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Journal of Chromatography A, 828 (1998) 199–207

JOURNAL OF CHROMATOGRAPHY A

Optimisation and routine use of generic ultra-high flow-rate liquid chromatography with mass spectrometric detection for the direct on-line analysis of pharmaceuticals in plasma

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Abstract

The use of ultra-high flow-rate chromatography coupled to mass spectrometry offers great potential for the rapid, on-line analysis of pharmaceutical compounds in plasma as it permits high throughput direct analysis of plasma samples without any time-consuming sample preparation such as solid-phase extraction. The coupling of mass spectrometry with high-performance liquid chromatography often results in enhanced selectivity and sensitivity compared to, for example, ultraviolet absorbance detection. This can remove the need for complete resolution of the analyte from endogenous materials in the matrix. The use of large particle size stationary phases, and therefore, the ability to use large porosity column end frits, coupled with the added selectivity and sensitivity of the mass spectrometer allows an on-line analysis approach to be used for the direct analysis of pharmaceuticals in biological matrices with extremely high throughput. This paper presents an overview of the manner in which we have optimised this technique for the analysis of plasma samples, in terms of gradient profile, system configuration and optimal injection volume for maximum throughput and robustness. The nature of the mobile phase flow is also discussed.

Keywords: Ultra-high flow-rate liquid chromatography; Flow-rate; Pharmaceutical analysis; Isoquinolines

1. Introduction

The ability to determine accurately the concentration of pharmaceuticals in biological matrices is essential in defining the pharmacokinetics of a pharmaceutical compound. Ever increasing numbers of candidate new pharmaceutical entities are entering the early stages of research and development since the advent of combinatorial chemistry approaches to molecule synthesis and this is increasing the number of samples received by the bioanalysts. Not only is the number of samples increasing, but also there is less time available for method development. Therefore, high throughput, generic approaches to bioanalysis are required to meet the requirements of modern drug development.

Most approaches to the analysis of pharmaceuticals in biological matrices involve pre-treatment of the sample to extract the analyte of interest from materials endogenous in the sample matrix. Typically, this has been achieved by techniques such as liquid–liquid extraction, solid-phase extraction, protein precipitation or column switching [1–4]. Liquid–liquid and solid-phase extraction protocols frequently include an evaporation and reconstitution step and are often time consuming. Column switch-

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00579-2

ing approaches avoid these problems but often lead to extended chromatography times. Protein precipitation is simple and quick but results in relatively dirty sample solutions and can restrict the injection volume.

To some degree the need for extensive sample preparation prior to chromatographic analysis has been reduced by the ability to interface liquid chromatographs and mass spectrometers via an atmospheric pressure ionisation interface. This has yielded not only enhanced sensitivity but also enhanced selectivity which in turn has reduced the need for complete resolution of the analyte from the endogenous materials. This has led to on-line approaches to analysis of biological samples, often using automated equipment [5]. These approaches are frequently successful but do not typically offer high enough sample throughput.

A new approach for extremely fast bioanalysis with no or minimal sample pre-treatment is turbulent flow chromatography [6]. This technique utilises large-particle-size stationary phases and extremely high flow-rates. Providing the flow-rate, and hence the linear velocity, is high enough the mobile phase flow ceases to be laminar and becomes turbulent. Under turbulent conditions, observed reduced plate heights are significantly lower than would be predicted by the conventional Van Deemter equation. In our own laboratory, we have used a similar approach operating at flow-rates of 4 $ml\,min^{-1}$ on 1 mm internal diameter columns packed with stationary phases of 30–50 μm particle size and have found this approach to be extremely useful for bioanalysis. Indeed we recently published our work [7] describing our conditions as turbulent. However, as will be discussed later, we now have evidence suggesting that our conditions do not represent a situation of true turbulent flow, which is why we now prefer the terms ultra-high flow-rate liquid chromatography and direct on-line bioanalysis rather than turbulent flow chromatography. In this paper we present an overview of the manner in which we have optimised this technique for the analysis of plasma samples, in terms of gradient profile, system configuration and optimal injection volume for maximum throughput and robustness. The analysis in plasma of the highly protein bound novel isoquinoline compound under development within GlaxoWellcome which was used as an example in our previous paper [7] will be again used as an example in this work.

2. Experimental

2.1. General

2.1.1. Chemicals

The novel isoquinoline and the $M+6$ deuterated internal standard were synthesized by GlaxoWellcome Research and Development (Stevenage, UK). Acetonitrile (HPLC grade) was supplied by Rathburn (Walkerburn, UK). Formic acid (analytical grade) and water (HPLC grade) were supplied by Fisons (Loughborough, UK).

2.1.2. Equipment

All experiments were conducted using a Hewlett–Packard model 1090 liquid chromatograph (Hewlett–Packard, Palo Alto, CA, USA) coupled to a PE-Sciex API III+ triple quadrupole mass spectrometer (Sciex, Concord, Canada) fitted with a TurboIonspray ionisation source.

2.1.3. Preparation of stock solutions of analyte

Two stock solutions of the novel isoquinoline were used for the validation exercise; these were designated A and B. The stock solutions were prepared by dissolving duplicate weighings of approximately 10 mg of the analyte authentic standard in 10ml of dimethylformamide. Dilutions of each stock were made with control human plasma. Dilutions of stock A were used in the preparation of calibration standards and dilutions of stock B were used in the preparation of the validation (VCs) and quality control samples (QCs). These stock solutions were stored for 1 month at 0–4°C before disposal.

2.1.4. Preparation of internal standard

The $M+6$ deuterated analyte was used as the internal standard. The internal standard was prepared by dissolving approximately 10 mg of isotope in 10 ml of dimethylformamide. This solution was further diluted in distilled water to give a 500 $ng\,ml^{-1}$ working solution of internal standard.

2.1.5. Preparation of calibration standards and quality control samples

Duplicate calibration standards at 5, 10, 20, 50, 100, 500, and 1000 ng ml^{-1} were prepared by spiking control human plasma with appropriate volumes of standard solutions containing the analyte to achieve the required concentration. Additionally independently spiked VCs at 5, 20, 100 and 1000 ng ml^{-1} were prepared for the validation of the method. Calibration standards, QCs, and VCs were stored at −20°C prior to analysis.

2.1.6. Assay procedure

For the analysis of the novel isoquinoline an aliquot of the plasma standard or sample was transferred to an autosampler vial and mixed with an equal volume of internal standard solution. Chromatography was performed using either a 50×1 mm Waters Oasis HLB extraction column (Waters, Milford, MA, USA) or a 50×1 mm Prime C_{18} column (Capital HPLC, Broxburn, UK) maintained at 40°C. The injection volume was 50 μl. Endogenous materials are separated from the analyte of interest and eluted from the column with a mobile phase of 100% aqueous formic acid (0.1%) for 0.2 min at 4 ml min^{-1}. During this time the eluent was diverted to waste to avoid soiling the mass spectrometer interface. For the remainder of the analysis the eluent was split with 3600 μl min^{-1} discarded to waste and 400 μl min^{-1} transferred to the mass spectrometer. Over the period 0.2 to 0.8 min, the solvent composition was rapidly changed from 100% formic acid (0.1%) to acetonitrile–formic acid (0.1%) (95:5). The solvent compositon was held at 95% acetonitrile from 0.8 to 0.9 min and subsequently returned to 100% formic acid (0.1%) by 1.0 min. The system was then allowed to re-equilibrate at 100% formic acid (0.1%) for the next 0.2 min giving a total run time of 1.2 min. Analysis of samples using this ultra-high flow-rate HPLC coupled to the PE-Sciex API-III+ were performed using a TurboIonspray interface operating in positive ion mode, at 550°C with nitrogen as both the nebulizer and auxillary gas. Argon was used as the collision gas at the indicated target thickness of $200 \cdot 10^{13}$ atoms cm^{-2}, with a collision energy of 25 eV. For determination of concentrations of the novel isoquinoline, analyte and internal standard were detected by tandem mass spectrometery (MS–MS) using selected reaction monitoring (SRM) of the transitions m/z 432⇒93 and m/z 438⇒99 for the analyte and deuterated internal standard, respectively, with a dwell time of 200 ms per transition.

2.2. Intra-assay precision

Validation controls were analysed along with two calibration lines, one at the beginning and one at the end of the analytical run, using ultra-high flow-rate LC–MS as described, coupled to the PE-Sciex API-III+ mass spectrometer. LC–MS–MS (SRM) peaks were integrated by the PE-Sciex MacQuan processing software. The calibration curve was constructed by plotting the area ratios of the analyte to internal standard against the nominal concentration using a weighted $(1/x)$ linear regression model. Concentrations of the analyte in the samples were subsequently interpolated from the curves. The intra-assay variability of the methods were determined using the relative standard deviation of replicate assays ($n=6$) for each of four selected concentrations on a single occasion. The intra-assay accuracy of the method was determined by comparing the mean measured concentrations with the nominal concentrations.

3. Results and discussion

3.1. Throughput

With a total sample analysis time of 1.2 min, including re-equilibration, there is a theoretical throughput of 50 samples per hour. To achieve this high throughput it is necessary that the autosampler completes its needle wash cycle and pre-loads the next sample such that it is ready for immediate injection on completion of the previous sample. It, therefore, becomes incumbent on the manufacturers of HPLC autosamplers to ensure that they have the capability to meet the requirements of this and other emerging high throughput techniques. In terms of further increasing this throughput, the 0.2 min initial aqueous elution which is discarded to waste and the final 0.2 min re-equilibration step are equivalent to more than 30 column volumes each (at 4 ml min^{-1}

on a 50×1 mm column assuming 40% of volume is occupied by the packing material). It is conceivable that this is excessive for both the elution of endogenous materials and for re-equilibration, but particularly in the former case, we prefer to ensure that the analyte is well separated from the endogenous materials before the direction of the flow is switched and the mass spectrometer brought into line to ensure no fouling of the source. Forming a linear gradient changing from 0% organic solvent to 95% organic solvent in 0.6 min at 4 ml min^{-1} is not challenging for modern binary pumping systems. This part of the elution profile could be accelerated. However, further reductions in the total analysis time per sample would put further strains on the capability of the autosampler to 'keep up' and could also lead to loss of resolution between the analyte and some endogenous substances which are retained by the column but are eluted before the analyte of interest. If there are no instrumental limitations it would be predicted on the basis of Snyder's equation [8] (where k'_{av} is proportional to gradient time multiplied by flow-rate over column length) that resolution could be maintained by increasing the flow-rate (system pressure permitting) whilst reducing the gradient time.

3.2. Flow considerations

In our previous work we estimated that our mobile phase flow-rate, 4 ml min^{-1}, was sufficiently high for the chromatography to exhibit turbulent rather than conventional behaviour. We no longer consider this to be the case. There are a number of parameters which can be calculated in determining whether or not the mobile phase is exhibiting turbulence. Firstly, let us consider the Reynold's number, Re, as an indicator of flow stability. The Reynold's number is defined by the equation

$$\mathrm{Re} = \frac{\rho u d}{\eta}$$

where ρ = density of the mobile phase, u = linear velocity of the mobile phase, d = diameter of the tube for open tubular systems or diameter of the particles for packed bed systems, η = the viscosity of the mobile phase.

We estimate that u for our system is approximately 0.14 m s^{-1} based on a 4 ml min^{-1} flow-rate and a 1 mm internal diameter column and assume a density of 1 g ml^{-1} and a viscosity of 0.001 kg m^{-1} s^{-1} for the mobile phase. Then Reynold's number for the system using the Oasis HLB column, with approximately 31 μm particle size, is 4.3. For turbulence to be an important chromatographic factor it has been reported that a Reynold's number in the region of 10 or higher is required [9]. This value will be lower, possibly as low as 3, for higher values of column/particle diameter ratio. In the case of the Oasis HLB column this ratio is approximately 32 and for the Prime C_{18} column this ratio is 20. We suggest that these ratios are not high enough to reduce the Reynold's number required for turbulence to below the value measured in our system.

Similarly, another parameter that can be relatively easily calculated is the reduced velocity, ν, which is given by the equation:

$$\nu = \frac{u d_p}{D_m}$$

where u = the linear velocity, d_p = the particle diameter, D_m = diffusion coefficient.

Taking a value of $1 \cdot 10^{-5}$ cm^2 s^{-1} for D_m, which is reasonable for typical small molecule drugs (molecular mass 300–500), we obtain a reduced velocity of 4340 for the Oasis HLB column and 7000 for the Prime C_{18} column. Knox has stated [9] that for liquid mobile phases 'turbulence need not be considered at reduced velocities below 10^3 to 10^4.' Again, therefore, in our systems there is no definite establishment of turbulent flow.

One experiment which can be very simply performed is to measure the mobile phase flow-rate delivered against the pressure drop across the system. At the point where Poiseuille's formula does not apply, that is, at the onset of turbulence, the relationship will cease to be linear [10]. As can be seen from Fig. 1, no evidence of non-linearity was found with flow-rates up to 9.5 ml min^{-1} on the 50×1 mm Oasis HLB column.

3.3. Robustness

The injection of large aliquots of plasma, 50 μl, directly onto columns of dimensions 50×1 mm represents the injection of approximately 2.5 column

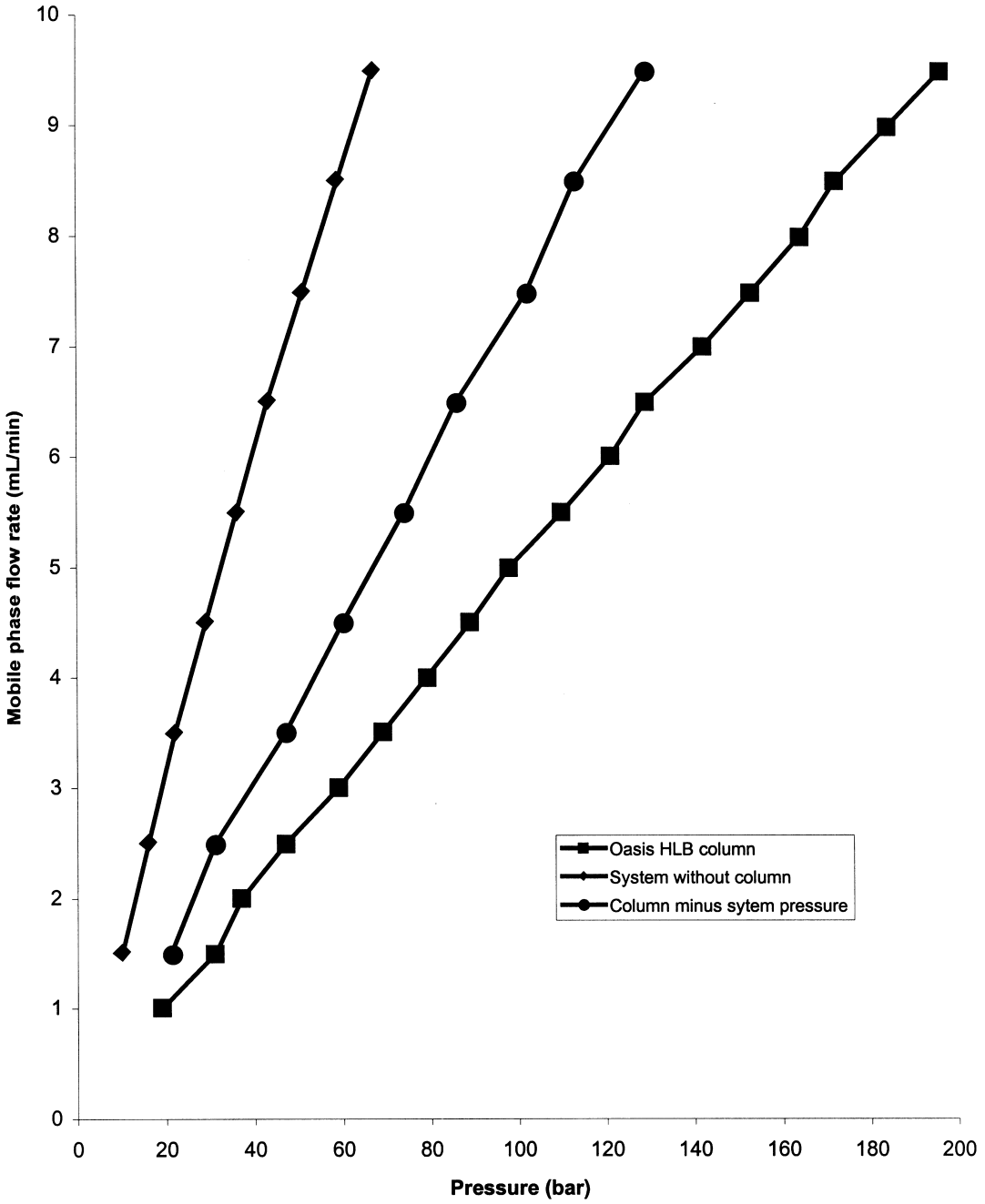

Fig. 1. The relationship between the volumetric flow-rate and the pressure drop across the system. Mobile phase 100% aqueous formic acid (0.1%); flow-rate 4 ml min^{-1}; column 50×1 mm Oasis HLB.

volumes for each sample. Although the initial part of the elution gradient is diverted to waste and, therefore, does not lead to any fouling of the mass spectrometer source, this injection protocol does expose the stationary phase to proteinaceous and other endogenous materials. It has been our ex-

perience that after approximately 100 injections deterioration of the chromatographic performance occurs, in terms of both broadened peak shapes and increased system back pressure. If the number of injections is increased much beyond this the system tends to fail, usually as a result of over-pressuring. We have, therefore, recommended that the column be treated as a consumable item in the same way as, for example, a 96-well solid-phase extraction plate. To make this an economically viable assay system it is necessary that the cost of the columns is kept in the same region as that of 96-well solid-phase extraction plates. Under these circumstances, the fact that the samples are analysed and the required concentration data obtained in less time than with any other bioanalytical strategy to our knowledge, means that there are still economies to be made in terms of analyst's time when using this technique. One way to enhance column lifetime would be to restrict the injection to say 10 μl per sample, but this of course means a reduction in the concentration sensitivity of the resulting assays, in many cases making them less sensitive than is required for our typical toxicokinetic applications. However, it is possible that a large amount of the stress experienced by the stationary phase under the 50 μl injection protocol can be simply alleviated. When making large volume injections onto relatively small columns there is a substantial pressure drop at the moment of injection. With a protocol calling for these injections to be made as frequently as every 1.2 min, the column experiences a lot of these pressure shocks within a short period of time. This can lead to the stationary phase packing down and even being physically damaged. In turn, this can lead to fines blocking the column end frits or meshes and the system over-pressuring. The column can be protected from these shocks, however, by placing a pulse damper in the chromatographic sytem immediately prior to the analytical column. Using this approach we have extended the usable lifetime of our columns 2–3 fold. A schematic representation of the liquid chromatography system is given in Fig. 2.

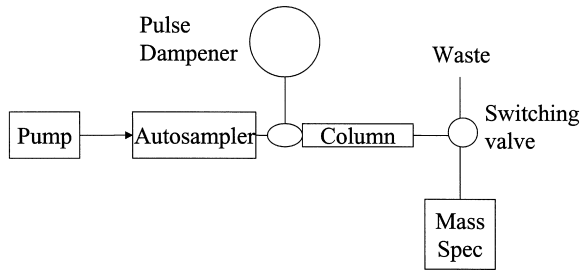

Fig. 2. Schematic representation of the chromatographic system used for ultra-high flow-rate on-line bioanalysis.

3.4. Analysis of a novel isoquinoline compound in plasma by ultra-high flow-rate on-line bioanalysis using an internal standard procedure

Sample chromatograms of blank plasma and a 5 ng ml^{-1} plasma standard for the analysis of the isoquinoline with a 1.2 min chromatography time are shown in Fig. 3. The peak area ratios of analyte to internal standard were linear over the calibration range 5–1000 ng ml^{-1}. Linear regression with $1/x$ weighting was used in the construction of the calibration lines. A typical calibration line is shown in Fig. 4. The limit of quantification was taken to be 5 ng ml^{-1}, the lowest calibration standard used. This value gave acceptable accuracy and precision data with a signal-to-noise ratio of approximately 4:1. The method displayed good intra-assay accuracy and reproducibility over the calibration range as shown by the data presented in Table 1.

4. Conclusions

The use of ultra-high flow-rates on large particle size stationary phases has been shown to be of great benefit for high throughput bioanalysis. The ability of this technique to directly analyse plasma or serum samples without the need for time-consuming sample preparation coupled with the extremely fast chromatographic analysis offers large savings in terms of total analysis time per sample. An example assay of a highly protein-bound pharmaceutical molecule with a sample throughput of 50 samples per hour has been validated over the range 5–1000 ng ml^{-1} and has been shown to be accurate and precise. Under our assay conditions we have shown that we are not operating under conditions of turbulent flow, yet the usefulness of the described approach for bioanalysis is not diminished by the absence of the improved chromatographic efficiency that turbulence would offer. With the inclusion in the chromatographic

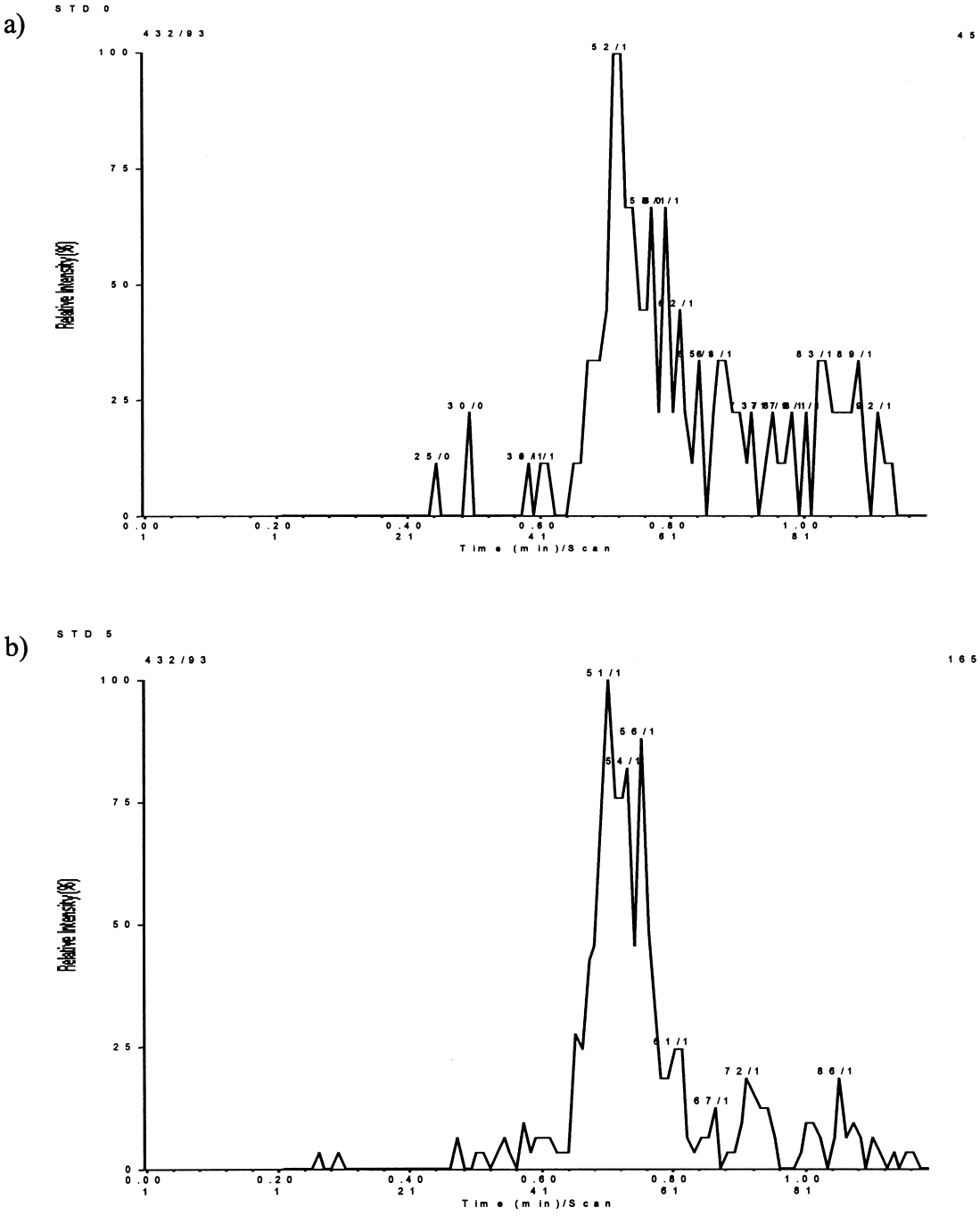

Fig. 3. Typical chromatograms of (a) blank plasma and (b) plasma spiked with the isoquinoline standard at 5 ng ml^{-1}. Chromatographic conditions: Column Waters Oasis HLB 50×1 mm; mobile phase 0–95% acetonitrile gradient in aqueous formic acid from 0.2–0.8 min; temperature 40°C; injection volume 50 μl.

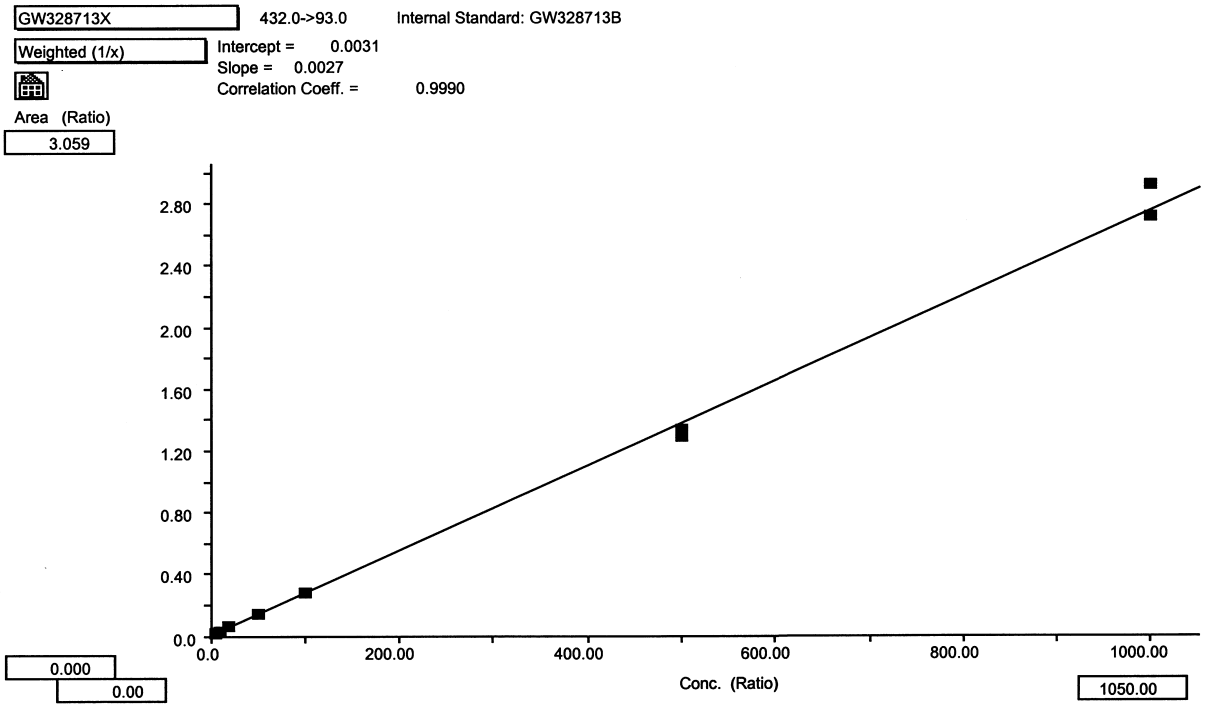

Fig. 4. A sample calibration line of isoquinoline analyte spiked into plasma at concentrations from 5–1000 ng ml^{-1}. Chromatographic conditions: Column Waters Oasis HLB 50×1 mm; mobile phase 0–95% acetonitrile gradient in aq. formic acid from 0.2–0.8 min; temperature 40°C; injection volume 50 μl.

system of a pre-column pulse dampener column lifetimes can be extended sufficiently, even under conditions of fairly high injection volumes (approximately 2.5 column volumes), to make this technique an attractive and economic option in the bioanalyst's arsenal.

Table 1
Results of the intra-assay validation of the analysis of a novel isoquinoline based pharmaceutical in plasma by ultra-high flow-rate on-line bioanalysis using an internal standard procedure

Replicate	Concentration of validation standard (ng ml^{-1})			
	5	20	100	1000
1	4.74	21.8	104	933
2	4.22	20.3	114	952
3	4.83	19.7	105	982
4	4.22	20.3	106	936
5	5.23	20.8	99.7	998
6	4.02	20.1	96.7	1030
Mean	4.54	20.5	104	972
S.D.	0.46	0.73	5.94	38.4
R.S.D. (%)	10.2	3.56	5.70	3.95
Bias (%)	−9.13	2.50	4.23	−2.82

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