ELSEVIER

Journal of Chromatography B, 658 (1994) 93–101

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Assay of zatebradine in plasma by fully automated sample clean-up, capillary gas chromatography and ammonia chemical ionisation mass spectrometry

J. Schmid*, A. Bücheler, B. Müller

Department of Pharmacokinetics and Drug Metabolism, Dr. Karl Thomae GmbH, Birkendorferstrasse 65, D-88 397 Biberach, Germany

Received 26 January 1994

Abstract

A method has been developed for the measurement of zatebradine (UL-FS 49), a heart-rate lowering drug, suitable for the treatment of stable angina pectoris. The method comprises a fully automated liquid–solid extraction using a Zymark Benchmate, a capillary gas chromatography and ammonia chemical ionisation (CI) mass spectrometry using hexadeuterated zatebradine for the internal standard. The assay has a mean between-batch imprecision of 4.9% and a mean inaccuracy of 1.5%. The calibration curve covers the range of 1–30 ng/ml. About 60 samples can be handled per day. The assay has been successfully applied to human pharmacokinetic studies.

1. Introduction

Zatebradine (UL-FS 49 CL) is a heart-rate lowering compound used for the management of chronic stable angina pectoris [1–4]. Although a HPLC column-switching assay with a high sample capacity exists [5], this assay has a limited sensitivity and is also susceptible to interferences from plasma components. The GC–MS assay described here was developed to check the specificity of the HPLC procedure and to obtain an assay that allows a more precise calculation of the pharmacokinetic parameters even from single-dose human pharmacokinetic experiments. Since in a clinical Phase III trial a lot of studies are conducted a fully automated sample preparation procedure had to be established.

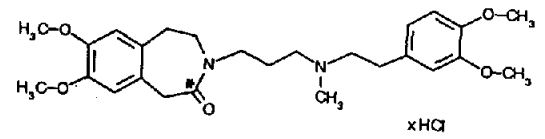
2. Experimental

2.1. Reagents

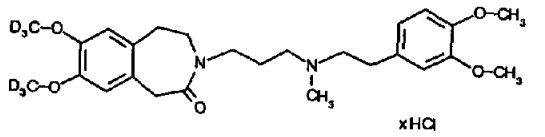
Zatebradine (internal laboratory code UL-FS 49 CL) (1,3,4,5-tetrahydro-7,8-dimethoxy-3[3-[[2-(3,4-dimethoxyphenyl) - ethyl]methylamino]-propyl] - 2H - 3 - benzazepin-2-on-hydrochloride) (Fig. 1), formula $C_{26}H_{37}ClN_2O_5$ (hydrochloride), molecular mass 492.7, and the deuterated compound 1,3,4,5-tetrahydro-7,8-dimethoxy[d_6]-3[3 - [[2 - (3,4 - dimethoxyphenyl) - ethyl]methylamino] - propyl] - 2H - 3 - benzazepin - 2 - on - hydro-

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00199-F



UL-FS 49 Cl I



[D6]UL-FS 49 Cl II

Fig. 1. Structure of zatebradine and its internal standard, hexadeutero zatebradine. * indicates the position of the ^{14}C-label.

chloride, were synthesised in Thomae Research laboratories and were of analytical grade. The ^{14}C-labelled zatebradine (specific activity 344.6 MBq/mmol = 0.699 MBq/mg = 18.89 μCi/mg) had a purity >99% and was synthesized in the Isotope Laboratory, Dr. Karl Thomae GmbH. It was labelled in the benzazepin ring of the molecule (Fig. 1).

Deionised water was prepared on site. Methanol, HPLC-quality (No. 8402), was from J.T. Baker (Deventer, Netherlands). Anhydrous sodium acetate, p.a. quality (No. 6268) was from Merck (Darmstadt, Germany) and was used as a buffer (0.8 g/l) in water. Toluene, nanograde (No. 8092), was from Mallinckrodt (Wesel, Germany). Bond Elute C_2 columns (3 ml) (No. 1210-2027) were from Varian (Harbor City, CA, USA).

2.2. Glass and plastic vessels

Glass tubes (10 ml) for single use were used for the extraction. The plasma samples were stored in tubes No. 3810 (1.5 ml) from Eppendorf (Hamburg, Germany) and frozen at −20°C.

2.3. Preparation of solutions

Internal standard solution

Zatebradine-d_6-HCl (10 mg) as weighed and dissolved in 100 ml water. This stock solution was diluted with water to a final concentration of 60 ng zatebradine-d_6-HCl/ml. For the assay 0.5 ml stock solution was added to the plasma samples, which corresponds to 30 ng per sample.

Preparation of standard samples

Zatebradine (10 mg) (as hydrochloride!) was weighed and dissolved in 100 ml water. This solution was further diluted with water to a concentration of 1.0 μg zatebradine/ml. With this *aqueous* stock solution a stock solution in *human plasma* was prepared containing 30 ng zatebradine/ml. The plasma stock solution was equilibrated at 37°C for 2 h. The stock solution was diluted with human blank plasma to the following concentrations: 20, 10, 5, 3 and 1 ng/ml plasma.

2.4. Equipment

Processing equipment

Benchmate Workstation: Zymark (Idstein, Germany). Centrifuge: type Microliter No. 2020, 15000 rpm (ca. 12000 *g*) Hettich (Tuttlingen, Germany). Evaporator: Buchler Vortex-Evaporator (Fort-Lee, NJ, USA). Pipettes: type Varipette (No. 4710) Eppendorf (Hamburg, Germany). Metering device: Brand (Wertheim, Germany).

Analytical equipment

Gas chromatograph: HRGC 5160 Mega Series, Carlo Erba (Milan, Italy). Injector: Grob-type; Lauber, Ciba-Geigy (Basel, Switzerland). Thermolite Septa. (No. 20368) Restek (Sulzbach, Germany). Column: DB-1, 15 m × 0.32 mm I.D., 0.25-μm film thickness, Fisons (Wiesbaden, Germany). Autosampler: (ALS 3940) Dani (Monza, Italy). Mass spectrometer: Finnigan 4500 modified according to Lauber by Cueni CCT (Basel, Switzerland).

GC–MS system parameters

Gas chromatograph: injector temperature: 250°C; starting oven temperature: 100°C; heating rate 25°C/min to 200°C, then 20°C/min to final temperature 320°C. Hold time: 8 min. Injection volume: 2 μl; split for 45 s closed. Gas and

flow-rate: Hydrogen type 4.5; 50 kPa pressure; gas velocity: 50 cm/s (at 160°C).

The retention times under the above conditions were: UL-FS 49: 10.38 min, the internal standard (UL-FS 49-d_6): 10.36 min.

Mass spectrometer source temperature: 130°C; vacuum: $6.66 \cdot 10^{-3}$ Pa ($5 \cdot 10^{-5}$ Torr); CI gas: 0.50 Torr NH_3. Electron energy: 43 eV; emission: 0.49 mA; SEV: 1400 V, preamplifier: 10^{-7} A/s; dynode: 3.1 kV; manifold temperature: 65°C; total scan time: 0.532 s; mass intervals: m/z 456.8–457.3 = 0.419 s; m/z 462.8–463.3 = 0.105 s.

2.5. Processing and extraction procedures

Plasma samples were thawed at 30°C in a water bath, shaken and mixed for 10 s. Samples were spun down for 3 min, in the Microliter centrifuge, and 1.0 ml of the supernatant was pipetted into a 10-ml glass tube. The extraction procedure by the Zymark Benchmate 2.4 was run as follows: Step 1: add 0.5 ml of internal standard solution containing 30 ng zatebradine-d_6; step 2: vortex-mix for 5 s at speed 2; step 3: condition column with 5 ml of methanol; step 4: condition column with 5 ml of sodium acetate buffer pH 7.3; step 5: load all of the sample (>1.5 ml) onto the column; step 6: rinse column with 1 ml of sodium acetate buffer pH 7.3; step 7: rinse column with 1 ml of water; step 8: rinse column with 1 ml of MeOH–H_2O (4:6, v/v; step 9: dry column with gas for 120 s; step 10: elute and collect analyte by 2 ml methanol; step 11: wash syringe with 3 ml of water; step 12: end.

The methanol extracts were evaporated to dryness at 40°C in the Vortex and then dissolved in 30 μl toluene containing 5% isopropanol.

2.6. Validation

Recovery experiments

In order to determine the recovery of the assay, samples containing 113, 56, 11.5 and 4.5 ng/ml [^{14}C]-zatebradine were processed and all extracted phases were analysed by liquid scintillation counting.

The method was checked for plasma (EDTA and heparinized) from man and mouse. For the investigation of blanks human (EDTA) plasma of five different volunteers was used. In a further clinical study plasma of 12 volunteers was assayed.

Long term stability of samples

In order to determine the stability in the frozen state, spiked plasma samples containing 30, 10, 3 and 1 ng/ml were prepared and frozen in Eppendorf vessels at −20°C in portions of 1.1 ml. Furthermore, *ex vivo* plasma samples of a study stored at −20°C, were re-analysed after 2 months.

Quality control of the assay

Quality control samples (QC) were spiked with 25, 8 and 2.5 ng/ml zatebradine. These samples were analysed in duplicate at each batch during the studies.

Limit of quantitation

The lower end of the calibration curve was set as limit of quantitation.

2.7. Human pharmacokinetic studies

The pharmacokinetics of zatebradine were investigated in elderly healthy volunteers. Male volunteers (12) received 5 mg zatebradine tablets twice daily for 4 days. The study was designed as an open, not randomised study. Blood (10 ml) was taken at predetermined times in the steady state, and EDTA plasma was prepared by centrifugation and stored at −20°C until analysis.

2.8. Statistics

From the quality control samples between-batch and within-batch variation was calculated by analysis of variance. For the estimation of the precision (reproducibility) of the assay under routine conditions, duplicates of a bioavailability study were analysed according to the method of Haeflinger and Wall [6]. The detection limit was also calculated using the method of confidence intervals of the calibration curve [7].

3. Results

3.1. Principle of the method

The method consists of 3 steps: (a) Automated solid-phase extraction with C_2 columns using a Benchmate. (b) Capillary GC using a deuterated standard. (c) Ammonia CI-mass spectrometry resulting in high sensitivity and specificity.

3.2. Extraction procedure and recovery

Using the Benchmate Workstation, the recovery of the ^{14}C-labelled zatebradine from human plasma was between 83.4 and 87.9% in the concentration range of 113 to 4.5 ng/ml. The precision of the recovery was remarkably low (Table 1).

The whole cycle lasted 12.8 min, so in one day 60 samples can be processed. A critical component of the Benchmate workstation was the three-way SPE-valve, which has to be cleaned every 300 samples to prevent clogging. The automated solid-phase extraction with C_2 reversed-phase columns using a step gradient resulted in clean extracts (see Figs. 4, 5).

Table 1
Recovery of zatebradine from human plasma at different concentrations, determined with ^{14}C-labelled compound

Initial dpm	4748	2364	485	189
ng/ml	113	56	11.5	4.5
Found (%)	83.4	83.5	83.2	87.9
C.V.% ($n = 8$)	1.9	2.6	3.1	2.8

3.3. Chromatographic system

Zatebradine could be chromatographed with a short capillary column of low film thickness (0.25 μm), using splitless injection. Peak shape was very good (see Figs. 4, 5).

3.4. Mass spectrometry and internal standard

For quantitation of zatebradine we used chemical ionisation with ammonia as reagent gas, as these conditions result in a low fragmentation and therefore in a high yield of the molecular ion [M + 1] (Figs. 2 and 3).

The hexadeuterated internal standard contributed for *ca.* 0.7% to the trace of the M + 1 ion of

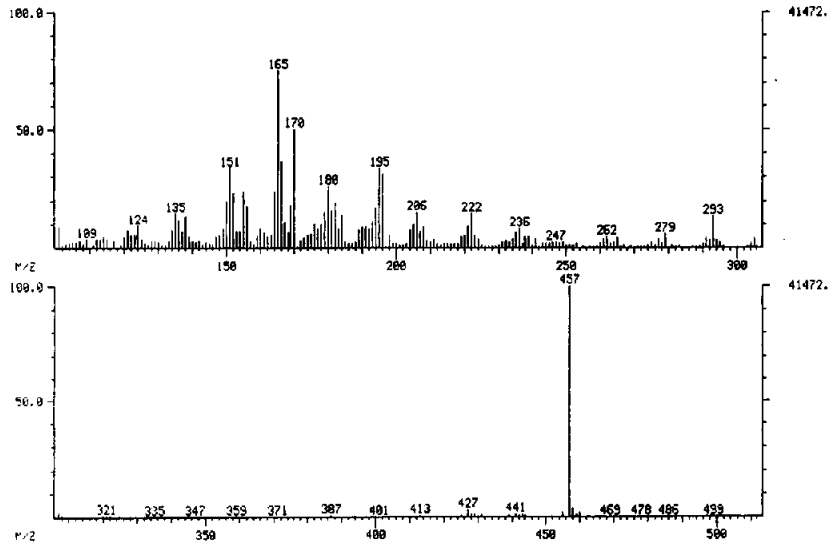


Fig. 2. Ammonia CI-mass spectrum of zatebradine.

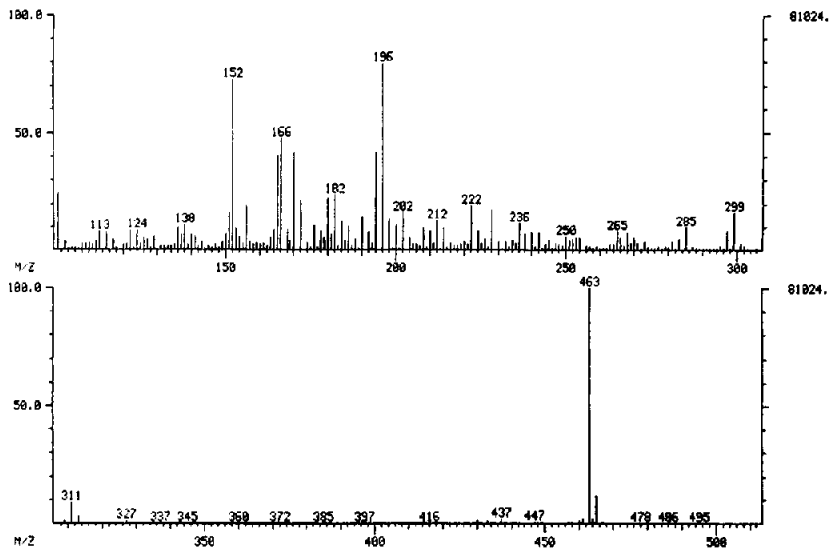


Fig. 3. Ammonia CI-mass spectrum of the internal standard (hexadeutero zatebradine).

the undeuterated standard. The undeuterated standard did not contribute to the mass of the deuterated standard.

3.5. Chromatograms

A GC–MS chromatogram of a human plasma sample spiked at a concentration of 10 ng/ml in comparison to a blank plasma is given in Fig. 4. The GC–MS chromatograms of two different *in vivo* samples, showing levels of 1.4 and 14.9 ng/ml of zatebradine are given in Fig. 5 (top and bottom). The low concentration samples show that the calibration curve could be extended to concentrations below 1 ng/ml, if necessary.

3.6. Calibration curve

Using spiked human plasma at a concentration range of 1–30 ng/ml a linear curve was obtained. The concentration of zatebradine was calculated by reference of the peak-area ratios (zatebradine/internal standard) against an independently prepared set of calibration standards which were analysed in duplicate with each batch. Regression analysis with weighting ($1/y$) was used to assess the closeness of the fit of the observed data to the actual concentrations. The calibration curve is described by the equation: y(ratio zatebradine/standard) = 0.0398x(concentration) − 0.000898. The correlation coefficient r^2 was 0.9981, indicating a good correlation.

3.7. Limit of detection and quantitation

The lowest calibration standard was 1 ng/ml, with an imprecision (within-batch) of 3.2% and an inaccuracy of 6.0%. Using the method of confidence intervals (95%) of the calibration curve [7], the limit of quantitation was 0.2 ng/ml; the limit of detection was 0.1 ng/ml.

3.8. Precision and accuracy

The within-batch precision of the GC–MS assay was determined by calculating the coefficient of variation of the ratios (zatebradine/internal standard) for the individual concentrations of the calibration curve. The within-batch precision of the concentration was 3.3% at 1 ng/ml and 2.1% at 30 ng/ml. The within-batch precision data was furthermore calculated from quality controls and are summarised in Table 2. The between-batch precision was also calculated from

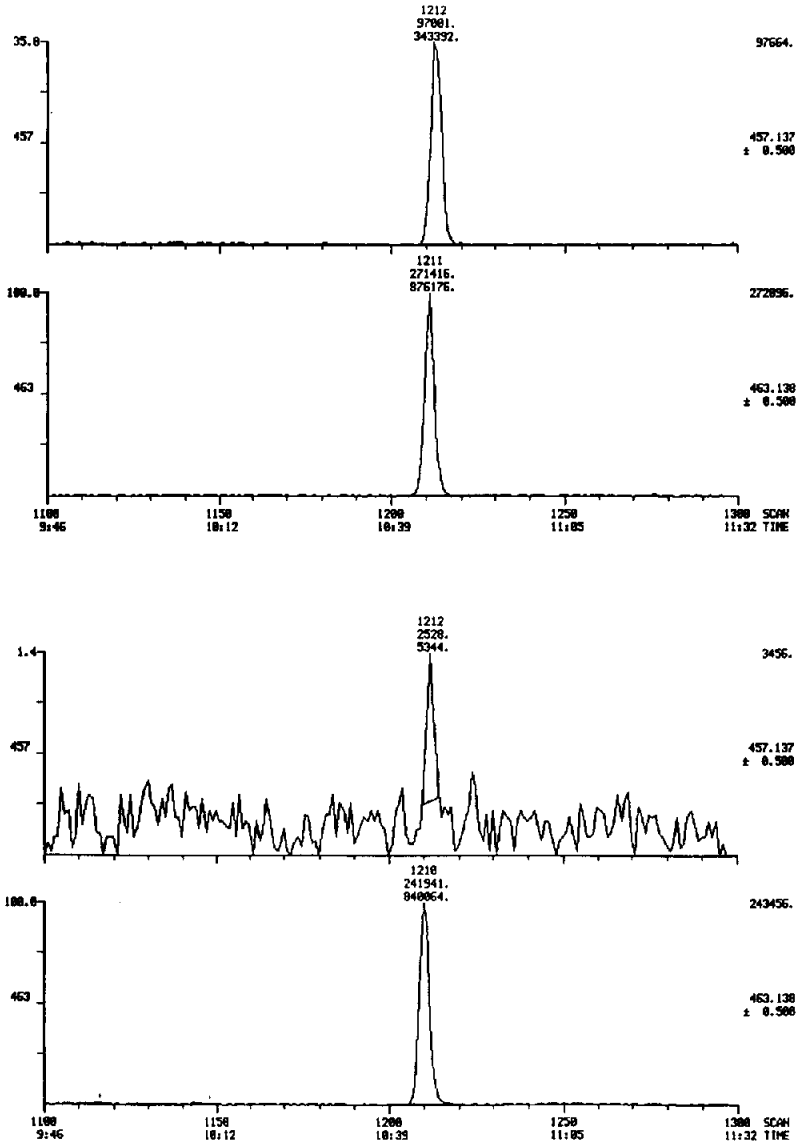


Fig. 4. Chromatograms of human plasma samples. (Top) Plasma spiked with 10 ng/ml zatebradine, (bottom) expanded scale chromatogram of blank plasma.

quality control samples over a whole pharmacokinetic study and was between 3.8% and 6.6%. The inaccuracy was between −2.8% and −1.1% (Table 2). About 10% of the samples of the kinetic study were analysed in duplicates. The results were analysed according to Haefliger and Wall [6] in order to obtain a good impression about precision under routine conditions. The precision over the whole study and the whole range of plasma levels (1–60 ng/ml) was in the range of 2.7–4.2%. The values are summarised in Table 3.

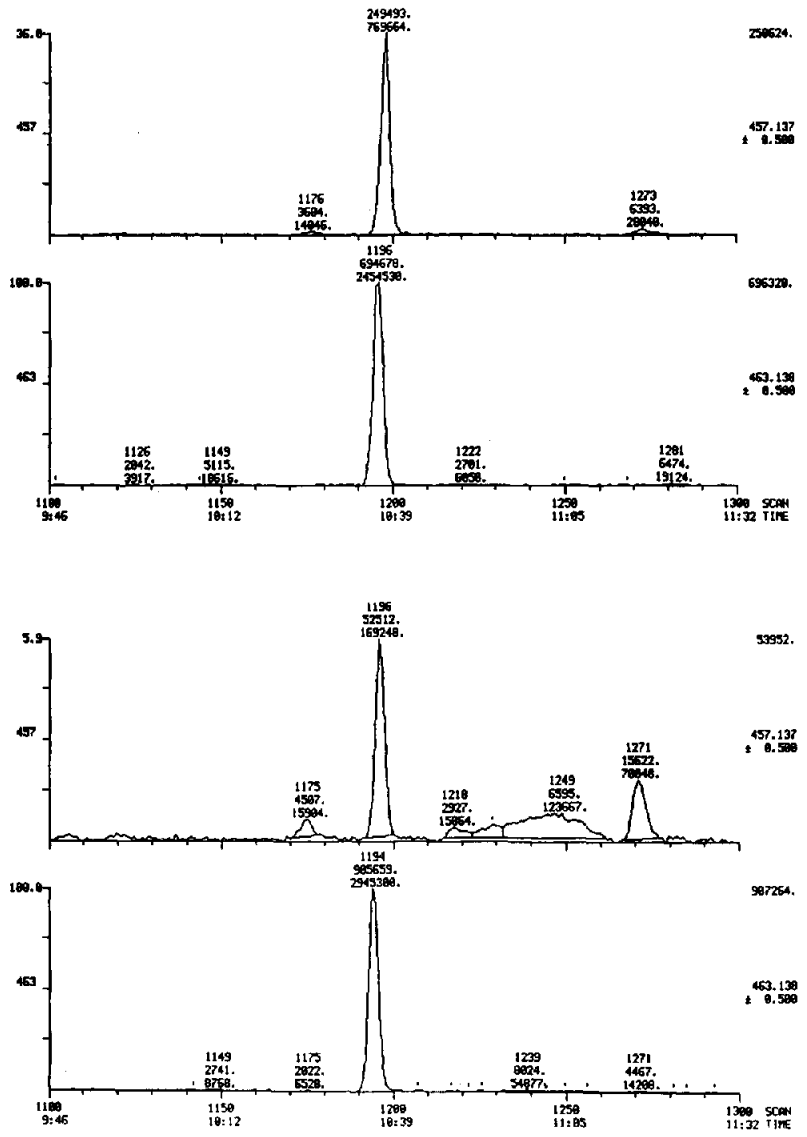


Fig. 5. Human plasma samples analysed during a kinetic study. (Top) Plasma containing 7.8 ng/ml zatebradine, (bottom) plasma containing 1.4 ng/ml zatebradine.

3.9. *Blanks and specificity*

Blank plasma showed a small interference (Fig. 4) which is due to the 0.7% undeuterated compound in the internal standard as discussed above. Because this is accounted for by the calibration curve, blank values have a concentration of 0 ng/ml. To confirm the nature of the interference, plasma of 5 individuals was measured with a different, structurally homologous internal standard; no interferences were observed.

Table 2
Summary of assay validation obtained from quality control samples

Concentration (ng/ml)		n	Inaccuracy (%)	Imprecision (%)	
Added	Found			Between-batch	Intra-batch
2.5	2.43	14	−2.8	3.76	3.16
8.0	7.91	15	−1.1	6.63	3.08
25.0	24.73	15	−1.1	4.29	1.69

During a pharmacokinetic study in 12 volunteers, no interferences due to endogenous compounds were observed in the individual predose samples. The assay is specific with respect to all known metabolites, because these differ from the parent compound with respect to their structure and molecular mass: The main metabolites in human plasma are the dimethoxyphenyl acetic acid (DMPA), the N-demethylated product, and O-demethylated compounds [4]. Due to the mild extraction conditions (pH 7.3), no cleavage of putative conjugates is expected.

3.10. Stability of plasma samples

In spiked plasma zatebradine is stable under the following conditions: At least 10 months at −25°C; during two freeze-thaw circles; for at least 24 h at room temperature (25°C). *Ex vivo* plasma samples containing zatebradine and its metabolites are stable for at least two months. In the autosampler the extracted samples were stable for at least 48 h.

3.11. Pharmacokinetics

The plasma profile found in the young (mean age = 34, range: 25–50) and elderly (mean age = 68, range: 64–73) volunteers under steady state conditions is given in Fig. 6. Maximal plasma levels were 23.4 ± 13.6 ng/ml. The morning troughs [i.e. plasma levels at the morning of day 4 (72 h in Fig. 6) before administration of the 5th dose] were 5.3 ± 1.8 ng/ml. The dominant half

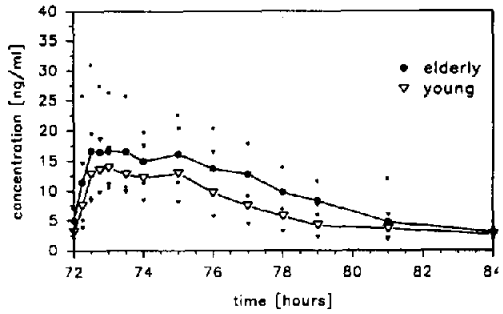


Fig. 6. Mean plasma levels (geometric means ± standard deviation) in elderly and young volunteers.

Table 3
Performance data of duplicates under field conditions. Calculated according to Haeflinger and Wall [6]

Concentration range (ng/ml)	Mean C.V. (%)	No. of duplicates with C.V. < 5%	Total No. of duplicates
1–5	2.72	6	8
5–15	4.18	14	19
15–25	3.29	17	19
25–60	2.78	11	13

life in the elderly volunteers was 3.75 ± 0.57 h, the clearance was 711 ± 254 ml/min.

4. Discussion

Up to now *ca.* 4500 plasma samples have been analysed with this method. The use of reversed-phase material which has only moderate, but sufficient for zatebradine, retention power (C_2 instead of C_{18} material), results in clean extracts.

The use of a Zymark Benchmate to perform the extraction procedure allows full automation and to run batches over-weekend. The greatest advantage of this equipment is not its speed, as one cycle needs *ca.* 12.8 min, but that all steps are controlled by weighting and all steps are stored on disc. Thus, irregular pipetting can be easily traced. Initially, clogging of valves by plasma proteins presented a problem. This could be solved by adding a wash step with water after the elution with pure methanol.

The use of capillary gas chromatography and ammonia CI-mass spectrometry results in a high selectivity of the assay. The lowest calibration standard was 1 ng/ml and the limit of quantitation has the same value. However, the coefficient of variation (C.V.) (within-day) of 3.2% at this level demonstrates that the potential of the method is not at its end. Furthermore, only 2-μl aliquots of the 30-μl extract are injected onto the chromatographic system.

Acknowledgements

We thank Mr. H. Switek for the synthesis of the labelled zatebradine and Dr. G. Trieb for the statistics.

References

[1] W. Kobinger and C. Lilie, *Eur. J. Pharmacol.*, 104 (1984) 9.
[2] H. Franke and C.A.P. Su, *Eur. Heart J.*, 8 (Suppl. C) (1987) 91.
[3] D.N. Duong, J.H.G. Jonkman, P.B.M. Zuiderwijk, R.G.L. van Tol and P.J.G. Cornelissen, *J. Clin. Pharmacol.*, 36 (Suppl.) (1989) 3.
[4] W. Roth, E. Bauer, G. Heinzel, P.J.G. Cornelissen, R.G.L. van Tol, J.H.G. Jonkman and P.B.M. Zuiderwijk, *J. Pharm. Sci.*, 82 (1993) 99.
[5] R. Brickl, HPLC-method and stability of zatebradine (UL-FS 49 CL) in plasma, internal report, Dr. Karl Thomae GmbH, U91-0659, December 19, 1991.
[6] P. Haeflinger and M. Wall, *Z. Analyt. Chem.*, 307 (1981) 271.
[7] W. Funk, V. Dammann, C. Vonderheid and G. Oehlmann (Editors), *Statistische Methoden der Wasseranalytik*, VCH Verlagsgesellschaft, D-6940 Weinheim, 1985, Ch. 4, p. 61.