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Determination of felodipine in plasma by capillary gas chromatography with electron capture detection

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Abstract: Felodipine in plasma was extracted with toluene and determined by automated capillary gas chromatography with electron capture detection. Undesirable oxidation of the dihydropyridine derivative in the injector and in the column was avoided by the use of glass materials with the inner surfaces treated by high-temperature silylation. The day-to-day reproducibility of the method was represented by a relative standard deviation (RSD) of 5% for a felodipine concentration of 25 nmol/l. The minimum determinable concentration (giving better than 15% RSD) was 1–2 nmol/l (0.4–0.8 ng/ml).

Keywords: *Felodipine; dihydropyridine; capillary gas chromatography; electron capture detection; high-temperature silylation.*

Introduction

Felodipine (Fig. 1) is a new derivative of dihydropyridine that has been developed for use as a selective vasodilator in cardiovascular disorders, primarily in arterial hypertension [1].

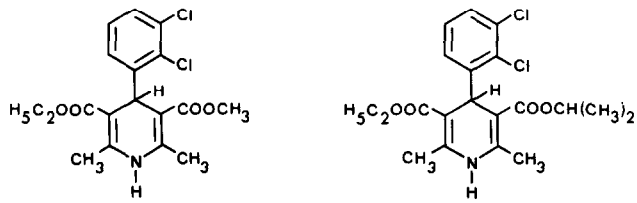


Figure 1
Structure of felodipine (left) and the internal standard H 165/04 (right).

Methods for the determination of the dihydropyridines nifedipine, nicardipine and nitrendipine in biological samples include gas chromatography with mass spectrometric detection [2–4], electron capture detection [5–10], thermoionic detection [11] or flame ionization detection [12], and liquid chromatography with spectrophotometric detection [13–15]. A radioceptor assay has also been reported for the determination of nitrendipine in serum [15].

The limited stability of the dihydropyridines against oxidation was pointed out by Higuchi *et al.*, who observed partial oxidation of nicardipine [5] and nifedipine [2] after injection on their packed OV-1 columns, which made quantitative determination difficult. Chromatography was therefore carried out after oxidation with nitrous acid to the corresponding pyridine derivative. Consequently, the sum of the dihydropyridine and possible pyridine derivatives was measured. Later the method was made selective for the dihydropyridine derivative by pre-separation from the pyridine derivative on thin layer plates [4].

Nevertheless Jacobsen *et al.* [6] and others [8, 9, 12] have determined intact nifedipine, using packed OV-17 or OV-101 columns. Of these only Dokladalova *et al.* [8] observed partial oxidation of nifedipine. They found about 2 ng/ml of the pyridine derivative in samples spiked with 200 ng/ml of nifedipine.

At an early stage of the present work, it was realized that: (i) oxidation of felodipine may occur in the gas chromatographic injector and column; and (ii) that the pyridine derivative is a metabolite which occurs at a level (relative to that of felodipine) of less than 2% in rat plasma, 12–35% in dog plasma and 100–220% in human plasma. It was evident that a method for the determination of felodipine in plasma with good precision and accuracy was needed in which oxidation of felodipine in the injector and on the chromatographic column was controlled.

Typical maximum concentrations of felodipine in the plasma of patients are 20–40 nmol/l (for a 10-mg dose); the plasma concentrations 24 h after intake are about 2 nmol/l. A minimum determinable concentration (corresponding to a relative standard deviation better than 15%) of 1 nmol/l was considered desirable. It was also considered important that the method should be suitable for a large number of samples.

Experimental

Chemicals

Felodipine (5-ethyl-3-methyl-4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate) (Fig. 1) was obtained from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden), which also supplied the other dihydropyridine and pyridine derivatives used in this work, including the internal standard H 165/04 (3-ethyl-5-(1-methylethyl)-4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate) (Fig. 1). Stock solutions were about 100 μM in methanol and were stored at −18°C. Toluene was HPLC grade from Rathburn (Walkerburn, UK), redistilled when necessary.

Apparatus

The gas chromatograph employed was either a Varian model 3700 or a Hewlett–Packard model 5730 equipped with a ^{63}Ni electron capture detector. For use with capillary columns, the Varian instrument was equipped with a DANI PC-IN 68/156 injector permitting up to 5 μl of sample to be injected in the splitless mode. A model 18740B capillary injector system was used on the Hewlett–Packard instrument. Both instruments were used with autosamplers.

Capillary column. Capillary columns were prepared from 25 m × 0.32 mm i.d. fused silica tubing (Quartz & Silice, Courbevoie, France). Capillaries for coating with CP^{tm}Sil 5 were rinsed with methanol and dried under nitrogen flow at 300°C. A plug of

octamethylcyclotetrasiloxane (ICN Pharmaceuticals, Plainview, NY, USA) was pushed through the capillary, which was then sealed, heated slowly and held at 400°C for 4 h. Capillaries for coating with CP^{tm}Sil 8 were rinsed overnight with 5–10 ml of 1% hydrochloric acid at room temperature and dried at 300°C. They were silylated with diphenyltetramethyldisilazane (Fluka, Buchs, Switzerland), following the same procedure as above. After rinsing with toluene, methanol and diethyl ether, the capillaries were statically coated with CP^{tm}Sil 5 or CP^{tm}Sil 8 (Chrompack, Middleburg, The Netherlands) in *n*-pentane (2 mg/ml).

Procedure for plasma samples

The plasma sample (1 g) was mixed with 1 ml of water and 1.00 ml of toluene containing the internal standard (about 100 nmol/l) in a test tube. After mechanical shaking for 45 min, the sample was centrifuged, the aqueous phase was frozen and the extract decanted into a sample vial. A 3-μl aliquot was injected onto the gas chromatograph.

Standards were prepared daily by adding 10 μl of a 4-μmol/l methanolic solution of felodipine to 1 g of blank plasma. These reference samples containing 40 nmol/l of felodipine were analysed together with the other samples. The peak height ratios of felodipine to the internal standard were used for quantitative evaluation.

Results and Discussion

Gas chromatography

Felodipine may elute as a tailing peak both on packed and open tubular capillary columns. In addition, on-column oxidation of the dihydropyridine to the corresponding pyridine derivative will give rise to a more or less easily observable plateau (or raised baseline) in front of the main peak (Fig. 2). Oxidation may also occur in the injector, resulting in a distinct peak for the pyridine derivative, as illustrated in Fig. 2. Oxidation cannot be eliminated solely by using oxygen filters in the carrier gas line. Whereas

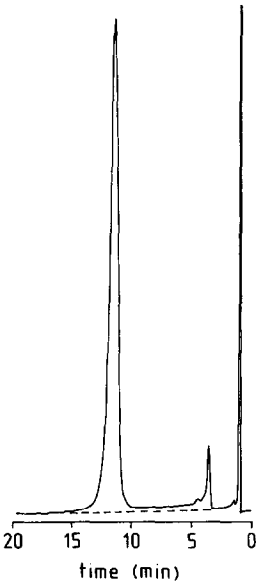


Figure 2
Chromatogram obtained for 40 pmol of felodipine injected onto a 2 m × 2 mm i.d. column of borosilicate glass, silanized with dimethyldichlorosilane at room temperature and packed with 3% OV-17 on GasChrom Q. The peak at 4.4 min and the plateau between 4.4 and 12 min are attributable to the pyridine derivative H 152/37 formed in the injector and on the column, respectively.

oxidation in the injector is caused by active glass or fused silica surfaces, oxidation on the packed column may be promoted by the support material as well as by the wall of the column.

Silylation at room temperature with dimethyldichlorosilane in toluene or by injecting silyl reagents onto the gas chromatograph has no effect on the factors causing oxidation. However, acid washing at room temperature followed by static gas phase silylation with hexamethyldisilazane at 400°C gives efficient and durable deactivation, suitable for injector liners and glass tubes for packed columns. Quick and efficient but less durable deactivation can be obtained by treatment with benzyltriphenylphosphonium chloride (10 mg/ml in dichloromethane) [16].

Suitably inert capillary columns may be prepared by high-temperature silylation [17, 18] of fused silica capillaries, followed by coating with non-polar silicone phases such as CP™Sil 5, a methyl silicone phase, or CP™Sil 8, a methyl silicone with 5% phenyl substitution. Capillary columns of acceptable quality are also commercially available.

Oxidation in the injector may be avoided by the use of cold on-column techniques [11, 19]. However, they are not currently compatible with automatic injectors. Moreover, they introduce all the non-volatile components in biological extracts onto the column.

Detection

Felodipine possesses good electron-capturing properties due to the 2,3-dichlorophenyl group. The response is only slightly dependent on the detector temperature between 300°C and 380°C, and is about 2.5 times lower than the response to nifedipine. With the instruments used and in the temperature-programmed mode, 0.5–1 fmol on-column (0.2–0.4 pg) could be detected with a signal-to-noise ratio of 3:1.

Extraction properties of felodipine

The hydrophobic nature of felodipine is reflected by the high values of the distribution constant K_D for partition between organic solvents and water. For toluene/water at room temperature the measured distribution constant, log K_D, was 4.52 ± 0.06 (n = 3). For cyclohexane/water, log K_D was 3.20 ± 0.06 (n = 3).

Extraction from plasma

Extraction from plasma was slower than from water. After 10 min of mechanical shaking with toluene, the recovery from aqueous buffer solution was complete. The recovery from plasma was 92–96% but increased to 98–99% after 40 min. With cyclohexane, however, the recovery after 40 min was only about 50%. Prolonged shaking did not increase the recovery.

For routine work, shaking with toluene for 45 min at 220 rpm was adopted. The dilution of 1 ml plasma with 1 ml water served to facilitate phase separation as well as to speed up the extraction process (cf. [20]). It seemed unwise to add any internal standard to the plasma sample prior to extraction to compensate for incomplete extraction, because extraction rates are different for analogues that differ by only one methylene group. Therefore the internal standard was added directly to the extraction solvent. Plasma samples with known amounts of felodipine were used as reference samples.

In an attempt to improve phase separation, sodium chloride was added to the sample, but without any beneficial effect. It was noticed that when the salt was added prior to extraction, the extraction rate decreased drastically: this effect was probably due to a salting-out effect. The fraction of felodipine in the plasma water, which corresponds to

1–3% of the total concentration of felodipine in human plasma, is lowered and the transfer of the substance from plasma proteins via the plasma water to the organic phase is hindered. The slow extraction from plasma of some strongly lipophilic drugs has been discussed by Vessman [20].

Chromatographic analysis of plasma samples

Provided that the chromatographic resolution is complete, the signal-to-noise performance of capillary chromatography allows accurate determination of 3 fmol (1 pg) injected on-column. It is then possible to analyse a plasma sample that contains 1 nmol/l of the drug by injecting only 3 μl of the plasma extract without any preconcentration. Thus 1 ml of plasma is extracted with 1 ml of toluene, the plasma is frozen and the extract transferred into autosampler vials. About 0.6 ml of the extract is recovered which is enough for repeated injections with the autosampler.

When the chromatographic separation is incomplete, this will determine the actual value of the minimum determinable concentration (m.d.c.). The m.d.c. is here defined as the lowest concentration which can be determined with a relative standard deviation (RSD) of less than 15%. With 2 m packed OV-101 or JXR columns, m.d.c. values of 5–10 nmol/l were achieved for about 95% of all sample batches; other batches showed interfering peaks from the plasma. With a capillary column coated with the methyl silicone phase CP^{tm}Sil 5, an m.d.c. of 2 nmol/l was achieved for most sample batches, although intermittent plasma interference still occurred. By using a methyl silicone phase with 5% phenyl substitution (CP^{tm}Sil 8), the chromatographic separation was further improved so that an m.d.c. of 1–2 nmol/l could be maintained for routine work. Close control of the instrumental performance was, however, necessary to enable the lower limit to be achieved consistently.

Figure 3 illustrates the chromatograms obtained for blank plasma, spiked blank plasma and for an authentic plasma sample. The latter also shows the pyridine metabolite H 152/37. Resolution between felodipine and the internal standard is seen to be satisfactory.

The precision of the analysis, as measured by repeated injections of the same sample, was 2–3% RSD (n = 10) for plasma concentrations above 2 nmol/l. The lifetime of the CP^{tm}Sil 5 columns was 5–10 months in continuous use, corresponding to 3000–6000 injections. The CP^{tm}Sil 8 columns could be used for 1000–3000 injections. On occasions about 0.5 m was removed from the inlet end of the column in order to remove contaminants. The injector liner was cleaned monthly and the septum was replaced every two weeks.

Method validation

Selectivity. Urinary metabolites have been investigated by Weidolf *et al.* [21] and plasma metabolites are currently under investigation. Figure 4 describes some potential metabolites of felodipine, among which only H 152/37 has been found and identified in plasma. Metabolites which can be extracted under the conditions employed are those with no free carboxylic group. They were injected onto an OV-101 packed column at 240°C. Retention times relative to felodipine ranged from 0.39 to 0.89. Thus none of the metabolites was found to interfere with the determination of felodipine.

Linearity. The electron capture response (as peak height ratios, h/h_{is}) was linear

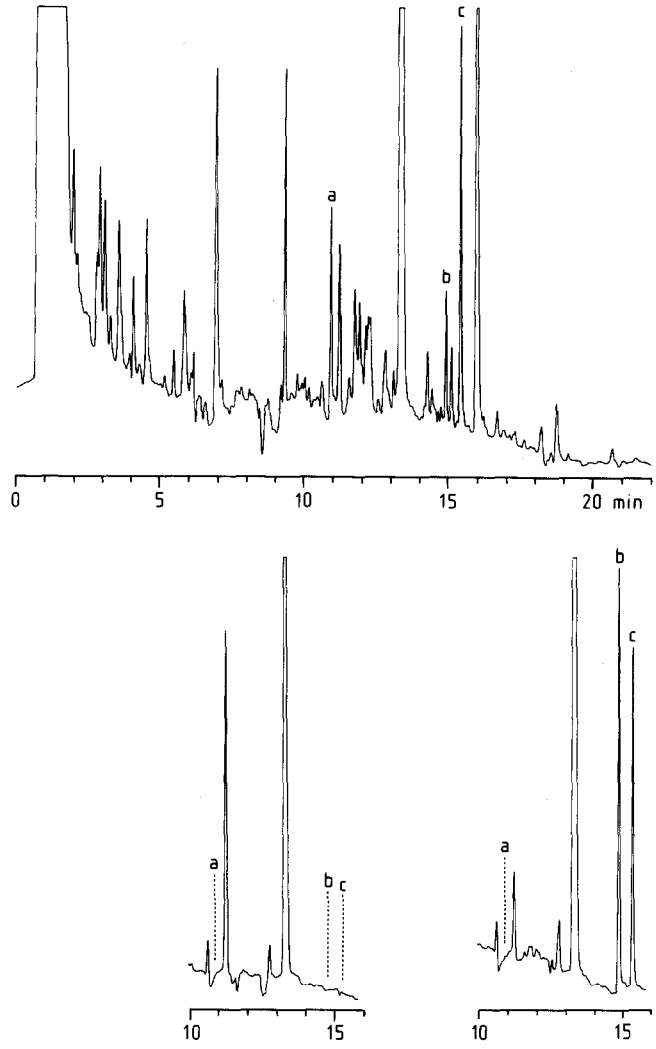


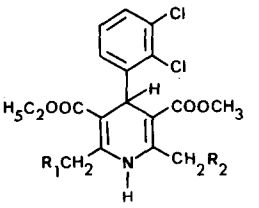
Figure 3
Chromatograms for human plasma. a: metabolite H 152/37; b: felodipine; c: the internal standard H 165/04. Upper chromatogram: authentic sample containing 11 nmol/l of felodipine. Lower, left: plasma blank. Lower, right: plasma spiked with 40 nmol/l of felodipine (b). Chromatographic conditions: 3 μl was introduced by splitless injection onto a 25-m CP™Sil 8 column; 2 min after injection the column temperature was programmed at 8°C/min to 270°C. Carrier gas: He at 1.2 bar pressure. The injector and electron capture detector were maintained at 270°C and 350°C, respectively.

between 2 nmol/l and 100 nmol/l which corresponds to the therapeutic range: $y = 0.00936\ x + 0.0213$; SE in gradient = 0.591×10^{-4}; $n = 24$.

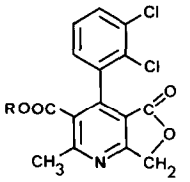
Within-run precision. The within-run RSD of spiked plasma samples was 4–8% at 2 nmol/l ($n = 5$) and 2–4% at 40 nmol/l ($n = 5$).

Between-run reproducibility. Several batches of control samples containing 25 nmol/l of felodipine were analysed, each over a period of about 2 months. The RSD values were close to 5% ($n = 40$). Over a period of one month, 21 authentic plasma samples with a

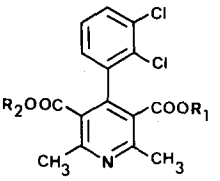
	R_1	R_2	Relative retention
Felodipine	H	H	1.00
H 167/98	H	OH	0.886*
H 182/24	OH	H	0.894*



	R	Relative retention
H 182/26	CH_3	0.60
H 152/77	C_2H_5	0.71



	R_1	R_2	Relative retention
H 152/37	CH_3	C_2H_5	0.39
H 152/66	CH_3	H	- - - -
H 167/87	H	C_2H_5	- - - -
H 152/57	H	H	- - - -



*These hydroxylated substances undergo partial cyclization when injected to form the lactones H 152/77 or H 182/26.

Figure 4
Structure of some substances related to felodipine and their chromatographic retention at 240°C on a column packed with 3% OV-101 on GasChrom Q.

found concentration of felodipine of 0.5–2.0 nmol/l were reanalysed. The mean day-to-day reproducibility for concentrations in this range, expressed as standard deviation, was ±0.25 nmol/l (n = 21).

Stability of felodipine in plasma. Plasma samples spiked with 76 nmol/l of felodipine and stored 0–21 weeks at −18°C were analysed. Linear regression analysis of found concentrations versus time for 71 samples showed a very small positive slope, 0.031%/week, which was not statistically significant (t = 0.49, $2{\cdot}p$ = 0.63). The 95% confidence interval of the slope varied from −0.2%/week to + 0.4%/week. Storage of samples for 5 months at −18°C was considered to be acceptable.

Conclusion

The use of capillary gas chromatography and electron capture detection provides high separation efficiency together with sensitive detection of felodipine. This permits a simple procedure for its determination in plasma samples containing 1 nmol/l or more of the drug, without the need for clean-up or preconcentration of extracts. The oxidation of felodipine in the capillary injector and column is minimized by the use of high-temperature silylated materials.

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