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Journal of Chromatography A, 1018 (2003) 203–212
JOURNAL OF CHROMATOGRAPHY A
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Performance and reliability of splitless microliter gradient pumps in a metabolic stability study using cytochrome P450/3A4 and capillary liquid chromatography–mass spectrometry

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Received 30 December 2002; received in revised form 4 April 2003; accepted 1 July 2003

Abstract

The performance of two commercially available capillary LC pumps (MicroPro (Eldex, USA), Evolution 200 (ProLab, Switzerland)) generating really splitless gradients in the microliter per minute range was tested in detail concerning their applicability for routine drug discovery. A standard method to study metabolic stability against CYP450 isoform 3A4 was selected. This method was transformed into a fast splitless capillary LC–MS method. Both pumps generated reproducible gradients at flows of 5–10 μl/min within 10–15 min. Although gradient formation of the MicroPro system was very reproducible, its equilibration time was too long for fast gradients around 5 μl/min. The Evolution 200 pump offered a good performance with 180 μm i.d. columns at a flow rate of 6 μl/min. The precision of the retention time of the internal standard (ISTD) varied between 1.4 and 3.4% ($n = 131$–152, three different columns tested). Up to 800 injections of sufficient performance on one column and a stable enough response of the ISTD for 16 h sequence duration were obtained. Accuracy between 95 and 105% and precision ≤8.4% for 1′-hydroxylated midozolam were reached. The IC_{50} values of the miniaturized assay (drug candidate BAL4815 1.7 ± 0.5, itraconazole 0.46 ± 0.06, and ketoconazole 0.12 ± 0.01 μM) agreed well with those of the conventional approach. Details concerning method optimization and limitations in operation are discussed in detail. Still, the overall performance of the capillary LC pumps cannot cope completely with that of conventional HPLC pumps in terms of user-friendliness.

Keywords: Pumps; Midazolame; Ketoconazole; Cytochromes

1. Introduction

Packed columns with inner diameters (i.d.) in the micrometer or low milimeter range have several advantages for high-performance liquid chromatographic (HPLC) separations. Compared to conventional columns of 1.5–4.6 mm i.d., lower detection limits are obtained in combination with concentration sensitive detectors and less sample volume is required. Furthermore, higher ionization efficiency is obtained for mass spectrometry (MS) employing pneumatically assisted electrospray ionization (ESI), the most

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doi:10.1016/j.chroma.2003.07.013

widely applied ionization method in HPLC–MS [1,2]. Microcolumn methods are classified according to the internal diameter of the columns as nano-HPLC (10–150 μm), capillary-HPLC (150–500 μm), or micro-HPLC (0.5–1.5 mm) [3].

Protein and peptide analysis is still the primary application field of capillary- and nano-HPLC–MS combined with ESI [2]. Furthermore, analysis of pharmaceuticals in biological fluids is a growing field of microcolumn-HPLC–MS [4]. Novel, more active drugs demand analytical methods with increased sensitivity. Results in the literature demonstrate that quantification limits in the lower picogram per mililiter range are achievable using 0.4–1 ml plasma aliquots and column switching capillary-HPLC systems combined with ESI-MS–MS [5,6]. In addition, microcolumn-HPLC allows to obtain pharmacokinetic (PK) profiles from less sample volume. This enables in vivo studies with mice instead of larger rodents leading to a reduced consumption of drug, reagents and solvents [7]. Moreover, in vitro experiments can be scaled down.

Despite all these advantages, microcolumn-HPLC is still not routine in drug discovery. Up to now, the usual method for generating flows smaller than 50 μl/min was flow-splitting, since suitable pumps were still in the stage of development. A conventional HPLC pump was coupled to a T-piece, and the column and a flow restriction was mounted at either outlet. An advanced splitting device, such as the “Accurate” system from LC Packings (Amsterdam, The Netherlands), allows to compensate viscosity changes in the gradient mode by a micro processor [3]. However, this does not eliminate changes in the split ratio by a changed pressure resistance of the separation column by matrix deposits or by injection of viscous samples. For example, a significant increase of the retention time of the analyte was observed by Fraser et al. after only 60 consecutive injections of precipitated blood samples [7]. New systems which actively monitor the flow after the split, opens the possibility to adjust the split ratio if necessary (see, e.g. Agilent 1100 Capillary LC, Palo Alto, USA). Still then, hardly visible micro leaks may cause systematic deviations since no pressure drop and change of solvent consumption is observed when employing gradients.

During the past 2 years pumps have become commercially available, which should allow to generate solvent gradients at a flow of a few microliter per minute without splitting. The aim of this work was to investigate, if these new devices allow to develop a robust capillary HPLC–MS method to study metabolic stability against cytochrome P450 (CYP) isoform 3A4 enzyme with a comparable sample throughput and robustness as for HPLC on conventional columns. In a first step, different micro flow pumps were tested concerning the generation of reproducible gradients from 5 to 90% organic phase within 5–10 min at flow rates between 1 and 10 μl/min. Columns of 500 μm i.d. and restriction capillaries were used. A pre-evaluation excluded the *Ultra-Plus II* (Micro-Tech Scientific Inc., Vista, CA, USA) and the *CapLC* pump (Waters, Milford, MA, USA). The first did not fulfil the specifications and the second had a too high dwell volume (17 μl). A reduced phase one drug testing system (one isoform instead of seven) and a capillary of 180 μm i.d. were selected. The metabolisation of midazolam (MDZ; 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-*a*][1,4]benzodiazepine) by CYP 3A4 isoform was chosen. The aim was to evaluate the benefits and limitations of the splitless pump system and to investigate the possibilities to develop a robust and reproducible method analysing protein-precipitated samples from in vitro microsomal incubations in series for at least 12 h. Although, only 1′-hydroxylated midazolam is really necessary to determine IC_{50} values, (CYP3A4 highly prefers 1′ hydroxylation [8]), 4′-hydroxylated derivatives were included to investigate separation performance. Observed problems during method development and results of a comprehensive method validation are presented and discussed in detail.

2. Experimental

2.1. Chemicals

2.1.1. Origins

Midazolam, the 1-hydroxy derivative (1-OH-MDZ), the 4-hydroxy derivative (4-OH-MDZ), the 1,4-dihydroxy derivative (1,4-DiOH-MDZ), the 1-methyl derivative (MMDZ) and itraconazole (4-[4-[4-[4-[[2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3H-1,

2,4-triazol-3-one) were obtained from F. Hoffmann-La Roche (Nutley, NY, USA and Minato-ku, Tokyo, Japan). An azole drug candidate BAL4815 was obtained from Basilea Pharmaceutica Ltd. (Basel, Switzerland). Ketoconazole ((±)-*cis*-1-acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine) was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Pooled human liver microsomes were obtained from Gentest (Woburn, MA, USA). Nicotinamide adenine dinucleotide phosphate (NADP) was delivered by Sigma. Glucose-6-phosphate dehydrogenase from yeast (Grade II) was obtained from Roche Diagnostic (Mannheim, Germany).

$MgCl_2$ (purissimum), ammonium acetate (NH_4OAc, analytical grade), and ammonia solution 32% (extra pure) were provided by E. Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA, for protein sequence analysis) and dimethyl sulfoxide (DMSO, ≥99% GC) were obtained from Fluka (Buchs, Switzerland). Acetonitrile (ACN, 190 far-UV) was purchased from Romil (Cambridge, England) and methanol (pestipur) from SDS (Peypin, France). Water was processed by an Elgastat Maxima HPLC water purification unit (Elga Ltd., Bucks, England). He of 99.996% (Carbagas, Switzerland) and N_2 of 99.5% purity (NM30L high purity nitrogen generator, Peak Scientific Instruments, Ichinnan, Great Britain) were used.

2.1.2. Standards

Calibration solutions at 5, 7, 10, 50, 100, 150, 200, 250, and 300 ng/ml were prepared by spiking 198 μl of human liver microsome mixture (same composition as the stopped reaction solution described below) with 2 μl of the working standard solutions prepared in DMSO containing MDZ, 1-OH-MDZ, 4-OH-MDZ and 1,4-DiOH-MDZ. Quality controls (QCs) of 45 and 230 ng/ml of the same analytes were made analogously.

2.2. Instrumentation

The systems MicroPro (Eldex, Napa, CA, USA) and Evolution 200 (ProLab Instruments GmbH, Reinach, Switzerland) were tested extensively at the end of 1999. Both are dual channel pumps delivering a splitless binary gradient flow. The MicroPro is a two syringe non-reciprocating and the Evolution 200 a four syringe reciprocating pump. Although the manufacturer defines the Evolution 200 as a piston pump, it is technical more like a syringe pump since there is only one valve per piston (see Fig. 1A).

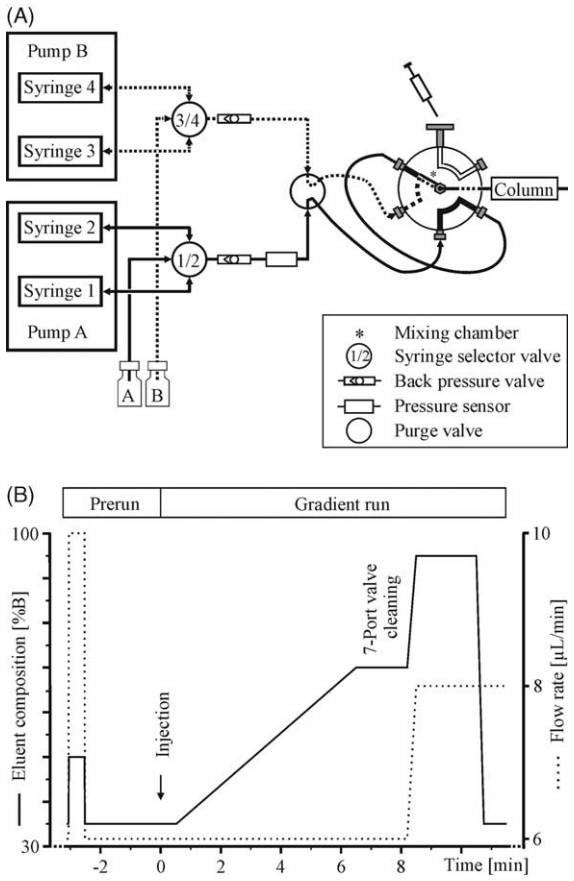

Fig. 1. (A) Schematic solvent flow path of the pump system Evolution 200 with 7-port injection valve in the inject position. (B) Gradient run scheme with additional pre-run phase for fast pressurization and equilibration ("piston initialization" mode on) as well as sequence for 7-port valve cleaning at constant eluent composition (see LC method MDZ).

The 7-port valve of the Evolution 200 pump was used for the MDZ method. This port had an internal mixing chamber and an external 1 μl loop rinsed by the polar eluent A only (see Fig. 1A). Samples were transferred with a PAL autosampler (CTC Analytics, Zwingen, Switzerland) overfilling five times the loop. The overall dead volume of transfer capillary (64 μm i.d.,

7.5 cm), precolumn filter (230 nl), and column inlet capillary (100 μm i.d., 7.7 cm) was ∼1.1 μl.

An ion trap mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA, USA) using ESI in the positive ion mode (ESI(+)) was employed.

2.3. Procedures

2.3.1. Gradient performance using acetone and UV detection

Conditions for test programme 1 are given in Table 1. The T-piece was connected to the restriction capillary coupled to the UV cell capillary (35.6 cm × 75 μm, 1.57 μl dead volume). Solvents were degassed with He for 5 min.

The instrument conditions of test programme 1 without gradient were used for the stepwise gradient test. The concentration of B was increased from 10 by 10% steps within 1 s for 1 min intervals up to 90% B. A 6 s return to 10% B was followed by 2 min equilibration. Since, the relative UV response varied somewhat with eluent composition, the theoretical gradient profile was plotted with corresponding corrections.

2.3.2. Gradient performance for selected drugs, midazolam and related substances

The HPLC separation conditions for the drug mixture (test programme 2) and the MDZ method are given in Table 1. The concentrations of the nine pharmaceutical compounds were 1–20 ng/μl. For injection an internal loop (200 nl) was used.

To achieve a dead time t_0 of <1 min for the MDZ method, which is essential for fast gradient, the same small and short transfer capillaries and the internal 7-port valve loop were employed as described under Section 2.2. In addition, the outlet capillary of the column was directly connected to the ESI source (see Section 2.3.6 for details). This reduced t_0 to 0.6 min even with a precolumn filter of 230 nl dead volume. The mobile phase was degassed with the Evolution 200 built-in degasser.

Table 1
Test programmes for evaluation of micro-flow pumps

	Test programme 1	Test programme 2	MDZ method
Flow rate (μl/min)	5	10	6
Eluents	A: water	A: 10 mM NH_4HCOO, pH 3	A: 10 mM NH_4OAc, pH 8.0 ACN (95:5, v/v)
	B: methanol with 2% acetone	B: ACN	B: ACN–methanol (95:5, v/v)
Gradients	0–1 min, 2% B 1–2 min, to 70% B 2 min, isocratic 4.0–4.1 min, to 2% B 5.9 min, isocratic	0–5 min, 5% B 5–10.5 min, from 5 to 70% B 1 min isocratic 11.5–12.5 min, to 5% B 5.5 min, isocratic	0–0.5 min, 35% B 0.5–6.5 min, to 70% B 6.5–8.2, isocratic 8.2–8.5 min, to 95% B and 8 μl/min 2 min isocratic 10.5–10.8 min, to 35% B 0.2 min, isocratic
Restriction capillary/column	55 cm × 25 μm i.d. fused silica (275 nl)	50 mm × 500 μm i.d., C_{18} phase ODS-4 HE, 3 μm spherical particles, 120 Å pore, monomeric, hydrophilic end-capped (GROM, Herrenberg/Kayh, Germany)	50 mm i.d. × 180 μm, C_{18} phase ODS-4 HE, 3 μm spherical particles, 120 Å pore, monomeric, hydrophilic end-capped (GROM)
Mixing chamber	Valco T-piece (≤1 μl)	Pump dependent	7-port valve internal (<300 nl)
Detection	UV detection (280 nm), capillary flow cell (35 nl, 8 mm path length)	Mass spectrometry	Mass spectrometry

2.3.3. Microsomal incubations

All volume additions were carried out with a Biomek 2000 pipetting workstation (Beckman, Fullerton, CA, USA). Incubation mixtures consisted of 0.45 mg/ml pooled human liver microsomes, 0.1 M potassium phosphate buffer (pH 7.4), NADPH generating system (1.07 mM NADP, 2.61 mM glucose 6-phosphate, 2.68 mM $MgCl_2$ and 1.15 U/ml glucose 6-phosphate dehydrogenase), 5 μM midazolam as probe substrate and the inhibitor substrate in a total volume of 200 μl. Concentrations of the inhibitor itraconazole and of the drug candidate were 0.069, 0.210, 0.620, 1.85, 5.56, 16.7, and 50.0 μM. A negative control incubation was included and ketoconazole was used as a positive control inhibitor at 10 times smaller concentrations. First, a solution of microsomes, phosphate buffer and midazolam was prepared, and 100 μl portions were transferred to 0.75 ml 96 well plate tubes (Matrix Technologies, Hudson, NH, USA). After 5 min pre-incubation at 37 °C, the inhibitors were added (2 μl each in DMSO). Addition of 100 μl generating system started the reaction, which was terminated after 15 min by 100 μl of cold methanol (−20 °C). Incubations were carried out in duplicate per inhibitor.

IC_{50} values were calculated from the sigmoidal fit function of Origin 5.0 (Mirocal Software, Northampton, MA, USA).

2.3.4. Clean-up

Aliquots (40 μl) of terminated reaction mixtures, standards and controls were transferred to 200 μl tubes of a "pcr" 96 microtiter plate (Eppendorf, Hamburg, Germany) washed with acetonitrile before use. An amount of 160 μl of a 25 ng/ml internal standard MMDZ (ISTD) solution in acetonitrile was added. Then, the plate was sealed with a thermo sealer (Heat Sealer, Eppendorf, Hamburg, Germany) and polypropylene sealing foil (Easy Peel, ABgene House, Surrey, UK), mixed for 1 min with a Vortex Genie 2 (Scientific Industries, Bohemia, NY, USA) and centrifuged for 10 min at 0 °C and 4000 rpm in a Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany). A total of 190 μl of the supernatant was transferred to a new microtiter plate and evaporated to dryness under a stream of 40 °C nitrogen with a micro-DS96 evaporation device (Porvair Science, Shepperton, UK). The residue was resolved in 30 μl of 10 mM NH_4OAc (pH 8.0) and the sealed plate centrifuged for another 5 min before analysis to move the liquid to the vial bottom.

2.3.5. Operation of the micropump Evolution 200

Software controlled compression steps were applied after refill of one syringe to minimise the pressure drop when switching to the full one. They had to be optimised manually and adjusted to gradient and corresponding viscosity changes during the gradient by selecting the parameters *m* and *b* of the compression step function "steps = ($m \times$ actual measured pressure) + *b*". This optimization can be quite time consuming (>1 h) due to the waiting period for a switch to the next syringe and several controls and readjustments at such low flows.

A 180 μm i.d. capillary and a flow of 6 μl/min was chosen for the MDZ method in order to operate the Evolution 200 close to the minimum flow rate necessary for fast and precise gradients. The pump was employed as a dual channel non-reciprocating syringe pump to avoid the time consuming optimization of compression steps. The syringes were refilled automatically before each run and all analytes eluted within one syringe volume. In this operation mode the initial pressurization is part of each run.

To accelerate this initial pressurization of the mobile phase about twofold, a short pre-run gradient was started before injection using the batch mode of the control software (Fig. 1B). It allowed to set the compression step for solvent channel A and B. During this pre-run, the pump was operated at 10 μl/min for the first 30 s at 50% B and then changed to 35% B and 6 μl/min within 1 s followed by a hold time of 1.5 min. After another minute, the sample was injected and the separation started. The pump automatically changed to initial conditions and refilled the syringes at the end of a gradient run or the beginning of a sequence. The hold time before the next injection varied and consequently the eluent volume available for a separation. To ensure constant start conditions, another pre-run was initiated.

At 70% B the 7-port valve was rinsed for 2 min with eluent A followed by three injections of 25 μl of methanol (see Fig. 1B). The port was switched twice to rinse its dead volume before every injection.

2.3.6. Midazolam analysis by capillary-LC–ESI-MS

Mass spectra were recorded in the full scan mode (mass range *m/z* 250–600). The following source parameters were applied: nitrogen sheath gas flow, 50 arbitrary units (arb, corresponding to ca. 300 ml/min); auxiliary gas flow, 7 arb; spray voltage, 4.5 kV. Voltages of the following devices were optimized by the autotune program to achieve maximum transmission of the $[M+H]^+$ ions of MDZ. The temperature of the heated capillary was held at 155 °C.

The column outlet capillary (1.4 μl, 100 μm i.d., 17.5 cm) was directly guided to the ESI spray tip. To allow insertion of 0.365 mm outer diameter (o.d.) fused silica capillaries, the ESI source had to be modified. The hole of the nozzle tip was widened to a diameter of 720 μm. The nozzle inlet was abraded by 0.7 mm in order to fit the customized Hamilton needle (gauge 22, length 67.5 mm, pointstyle 3, hub diameter 4 mm, distance hub edge to needle end 7 mm, Hamilton, Bonaduz, Switzerland).

An acidic sheath liquid (acetonitrile/H_2O 90:10 (v/v) with trifluoro acetic acid, pH 2) was added to the basic eluent at a flow rate of 4 μl/min to protonate analytes. For this purpose, a flow of 300 μl/min was split 1:75 (HP1050 pump, Hewlett-Packard, Palo Alto, USA.). A flow of 4 μl/min was a good compromise between loss of sensitivity due to dilution and gain of sensitivity due to better ionization. Best sensitivity was reached with 10% acetonitrile added and a pH 2.0 adjusted with trifluoro acetic acid. Compared to a neutral eluent, the signal-to-noise ratio for midazolam was twice lower.

3. Results and discussion

3.1. Pump evaluation

3.1.1. Linear control of gradient with acetone

Many sequences of fast linear gradients with 30 runs each were carried out to compare the stability of eluent composition (test programme 1 in Table 1). UV absorption of the organic mobile phase containing acetone was monitored at a flow rate of 5 μl/min. Syringe pumps are known for their time-consuming flow equilibration [9]. As shown in Fig. 2, even with an isocratic pre-run, the MicroPro pump with a 2000 μl syringe volume needed at least three gradient runs until the best possible precision was obtained. Then, the specified gradient precision of 0.5% ($n = 5$) was reached for 5, 50, and 95% change of the gradient. This specification was also passed for a flow of 1 μl/min, however, with ca. five times longer equilibration times.

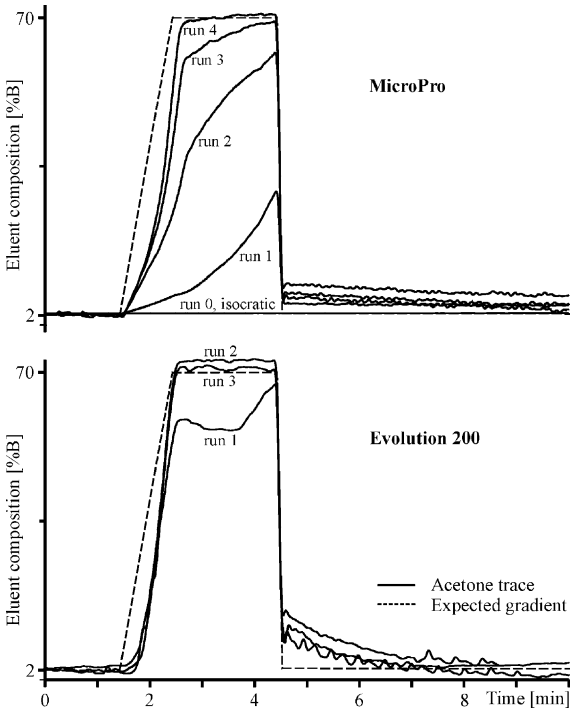

Fig. 2. Time dependence of change of eluent composition for a very steep gradient (see test programme 1) for the pumping systems MicroPro and Evolution 200 at 5 μl/min.

The precision of the gradient varied between 0.2 and 2.0% ($n = 5$) at 5 μl/min after only one initial gradient (Fig. 2) for the Evolution 200 with 35 μl syringe volumes. The pressure profile followed the programmed gradient quite well with a difference of 21.5×10^5 Pa between start and maximum pressure. The wave-type behavior of the baseline might be due to an insufficient piston guiding. It had no influence on the noise level of the ESI detection (see Fig. 5A and C).

3.1.2. Control by a step gradient

The Evolution 200 pump could generate fast gradients and was, therefore, evaluated further with a step gradient (Fig. 3). Start and stop of the plateaux were determined by the maxima and minima of the second derivative. The step heights were calculated from the

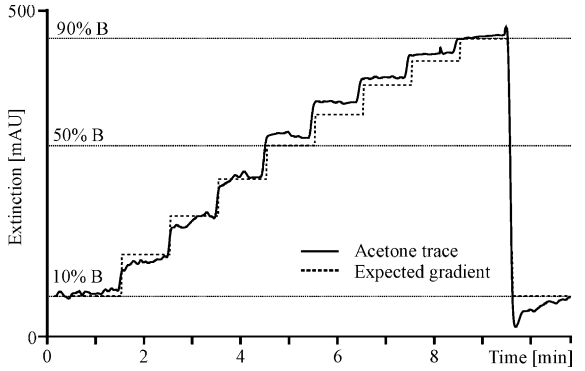

Fig. 3. Stepwise increase of solvent B by 10% for the Evolution 200 pump at 5 μl/min. The ideal gradient profile is given as a dotted line. Non-linear UV response is corrected.

mean of absorbance at the start and stop times of a respective plateau. They deviated from theoretical values by up to 31%. The mean time of the signal increase phase was 3.5 ± 0.4 s ($n = 8$), and the mean duration of the plateau phase was 56 ± 1 s ($n = 7$). This was found acceptable regarding the low flow rate and short runtime of the splitless system. The steps were shorter (ca. 45 s) and the edges rounder at a flow of 3 μl/min.

3.1.3. Gradient performance with a reference drug mixture

Determination of precision of retention time and peak area was carried out with nine drug discovery compounds (see Fig. 4 and test programme 2 in Table 1). These compounds were chosen due to different pK_a values resulting in a high risk of variations of retention times by small pH changes during the gradient.

Relative standard deviations of the retention times were <1% (C1–C9) for the MicroPro pump and ≤1.7% for the Evolution 200 (see Table 2). The precision of signal areas varied between 2 and 7% (C1–C9) for MicroPro and between 4 and 14% (C1–C9) for the Evolution 200. However, only two values were >8% (C1, C9).

3.1.4. Gradient performance with midazolam and related substances

The Evolution 200 was chosen for the development of the method to study metabolic stability against CYP450/3A4, since the pump was capable to generate

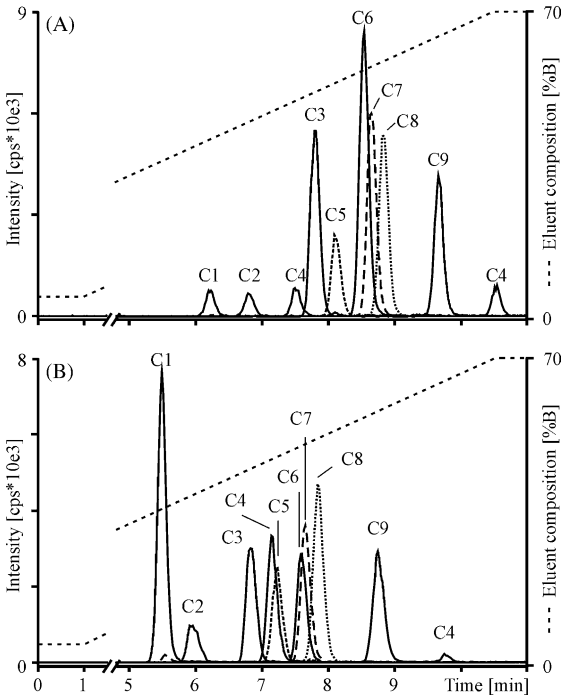

Fig. 4. Combined mass chromatograms of a mixture of nine pharmaceutical compounds (C1 to C9, test programme 2, see Table 1) separated by MicroPro (A) and Evolution 200 (B).

fast gradients. As expected, the chromatographic behavior of midazolam and its derivatives was strongly dependent on the pH of the mobile phase. Acidic conditions split 1,4-DiOH-MDZ into two peaks. Neutral

Table 2
Relative standard deviations ($n = 5$) of retention time (t_R) and signal area of the analytes C1 to C9 relative to compound C5 obtained for the two investigated pumps with test programme 2

Analyte	MicroPro		Evolution 200	
	t_R R.S.D. (%)	Signal area R.S.D. (%)	t_R R.S.D. (%)	Signal area R.S.D. (%)
C1	0.5	2.1	0.9	10.4
C2	0.8	7.3	1.7	7.8
C3	0.3	3.5	1.4	7.4
C4	0.3	4.5	1.3	5.9
C5	0.3	2.8[a]	1.1	6.3[a]
C6	0.1	4.1	0.6	3.9
C7	0.1	3.8	0.8	5.4
C8	0.1	3.8	0.8	5.4
C9	0.3	4.1	0.6	14.1

[a] Variation of absolute values.

Table 3
Overall inter-assay precision for 1-OH-MDZ in the stopped medium obtained with four incubation series and ketoconazole as inhibitor

Spiked amount ketoconazole (μM)	Mean concentration 1-OH-MDZ (ng/ml)	R.S.D. (%)
5.00	6	10.4
1.67	17.5	14.7
0.560	39.7	2.7
0.185	75.4	12.7
0.062	108	12.1
0.021	142	15.3
0.007	160	7.8
Control	156	8.0

pH caused a steady increase of retention (ca. 15% after 140 injections). However, a basic pH of 8.0 gave stable retention times even with microsomal samples (increase of 2–6% after ca. 140 injections (repeated on two columns)).

3.2. Application to cytochrome P450/3A4 inhibition study

3.2.1. Precision and accuracy

Precision for the inhibitor ketoconazole was determined with four incubation series (two incubation experiments at eight concentrations and duplicated measurements). Relative standard deviations were 2.7–15.3% for all concentration levels (Table 3), which is well below the limit of 20% requested in drug discovery. The inter-assay accuracy and precision of the quality control samples are given in Table 4. They were determined for each analyte at two different concentration levels (45 and 230 ng/ml). Accuracy was between 85 and 105% except for low-level controls of 1,4-DiOH-MDZ and 4-OH-MDZ. Precision varied between 0.9 and 9.9% excluding the first eluting analyte 1,4-DiOH-MDZ. The accuracy (95–105%) of 1-OH-MDZ was clearly within the requested range of 80–120% and its precision (≤8.4%) below the limit of 20%, enabling a determination of IC_{50} values.

The unsatisfactory data for 4′-hydroxylated midazolames are due to their instability in solution. 4′-Hydroxylated midazolams (4-OH-MDZ and 1,4-DiOH-MDZ) were not stable when dissolved in eluent A (10 mM NH_4OAc (pH 8.0)/acetonitrile 95:5 (v/v)), in methanol or in the aqueous phase at pH 8.0. Within 26 h, a signal decrease of 34% (4-OH-MDZ) and 26% (1,4-DiOH-MDZ) was observed for the standards solved in eluent A and kept at 21 °C. Later eluting degradation products were also present (base ion *m*/*z* 343 and 359).

3.2.2. Linearity and limits of quantification

The method allowed a detection of midazolam as 1′-hydroxylated metabolite 1-OH-MDZ down to 1% of the starting concentration corresponding to 10.9 ng/ml in the stopped incubation solution. A limit of quantification (LOQ, signal-to-noise ratio (S/N) 10:1) of 7 ng/ml was obtained with 40 μl stopped medium (26.7 μl reaction mixture) in the full scan mode (scan range *m*/*z* 250–600). The limit of detection (LOD, S/N 3:1) was 1 ng/ml for 1.3 pg injected (Fig. 5). Only a twofold gain in sensitivity was observed, when the ion trap was operated in the selected reaction monitoring (SRM) mode. Furthermore, SRM with an ion trap requires a complete fill and isolation cycle for each ion to be determined, which is in the

Table 4
Inter-assay precision and accuracy ($n = 3$) of the quality control samples at 45 and 230 ng/ml for all four analytes

Analyte	Concentration added (ng/ml)	Mean concentration found (ng/ml)	Accuracy (%)	R.S.D. (%)
1-OH-MDZ	45	47	105	8.4
	230	221	96	3.9
MDZ	45	47	104	9.9
	230	214	93	0.9
4-OH-MDZ	45	82	183	3.1
	230	197	86	5.7
1,4-DiOH-MDZ	45	58	128	23
	230	196	85	21

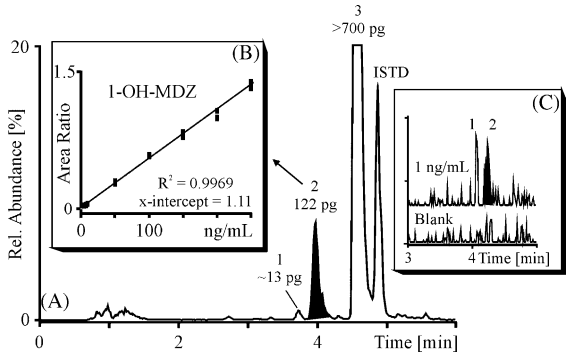

Fig. 5. ESI(+) base ion chromatogram (A) of a stopped microsome medium from the metabolic study (1 μl injected) recorded for the mass range *m*/*z* 325–359. Linear range (B, double determination for each concentration) and signal at 1.3 pg injected for 1-OH-MDZ corresponding to the limit of identification. The mass trace at *m*/*z* 342.2 is shown in (C).

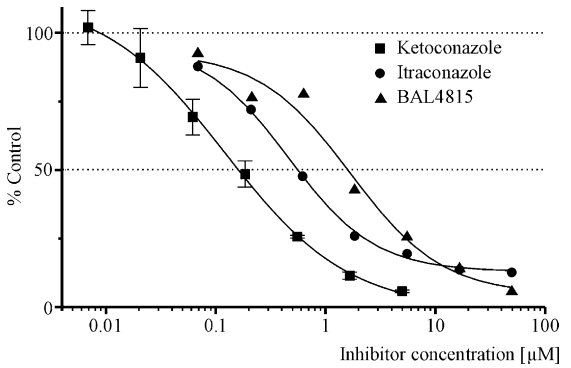

Fig. 6. Experimentally determined inhibition curves for ketoconazole ($n = 4$), itraconazole ($n = 1$), and drug candidate BAL4815 ($n = 1$). Error bars are given for ketoconazole.

order of 450 ms resulting in a total record time of 1.8 s. Therefore, it was not possible to detect all five analytes within one SRM run due to an insufficient number of data points per chromatographic signal.

Yin et al. [10] reported a comparable LOD of 1.7 ng/ml for 1-OH-MDZ with a triple quadrupole (TQ) MS in the SRM mode. Due to the use of a 2 mm i.d. column, they had to inject about 33 times more analyte (absolute 43 pg). Since their MS technique is at least 10 times more sensitive than an ion trap mass spectrometer in the full scan or SRM mode [11], the combination of capillary-LC and TQ-MS would result in a further considerable gain in mass sensitivity.

The linear range for 1,4-DiOH-MDZ, 4-OH-MDZ, 1-OH-MDZ, and MDZ was 7–300 ng/ml ($R^2 > 0.997$, see Fig. 5 for an example). This is sufficient for the determination of the IC_{50} values as well as comparable to the data of Plumb et al. [12]. A linear range of three orders of magnitude for 1-OH-MDZ was reported by Yin et al. using a quadrupole MS/MS method [10].

3.2.3. Results of inhibition study

The drug candidate had a weaker inhibitor activity on midazolam hydroxylase than itraconazole and ketoconazole (1.7±0.5, 0.46±0.06, and 0.12±0.01 μM, respectively, see Fig. 6). The results were in good agreement with those determined at Basilea by a conventional HPLC-TQ-MS–MS system for the drug candidate and itraconazole (1.4 and 0.46 μM, unpublished data), but their value for ketoconazole was three times lower. These variations are not unusual for strong inhibitors such as ketoconazole and can be attributed to differences in assay setup, for example microsomal concentrations (0.2 mg/ml instead of 0.45 mg/ml), reaction times (10 min instead of 15 min), and substrate concentrations (10 μM instead of 5 μM). Yin et al. [10] determined an IC_{50} for ketoconazole of 0.09 μM for a 0.3 mg/ml final microsomal concentration, 10 min reaction time, and 10 μM midazolam. The observed differences are acceptable taken into account the screening purpose of the method and the overall limited comparability of IC_{50} values.

3.2.4. Method perfomance

The whole work-up enriched the analytes by a factor of 1.3. To prevent clogging of the column with precipitated proteins, the supernatant was transferred to new vials after centrifugation. The overall analysis time was ca. 17 min, which is similar to 15 min reported for the analysis of seven different probe substrates on a 2 mm i.d. system [10]. About 25% of the analysis time was spent to overcome practical limitations of the pump and the 7-port valve (pre-run time, additional rinsing phase).

Up to 800 separations of sufficient performance were possible on one 180 μm i.d. capillary column. This is comparable to reports in the literature (>500 injections) [12]. A good precision of the retention time of the ISTD was obtained for all three columns tested (1.4–3.4% for $n = 131$–152). The response of the

ISTD was stable enough to allow the analysis of 60 samples (decrease by ∼30% within 16 h).

4. Conclusions

Splitless gradient systems for capillaries start to show a state of maturity. Both examined pumps generated reproducible gradients from 10 to 5 μl/min within 10–15 min. Furthermore, a direct detection was possible of micro leaks or pressure changes of the column. A gradual built-up of matrix deposits in the capillary did not lead to significant shifts of retention as frequently observed for split systems.

The flushing of the dead volume takes much more time for a micro pump than for a conventional system, since the ratio flow rate to internal volume is less favorable for the first one. Therefore, optimization of pressurization and system equilibration is more time consuming. Low internal dead volumes are decisive for fast gradients usually applied in quantitative LC/MS(/MS) and essential for applications at low flow rates. The Evolution 200 system had the smallest internal volume and needed at least 2.5 min to reach a stable working pressure for flows around 5 μl/min for an initial pressurization.

At flows <10 μl/min, the microflow pumps still cannot cope with the performance of conventional HPLC pumps. Despite different electronic improvements of the Evolution 200 in the past 2 years, the optimization of the compression steps is still complex and increasingly time-consuming the lower the flow rate. This can be omitted by using it as a non-reciprocating syringe pump at the expense of longer equilibration times and volume limitations. Despite some weaknesses, the applicability of splitless micro HPLC pumps at flow rates <10 μl/min could be demonstrated for routine analysis with a method to study metabolic stability against CYP450 isoform 3A4. Currently, the additionally necessary time for method optimization and limitations in operation jeopardise the advantages of micro HPLC compared to conventional HPLC.

Acknowledgements

F.K. thanks F. Hoffmann-La Roche Ltd. and Basilea Pharmaceutica Ltd. for funding part of this work. Special thanks to M. Donzelli for the incubation experiments and to I. Walter and S. Masur for technical assistance. We also thank W. Döbelin and J. Maier-Rosenkranz for their continuous support.

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