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Simultaneous determination of midazolam and its metabolites 1-hydroxymidazolam and 4-hydroxymidazolam in human serum using gas chromatography–mass spectrometry

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Abstract

A method for the quantitation of midazolam and its metabolites 1-hydroxymidazolam and 4-hydroxymidazolam from human serum capable of monitoring concentrations achieved under therapeutic conditions is presented. The substances were extracted under basic conditions with toluene and the hydroxy metabolites transformed to their *tert*-butyldimethylsilyl derivatives with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide. The samples were measured by gas chromatography–mass spectrometry. The limits of detection are 0.2 ng ml^{-1} for midazolam and 0.1 ng ml^{-1} for 1-hydroxy- and 4-hydroxymidazolam. The coefficients of variation are 3.9% at 5 ng ml^{-1} for midazolam, 6.7% at 2 ng ml^{-1} for 1-hydroxymidazolam and 8.8% (22.2%) at 0.5 (0.2) ng ml^{-1} for 4-hydroxymidazolam.

Keywords: Midazolam; Hydroxymidazolam

1. Introduction

Midazolam is a short acting benzodiazepine which is administered parenterally to induce anaesthesia and orally to induce sleepiness and relief of apprehension prior to surgeries. The drug is metabolized extensively in the liver by the isoenzymes cytochrome P450 3A3, P450 3A4 and P450 3A5 to the 1-hydroxy- and 4-hydroxy analogues [1] (Fig. 1). Therefore, midazolam can also serve as a probe for the inter- and intraindividual variability of cytochrome P450 3A activity [2]. Therefore, it is desirable to measure the two metabolites as well as the parent drug in human serum.

Several chromatographic methods have been reported for the analysis of midazolam and its 1-hydroxy metabolite in human plasma using HPLC [3–5] or GC [6–9]. Methods for the additional determination of the 4-hydroxy metabolite by HPLC [10–14] and GC [15–17] have also been reported. In none of these methods is the limit of determination for 4-hydroxymidazolam lower than 1 ng ml^{-1} (achieved in Ref. [15]). This is not sufficient to quantitate this compound in serum samples from patients receiving midazolam in therapeutic oral doses.

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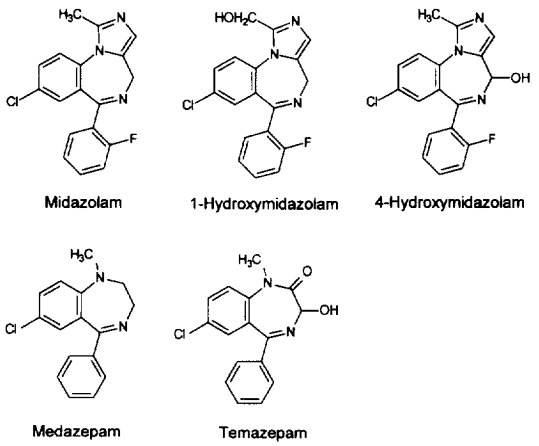

Fig. 1. Chemical structures of midazolam, its metabolites and the substances used as I.S.

The method presented here is very sensitive and specific. It can simultaneously determine human serum concentrations of midazolam and its 1-hydroxy and 4-hydroxy metabolites in therapeutic ranges.

2. Experimental

2.1. Apparatus

A gas chromatograph (HP 5890 Series II plus) with electronic pressure programmer, automatic sampler (HP 7673), split–splitless injector and a mass selective detector (HP 5972) was used. Data were collected and analyzed using a Hewlett-Packard DOS ChemStation version C.01.05 (all Hewlett-Packard, Waldbronn, Germany). The analytes were separated on a RTX-1 capillary column with dimensions 15 m×0.25 mm I.D. and d_f 0.5 μm (Restek, Bad Soden, Germany).

2.2. Chemicals

Midazolam, 1-hydroxymidazolam and 4-hydroxymidazolam were kind gifts from Hoffmann-La Roche (Basel, Switzerland), Temazepam was purchased from Sigma (Deisenhofen, Germany), Medazepam was obtained from Arzneimittelwerk Dresden (Radebeul, Germany). *N*-(*tert*-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylsilylchloride (TBDMSTFA) was purchased from Aldrich (Steinheim, Germany). Toluene, methanol and NaOH in grade ACS (quality norm of the American Chemical Society) and amyl alcohol in quality grade 'zur Analyse' were purchased from Merck (Darmstadt, Germany), ethyl acetate in reagent grade was obtained from Baker (Gross-Gerau, Germany). Helium, purity >99.9996, was purchased from Messer-Griessheim (Magdeburg, Germany).

2.3. Sample collection

The blood samples (about 10 ml) were collected into polypropylene tubes. To separate serum from blood cells, the samples were centrifuged for 10 min at 2400 g. After centrifugation, the serum samples were frozen at −20°C until analysis.

2.4. Sample preparation

To 1 ml of serum, 50 μl of the I.S. solution (medazepam and temazepam, each 10 μg ml^{-1} in methanol) and 100 μl of 0.1 *M* NaOH were added. The sample was extracted twice with a mixture of 2 ml toluene and 20 μl amyl alcohol. After phase separation, the organic phase was evaporated, and the residue was derivatized with 50 μl TBDMSTFA for 30 min at 60°C in a tightly closed vessel. After this, the excess of the TBDMSTFA was evaporated in vacuum, the residue was reconstituted in 50 μl ethyl acetate, transferred into autosampler vials and forwarded to the GC–MS analysis.

2.5. Calibration samples

An initial solution for calibration was made by dissolving 25 mg midazolam, 10 mg 1-hydroxymidazolam and 1 mg 4-hydroxymidazolam in 100 ml methanol. This solution was diluted by a factor of 100 with methanol. Drug-free human serum was spiked with 1–50 μl of this solution to yield calibration samples in the concentration range of 2.5–125 ng ml^{-1} midazolam, 1–50 ng ml^{-1} 1-hydroxymidazolam and 0.1–5 ng ml^{-1} 4-hydroxymidazolam.

Table 1
Retention times and selected ions

Compound	t_r (min)	Selected ions (m/z)	Time window (min)
Medazepam	8.7	242,270	7.0–10.0
Midazolam	11.1	310,325	10.0–12.0
Temazepam[a]	12.6	283,357	12.0–13.0
4-Hydroxymidazolam [a]	13.4	398,400,440	13.0–14.5
1-Hydroxymidazolam [a]	13.9	398,400,440	13.0–14.5

[a] TBDMSTFA derivative.

2.6. Chromatographic conditions

The carrier gas for the chromatographic separation was helium at a flow-rate of 1.2 ml min^{-1}. This flow-rate was held constant over the run time. The temperature program started at 85°C, was held constant for 1 min, then the temperature was raised by 30°C min^{-1} up to 200°C, then by 10°C min^{-1} up to 310°C and hold constant for 2 min. The total run time was 17.8 min. The injector and MS interface temperatures were 300°C. A 2 μl volume was injected in the splitless mode of the injector. The splitless time was 1 min, then a splitflow of 20 ml min^{-1} was applied. The retention times achieved under these conditions are listed in Table 1.

The mass spectrometric detector was working in the selected ion monitoring mode and the ions were formed by electron impact ionization with an energy of 70 eV. The relative masses of the observed ions and the time windows related to them are summarized in Table 1.

3. Results and discussion

3.1. Chromatography and mass spectrometry

The two metabolites of midazolam, 1-hydroxymidazolam and 4-hydroxymidazolam, are quite polar and can not be transferred from a split–splitless injection port onto the analytical column with satisfying results. The required very high sensitivity can only be achieved by means of a derivatization procedure. Temazepam (Fig. 1) is used as the I.S. for the two hydroxy metabolites, because each compound carries one hydroxy moiety, which reacts with TBDMSTFA to yield the *tert*-butyldimethylsilyl derivatives. Medazepam (Fig. 1) works as I.S. for midazolam, since both substances do not react under the described conditions with TBDMSTFA and remain unchanged.

We chose TBDMSTFA as the derivatizing reagent for the I.S. temazepam and the two hydroxy metabolites, as the resulting *tert*-butyldimethylsilyl derivatives exhibit mass spectra with an easy fragmentation pattern and high mass base peaks, which facilitates the high-sensitivity detection. In all three compounds, the base peak in the mass spectrum corresponds to the loss of a *tert*-butyl radical, which results in the peak at 398 m/z for the two hydroxy metabolites and 357 m/z in the case of temazepam. The fragment ions 400 m/z and 440 m/z, which are monitored in the time window of the hydroxy metabolites, too, serve as qualifiers for the peak identification. The mass spectra of medazepam and midazolam correspond to those already published by Pfleger et al. [18].

Fig. 2 shows chromatograms obtained under the described conditions, the upper one from human serum spiked with the lowest calibration concentrations, the lower one from drug-free human serum. All substances exhibit sharp and symmetrical peaks and do not interfere with endogenous substances. In the case of 4-hydroxymidazolam the detector's sensitivity limit is nearly reached at this concentration level (0.1 ng ml^{-1}), but detection is still possible.

Fig. 3 shows concentration–time curves of midazolam and its metabolites from a healthy volunteer receiving a single oral dose of 15 mg midazolam. The sensitivity of the assay described here is sufficient to monitor midazolam and its metabolites over a 9 h period after dosing.

3.2. Extraction efficiency

The recovery of midazolam and its two hydroxy metabolites is determined by comparing peak areas in chromatograms of extracted samples with those of pure solutions at concentrations corresponding to 100% recovery. A trial with 10 sample pairs results in an extraction yield of 83% for midazolam, 92% for 1-hydroxymidazolam and 96% for 4-hydroxymidazolam.

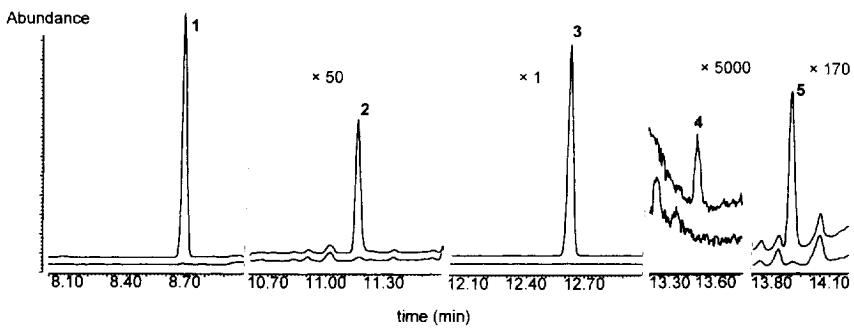

Fig. 2. Chromatograms obtained from drug-free human serum and human serum spiked with: (1) 500 ng ml^{-1} medazepam, (2) 2.5 ng ml^{-1} midazolam, (3) 500 ng ml^{-1} temazepam, (4) 0.1 ng ml^{-1} 4-hydroxymidazolam, and (5) 1 ng ml^{-1} 1-hydroxymidazolam. The abundance units are arbitrary with different scaling factors for each part of the chromatograms. The ion traces depicted are: (1) 242 *m/z*, (2) 310 *m/z*, (3) 357 *m/z*, (4) 440 *m/z*, and (5) 398 *m/z*.

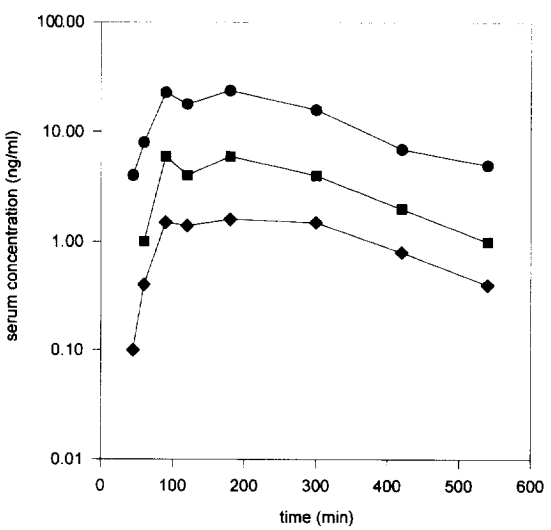

Fig. 3. Concentration–time curves of midazolam (●), 1-hydroxymidazolam (■) and 4-hydroxymidazolam (♦) after a single oral dose of 15 mg midazolam in a healthy volunteer.

3.3. Quantitation and precision

The parameters of the calibration functions of midazolam, 1-hydroxymidazolam and 4-hydroxymidazolam are summarized in Table 2.

The limit of detection, which is defined here as $S/N>3$, is 0.2 ng ml^{-1} for midazolam and 0.1 ng ml^{-1} for 1-hydroxymidazolam as well as for 4-hydroxymidazolam. The limit of quantitation is defined here as three times the limit of detection. The values are 0.6 ng ml^{-1} for midazolam and 0.3 ng ml^{-1} for the two hydroxy metabolites.

Table 3 contains the data of the intra-day precision trial and Table 4 contains those of the inter-day precision trial. The 0.2 ng ml^{-1} concentration level for 4-hydroxymidazolam is beneath the limit of quantitation, which results in a quite high R.S.D. of 22.2% in the intra-day precision and of 25.5% in the inter-day precision.

Table 2
Parameters of the calibration functions

Compound	Range (ng ml^{-1})	n	Slope (±S.D.)	Intercept (±S.D.)	r
Midazolam	2.5–125.0	7	2.86E−3 ±1.83E−5	1.47E−3 ±9.87E−4	0.9999
1-Hydroxymidazolam	1.0–50.0	7	3.65E−3 ±2.71E−5	8.48E−4 ±5.87E−4	0.9998
4-Hydroxymidazolam	0.1–5.0	7	1.16E−3 ±2.77E−5	8.48E−5 ±5.99E−5	0.9983

Table 3
Intra-day precision and accuracy

Compound	Concentration (ng ml^{-1})	n	R.S.D.	Accuracy
Midazolam	100.0	10	2.7%	−1.23%
	5.0	10	3.9%	−1.88%
1-Hydroxymidazolam	40.0	10	3.5%	1.53%
	2.0	10	6.7%	−5.95%
4-Hydroxymidazolam	4.0	10	4.2%	−1.65%
	0.5	9	8.8%	7.40%
	0.2	10	22.2%	−8.00%

Table 4
Inter-day precision and accuracy

Compound	Concentration (ng ml^{-1})	n	R.S.D.	Accuracy
Midazolam	125	8	1.3%	−0.2%
	5	8	9.1%	5.3%
1-Hydroxymidazolam	50	8	2.4%	−0.8%
	2	8	9.5%	−1.6%
4-Hydroxymidazolam	5	8	3.1%	2.0%
	0.4	8	10.2%	14.3%
	0.2	8	25.5%	14.7%

4. Conclusions

The method described here is sufficiently sensitive to determine besides midazolam and its main metabolite 1-hydroxymidazolam its minor metabolite 4-hydroxymidazolam in human serum at concentration levels achieved under therapeutic conditions. A typical serum concentration–time curve of midazolam and its two metabolites after a single oral dose of 15 mg midazolam is depicted in Fig. 3. As can be seen, even 9 h after dosing the quantitation of midazolam and its metabolites is possible.

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