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Short communication

Simultaneous determination of fentanyl and midazolam using high-performance liquid chromatography with ultraviolet detection

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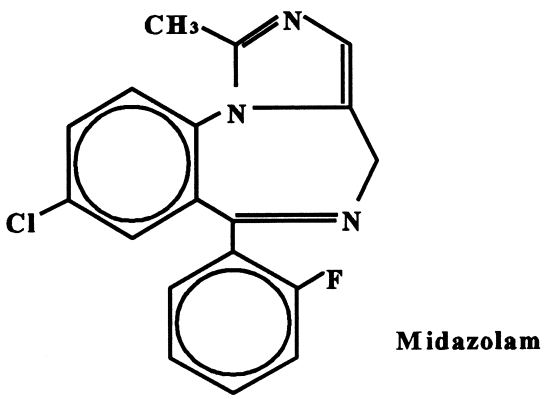
Abstract

When measuring fentanyl and midazolam simultaneously in the same plasma sample with standard high-performance liquid chromatography–ultraviolet (HPLC–UV) detection, overlap of the fentanyl peak by the midazolam peak occurs, which makes fentanyl determination impossible. We tested the hypothesis that by acidifying the methanol mobile phase with 0.02% perchloric acid, 70%, it would be possible to separate both peaks. The UV detector was set at 200 nm. Calibration curves for fentanyl (range 0–2000 pg/ml) and midazolam (range 0–400 ng/ml) were linear ($r>0.99$). The detection limits were 200 pg/ml (fentanyl) and 10 ng/ml (midazolam). Precision and accuracy for intra- and inter-assay variability as well as in-line validation with quality control samples (QCS) were acceptable (< 15 and 20%, respectively), except for fentanyl QCS of 200 pg/ml (17.8% precision). Although less sensitive than gas chromatography–mass spectrometry (GC–MS), reliable measurements of fentanyl, simultaneously with midazolam, can be performed with this HPLC–UV system.

Keywords: Fentanyl; Midazolam

1. Introduction

Administration of a combination of benzodiazepines and opiates has proven to be successful when sedation and analgesia are required during surgery or to support patients undergoing mechanical ventilation in the intensive care unit. In many hospitals, midazolam (Fig. 1) is the benzodiazepine of choice because of its rapid onset of sedation and short elimination half-life. The combination of midazolam with fentanyl (Fig. 2), an opiate with an intrinsic analgesic potency that is 100 times higher than that



Midazolam

Fig. 1. Structure of midazolam.

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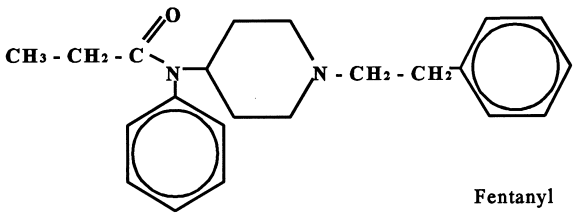

Fig. 2. Structure of fentanyl.

of morphine, induces an additional sedative effect and reliable analgesia [1–3]. The need to measure plasma concentrations of both drugs depends on the clinical setting. In the case of an intensive care patient with a persisting depressed mental state for several days after stopping midazolam and fentanyl infusion, the question will be raised of whether this is caused by persisting drug action or by neurological damage. Plasma concentration measurements of the drugs and major active metabolites can be of help in giving the correct clinical diagnosis. There is also a need for information on drug concentrations when both drugs are administered in experimental settings.

Reliable methods exist for measuring fentanyl and midazolam when they are given separately [4–8]. However, if both drugs are administered simultaneously, measuring them separately requires twice the volume of blood and twice the analysis time and equipment. We therefore wanted to measure both drugs in the same sample in a single high-performance liquid chromatography (HPLC) system using UV detection. Measurement of midazolam plasma concentrations did not present problems because its values were relatively high compared to the 100-times-lower fentanyl concentrations. However, determination of the fentanyl peak was not possible because only poor separation from the midazolam peak was obtained. Therefore, another mobile phase was looked for in order to improve the separation. We postulated that an acidic eluent could better separate the midazolam and fentanyl peaks. Flanagan and Jane [9] described the influence of variation of the eluent-pH on the retention time of basic and acidic drugs. Low pH values appeared to decrease the retention time of basic drugs, while an increase in retention time occurred for acidic drugs. These differential effects on retention time could possibly be explained by differences in protonation, as the pK_a value in water of midazolam is 6.2 and of fentanyl is 8.4. Accordingly, by adding an acid to the eluent, it was expected that the retention time of fentanyl would be decreased because of increased protonation, and that there would be an increase in the retention time of midazolam, caused by decreased protonation.

2. Experimental

Stock solutions containing 10 μg/ml of fentanyl (0.05 mg/ml, Hospital Pharmacy, Academic Medical Center) and midazolam (1 mg/ml; Roche, Basel, Switzerland), alfentanyl (0.5 mg/ml, Janssen Pharmaceutica, Tilburg, The Netherlands) and flurazepam were prepared in methanol (LiChrosolv grade 6007 from Merck, Darmstadt, Germany) and stored at −20°C. Fentanyl, midazolam, alfentanyl and flurazepam appeared to remain stable under these conditions for at least two months. Working solutions containing 5 ng/ml of fentanyl and of alfentanyl were prepared by a further 1:1 dilution of the stock solution in water. For midazolam and flurazepam, working solution concentrations of 5 μg/ml were used. The mobile phase was a mixture of methanol and 0.02% perchloric acid, 70%.

The chromatographic system consisted of a Spectraflow 400 pump (Applied Biosystems, Rotterdam, The Netherlands), a Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, USA) with a Rheodyne 100 μl sample loop (Chrompack, Bergen op Zoom, The Netherlands), and a Spherisorb silica analytical column (5 μm, 250×4.6 mm; Hewlett-Packard, Amstelveen, The Netherlands). The detector was an UV detector with variable wavelength (Spectroflow 757 A, Applied Biosystems Separations, H.I. Ambacht, The Netherlands). Chromatograms were recorded on a Kipp & Zonen BD40 recorder (Delft, The Netherlands) and with a Datajet integrator (Spectra Physics, H.I. Ambacht, The Netherlands).

To 1 ml of plasma, 100 μl of the internal standard, flurazepam, and 200 μl of 0.5 *M* KOH were added. After the samples had been vortex-mixed for 30 s, 3 ml of cyclohexane were added. The sample was again vortex-mixed for 2 min and finally centrifuged for 10 min (3000 *g*). A 2-ml volume of the organic layer was transferred to a clean tube and evaporated to dryness under a continues nitrogen stream in a

waterbath at 40°C. The samples were redissolved in 350 μl of mobile phase and centrifuged for 3 min, and 100 μl was injected onto the analytical column. The flow-rate of the mobile phase was 1 ml/min. The sensitivity of the UV detector was set at 0.02 AU, λ=200 nm, with a time constant of 2 s.

For calibration curves, samples with concentrations in the 0–2000 pg/ml range for fentanyl and 0–400 ng/ml range for midazolam were prepared by adding 4–400 μl of fentanyl standard solution and 4–40 μl of midazolam standard solution, together with 50 μl of internal standard solution to 1 ml blank human plasma samples.

3. Validation results

In Fig. 3, representative chromatograms of a blank sample and of simultaneously analysed fentanyl (500 pg), alfentanyl (50 ng), midazolam (100 ng) and flurazepam (100 ng) samples, respectively, are depicted. Good separation was obtained as the retention times of fentanyl, alfentanyl, midazolam and flurazepam were 3.90, 4.53, 6.22 and 8.64 min, respectively. No interfering peaks were encountered in the blank samples.

3.1. Recovery

By comparing peak heights for fentanyl, midazolam, alfentanyl and flurazepam, the two internal standards, in plasma samples spiked with known concentrations, with the peak heights obtained by injecting a stock solution with the same concentrations, we obtained almost 100% recovery for the four compounds.

3.2. Calibration

The calibration curves for fentanyl were obtained by spiking drug-free plasma samples with a standard fentanyl solution to produce concentrations of 0, 200, 500, 1000 and 2000 pg/ml. For midazolam, the concentrations were 0, 50, 100, 200 and 400 ng/ml. Peak heights were plotted against the corresponding drug concentrations after the extraction and assay procedures. Linear regression analysis of the calibration plots resulted in the following equations

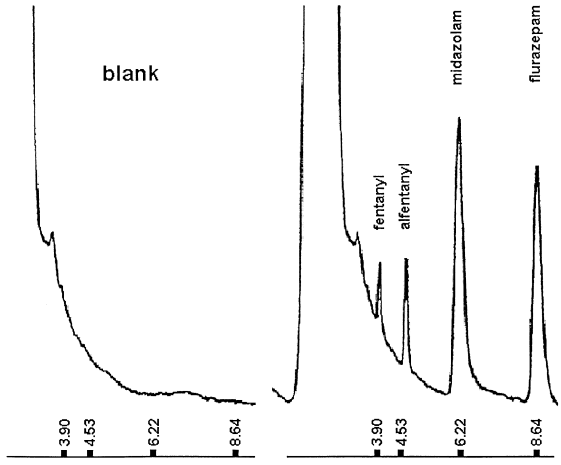

Fig. 3. HPLC–UV chromatogram of fentanyl, midazolam, flurazepam and alfentanyl.

(mean, n=6): y=0.9509x−0.01199 for fentanyl, and y=0.00399 x+0.0303 for midazolam, y representing the peak height ratio of the drug concentration to the internal standard and x the concentration of the samples. Assay linearity in the range of the fentanyl and midazolam concentrations to be expected was concluded from mean correlation coefficients of r= 0.996 for fentanyl and r=0.996 for midazolam.

Samples from patients that were found to be outside the range of concentrations used for the calibration curves were diluted with blank serum and processed again.

3.3. Assay precision and accuracy

3.3.1. Intra-assay precision and accuracy

In order to determine the intra-assay precision and accuracy, a series of four fentanyl plasma concentrations, ranging from 200 to 2000 pg/ml, was tested six times on the same day (within-day variability). In addition, four concentrations of midazolam, ranging from 50 to 400 ng/ml, were tested six times. As shown in Table 1. the deviations for all concentrations of both drugs was never higher than 10.1%.

3.3.2. Inter-assay precision and accuracy

The between-day precision and accuracy was determined by measuring the same four plasma concentrations of fentanyl and midazolam as shown in Table 1 on 6–14 different days. From Table 2, it

Table 1
Intra-assay variability of fentanyl (F) and midazolam (M)

Concentration (ng/ml)	Concentration found (ng/ml) (mean ±SD, n=6)	Precision (% deviation)	Accuracy (% deviation)
Fentanyl			
0.2	0.22±0.02	7.3	9.2
0.5	0.51±0.05	10.1	2.0
1.0	0.98±0.06	5.9	−1.8
2.0	2.01±0.04	1.9	0.3
Midazolam			
50	48.7±4.1	8.4	−2.6
100	103.5±3.2	3.1	3.5
200	208.6±11.2	5.4	4.3
400	394.9±5.8	1.5	−1.3

appears that both precision and accuracy were within acceptable limits (<15 and <20%, respectively).

3.4. Quality-control samples

For within-study validation we measured freshly prepared spiked QCS (9–18 times per concentration) with fentanyl concentrations of 200, 500, 1000 and 2500 pg/ml. For midazolam the QCS contained concentrations of 50, 100, 200 and 400 ng/ml and these were measured 12 times per concentration. The stock solutions for these samples were different than those that were used for the calibration curves. During the study period, all samples were stored under the same conditions. The results for both drugs are shown in Table 3. The precision of 17.8% for the lowest fentanyl concentration of 200 pg/ml was just above the acceptable level of 10–15%, however, the accuracy was good. For midazolam, all tested concentrations were measured with acceptable precision and accuracy.

4. Limits of quantitation

When the limit of quantitation is defined by a criterion for precision and for accuracy of less then 15% deviation from the mean, then, in our assay, the

Table 2
Inter-assay variability of fentanyl (F) and midazolam (M)

Concentration (ng/ml)	Concentration found (mean±SD) (ng/ml)	*n*	Precision (% deviation)	Accuracy (% deviation)
Fentanyl				
0.2	0.22±0.02	9	9.0	10
0.5	0.47±0.05	6	10.8	−5.3
1.0	1.01±0.02	6	2.2	0.8
2.0	2.00±0.04	6	2.1	0.1
Midazolam				
10	9.99±1.3	14	13.4	−0.13
50	48.9±4.3	6	8.8	−2.1
100	104.3±5.5	6	5.2	4.3
200	200.9±6.5	6	3.2	0.5
400	396.4±2.0	6	0.5	−0.9

Table 3
In-line validation with quality control samples (QCS) of fentanyl and midazolam

Concentration added (ng/ml)	(Mean±SD) (ng/ml)	*n*	Precision (% deviation)	Accuracy (% deviation)
Fentanyl				
0.2	0.21±0.04	18	17.8	4.5
0.5	0.47±0.03	18	5.8	−6.7
1.0	1.03±0.05	9	4.7	3.3
2.5	2.43±0.17	9	7.1	−2.8
Midazolam				
50	52.5±7.2	12	13.8	5.1
100	107.9±10.0	12	9.2	7.9
200	205.9±9.1	12	4.4	3.0
400	396.3±4.5	12	1.1	−0.9

limits of quantitation for fentanyl and for midazolam were 200 pg/ml and 10 ng/ml, respectively (see Table 2).

5. Discussion

When we used our standard method for midazolam [6] with HPLC–UV, it appeared to be impossible to detect fentanyl, due to overlap of both peaks. In this assay, the eluent contains almost 100% methanol. This means that the pH and pK_a values of the solutions and drugs, which are normally measured in water, are not applicable. We decided to add 0.02% perchloric acid 70% to the methanol eluent in order to create acidity. This appeared to have a marked influence on the retention time of both midazolam and fentanyl and now both peaks could be detected in the same chromatogram.

The exact reason for this shift in retention times is not fully understood. Although decreased protonation of acidic drugs by adding acid to the solution will cause more attachment on the HPLC column and, therefore, an increase in retention time, however, it has been suggested that other processes could play a role. Creating an acidic, neutral or basic eluent also induces variations in ionisation of the silica surface of the column, which consists of siloxane and silanol moieties. For emepromium (a quaternary ammonium compound), it was shown that the increase in retention time with increasing pH was similar to the ionisation profile of the silica silanols. This indicates a possible pH-dependent effect on retention time based on both changed ionisation of the drug as well as the column surface [9].

We are aware of the fact that our assay for fentanyl is less sensitive than that using gas chromatography–mass spectrometry (GC–MS) [4]. However, GC–MS for quantification of fentanyl is far more expensive than an HPLC–UV method.

It should be noted that the columns used in our study (as well as many other columns) are sensitive to low pH values. We therefore had to change the column frequently.

In our clinical pharmacological experiments with fentanyl and midazolam infusions, we measured fentanyl concentrations between 200 and 4900 pg/ml and midazolam concentrations between 10 and 1480 ng/ml and the above-described method for the simultaneous quantification of fentanyl and midazolam appeared to be suitable for the determination of the pharmacokinetic parameters of both drugs.

We conclude that, although many different HPLC assays for midazolam can be found in the literature, and fentanyl measurements can best be performed using GC–MS, thusfar no method for the simultaneous quantification of both drugs has been described. Simultaneous measurement of two drugs in the same system reduces costs and time. During the development of a modified HPLC method with UV detection, we found an adequate solution, based on acidification of the eluent, for overlapping retentions of midazolam and fentanyl. Using this assay, we

were able to quantify both drugs with good precision and accuracy and with sufficient sensitivity for pharmacokinetic studies in man.

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