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COMPARISON OF HIGH-SELECTIVITY GAS CHROMATOGRAPHIC METHODS, INCLUDING COLUMN SWITCHING, FOR THE DETERMINATION OF FELODIPINE IN PLASMA

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SUMMARY

The selectivity required for the determination of low concentrations of felodipine in plasma was achieved by either (1) mass-selective detection, (2) optimization of stationary phase selectivity or (3) column-switching gas chromatography (GC) with a dual-oven chromatograph, the latter two with electron-capture detection. The three approaches were evaluated in terms of selectivity, detectability, precision and suitability for routine applications with automated injection.

Using mass-selective detection, the detectability in plasma samples was limited by the performance of the mass spectrometer. The detection limit (signal-to-noise ratio = 3) was 4.7 pmol (1.8 pg) of felodipine. Pre-concentration of extracts permitted quantitation in plasma down to 0.2 nmol/1. Using electron-capture detection, the detectability was determined by the selectivity and bleeding characteristics of the columns. For single-column separation, a 35% phenyl phase was selected. The detection limit was 3.0 fmol (1.2 pg). The limit of quantitation in plasma was 1 nmol/1.

In column-switching GC, bleeding products from the first column will separate on the second column and may interfere in separations for trace analysis. Bleeding products from a 50% phenyl phase (DB-17) were characterised by GC–mass spectrometry. With a dual-column system, employing a DB-17 (50% phenyl) column for selective introduction on to a CP-Sil 5 (0% phenyl) column, the signal-to-noise ratio was limited by the low-bleeding second column, provided that the bleeding products from the first column were adequately separated from felodipine. The detection limit in this instance was significantly lower 0.35 fmol (0.13 pg). Direct injection of plasma extracts permitted quantitation down to 0.4 nmol/l. All three methods were well suited for use with autosamplers.

INTRODUCTION

The required selectivity of a method increases with decreasing analyte concentration in a complex sample matrix, such as blood plasma. Apart from pre-chromatographic and chromatographic separation, the use of more or less selective detection methods is necessary, such as electron-capture (ECD), nitrogen-selective and mass-selective types. When separation is troublesome, a straightforward approach is

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to use mass-selective detection. If electron-impact ionization and positive ion monitoring do not give the desired sensitivity or selectivity, chemical ionization and negative ion monitoring may be exploited. For a number of drug substances, the ECD sensitivity (signal-to-noise per unit amount injected) compares favourably with that of mass spectrometry (MS) in the ion monitoring mode. The selectivity of ECD is, however, considerably lower and stricter requirements are therefore placed on the separation. Although elaborate sample work-up may solve the problem, instrumental solutions are generally more attractive. Improving the separation by increasing the number of theoretical plates above that obtained by a standard column ($N = 10^5$) is not very effective. Insted, a first approach would be to search for a stationary phase with optimal selectivity. This may take a considerable time when the sample contains a large number of unidentified components that may appear in varying concentrations. A second approach would be to use a two-column system with selective transfer (heart cutting). In both approaches a number of factors have to be considered in order to combine adequate separation with high signal-to-noise performance of detection. For separations performed well above 200°C, column bleeding is especially important. When different stationary phases are selected for selectivity reasons, quality criteria regarding inertness and column effeciency must also be met.

The potential of so-called multi-dimensional GC for the separation of complex mixtures has been well demonstrated during the last decade. Among applications, qualitative analyses dominate, quantitative aspects of trace analysis (target compound analysis) being less often treated[1–7]. Although the heart-cut technique seems ideally suited for sensitive determinations of single compounds, signal-to-noise optimization of a dual-column instrument is more complex than for conventional systems.

Felodipine is a dihydropyridine-type calcium antagonist developed for use as a selective vasodilator in cardiovascular disorders, primarily in arterial hypertension[8]. Plasma concentrations during therapy are in the range 2–25 nmol/l (0.8–10 ng/ml). For an assay based on gas chromatography with electron-capture detection of the unchange substance the lower limit of determination is 2 nmol/l[9]. For pharmacokinetic studies it was desirable to measure plasma concentrations down to 0.5 nmol/l in order to follow the elimination of felodipine for 48 h after a single dose.

This paper compares the three approaches described above to increase further the assay sensitivity for felodipine in plasma samples: mass-selective detection (GC–MS), stationary phase optimization and electron-capture detection (GC–ECD) and column-switching GC with electron-capture detection (GC–GC–ECD). No efforts were made to force the assay sensitivity considerably below 0.5 nmol/l. Instead, emphasis was laid on method reliability during the routine analysis of large sample series with automated instruments. If possible, a concentration step was avoided, with two aims: to save labour and to save the instruments from unnecessary contamination.

EXPERIMENTAL

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard 5970B mass-selective detector was coupled to a Hewlett-Packard 5890 gas chromatograph via an open-split coupling. Chromatography was

performed on a 25 m × 0.20 mm I.D. fused-silica capillary column with methylsilicone or SE-54 stationary phase. The inlet pressure was 1.5 bar (helium). The column temperature was 120°C for 1 min, then programmed at 30°C/min to 270°C, where it was held for 8 min. The mass-selective detector was operated in the selective-ion monitoring (SIM) mode at m/z 238 and 244. The detector voltage was 2.2 kV and the open-split and connection-line temperature was 160°C.

Gas chromatography with electron-capture detection (GC–ECD)

A gas chromatograph (Hewlett-Packard 5730), equipped with a split/splitless injector and a ^{63}Ni electron-capture detector, was used. The injector glass liner was deactivated either by treatment with benzyltriphenylphosphonium chloride (BTPPC), dissolved in dichloromethane or by high-temperature silylation with hexamethyldisilazane in order to avoid oxidation of felodipine in the injector[9]. The 35% phenyl–65% methylsilicone column (30 m × 0.32 mm I.D.) (SPB-35 from Supelco, Bellefonte, PA, U.S.A., film thickness 0.25 μm) was temperature programmed at 16°C/min from 120 to 240°C, where it was held for 32 min.

Column-switching gas chromatography with electron-capture detection (GC–GC–ECD)

A SiChromat 2 dual-oven instrument (Siemens, Karlsruhe, F.R.G.) was equipped with two capillary columns, connected via a six-port coupling (Live-piece). A flame ionization detector was used as monitor detector. The instrument configuration is shown in Fig. 1. The final set-up included a column coated with a phenylmethylpolysiloxane phase in the first oven (DB-17, 30 m × 0.32 mm I.D., film thickness 0.15 μm, from J & W Scientific) and, in the second oven, a laboratory-made methylpolysiloxane column [25 m × 0.32 mm I.D., deactivated with octamethylcyclotetrasiloxane) and coated with 0.15 μm CP-Sil 5 (Chrompack, Deventer, The Netherlands)]. Flow switching and temperature programmes are shown in Fig. 2.

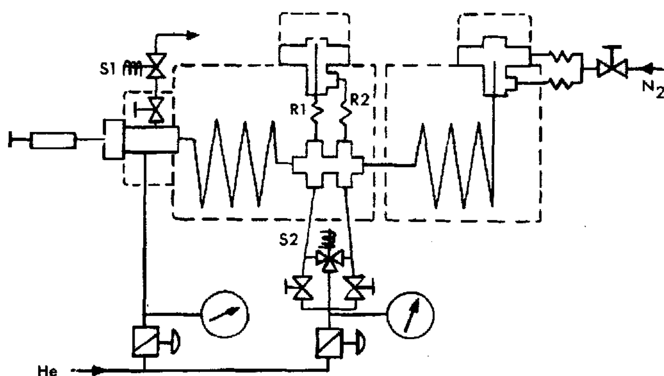

Fig. 1. Configuration of dual-column gas chromatograph with a flame ionization detector as monitor detector and electron-capture detection of separated heart cuts. Carrier gas pressure over first column, 2.15 bar; over column interface, 1.45 bar. S1 = solenoid valve for closing split, S2 = solenoid valve controlling the pressure difference over the interface; R1 and R2 = deactivated fused-silica capillaries (40 cm × 0.15 μm I.D.; helium flow-rate 20 ml/min.

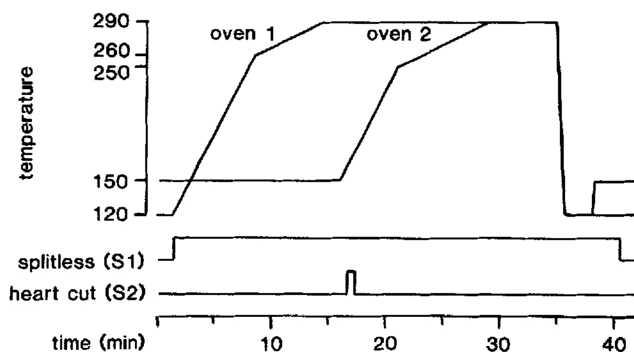

Fig. 2. Time programme for oven temperatures and the two valves, S1 and S2, used for splitless injection and column switching, respectively.

Mass spectrometric investigation of column bleeding

Bleed components from the DB-17 column held at 290°C were trapped on the CP-Sil 5 column held at 100°C when both columns were mounted in the SiChromat 2 instrument. The split/splitless injector had been cooled down and the columns were thoroughly conditioned before the DB-17 column effluent was transferred to the second column during a 30-min period. Then the second column was transferred to a GC–MS instrument (Model 44S, Finnigan-MAT, Bremen, F.R.G.). Repetitive scans were registered during a temperature programme similar to that used in the SiChromat 2 instrument for felodipine determinations.

Analysis of plasma samples

For GC–MS analysis, 1-ml plasma samples were extracted with 1 ml of toluene, to which deuterated felodipine ([$^2H_6$]felodipine; Department of Organic Chemistry, AB Hässle, Mölndal, Sweden) had been added. About 0.75 ml of the extract was recovered and concentrated to 100 μl. The volume injected was 5 μl (splitless).

For GC–ECD and GC–GC–ECD analysis, 0.5 ml of plasma was extracted with an equal volume of toluene. The internal standard was a dihydropyridine analogue, H 165/04[9]. The volume injected was 3 μl for the single-column instrument and 2 μl for the dual-column instrument. Autosamplers (Hewlett-Packard 7672A or Varian 8000) were used on all instruments.

RESULTS AND DISCUSSION

Comparison of the three methods

Signal-to-noise measurements after injection of 20–0.9 fmol of felodipine gave the lowest detection limit with GC–GC–ECD (Table I). Linearity was better with mass-selective detection than with electron-capture detection, where a tendency towards increased response at lower concentations was observed.

The minimum determinable concentration in plasma was a function of selectivity, detector sensitivity and the amount of sample injected. The last condition was deliberately chosen differently in order to obtain practical methods as discussed in the Introduction. The repeatability of the assay was within 4.0% (relative standard

TABLE I

PERFORMANCE OF INSTRUMENTS AND ANALYTICAL METHODS WITH MASS-SELECTIVE DETECTION (GC–MS), ELECTRON-CAPTURE DETECTION (GC–ECD) OR COLUMN-SWITCHING GC SEPARATION WITH ELECTRON-CAPTURE DETECTION (GC–GC–ECD)

Performance	*Parameter*	*Technique*		
		GC–MS	*GC–ECD*	*GC–GC–ECD*
Instrumental performance	Amount injected (fmol)	20	8.0	0.9
	Signal-to-noise ratio (S/N)	13	8	8
	Detection limit (S/N = 3)			
	fmol	4.7	3.0	0.35
	pg	1.8	1.2	0.13
		*A**	*B***	*C****
Assay performance	Level (nmol/l)	0.75	2.0	1.6
	Imprecision (%, R.S.D.)	6.5($n=6$)	6.4($n=7$)	3.7($n=10$)
	Level (nmol/l)	20	20	–
	Imprecision (%, R.S.D.)	2.1($n=8$)	3.9($n=5$)	–
	Minimum determinable concentration: nmol/l	0.2	1.0	0.4
	pg/ml	80	400	150
	Time between injections (min)	22	48	46

* Plasma extract concentrated by a factor 7; 5 μl injected.
** Direct injection of plasma extract; 3 μl injected.
*** Direct injection of plasma extract; 2 μl injected.

deviation) for all three methods at levels well above the minimum determinable concentration. The time between two injections was considerably shorter with GC–MS (Table I).

GC–MS

Quantitation in plasma samples was limited by detector noise with no correlation to the sample as long as the aliquot injected corresponded to 40 μl of plasma or less. The detection limit was slightly higher than that reported by Tokuma *et al.*[10] for felodipine using methane chemical ionization and negative ion detection (see Fig. 3).

GC–ECD

After having tested stationary phases with varying degrees of phenyl substitution, we selected a commercial column, SPB-35 with 35% phenylgroups, for optimal separation of felodipine from the plasma background. Column bleeding at elevated temperatures, causing increased detector noise, occurred with this column and with all other columns containing more than 5% phenyl. A low elution temperature was necessary to minimize noise and for maximum separation, resulting in a fairly long separation time (see Fig. 4). Automated peak integration permitted quan-

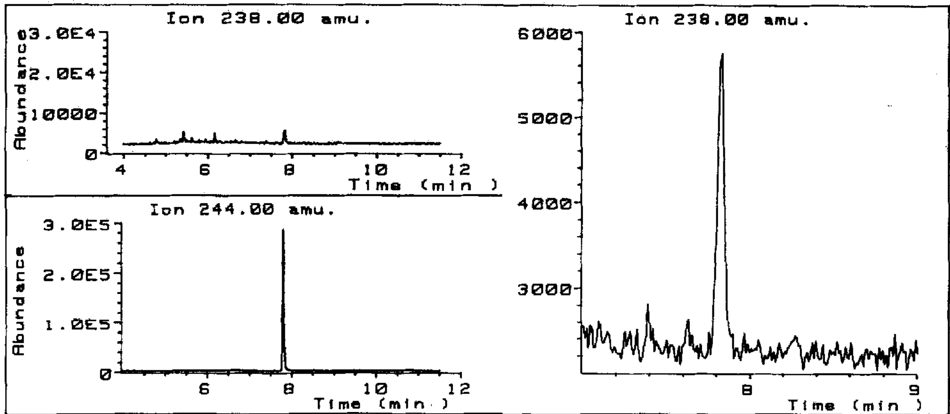

Fig. 3. Selected-ion monitoring at m/z 238 for felodipine and m/z 244 for [$^{3}H_{6}$] felodipine. Felodipine concentration in plasma: 0.5 nmol/l.

tification down to 1.0 nmol/l. Quantification down to 0.5 nmol/l required either manual peak-height measurements or pre-concentration of extracts to half their volume.

GC–GC–ECD

Optimized instrument set-up. The instrument was set up with two columns suitable for operation at up to 300°C and with a significant polarity difference. We chose a 50% phenyl phase (DB-17) for the first column and an unsubstituted methylpolysiloxane (CP-Sil 5) for the second column. The electron-capture detector operted with nitrogen as the purge gas. In the normal set-up, the detector also receives

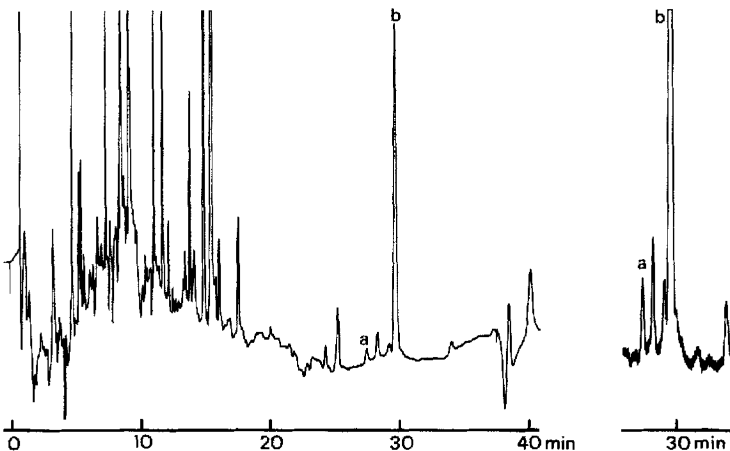

Fig. 4. Electron-capture detection of a plasma extract, separated on a 30 m × 0.32 mm I.D. SPB-35 column. (a) Felodipine; (b) internal standard. Felodipine concentration in plasma: 2.0 nmol/l. Right: same sample reinjected with four times lower attenuation of signal.

20–30 ml of carrier gas, the main part via a capillary restrictor from the column interface. As nitrogen, recommended by the manufacturer as the carrier gas for GC–ECD, would give excessively lengthy separations, helium was chosen. Gas flowing through retrictor R2 from the interface (Fig. 1) was led to the monitor detector to avoid high helium concentrations in the electron-capture detector. Restrictor R2, which under proper pressure conditions only conducts pure gas, was connected as make-up gas at the bottom of the flame ionization detector. Restrictor R1, which at times conducts the effluent from the first column, was positioned with its end close to the flame tip. The time lag thus obtained for a solute coming through restrictor R2 allowed pressure balancing with the use of only one detector for both restrictors.

The more polar SPB-35 column was tested in both the first and second positions. When used in the second position, column bleeding at high temperature (290°C) caused an unacceptable increase in the background signal and noise. Therefore, the low-bleeding CP-Sil 5 column was used in the second position. A temperature programme from 120°C was used for the first column to permit splitless injection of the sample dissolved in toluene (Fig. 2). Felodipine and the internal standard were eluted at 290°C with retention times of 16.80 and 16.95 min, respectively, and were transferred with one 0.65-min long heart cut to the second column held at 150°C. A similar temperature programme was used to elute the selected components from the second column. A chromatogram obtained from a plasma sample is shown in Fig. 5.

Problems caused by heart cutting from a column with significant column bleeding. Fig. 6 shows that bleeding from the first column eluted as separate peaks from the seond, low-bleeding column. During the heart cutting, a sample of the column bleed, with a band width equal to the time of the heart cut, was transferred to the second column. By choosing the start temperature for the second column to be *ca.* 50°C lower than that required for proper focussing of the analytes (150 instead of 200°C), the bleed components were focused and were eluted as sharp peaks. In our case the

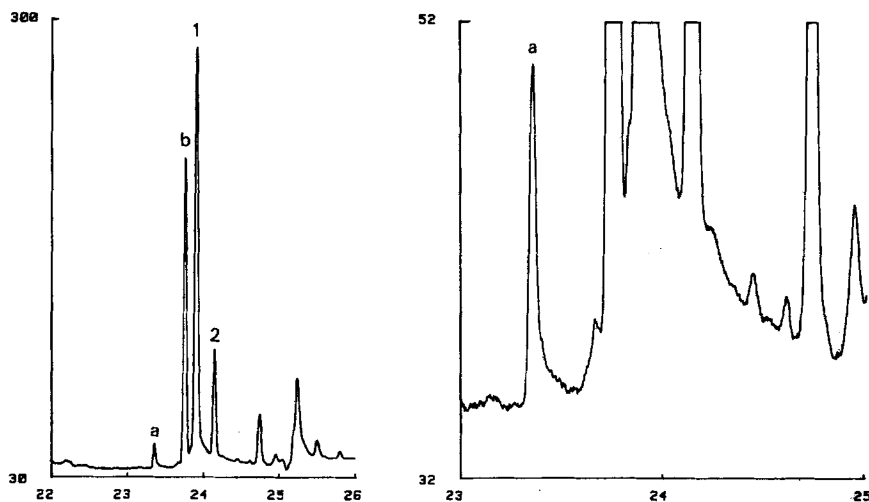

Fig. 5. Electron-capture detection of plasma extract after a two-stage separation where a 0.65-min heart cut was transferred to the second column. (a) Felodipine; (b) internal standard. Felodipine concentration in plasma: 1.6 nmol/l. Peaks 1 and 2 are the same as in Fig. 6.

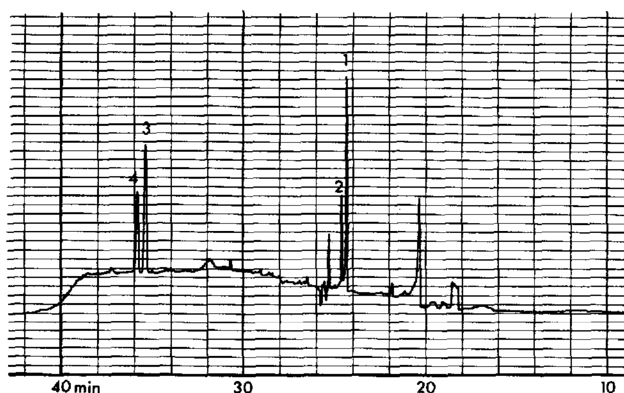

Fig. 6. Detector trace from an experiment as in Fig. 5, but without sample injection. Peaks 1–4 were identified by mass spectrometry as cyclic methylphenylsiloxanes (Table II).

chromatographic separation between felodipine, the internal standard and bleed components was adequate, so that felodipine was eluted and detected under conditions of very low detector noise, as shown in Table I.

Identification of column bleed components. Mass spectra were taken on the peaks shown in Fig. 6. Four of the peaks were identified as three- or four-membered cyclic siloxanes containing four or six phenyl groups and no, two or four methyl groups (Table II). The chemical structure of the DB-17 phase evidently contains diphenylsiloxane and dimethylsiloxane units, but not methylphenylsiloxane. Other phenyl phases, for example synthesized from methylphenyl monomers, would give other degradation products, resulting in a completely different pattern of disturbing peaks.

Artefacts in column-switching GC were observed by Ligon and May[7] in connection with very long periods for sample transfer between the columns. Thermal degradation products transfer between the columns. Thermal degradation products from stationary phases with low phenyl content were investigated by Schmidt *et al.*[11] using GC–MS.

TABLE II

MASS SPECTRAL DATA ON BLEED COMPONENTS ELUTED FROM A DB-17 COLUMN

Numbers refer to peaks in Fig. 6. B = base peak.

No.	*m/z*	*Tentative formula*
1	470(M$^+$), 455, 392, 377(B), 315	$Si_3O_3(CH_3)_2(C_6H_5)_4$
2	544(M$^+$), 529, 467, 251, 389(B), 373	$Si_4O_4(CH_3)_4(C_6H_5)_4$
3	594(M$^+$), 516, 439(B), 362	$Si_3O_3(C_6H_5)_6$
4	668(M$^+$), 653, 591, 575, 513, 497, 435, 218(B)	$Si_4O_4(CH_3)_2(C_6H_5)_6$

CONCLUSIONS

The high selectivity and sensitivity needed for the determination of felodipine in plasma at the 1 nmol/l level was achieved with all three methods, although differences between them were seen. The risk of unforeseen disturbing peaks should be smaller for the GC–MS method, followed by the coupled-column method (GC–GC–ECD). For the conventional GC–ECD method it might be necessary on rare occasions to check results using a different column. This method could not be used at levels below 0.5 nmol/l by injecting a larger portion of the sample, as could be done with GC–MS.

It was found that bleeding from the capillary column may be of major concern in trace analysis using coupled-column GC. Not only do bleeding products from the second column cause and increase in detector signal and noise during programming, but bleeding products form the first column contaminate the selectively transferred sample and will start to separate on the second column. In trace analysis, the actual amount of analyte in the injected sample may be considerably smaller than the amount of phase degradation products transferred together with the analytes. Although adequate separation may be achieved, as in this study, selective columns with very low bleeding are highly desirable in coupled-column GC used for trace analysis.

Excellent detectability was achieved with coupled-column GC and the desired assay sensitivity was achieved without pre-concentration of extracts. The pressure balancing necessary for proper switching did not require readjustment during operation over several weeks, and the instrument reliability was not different from that of conventional GC instruments.

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