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Short communication

Gas chromatographic determination of midazolam in low-volume plasma samples

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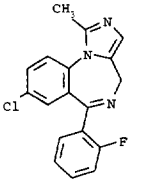
Abstract

A gas chromatographic method for the sensitive determination of midazolam in plasma volumes as low as 40 μl was developed, utilizing clinazolam as the internal standard. After liquid–liquid extraction at basic pH into 1-chlorobutane–dichloromethane (96:4) a 2- to 4-μl portion of the reconstituted extract was injected under electronic pressure control onto a 12 m×0.2 mm I.D. methyl silicone capillary column, and was exposed to a three-step temperature program from 120 to 310°C, to separate the analytes from the plasma constituents. The compound of interest was identified and quantified by means of a mass-selective detector. The assay was linear from 10 to 500 ng/ml using 40 μl of plasma (limit of quantification: 10 ng/ml) and was linear from 0.25 to 100 ng/ml using 500 μl of plasma (limit of quantification: 0.25 ng/ml). The intra-day precision for the 40-μl aliquots varied from 2.2 to 6.6%, the corresponding accuracy from −7.4 to −4.4%; the inter-day precision ranged from 5 to 7.2% and the corresponding accuracy from −7.2 to −5.1%.

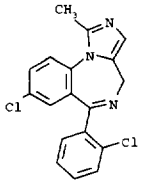
Keywords: Midazolam

1. Introduction

Midazolam, a triazolo-1,4-benzodiazepine (Fig. 1), is used therapeutically as a short-term hypnotic and for premedication in surgical anaesthesia. After single-dose application, complete concentration–time profiles from individual rats had to be measured to establish a pharmacokinetic model for midazolam. In the literature, quite a number of midazolam assays were reported (for examples, see Refs. [1–11]). Most of these methods used plasma volumes of 0.2 to 1 ml, and were labor-intensive and time-consuming. Therefore, they were inadequate for concentration–time profile monitoring in rats and it was necessary



Ro 21-3981, midazolam



Ro 21-3982, clinazolam, internal standard

Fig. 1. Structures of compounds used.

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to develop an assay capable of determining the compound in very low plasma volumes, while maintaining sufficient sensitivity, precision, accuracy and selectivity.

Since the volumes of rat plasma available were very limited, the assay was developed utilizing equivalent volumes of human plasma for standard preparation, and only a small number of rat plasma reference standards. Therefore, the assay validation was carried out in parallel for concentration ranges required for plasma level determinations in man (low doses) and rats (small volumes).

The presented gas chromatographic (GC) assay with mass-selective (MS) detection requires only a short sample preparation time, allows a high sample throughput, and the on-line analysis of about 70 samples per man-day.

2. Experimental

2.1. Materials

The free bases of midazolam and clinazolam were available in-house. All solvents and the trisodium phosphate were of HPLC or analytical reagent grade.

2.2. Preparation of standard solutions

For preparation of calibration standards, a stock solution was prepared by dissolving 10 mg of midazolam in 10 ml of ethanol. The working solution, I, for the midazolam standards was prepared by dilution of the stock solution with ethanol to give concentrations of 20 ng/μl. A set of working solutions prepared from I in ethanol allowed the spiking of interference-free human plasma to give calibration standards with concentrations of (a) 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml, using 0.5 ml of plasma, and (b) 10.0, 25.0, 50.0, 100.0, 250.0 and 500.0 ng/ml, using 40 μl of plasma.

For preparation of quality control (QC) standards, ethanolic working solutions were used to spike interference-free human plasma to give QC standards at concentration levels of (a) 1.0, 5.0 and 25.0 ng/ml, using 0.5 ml of plasma, and (b) 25.0, 100.0 and 250.0 ng/ml, using 40 μl of plasma.

To correlate the 40-μl assay to low-volume rat plasma samples, a blank plasma sample and a QC at a concentration level of 100.0 ng/ml were prepared from rat plasma in parallel with the human plasma-based standards and its concentration was determined throughout the study.

The internal standard, clinazolam (Fig. 1), was prepared with stock and working solutions in ethanol resulting in concentrations of (a) 25.0 ng/ml for the 0.5-ml plasma samples and standards and (b) 100.0 ng/ml for the 40-μl plasma samples and standards.

2.3. Extraction

Frozen plasma samples were thawed, vortex-mixed briefly and 500- and 40-μl aliquots transferred to disposable extraction tubes. A 200-μl volume of HPLC grade water was added to the 40 μl aliquots and the solutions were vortex-mixed again. A 50-μl volume of internal standard solution and 50 μl of concentrated trisodium phosphate solution (pH 12.5) were added to aliquots and standards. After addition of each of the liquids, the solutions were homogenized. For extraction, 3 ml of 4% dichloromethane in 1-chlorobutane were added, and the tubes vortex-mixed or mixed on the rotation shaker for 5 or 15 min, respectively. A centrifugation step at 10°C and 2500 *g* for 10 min followed subsequently. The organic layer was transferred to deactivated, conical tubes and was evaporated under a gentle stream of nitrogen at 50°C on a waterbath. The residues were carefully reconstited in 25 μl of butyl acetate, and 2 to 4 μl of the solutions were injected into the GC system.

2.4. Gas chromatography

For the chromatographic separation of the samples, a Hewlett-Packard (HP) 5890 series II GC (Palo Alto, CA, USA) was used with a HP Ultra-1 capillary column (12 m×0.2 mm I.D., 0.33 μm film thickness). The carrier gas was helium, split ratio 20:1. The split-splitless injector with electronic pressure control (EPC) was run at 290°C with an injection pressure of 103 kPa (0.75 min) and an elution pressure of 41.4 kPa. The GC oven was operated with a temperature program of 120°C (1 min), ramping to 240°C (0.5 min) with 30°C/min,

ramping to 280°C (4.3 min) with 15°C/min, and with 30°C/min ramping to 310°C (0.5 min). The MS detector was operated at 300°C interface temperature.

Injections were performed by means of a HP 7673A autosampler.

2.5. Mass spectrometry

The HP 5971A MS detector (electron-impact ionization at 70 eV) was tuned for optimum sensitivity on the masses 131, 264 and 414 of the PFTBA (perfluorotributylamine). In the selected-ion monitoring (SIM) mode, the masses 310 and 325 for midazolam, and the masses 326 and 341 for clinazolam, were monitored (see Fig. 2; mass axis calibration performed once every week). For quantitative purposes, the mass 310 was used for midazolam, and the mass 326 for clinazolam (see Fig. 3 and Fig. 4); the second mass of each compound was used as the qualifier. The peak-height ratio between the quantification and qualifier mass of each compound had to fall within 15% of the calibration standards to identify a peak as the compound of interest.

2.6. Calibration

The calibration standards for 40-μl aliquots had concentrations of 10, 25, 50, 100, 250 and 500 ng/ml. For 500-μl aliquots the concentration levels of the calibrators were 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng/ml. Drug-free plasma was spiked with the appropriate working solutions to prepare the required standards. The calibration curve was calculated by a $1/y^2$ weighted least-squares linear regression of the peak-height ratios of midazolam to clinazolam versus the targeted midazolam concentration.

3. Results

The ion chromatograms displayed in Fig. 3 show a predose plasma sample (40-μl aliquot), in Fig. 4 show a 0.25 ng/ml spiked human plasma sample (0.5-ml aliquot), and in Fig. 2 show a 12.6 ng/ml rat plasma sample (40-μl aliquot). The retention time for midazolam was approximately 7.8 min and for clinazolam was approximately 8.6 min (dependent on the actual column length).

Both calibration curves were linear (r^2=0.997/0.996) over the calibration range from 10 to 500 and from 0.25 to 100 ng/ml, respectively.

For inter- and intra-day precision and accuracy data, see Table 1. The results of the QC samples from three different analytical sequences were used for the calculation of these parameters.

The lower limit of detection (LOD) of midazolam in the 40-μl part of the assay was approximately 2 ng/ml and for the 500-μl part was approximately 60 pg/ml. The lower limit of quantification (LOQ) of midazolam was 10 ng/ml and 0.25 ng/ml, respectively. The overall recovery of midazolam was approximately 90% (for details, see Table 2).

4. Discussion

The assay described was mainly used for the determination of midazolam in rats, for which complete concentration–time profiles in individual animals could be determined (see Fig. 5); the predose level and levels found 1–120 min after intravenous dosing of 1 mg/kg midazolam are shown. Coextracted cholesterol fractions did not interfere with the midazolam determinations, but negatively influenced the lifetime of the columns used. As an indicator for this, the tendency towards peak-broadening, of test solutions, was checked very carefully. Overall, columns could be used for six months without performance losses, despite shortened retention times due to reduced column length.

The assay is simple, precise and selective and can be effectively used for midazolam determinations in low-volume and low-level plasma samples of human or rat origin.

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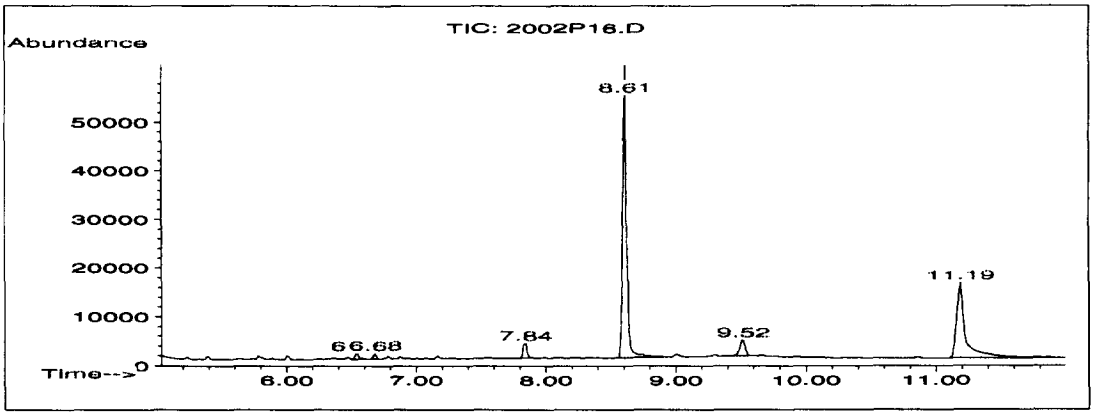
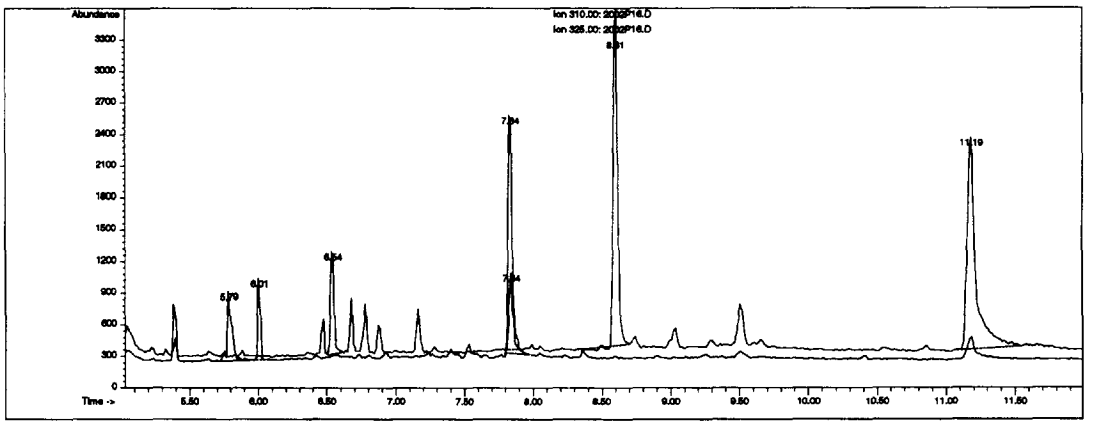


MIDAZOLAM



CLINAZOLAM - Internal Standard

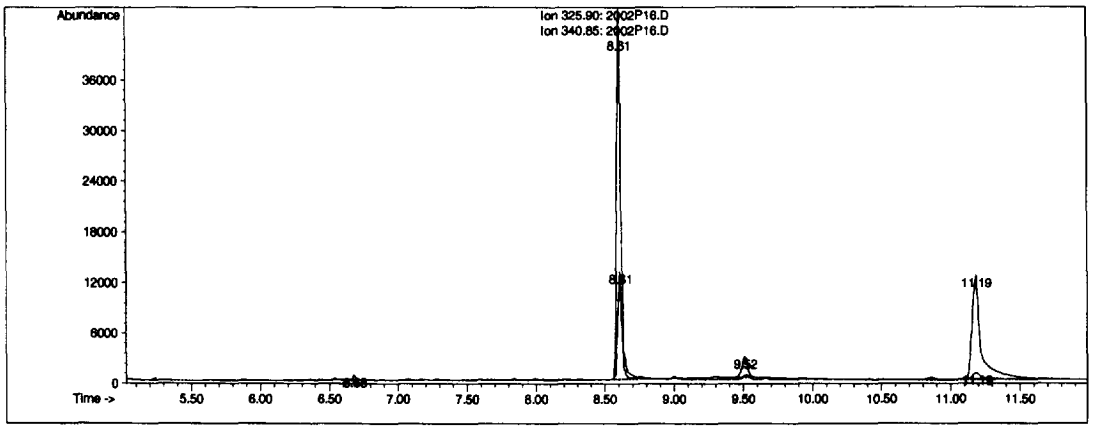
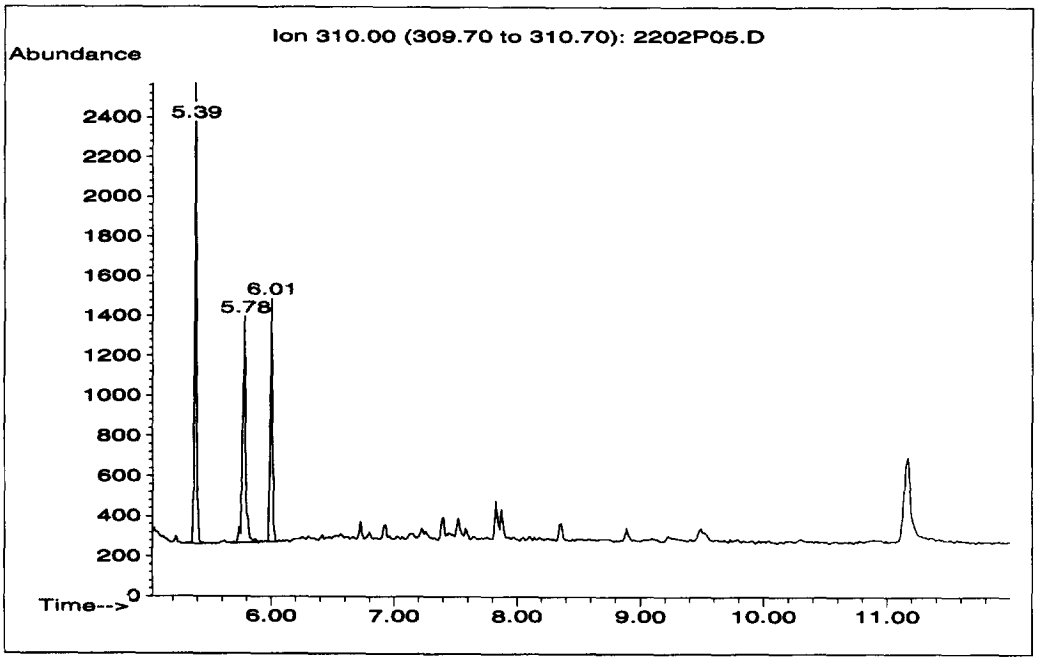


Fig. 2. 12.6 ng/ml rat plasma sample (40-μl aliquot).

MIDAZOLAM



CLINAZOLAM Internal Standard

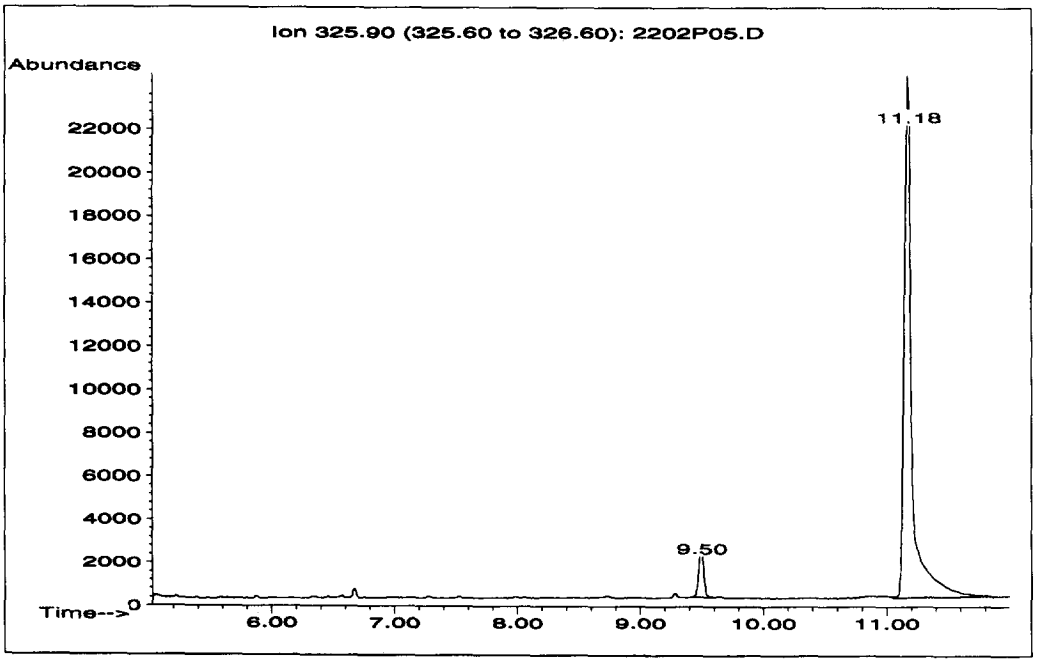
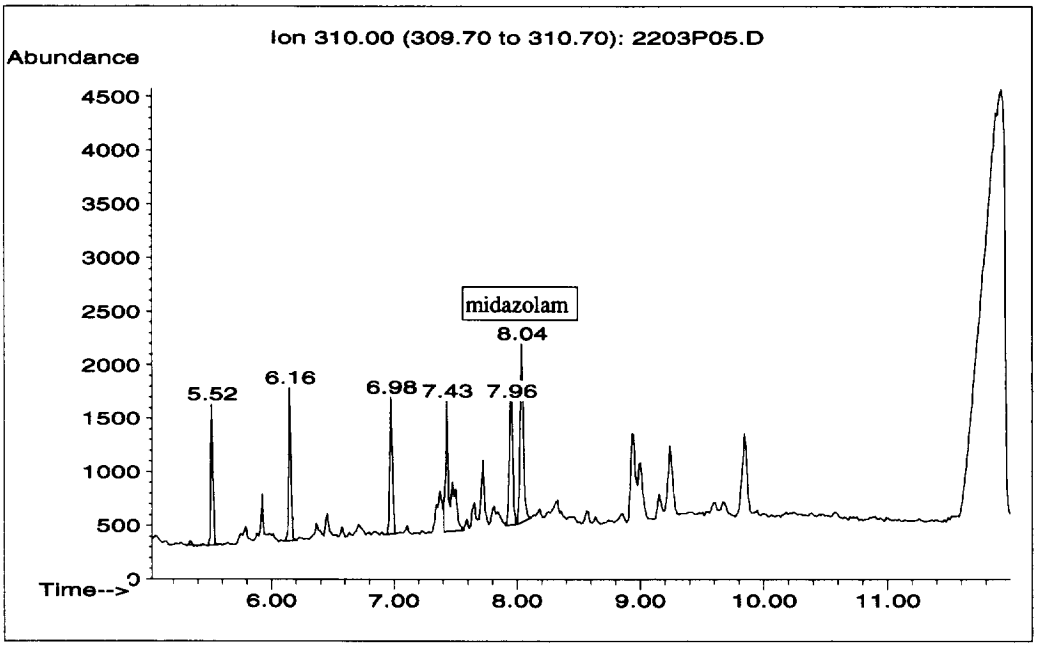


Fig. 3. Pre-dose rat plasma sample (40-μl aliquot).

MIDAZOLAM



CLINAZOLAM Internal Standard

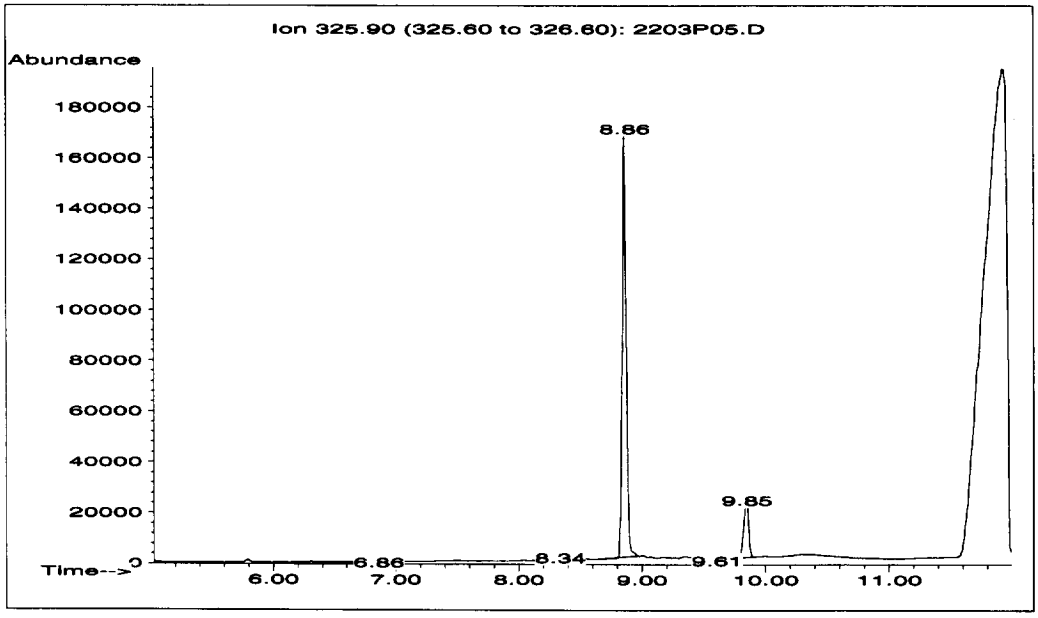


Fig. 4. 0.25 ng/ml calibration sample (500-μl aliquot).

Table 1
Intra- and inter-day precision and accuracy of the midazolam assay ($n=10$)

Aliquots (ml)	Calibration range (ng/ml)	Intra-day		Inter-day	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
40 μl	10–500	2.2 to 6.6	−7.4 to −4.4	5.0 to 7.2	−7.2 to −5.1
500 μl	0.25–100	3.3 to 4	−1.3 to 2.7	4.8 to 6.7	−1 to 2.9

Table 2
Recovery data

Level (ng/ml)	Recovery (%)	R.S.D. (%)
Utilizing 40-μl plasma aliquots		
25	75.2	7.3
65	90.4	3.4
250	90.8	3.5
Utilizing 500-μl plasma aliquots		
4	90.4	5.1
50	91.1	2.0

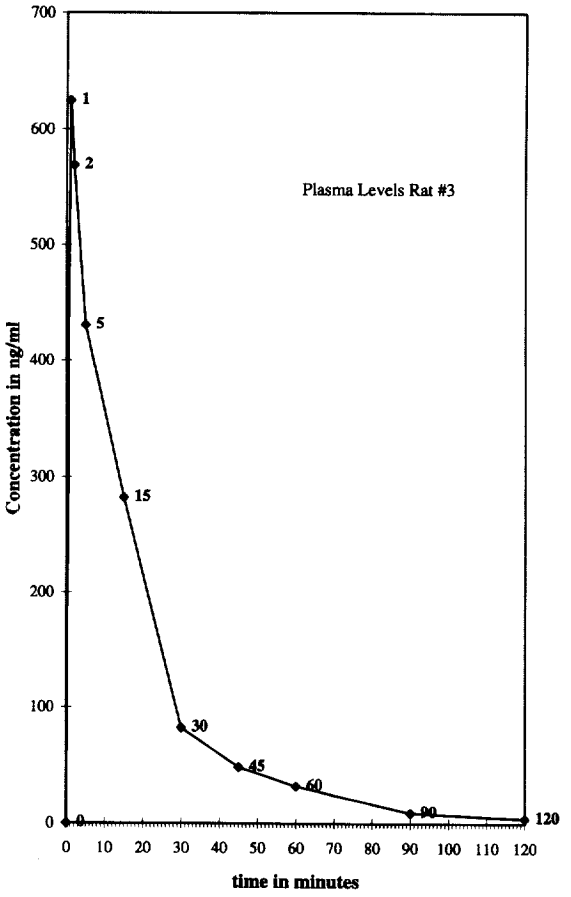


Fig. 5. Concentration–time profile of rat No. 3.

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