ELSEVIER

Journal of Chromatography B, 723 (1999) 211–219

JOURNAL OF CHROMATOGRAPHY B

# Determination of an ultrashort-acting antihypertensive dihydropyridine, clevidipine, in blood using capillary gas chromatography–mass spectrometry and of the primary metabolite using liquid chromatography and fluorescence detection

Christina Fakt*, Helene Stenhoff

*Bioanalytical Chemistry, Astra Hässle AB, S-431 83 Mölndal, Sweden*

Received 14 July 1998; received in revised form 16 October 1998; accepted 23 October 1998

**Abstract**

A method for the quantitative determination of a new ultrashort-acting dihydropyridine, clevidipine (butyroxymethyl methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate), in whole blood with capillary gas chromatography–mass spectrometry with negative ion chemical ionisation is presented. The rapidly metabolised drug is stabilised in blood using sodium dodecyl sulphate (SDS) which prevents ester hydrolysis. The analytical procedure involves liquid–liquid extraction prior to gas chromatographic determination with a limit of quantification of 0.5 nmol/l blood. The acidic primary metabolite (methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate), MI, can be determined with liquid chromatography and fluorescence detection using a similar sample work-up procedure. Ascorbic acid is then added before sampling to prevent oxidation. The limit of quantification for MI is 50 nmol/l blood. 

*Keywords:* Dihydropyridine; Clevidipine

## 1. Introduction

A new ultrashort-acting vascular selective calcium antagonist, clevidipine, intended for short-term reduction and control of blood pressure by intravenous treatment during cardiovascular surgery and in the postoperative period, has been developed and is subjected to clinical investigation. Clevidipine, butyroxymethyl methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate, is rapidly metabolised to the corresponding carboxylic acid, MI, (Fig. 1) with elimination half-lives in vivo and in vitro in human blood of approximately 1.5 and 6 min, respectively. The drug is pharmacologically active in the low nanomolar range and in order to perform pharmacokinetic studies a highly sensitive analytical method was required. It was also vital to find a way to inhibit the enzymatic hydrolysis of clevidipine.

Various analytical methods for the determination of dihydropyridines have been reported where a vast majority uses gas chromatography (GC) with electron capture (EC) detection or mass spectrometry

*Corresponding author. Fax: +46-31-776-37-60.

0378-4347/99/$ – see front matter 
PII: S0378-4347(98)00495-2

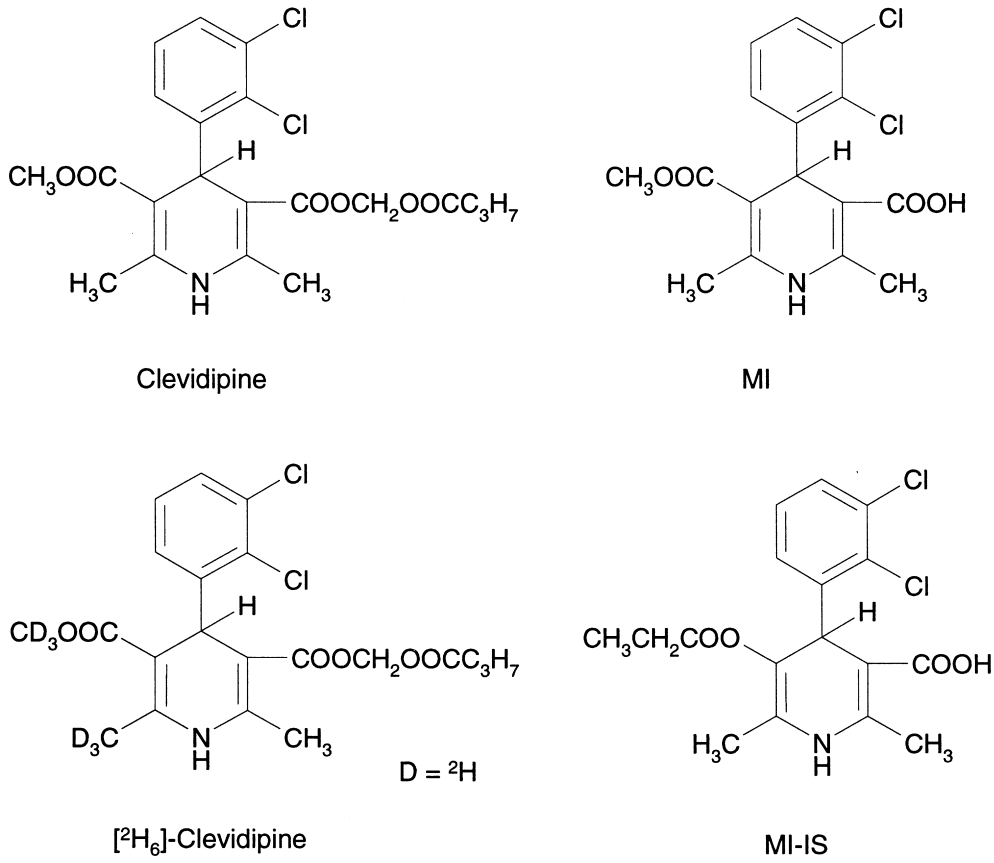


Fig. 1. Molecular structure of clevidipine, MI and their internal standards.

(MS) in electron ionisation (EI) mode or electron capture negative ion chemical ionisation (EC-NICI) mode. In a review article by Ahnoff and Persson [1], gas and liquid chromatography methods for 14 calcium antagonists are thoroughly discussed. Most papers were published during the 1980s but more recent papers show that GC–MS using EC-NICI is still the method of choice for quantification of low levels of dihydropyridines. In an article by Mück and Bode [2], published in 1994, an excellent overview of bioanalytical methods for nimodipine, another dihydropyridine calcium antagonist is given.

The primary metabolite, MI, with a half-life in vivo in human subjects of approximately 8 h is inactive and is also found in subjects given felodipine [3], a structurally related dihydropyridine. In this paper by Ohtake et al. [3] MI is determined in plasma by GC–MS after extraction with diethyl ether and derivatisation with diazomethane. In spite of the fact the metabolite has been found inactive, there was still a need to monitor the concentrations of MI in order to follow the accumulation due to the large difference in elimination half-life between clevidipine and the metabolite.

This work presents a procedure for sampling and liquid–liquid extraction of whole blood for analysis with capillary gas chromatography selected-ion monitoring mass spectrometry (GC–MS) with electron capture negative ion chemical ionisation (EC-NICI). By using a slightly different sampling and work-up procedure, the primary metabolite, MI can be determined by reversed-phase liquid chromatography and fluorescence detection and this analytical procedure is also presented.

## 2. Experimental

### 2.1. Chemicals and reagents

#### 2.1.1. Clevidipine

Clevidipine and the deuterated internal standard (Fig. 1) were obtained within Astra AB. Toluene was of glass-distilled grade and methanol of HPLC grade (Rathburn, Walkerburn, UK). Sodium dodecyl sulphate (SDS), GC grade, was from Sigma (St. Louis, MO, USA) and $NaH_2PO_4$ of analytical grade (e.g., Merck, Darmstadt, Germany).

Standard solutions of clevidipine were prepared in methanol to produce working standard solutions at two concentration levels of 100 and 2 nmol/l. The deuterated internal standard, was prepared in methanol to give a working internal standard solution with a concentration of 200 nmol/l. The high standard solution and the internal standard solution can be stored at −18°C for up to 6 months.

#### 2.1.2. MI

MI (H 152/81) and the internal standard MI-IS (H 172/99), Fig. 1, were obtained within Astra AB. Methyl-*tert*.-butyl ether (MtBE) and acetonitrile were of HPLC grade (Rathburn). Acetic acid (glacial), sodium acetate, ascorbic acid, $NaH_2PO_4$ and hydrochloric acid (Titrisol, 1.0 mol/l) were of analytical grade (e.g. Merck).

Standard solutions of MI and the internal standard, MI-IS were prepared in methanol to give working standard and internal standard solutions with final concentrations of 34 and 135 μmol/l, respectively. These solutions can be stored at −18°C for up to 3 months.

### 2.2. Apparatus

#### 2.2.1. Clevidipine

A Finnigan SSQ 710 mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph with the column interfaced directly into the ion source was used for the analyses.

Chromatography was performed on a 15 m×0.20 mm I.D. fused-silica capillary column with an SPB-20 stationary phase (0.20 μm, Supelco, Bellefonte, PA, USA). The inlet pressure of hydrogen was 12 p.s.i. (0.85 bar). The column temperature was held at 120°C for 0.6 min, then programmed at 40°C/min to 260°C and at 12°C/min to 310°C where it was held for 3 min. The injector was operated in the splitless mode at 280°C.

The mass selective detector was operated in the electron capture negative ion chemical ionisation (EC-NICI) selective ion monitoring (SIM) mode at $m/z$=455 and 461. The reagent gas was ammonia at an ion source pressure of 8 Torr (1.1 kPa). The electron energy was set at 100 V, the ion source temperature at 150°C and the connection line temperature at 280°C.

#### 2.2.2. MI

The chromatographic system consisted of an isocratic LC pump Gynkotec model 480 (Gynkotec, Munich, Germany), an autosampler, Spectra-Physics AS 3000 (Thermo Separation Products, Freemont, CA, USA) and a fluorescence detector, Shimadzu RF 551 (Shimadzu, Kyoto, Japan) set at an excitation and emission wavelength of 358 and 440 nm, respectively. The band width was 15 nm and the time constant 1.5 s. Data collection and quantification were performed automatically by a chromatography data system; VG Multichrom (Labsystem, Altrincham, UK). The guard column was a 15×3.2 mm I.D. Brownlee Aquapore Cyano 7 μm (Brownlee Labs, Santa Clara, CA, USA) and the analytical column was a 100×4.6 mm I.D. Microspher $C_{18}$, 3 μm (Chrompack, Middelburg, The Netherlands). The mobile phase was prepared by mixing 450 ml of acetonitrile and 100 ml acetate buffer (pH 5.0, $I$= 0.1) and diluting with water to 1000 ml. Flow rate 1.0 ml/min and room temperature were used.

### 2.3. Glassware

All glassware was washed in a laboratory dishwasher at pH 12 (Extran AP 12, Merck No. 7559), then rinsed at pH 2 and finally with deionised water and dried at 120°C.

### 2.4. Sampling procedure

About 1 ml of blood was collected as fast as possible in sample tubes containing 1.00 ml of SDS solution (10% in water). After immediate and thorough mixing for 5–10 s, the samples were deep

frozen and kept at −70°C until analysis. For appreciation of the dilution factor, the sample tubes were weighed before and after the sampling to determine the exact weight of the blood sample. In order to determine MI the sample tubes must also contain 100 μl of ascorbic acid (0.5 mol/l in 0.01 mol/l HCl) to prevent degradation.

### *2.5. Analytical procedure*

#### *2.5.1. Clevidipine*

The samples were allowed to thaw at room temperature, mixed and then 400 μl of the blood mixture was transferred to a centrifuge tube. A 50-μl volume of the working internal standard solution and 400 μl of $NaH_2PO_4$ (0.05 mol/l) were added. After mixing, 2 ml of toluene was added and the tubes were shaken for 30 min. The samples were then centrifuged (5 min, 1400 *g*) and the organic extract separated and evaporated to dryness at 35°C under a gentle stream of dry nitrogen. The residue was reconstituted in 100 μl of toluene and 2 μl was injected onto the chromatographic column.

#### *2.5.2. MI*

A total of 1.00 ml of the blood mixture was transferred to a centrifuge tube after thawing at room temperature and mixing. A 50-μl volume of the working internal standard solution and 1 ml of $NaH_2PO_4$ (0.1 mol/l) were added. After mixing, 5 ml of the extraction solution (20% MtBE in toluene) were added and the tubes were shaken for 30 min. The samples were then centrifuged (5 min, 1400 *g*) and 2 ml of the organic extract were separated and evaporated to dryness at 25°C under a gentle stream of dry nitrogen. The residue was reconstituted in 300 μl of mobile phase and 100 μl were injected onto the chromatographic column.

### *2.6. Quantification*

Blood standards were prepared by mixing 200 μl of blank blood, 200 μl of SDS solution and 50 μl of the working standard solutions. At least six blood standards were prepared from each working standard solution and analysed according to the procedure given above. The mean value of the peak height ratio of clevidipine to its internal standard in the blood standards was used for the quantitative evaluation of clevidipine in the analysed samples using two point calibration.

Similarly, blood standards for MI, were prepared by mixing 500 μl of blank blood with 500 μl SDS solution, 50 μl of ascorbic acid solution and 50 μl of the working standard solution and analysed according to the procedure given above. The mean value of the peak height or area ratio of MI to its internal standard MI-IS was used for estimation of the blood concentrations.

The obtained results were compensated for the dilution with the SDS solution (and ascorbic acid) and when a weight was converted into volume, the density for blood, 1.054 g/ml was used.

### *2.7. Recovery, precision and linearity*

The extraction recovery for clevidipine, the deuterated internal standard, MI and MI-IS from blood using the analytical procedure was evaluated. The peak heights for the added analytes were compared with those from pure solution added to a blank blood extract which then was processed in parallel to extract from the spiked blood sample. The precision at different concentration levels was estimated by determining the repeatability or within-day variation by analysis of eight to 10 replicates. Reproducibility or inter-day variation was estimated from quality control blood samples in duplicate ran each day of analysis. Linearity was studied by preparing at least three replicates of blood samples at eight concentration levels and processing them according to the analytical procedure. The concentration range examined comprised limit of quantification (LOQ) as the lowest concentration and the upper level exceeded the highest sample concentrations. Full standard curves were run intermittently during the course of routine analysis to control linearity.

## 3. Results and discussion

### *3.1. Blood sampling and storage*

Clevidipine is rapidly hydrolysed in blood by endogenous esterases in vivo and in vitro and to prevent this sodium dodecyl sulphate (SDS) was

used as an esterase inhibitor. Holm et al. [4] investigated the inhibition of the ester hydrolysis of two labile ultrashort-acting β-adrenoceptor blocking drugs and found SDS superior to other stabilising agents suggested in literature. An alternative might be to extract the labile compound into an organic solvent immediately after sample collection, but this would be inappropriate when collecting large number of samples in clinical practice. It must be pointed out that both sampling and handling of the samples are critical if reliable results are wanted. The blood must be collected directly into the sample tubes containing the SDS solution and immediately mixed to make sure that all the enzymes are brought in contact with the SDS. SDS-treated samples have so far proved satisfactory stability. Blood standards kept at room temperature for 4 h gave 100% recovery and authentic blood samples from animals (rats and dogs) stored at −70°C for 12 months showed no decrease in concentration. The hydrolysis product, MI, displayed a pronounced instability in the blood/SDS mixture by oxidation to the corresponding pyridine. To prevent this, ascorbic acid, a well know antioxidant, was added before sampling. However, stability tests showed that samples could not be kept thawed at room temperature for more than 1 h, thereafter a slight decrease in concentration of MI was detected. Blood samples could be stored at −70°C for up to 5 months.

### 3.2. Method performance

The recoveries of clevidipine and MI from blood at three concentration levels were about 85% and are, together with the relative standard deviations (C.V.), i.e. intra-day variation, shown in Table 1. The inter-day variation as estimated C.V. from quality control samples was typically 2% for clevidipine at 65 nmol/l and 1.5% for MI at 1500 nmol/l. The limit of quantification (C.V.<20%) for the proposed methods was 0.5 nmol/l blood (0.23 ng/ml) for clevidipine and 50 nmol/l (18 ng/ml) for MI. Accuracy at limit of quantification must be within ±15% according to our standard operation procedures. The standard curves showed linearity from limit of quantification to more than 1000 nmol/l for clevidipine and more than 30 000 nmol/l, for MI which exceeded the blood concentrations measured in clinical studies.

The methods were shown to be selective towards metabolites and endogenous compounds. Chromatograms from samples taken before and after dose are shown in Figs. 2 and 3.

### 3.3. Electron capture negative ion chemical ionisation

The analysis of clevidipine was initially performed using electron impact ionisation (EI) and mass spectra are shown in Fig. 4. The ions $m/z$ 310 and 316 were used for the quantification of clevidipine and the internal standard, respectively. With this ionisation technique the LOQ was 2.5 nmol/l blood which was not sensitive enough for complete pharmacokinetic evaluation of the drug. If the chemical background from the samples and from column bleeding could be lowered, this would improve the signal-to-noise ratio and thus the sensitivity by lowering LOQ. Matrix interference is of less importance when quantification is carried out using a

Table 1
Extraction recoveries and relative standard deviations (C.V.) of clevidipine, MI and their internal standards

| | Concentration (nmol/l blood) | Absolute recovery (%) | C.V. (%) | *n* |
|---|---|---|---|---|
| Clevidipine | 1.3 | 84.1 | 3.1 | 8 |
| | 31.2 | 86.0 | 2.7 | 8 |
| | 125 | 92.8 | 3.1 | 8 |
| [$^2H_6$]Clevidipine | 54 | 86.4 | | |
| MI | 217 | 84.3 | 4.7 | 10 |
| | 2600 | 82.2 | 2.2 | 10 |
| | 31200 | 85.4 | 1.5 | 10 |
| MI-IS | 4600 | 100.5 | | |

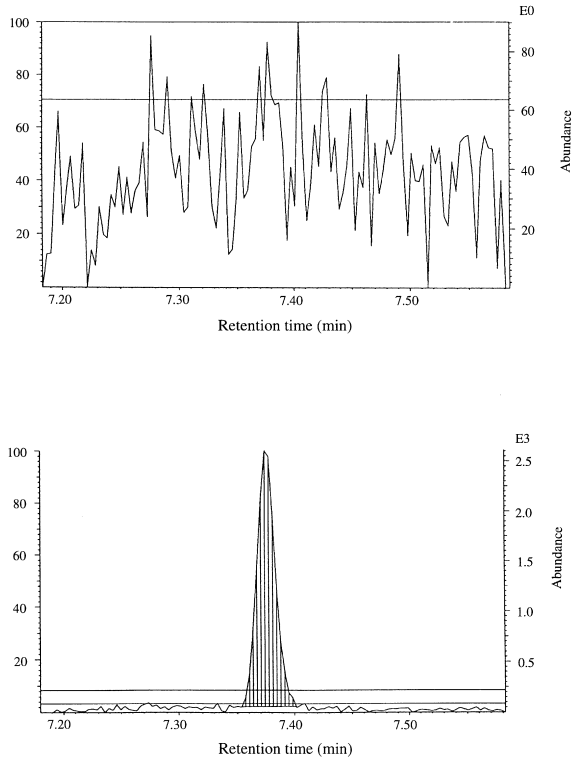


Fig. 2. Reconstructed ion monitoring ($m/z$ 455) chromatograms from blood samples taken before (upper) and after (lower) a dose of clevidipine. The measured concentration of clevidipine in the blood sample was 2.5 nmol/l. Note that each chromatogram has been normalised to the highest peak.

fragment ion at a high mass and one way to achieve this could be to use a softer ionisation technique like chemical ionisation.

Using ammonia as reaction gas for EC-NICI produced a stable signal with no problem with stability when analysing large batches of samples with between 80 and 100 injections in one run. The opposite was found when methane was used with sensitivity decreasing with every injection. Figure 5 displays the mass spectrum of clevidipine using electron capture negative ion chemical ionisation where the ion $m/z$ 455 corresponds to the molecular radical ion ($m/z$ 461 for the internal standard). With this ionisation technique, LOQ was decreased to 0.5 nmol/l blood and if necessary even further down to 0.1 nmol/l by using more sample (1 ml of blood mixture), reconstitution in a smaller volume of toluene (50 μl) and by injecting 3 μl onto the column. This was, however, not straightforward due to interfering peaks in the chromatogram and LOQ was routinely set at 0.5 nmol/l. The extract can be further cleaned by another liquid–liquid extraction, which was done when clevidipine was assayed by GC–MS in MtBE/toluene extracts primarily intended for analysis of MI. A total of 3 ml of the organic phase was shaken for 10 min with 0.5 ml of 0.1 mol/l $Na_2CO_3$ to remove interfering co-extracted compounds before evaporation and reconstitution in toluene.

### *3.4. Chromatography of clevidipine and MI*

An SE-54 stationary phase for capillary gas chromatography was first tested, but there was severe interference of the clevidipine peak from a huge cholesterol peak, which is why a more polar phase SPB-20 was chosen. MI itself was run on a $C_{18}$ liquid chromatographic column with 45% acetonitrile in acetate buffer, pH 5.0, as mobile phase. In certain preclinical safety studies clevidipine and MI were determined simultaneously by liquid chromatography with a LOQ of 50 nmol/l for both. Then a $C_8$ stationary phase and a mobile phase using a stepwise acetonitrile gradient from 45 to 65% in phosphate/citrate buffer, pH 5.7, was applied where MI eluted isocratically at 45% and clevidipine at 65% acetonitrile. The acidic metabolite, MI, could also have been determined together with clevidipine using GC–MS. This would require derivatisation of the carboxylic acid, and the obvious choice would be an alkylation to transform the acid into an ester, e.g. by using diazomethane as suggested by Ohtake et al. [3]. This was, however, never tried for two reasons. Performing derivatisations can be laborious and there is a risk of producing the same derivative from clevidipine itself. We also knew from experience that the difference in concentration between clevidipine and the metabolite would be so large that both concentrations could not be determined from the same injection using GC–MS. When a lower LOQ was required for clevidipine in pharmacokinetic studies we therefore used GC–MS only for clevidipine and kept a simplified isocratic LC method for MI.

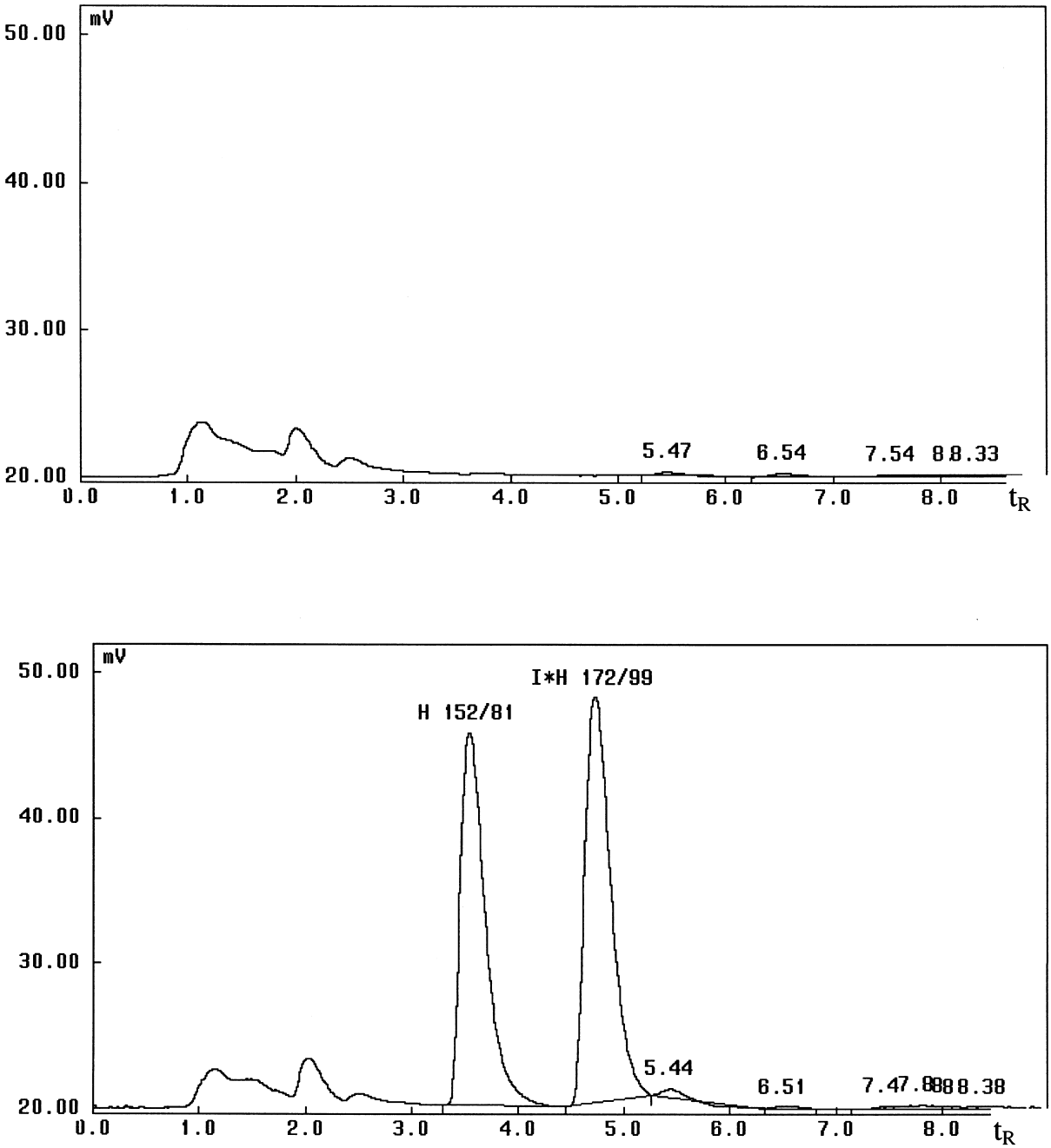


Fig. 3. Chromatograms (MI) from blood samples taken before (upper) and after (lower) a dose of clevidipine. The measured concentration of MI in the blood sample was 2380 nmol/l.

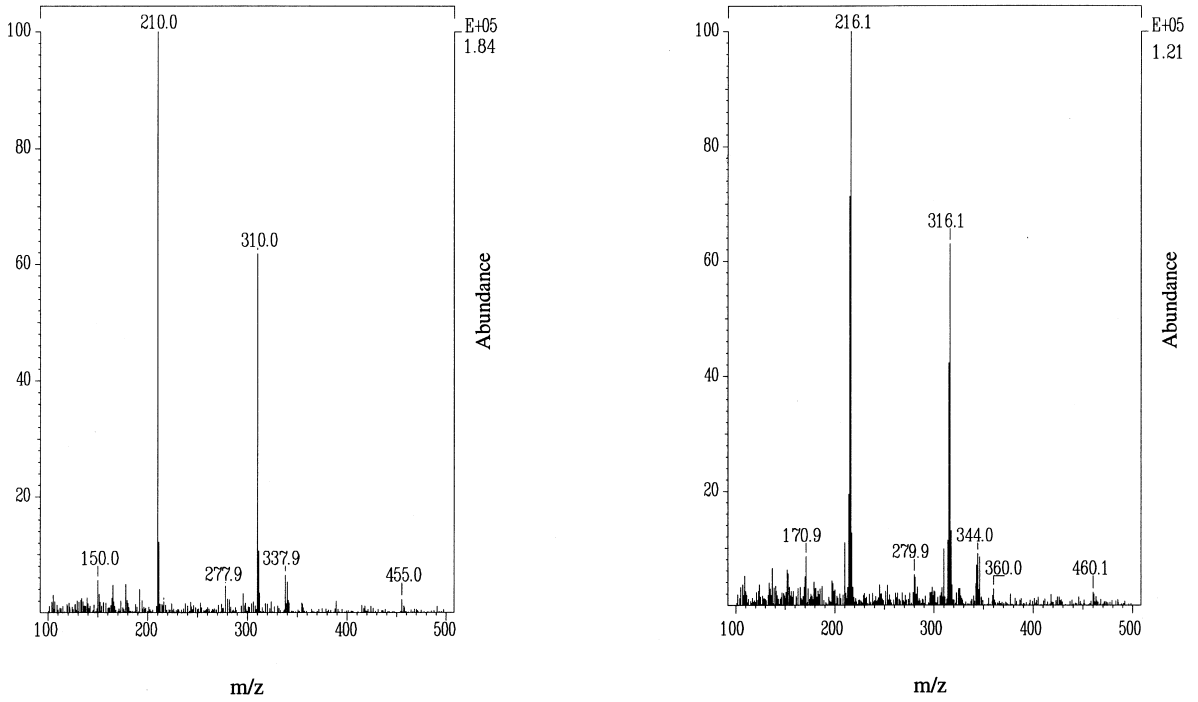


Fig. 4. Electron impact ionisation (EI) mass spectra of clevidipine (left) and the internal standard [$^2H_6$]-clevidipine (right).

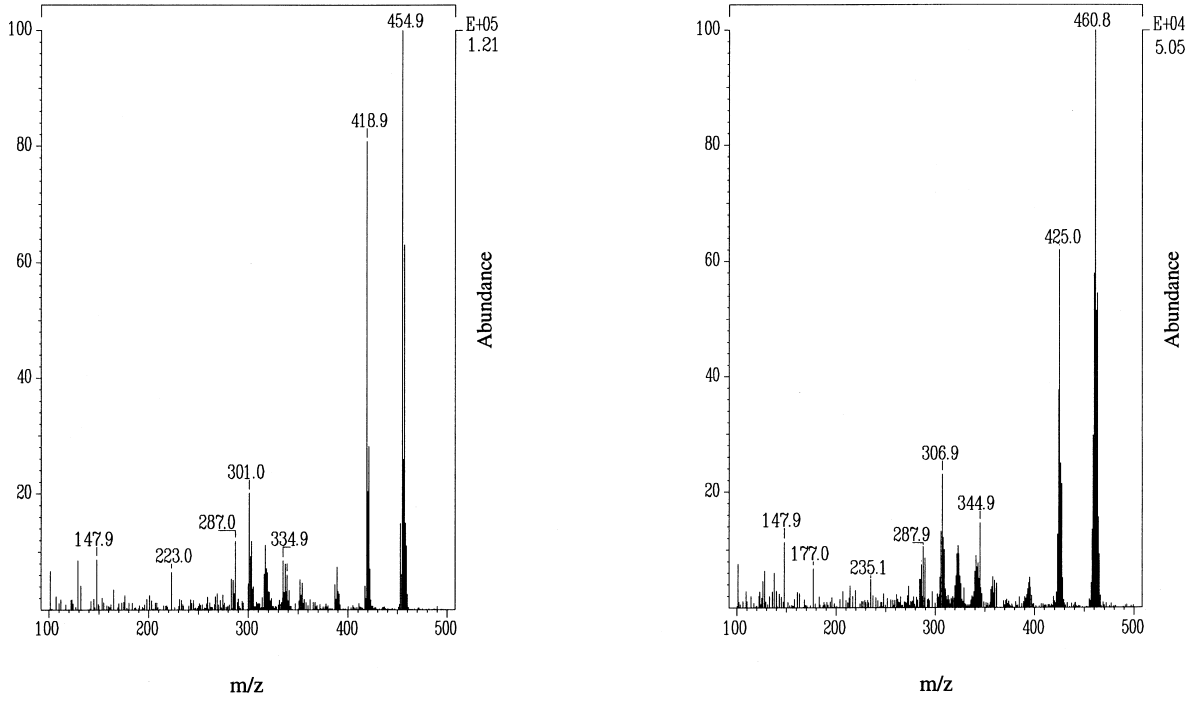


Fig. 5. Electron capture negative ion chemical ionisation (EC-NICI) mass spectra of clevidipine (left) and the internal standard [$^2H_6$]-clevidipine (right).

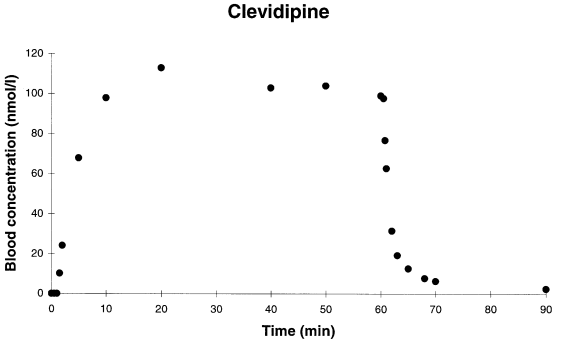


Fig. 6. Blood levels of clevidipine after a dose of clevidipine given to a human volunteer as a constant rate intravenous infusion during 60 min. The blood concentration is further monitored after stop of infusion for 30 min.

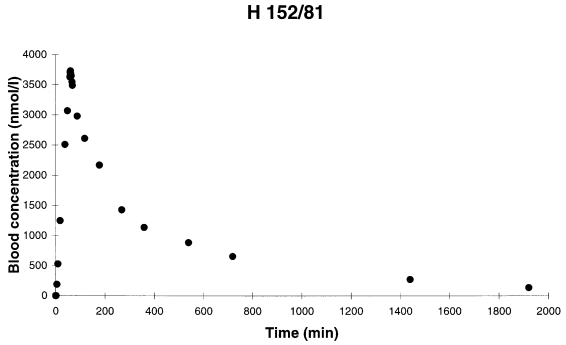


Fig. 7. Blood levels of MI after a dose of clevidipine given to a human volunteer (same as above) as a constant rate intravenous infusion during 60 min. The blood concentration is further monitored for 31 h after stopping the infusion.

## 4. Conclusions

The methods described have been applied to analysis of clevidipine and MI in blood from both animals and humans in pharmacokinetic studies. In Figure 6 the blood concentration versus time from a healthy volunteer receiving a constant rate intravenous infusion of clevidipine during 1 h is shown. As shown, the concentration of clevidipine was also monitored after stopping the infusion. Figure 7 shows the initial accumulation of MI and the slower elimination when the infusion of clevidipine was stopped.

## Acknowledgements

We would like to thank Dr. Bengt-Arne Persson for encouraging support and help in revising the manuscript.

## References

[1] M. Ahnoff, B.-A. Persson, J. Chromatogr. 531 (1990) 181.
[2] W. Mück, H. Bode, Pharmazie 49 (1994) 130.
[3] Y. Ohtake, T. Sakamoto, R. Nishioka, K. Uno, S. Tabata, Drug Invest. 4 (1992) 528.
[4] G.-B. Holm, K. Kylberg-Hanssen, L. Svensson, Clin. Chem. 31 (1985) 868.