymidazolam

1. Introduction

Cytochrome P4503A (CYP3A), a term that in adults reflects the collective activity of cytochrome P4503A4 (CYP3A4) and cytochrome P4503A5 (CYP3A5), plays a central role in the metabolism of a wide variety of drugs [1]. Considering the large inter-individual and intra-individual variability in CYP3A activity, a method allowing to assess its activity *in vivo* is valuable. Several methods for phenotyping CYP3A activity have been proposed, which include the administration of midazolam (MID), ^{14}C-labeled erythromycin, dapsone, alfentanil, nifedipine or lidocaine, or which measure the hydroxylation of endogenous cortisol (for a review see [1–3]). With regard to the latter, following oral administration, MID is oxidized to 1-OH midazolam (1OHMID) and 4-OH midazolam (4OHMID) by CYP3A [1] (Fig. 1), and it is a widely accepted probe for this isozyme [1–3]. However, a disadvantage of using midazolam derives from the requirement of using a therapeutic dose, i.e. in the mg range, due to the limit of quantification of standard analytical methods. This thus prevents its simple use because of the side-effects (drowsiness, sedation and even amnesic effects in some subjects). Previously published methods on the determination of MID and its metabolites used gas chromatography equipped with an electron capture detector with [4], a nitrogen-phosphorus detector [5], with a mass spectrometry detector [6] in the electron impact mode [7,8] or in the negative chemical ionization mode [9,10], or used a liquid chromatography–UV detection [11,12], or liquid chromatography–mass spectrometry [13–15]. The

* Corresponding author. Tel.: +41-21-643-64-38; fax: +41-21-643-64-44.
E-mail address: Chin.Eap@inst.hospvd.ch (C.B. Eap).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.12.014

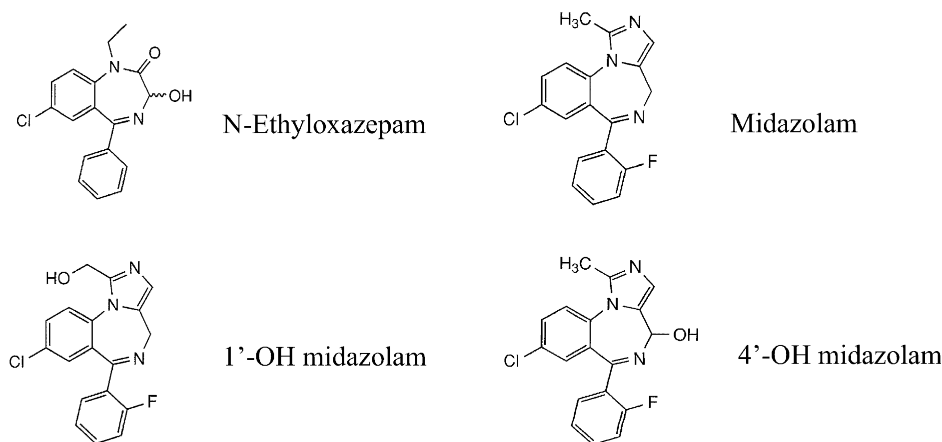

Fig. 1. Chemical structures of midazolam, 1-OH midazolam, 4-OH midazolam, and *N*-ethyloxazepam (internal standard).

described limits of quantification for MID, 1OHMID or 4OHMID were either in the ng/ml range [4,11,12] or in the hundredth(s) of pg/ml range [5–10,13–15].

We developed and validated a very sensitive gas chromatography–mass spectrometry–negative chemical ionization analytical procedure, which allows to measure levels of MID and its metabolites in the 10–100 pg/ml range, i.e. levels reached after the administration of one hundredth of the usual dose, 75 μg of midazolam, which is not expected to produce any central nervous effects.

2. Experimental

2.1. Reagents

Roche (Basle, Switzerland) supplied midazolam base (purity >98.5%), 1-OH midazolam base and 4-OH midazolam base (no information available on the purity of the latter two compounds). *N*-ethyloxazepam (EOZ, internal standard, purity >98.5%) was purchased from Lipomed (Alersheim, Switzerland) directly as a 1 mg/ml solution in methanol. *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane was from Fluka (Buchs, Switzerland). β-Glucuronidase (Helix pomatia) was from Sigma (No G-7770, Fluka, Buchs, Switzerland). All other reagents were of analytical or HPLC grade.

Stock solutions of MID, 1OHMID, 4OHMID were made at 1 mg/ml in methanol and stored at −20 °C. From these, solutions were prepared at 10 ng/μl in methanol. Working solution of EOZ was made at 0.02 μg/ml in methanol and stored at −20 °C. Working solutions (prepared each day) of MID, 1OHMID and 4OHMID were made at 1, 0.1, 0.01 and 0.001 ng/μl in methanol by dilution from the solution at 10 ng/μl. A calibration curve was prepared at 0.02, 0.05, 0.1, 0.5, 1, 2 and 5 ng/ml with blank plasma using the working solutions of 0.1 ng/μl (for the concentrations between 0.02 and 0.1 ng/ml), of 0.01 ng/μl (for the concentrations between 0.5 and 1 ng/ml) and of 0.1 ng/μl (for the concentrations between 2 and 5 ng/ml). Three control plasmas were prepared by diluting the working solution at 1 ng/μl with blank plasma to give a final concentration of 2 ng/ml or dilution of the working solution at 0.1 ng/μl to give the final concentrations of 0.5 and 0.1 ng/ml.

2.2. Instrumentation and chromatographic conditions

Analyses were performed on a Hewlett-Packard HP 6890 gas chromatograph equipped with a splitless capillary and an electronic control pressure system, and linked to a quadrupole HP 5973 mass spectrometer operated in negative chemical ionization mode (reagent gas: methane, purity 99.9995%), set to 40% of the total possible flow. The GC-MS capillary direct interface temperature was 280 °C. Splitless injections of 3 μl of ethyl acetate (see extraction condition) were made into a fused-silica OV1 capillary column (Macherey-Nagel, Oensingen, Switzerland), 25 m × 0.25 mm i.d., 0.25 μm film thickness, with helium (purity 99.999%, CarbaGas, Lausanne, Switzerland) as the carrier gas in constant flow mode (initial flow: 1.1 ml/min; nominal initial pressure: 8.6 psi; average velocity: 43 cm/s). The injection was made in pulsed splitless conditions; temperature: 300 °C; pressure: 8.7 psi; pulse pressure 40 psi; pulse time: 0.80 min; purge flow: 50 ml/min; purge time: 1 min, total flow: 54 ml/min, gas saver off. GC conditions were: equilibration time: 0.50 min, initial temperature 85 °C, initial time 1 min, heating rate 30 °C/min until 200 °C (final time: 0 min) and then 10 °C/min until 310 °C (final time: 1 min) and injector temperature 250 °C. Detections were performed in the selected-ion monitoring (SIM) mode for the singly charged negative ions at *m/z* 325.1 (MID), 455.2 (1OHMID), 323.0 (4OHMID), and 428.2 (EOZ), with a dwell time of 70 ms.

2.3. Extraction procedures

To a 10-ml Pyrex glass tube (tube A) which contains 1-ml (for blood samples collected after the oral administration of

75 μg of midazolam) volume of heparinized plasma sample, 50 μl of EOZ (IS) working solution, 100 μl of 0.1 M NaOH, and 1.4 ml of toluene-isoamylalcohol (99:1, v/v) were added. When blood samples are collected during a four hour period after the administration of therapeutic dose of midazolam (for example 7.5 mg), only 20 μl of plasma is used for extraction, diluted to 1 ml with blank plasma. Extraction was performed on a rotary shaker for 10 min. After centrifugation (8 min, 8 °C, 3300 g), the organic layer was transferred to a 1.5-ml flat bottomed glass injection vial (tube B) and evaporated to dryness under a stream of nitrogen at 40 °C. Another 1.4 ml of toluene-isoamylalcohol (99:1, v/v) were then added to tube A again. After shaking and centrifugation as above, the organic phase was transferred to the same injection vial as above-mentioned (tube B). After evaporation to dryness under nitrogen at 40 °C, 50 μl of *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide were added to the injection vial. The vials were immediately sealed with aluminum caps, thoroughly vortexed, and left for 30 min at 60 °C. The caps were then removed and the reagent was evaporated to dryness under a stream of nitrogen at 40 °C. The residues were then left at ambient temperature to cool down, reconstituted in 50 μl ethyl acetate and thoroughly vortexed. The resulting solutions were transferred to microtubes with insert and 3 μl of each sample was injected into the GC–MS system.

The use of total (free + conjugated) 1OHMID concentration for the 1OHMID/MID ratio is a better indicator of CYP3A activity than the use of free 1OHMID concentration because the enzymatic hydrolysis of the glucuronic conjugates allows the removal of the interindividual variabilities of glucuroconjugation [16]. Tests were thus carried out to obtain the best conditions for hydrolysis, by adapting a previously published method [17]. The crude

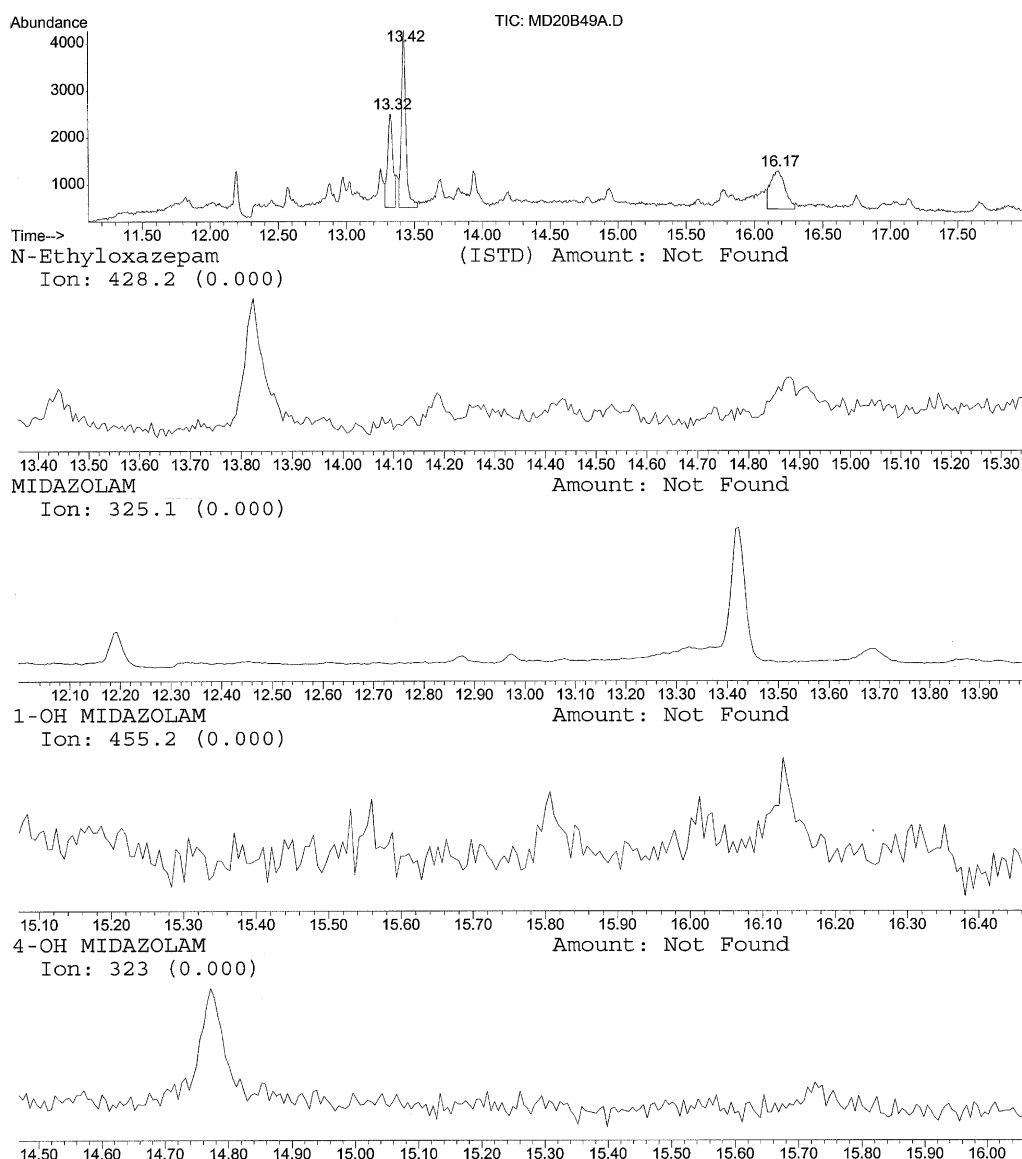

Fig. 2. SIM tracing of a 1-ml blank plasma.

β-glucuronidase was chosen over the pure β-glucuronidase for its more regular calibration curves and its more reproductive recovery (data not shown). Tests with different concentrations of β-glucuronidase over the range 0–6000 units showed a strong emulsion at and over 3000 units, which diminished the recovery of the internal standard (data not shown). Finally it was found that adding 1000 units to each ml of plasma gave a sufficient and reproductive incubation concentration (data not shown). Thus, for the determination of total (free + conjugated) 1OHMID and 4OHMID, 1 ml of plasma was incubated with 1 ml 0.1 M acetate buffer (pH 5.0; CH_3COOH 0.1 M adjusted to pH 5.0 with $CH_3COONa{\cdot}3H_2O$ 0.1 M), 50 μl of 4% sodium azide and 50 μl of a solution of β-glucuronidase at 20 000 units/ml at 37 °C overnight. After adding 300 μl of 0.1 N NaOH, the mixture was then treated as described above.

3. Results and discussion

We took advantage of the electron capturing ability of the halo substituted heteroaromatic substructure of midazolam to develop this very sensitive gas chromatography–negative ion chemical ionization method. A silylation reagent, which donates *tert*-butyldimethyl silyl moiety was used to derivatize the hydroxyl groups of 1OHMID, 4OHMID and EOZ. This procedure avoids possible spurious processes which may occur in the column of the gas chromatograph for the underivatized hydroxyl groups. SIM was used for the detection of the negative molecular ions $M^{-\bullet}$ of underivatized MID, of the *tert*-butyldimethylsilyl derivatives of 1OHMID and EOZ. In the case of the *tert*-butyldimethylsilyl derivatives of 4OHMID, the dominant peak of the mass spectrum corresponds to the loss

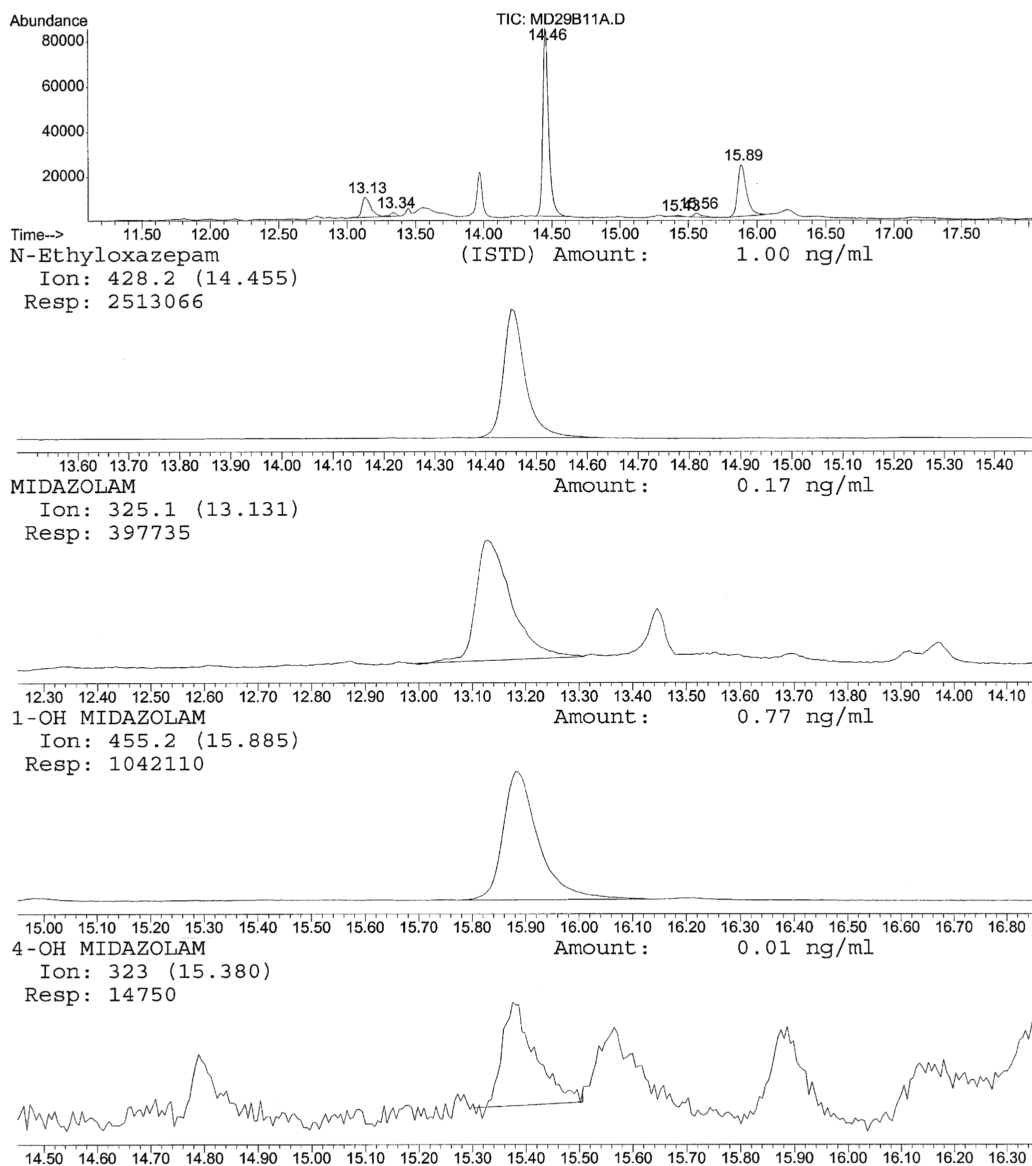

Fig. 3. SIM tracing of a 1-ml plasma from a volunteer receiving an oral dose of 75 μg of midazolam. Midazolam (ion 325.1, 13.13 min), 1-OH midazolam (ion 455.2, 15.89 min), 4-OH middazolam (ion 323.0, 15.38 min), *N*-ethyloxazepam (internal standard, ion 428.2, 14.46 min).

of a molecule of *tert*-butyldimethylsilyl alcohol from $M^{-\bullet}$ ions, this signal was thus used to monitor 4OHMID. The strong elimination of the *tert*-butyldimethylsilyl alcohol molecule from 4OHMID and not from 1OHMID can most probably be explained by the formation of a very stable ion in the former but not in the latter case. Indeed, the *tert*-butyldimethylsilyl group is on a saturated carbon for 1OHMID, while it is on a carbon of the heterocyclic skeleton for 4OHMID, the loss of the alcohol molecule leading to a completely delocalized (i.e. very stable) ion for 4OHMID. In all the cases, the signal associated with the major ^{35}Cl isotope has been retained in the SIM mode, corresponding to *m/z* values of 325.1, 455.2, 323.0 and 428.2 for MID, 1OHMID, 4OHMID and EOZ respectively.

Fig. 2 shows the SIM tracing of a blank plasma and Fig. 3 shows an example of chromatogram obtained from the analysis of a plasma sample drawn 30 min after the intake of an oral dose of 75 μg of midazolam in a healthy volunteer. The measured concentrations of MID and total (free + conjugated) 1OHMID were 170 and 770 pg/ml, respectively. Fig. 4 shows the concentration profile over time of MID and total 1OHMID after oral administration of 75 μg of midazolam in a subject. It should be mentioned that total 4OHMID can be measured in subjects receiving a therapeutic dose of midazolam, i.e. 7.5 mg, but cannot be detected in the majority of subjects receiving the very low dose, i.e. 75 μg. As MID and 1OHMID concentrations, but not 4OHMID concentrations, are needed for measuring CYP3A activity (determined by 1OHMID/MID ratio) [11,12,16,17], the fact that 4OHMID cannot be detected after the very low dose does not prevent its use for CYP3A phenotyping.

Table 1 shows a summary of the statistical data on the analysis of MID, 1OHMID, and 4OHMID. In summary, the coefficients of correlation of the calibration curves obtained from six separate experiments were between 0.996 and 1.0. As pure standards of the derivatized compounds, i.e. 1OHMID, 4OHMID and EOZ, are not available, recovery was calculated by dividing the mean areas ($n = 7$) obtained after the complete extraction and derivatization procedure of plasmas containing low (100 pg/ml), medium (500 pg/ml) and high (2 ng/ml) concentrations of these substances by the mean areas obtained after direct derivatization of the same quantities of the pure standards. The mean recoveries measured at three concentrations ranged from 76 to 87% for MID, from 76 to 99% for 1OHMID, from 68 to 84% for 4OHMID, and was of 82–109% for EOZ. The variability of the assays for the intra- ($n = 7$) and the interday experiments ($n = 8$), as assessed by the coefficients of variation (CV), measured at three concentrations for each substance, ranged from 1 to 8% for MID, from 2 to 13% for 1OHMID, and from 1 to 14% for 4OHMID. The percent theoretical concentrations, which represent the accuracy of the method, were all within ±8% for MID and 1OHMID, and within ±9% for 4OHMID at the concentrations of 500 pg/ml and 2 ng/ml. However, a larger (28%) percent theoretical concentration was calculated for 4OHMID at low concentrations, i.e. 100 pg/ml. It should be mentioned that the 1OHMID/MID ratio is used as an index of CYP3A4 activity and that this lower accuracy for the determination of 4OHMID at low concentration does not have any consequence for the phenotyping test. The limits of quantification, calculated with an extended calibration curve with a lowest concentration being of 5 pg/ml, and defined as the

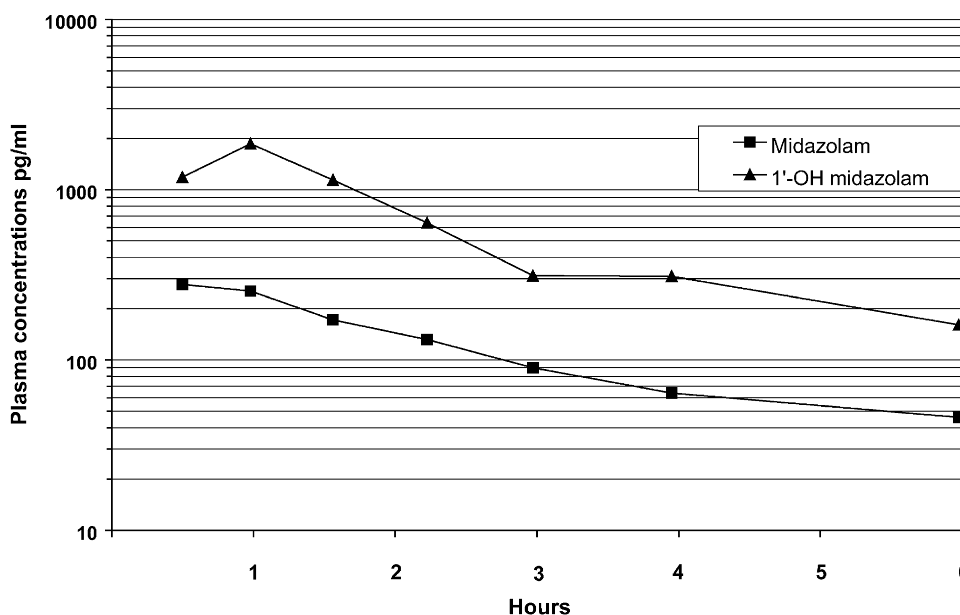

Fig. 4. Concentration profile of midazolam (■) and total (free + conjugated) 1-OH-midazolam (▲) after oral administration of 75 μg of midazolam in a healthy volunteer.

Table 1
Statistical data concerning the analysis of midazolam, 1′-OH midazolam and 4-OH midazolam

Parameter	Midazolam	1′-OH midazolam	4-OH midazolam
Calibration ($n = 6$)			
Range (ng/ml)	0.020–5.000	0.020–5.000	0.020–5.000
Slope, mean ± S.D. (CV)	0.65 ± 0.10 (15)	0.57 ± 0.10 (17)	0.52 ± 0.05 (10)
Coefficient of correlation, mean (minimal value)	0.999 (≥0.996)	0.999 (≥0.998)	1.0 (≥0.999)
Recovery ($n = 7$)			
Concentration used (ng/ml)	0.10	0.10	0.10
Recovery (in %), mean ± S.D. (CV)	84 ± 4 (5)	99 ± 8 (8)	84 ± 7 (8)
Concentration used (ng/ml)	0.50	0.50	0.50
Recovery (in %), mean ± S.D. (CV)	87 ± 7 (8)	90 ± 8 (9)	80 ± 4 (5)
Concentration used (ng/ml)	2.0	2.0	2.0
Recovery (in %), mean ± S.D. (CV)	76 ± 8 (11)	76 ± 9 (12)	68 ± 7 (10)
Intra-day variation ($n = 7$)			
Theoretical values (ng/ml)	0.10	0.10	0.10
Measured values (ng/ml), mean ± S.D. (CV)	0.104 ± 0.004 (3)	0.106 ± 0.004 (4)	0.128 ± 0.006 (5)
Percentage of theory	104	106	128
Theoretical values (ng/ml)	0.50	0.50	0.50
Measured values (ng/ml), mean ± S.D. (CV)	0.475 ± 0.012 (4)	0.484 ± 0.019 (4)	0.476 ± 0.011 (5)
Percentage of theory	95	97	95
Theoretical values (ng/ml)	2.0	2.0	2.0
Measured values (ng/ml), mean ± S.D. (CV)	1.859 ± 0.018 (1)	1.842 ± 0.044 (2)	2.147 ± 0.019 (1)
Percentage of theory	93	92	107
Inter-day variation ($n = 8$)			
Theoretical values (ng/ml)	0.10	0.10	0.10
Measured values (ng/ml), mean ± S.D. (CV)	0.103 ± 0.004 (4)	0.100 ± 0.013 (13)	0.102 ± 0.014 (14)
Percentage of theory	103	100	102
Theoretical values (ng/ml)	0.50	0.50	0.50
Measured values (ng/ml), mean ± S.D. (CV)	0.524 ± 0.029 (6)	0.524 ± 0.045 (9)	0.506 ± 0.055 (11)
Percentage of theory	105	105	101
Theoretical values (ng/ml)	2.0	2.0	2.0
Measured values (ng/ml), mean ± S.D. (CV)	1.991 ± 0.154 (8)	1.874 ± 0.235 (13)	1.927 ± 0.159 (8)
Percentage of theory	100	94	96
Limit of quantitation ($n = 8$)			
Theoretical values (pg/ml)	10.0	10.0	10.0
Measured values (pg/ml), mean ± S.D. (CV)	9.35 ± 1.07 (11)	11.35 ± 1.06 (9)	10.82 ±1.02 (9)
Percentage of theory	110	114	108

Standard deviation (S.D.), coefficient of variation (CV, in %).

concentrations for which the mean value of replicate determinations ($n = 8$) is within 20% of the actual value, the coefficient of variation less than 20%, and which gives a signal-to-noise ratio of at least 10, were found to be 10 pg/ml for the three substances. The limit of detection, defined as the concentration which gives a signal-to-noise ratio of at least 3 was found to be at least 5 pg/ml for all three substances.

The specificity of the assay was also evaluated: 200–500 ng of each of the following substances diluted in methanol were dried, derivatized, dried, reconstituted in 50 μl ethyl acetate, and injected into the GC–MS: methadone (500 ng), temazepam (500 ng), medazepam (500 ng), clovoxamine (200 ng), demoxepam (500 ng), tetrazepam (500 ng), rifampicine (200 ng) and ketoconazole (450 ng). No interferences were noted from these drugs. Equally, no interferences were observed from endogenous compounds following the extraction of plasma samples from 25 different human controls who were not receiving any medication. It should be emphasized that, due to the negative chemical ionization mode chosen, ions of high molecular weight or quasi molecular ions were produced, which minimizes potential interferences from other substances. Also EOZ, which is used as the internal standard, is not commercially available for prescription. The stability of MID and its metabolites were evaluated by analyzing spiked plasma samples stored at −20 °C for different periods of time. No loss was noted after storage of up to three months. Finally, the stability of the derivatized forms of 1OHMID, 4OHMID and EOZ was evaluated: No change was noted after storage of up to two days at room temperature (data not shown).

In summary, this selective and very sensitive method allows the quantification of MID and its metabolites in plasma following the administration of very low dose of midazolam. This assay was successfully used to validate the administration of 75 μg of midazolam as a CYP3A phenotyping test (Eap et al., manuscript submitted).

Acknowledgements

We gratefully acknowledge the editorial assistance of Mrs. V. Sari, and the bibliographic help of Mrs. J. Rosselet, Mrs. M. Gobin and Mrs. E. Ponce. We thank the firm Roche for providing us with MID and its metabolites. This work was supported in part by the Swiss National Research Foundation (project 3200-065427.01).

References

[1] S.A. Wrighton, K.E. Thummel, in: R.H. Levy, K.E. Thummel, W.F. Trager, P.D. Hansten, M. Eichelbaum (Eds.), Metabolic Drug Interactions, Lippincott Williams & Wilkins, Philadelphia, USA, 2000, p. 115 (Chapter 10).
[2] P.B. Watkins, Pharmacogenetics 4 (1994) 171.
[3] D.S. Streetman, J.S. Bertino Jr., A.N. Nafziger, Pharmacogenetics 10 (2000) 187.
[4] P. Heizmann, M. Eckert, W.H. Ziegler, Brit. J. Clin. Pharmacol. 16 (1983) 43S.
[5] J.T. Backman, K.T. Olkkola, P.J. Neuvonen, Clin. Pharmacol. Therapeut. 59 (1996) 7.
[6] S.N. de Wildt, G.L. Kearns, W.C. Hop, D.J. Murry, S.M. Abdel-Rahman, J.N. van den Anker, Clin. Pharmacol. Therapeut. 70 (2001) 525.
[7] J. Martens, P. Banditt, J. Chromatogr. B 692 (1997) 95.
[8] K.E. Thummel, D.D. Shen, T.D. Podoll, K.L. Kunze, W.F. Trager, P.S. Hartwell, V.A. Raisys, C.L. Marsh, J.P. McVicar, D.M. Barr, J.D. Perkins, R.L. Carithers Jr., J. Pharmacol. Exp. Therapeut. 271 (1994) 549.
[9] M.F. Paine, D.D. Shen, K.L. Kunze, J.D. Perkins, C.L. Marsh, J.P. McVicar, D.M. Barr, B.S. Gillies, K.E. Thummel, Clin. Pharmacol. Therapeut. 60 (1996) 14.
[10] M.D. Reed, A. Rodarte, J.L. Blumer, K.C. Khoo, B. Akbari, S. Pou, G.L. Kearns, J. Clin. Pharmacol. 41 (2001) 1359.
[11] B. Zhu, D.S. Ou-Yang, Z.N. Cheng, S.L. Huang, H.H. Zhou, Acta Pharmacol. Sin. 22 (2001) 634.
[12] J.A. Carrillo, S.I. Ramos, J.A.G. Agúndez, C. Martinez, J. Benitez, Therapeut. Drug Monitor. 20 (1998) 319.
[13] P. Marquet, O. Baudin, J.M. Gaulier, E. Lacassie, J.L. Dupuy, B. Francois, G. Lachâtre, J. Chromatogr. B 734 (1999) 137.
[14] A.D.M. Kashuba, J.S. Bertino, M.L. Rocci, R.W. Kulawy, D.J. Beck, A.N. Nafziger, Clin. Pharmacol. Therapeut. 64 (1998) 269.
[15] J. McCrea, T. Prueksaritanont, B.J. Gertz, A. Carides, L. Gillen, S. Antonello, M.J. Brucker, C. Miller-Stein, B. Osborne, S. Waldman, J. Clin. Pharmacol. 39 (1999) 1212.
[16] K.E. Thummel, D.D. Shen, T.D. Podoll, K.L. Kunze, W.F. Trager, C.E. Bacchi, C.L. Marsh, J.P. Vicar, C.L. Barr, J.D. Perkins, J. Pharmacol. Exp. Therapeut. 271 (1994) 557.
[17] J.P. Villeneuve, L.L. Ecuyer, S. De Maeght, P. Bannon, Clin. Pharmacol. Therapeut. 67 (2000) 242.