ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 645–651

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Single-step extraction of fluconazole from plasma by ultra-filtration for the measurement of its free concentration by high performance liquid chromatography

Paul Anthony Majcherczyk [a,*], Philippe Moreillon [a], Laurent Arthur Decosterd [b], Dominique Sanglard [c], Jacques Bille [c], Michel Pierre Glauser [a], Oscar Marchetti [a]

[a] *Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*
[b] *Division of Clinical Pharmacology, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*
[c] *Institute of Microbiology, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*

Received 11 October 2001; accepted 22 October 2001

Abstract

High performance liquid chromatography (HPLC) is the reference method for measuring concentrations of antimicrobials in blood. This technique requires careful sample preparation. Protocols using organic solvents and/or solid extraction phases are time consuming and entail several manipulations, which can lead to partial loss of the determined compound and increased analytical variability. Moreover, to obtain sufficient material for analysis, at least 1 ml of plasma is required. This constraint makes it difficult to determine drug levels when blood sample volumes are limited. However, drugs with low plasma-protein binding can be reliably extracted from plasma by ultra-filtration with a minimal loss due to the protein-bound fraction. This study validated a single-step ultra-filtration method for extracting fluconazole (FLC), a first-line antifungal agent with a weak plasma-protein binding, from plasma to determine its concentration by HPLC. Spiked FLC standards and unknowns were prepared in human and rat plasma. Samples (240 μl) were transferred into disposable microtube filtration units containing cellulose or polysulfone filters with a 5 kDa cut-off. After centrifugation for 60 min at 15000*g*, FLC concentrations were measured by direct injection of the filtrate into the HPLC. Using cellulose filters, low molecular weight proteins were eluted early in the chromatogram and well separated from FLC that eluted at 8.40 min as a sharp single peak. In contrast, with polysulfone filters several additional peaks interfering with the FLC peak were observed. Moreover, the FLC recovery using cellulose filters compared to polysulfone filters was higher and had a better reproducibility. Cellulose filters were therefore used for the subsequent validation procedure. The quantification limit was 0.195 mg l^{-1}. Standard curves with a quadratic regression coefficient $\geqslant 0.9999$ were obtained in the concentration range of 0.195–100 mg l^{-1}. The inter and intra-run accuracies and precisions over the clinically relevant concentration range,

* Corresponding author. Tel.: +41-21-3144055; fax: +41-21-3144060.
E-mail address: paul.majcherczyk@chuv.hospvd.ch (P.A. Majcherczyk).

PII: S0731-7085(01)00686-0

1.875–60 mg l^{-1}, fell well within the ±15% variation recommended by the current guidelines for the validation of analytical methods. Furthermore, no analytical interference was observed with commonly used antibiotics, antifungals, antivirals and immunosuppressive agents. Ultra-filtration of plasma with cellulose filters permits the extraction of FLC from small volumes (240 μl). The determination of FLC concentrations by HPLC after this single-step procedure is selective, precise and accurate.

Keywords: Plasma ultra-filtration; Single-step extraction; Fluconazole; HPLC

1. Introduction

Fluconazole (FLC) is a first-line antifungal drug. Measuring its concentrations in the plasma of patients is of clinical relevance when pharmacokinetics are unpredictable (e.g. in patients with rapidly declining renal function or on continuous veno-venous haemofiltration, in outpatients with questionable adherence, in patients treated with drugs interacting with the metabolism of FLC) or when treating infections due to fungi with decreased susceptibility. Moreover, monitoring FLC levels is an essential tool in experimental models, in particular for investigating the correlation between the minimum inhibitory concentration of FLC and the drug level needed for therapeutic success.

FLC concentrations in plasma can be measured either by HPLC or by bioassay. However, because of its robustness, HPLC remains the reference method and is required to validate the development of any bioassay [1–5]. Prior to the HPLC analysis, careful sample preparation to extract FLC from plasma needs labour intensive procedures using organic solvents and/or solid-phase extraction [1–4,6,7]. This entails several manipulations that can lead to an increased analytical variability due to an unpredictable partial loss of the measured compound. Furthermore, these methods require at least 1 ml of plasma making it difficult to determine drug levels when amounts of plasma are limited, for example, in children or in experiments with small animals.

HPLC sample preparation by ultra-filtration of plasma is a simple and reliable extraction method of the free circulating drug fraction of different antimicrobial and antiretroviral agents [8–10]. FLC is weakly bound to plasma proteins (approximately 10%) and circulates mainly as free drug [11]. Therefore, measuring its free level in plasma allows a good estimate of the circulating amount of FLC available for penetration in infected tissues. This study aimed to develop and validate a single-step ultra-filtration method for extracting FLC from small amounts of plasma to determine its concentration by HPLC in the clinically relevant concentration range of 1.875–60 mg l^{-1}.

2. Experimental

2.1. Sample preparation and storage

Spiked samples were prepared by serial dilution of FLC (kindly provided by Pfizer, Sandwich, United Kingdom) in both, pooled human and rat plasma containing 100 IU ml^{-1} sodium-heparin (5% vol/vol). FLC standards ranged between 0.195 and 100 mg l^{-1}. Quality controls contained 1.875, 3.75, 15 and 60 mg l^{-1} FLC. Samples were aliquoted (250 μl) and stored at −80 °C. FLC in plasma was stable after 24 h at room temperature and after 1, 2 or 3 freeze-thaw cycles.

2.2. Comparison of polysulfone and cellulose filters

Two types of filters with a 5 kDa cut-off, containing either polysulfone or cellulose membranes (Ultrafree MC centrifugal filter units, Millipore Co., Bedford, MA) were compared. 240 μl aliquots of quality control samples were transferred into disposable microtube filtration units pre-wetted with phosphate-buffered saline. After centrifugation at 15000*g* for 60 min at 15 °C, 160 μl of filtrate was collected and directly analysed in duplicate to measure the FLC concentration by HPLC. The HPLC system (Hitachi Instruments,

Ichige, Hitachinaka, Japan) consisted of the L-7200 autosampler, the L-7100 gradient pump, with low pressure mixing, and the L-7450A diode array detector. Column temperature was maintained at 30 °C using a peltier column oven (LabSource, Reinach, Switzerland). Samples were run in duplicate. The results were analysed using the D-7000 HPLC System Manager program (Hitachi). A 60 μl sample was injected into a reverse-phase C18 column (250 mm × 4 mm ID, 5 μm bead size; SuperPac Sephasil, Pharmacia Biotech, Uppsala, Sweden) and eluted isocratically with 0.1 M sodium acetate, pH 5.0, containing 30% methanol, at 30 °C with a flow rate of 1 ml min^{-1}. FLC was detected by measuring UV absorbance at 210 nm and quantified by the external standard method using the FLC peak area. The chromatogram was compared to that of ultra-filtered FLC-free plasma. Moreover, the FLC peak area obtained for each quality control plasma sample after ultra-filtration with both types of filter were compared to those measured after direct injection of water containing the same concentrations of FLC. For both filters, the recovery rate of each quality control concentration was calculated according to the formula: FLC peak area in the plasma ultra-filtrate/FLC peak area in unfiltered water × 100. For each tested FLC concentration (1.875, 3.75, 15 and 60 mg l^{-1}), recovery rates of triplicate experiments were expressed as mean ± standard deviation. Recovery results obtained for each tested concentration were pooled to calculate the average recovery and its coefficient of variation (standard deviation/mean recovery × 100). The recovery rates with the two types of filters were compared by a two-sided t-test. The significance level was set at a $P < 0.05$.

2.3. Validation of the ultra-filtration method using cellulose filters

Validation was performed according to the current recommendations for analytical method validation, that require a standard curve with five to eight points with reproducible linear or non-linear responses and statistical fits [12]. The present work used standard curves composed of eight points and calculated by quadratic regression. Each analytical run comprised eight standards and four quality controls, all in duplicate. Intra and inter-run accuracy (expressed by percent deviation calculated according to the formula: measured value/nominal value × 100) and precision (expressed by the coefficient of variation calculated according to the formula: standard deviation of measured values/mean measured values × 100) were determined for each quality control sample (1.875, 3.75, 15 and 60 mg l^{-1}). For both validation steps five analytical runs were performed. Finally, in order to test the selectivity of the method, the retention times of commonly used antibiotics, antifungals, antiviral and immunosuppressive drugs were compared to that of FLC. For this purpose, all drugs were diluted in water and directly injected in the HPLC. The experimental conditions were identical to those used to measure FLC concentrations in ultra-filtered plasma. The following compounds were tested: antibiotics—penicillin, amoxicillin, ampicillin, piperacillin, clavulanic acid, tazobactam, ceftriaxone, ceftazidime, cefepime, imipenem/cilastatin, meropenem, amikacin, gentamicin, vancomycin, teicoplanin, metronidazole, sulfamethoxazole, trimethoprim, erythromycin, clarithromycin, clindamycin, rifampicin, tetracycline, ciprofloxacin, levofloxacin; antifungals—FLC, itraconazole, 5-fluorocytosine, amphotericin B; antivirals—acyclovir, gancylovir, foscavir, ritonavir, nelfinavir, indinavir, amprenavir, lopinavir, saquinavir, nevirapine, efavirenz; immunosuppressants—cyclosporin A and tacrolimus.

Moreover, in order to provide a practical example, the plasma of a patient treated with FLC was analysed to determine its trough concentration.

3. Results and discussion

3.1. Comparison of polysulfone and cellulose filters

The chromatograms obtained after ultra-filtration of plasma alone (top panel) and of plasma containing 50 mg l^{-1} FLC (bottom panel) using polysulfone (left figures) or cellulose filters (right

figures) are shown in Fig. 1. Filtration with cellulose filters removed most of the plasma proteins, and the remaining low molecular weight proteins eluted over the first 7 min. No further plasma protein peaks were observed. FLC was well separated and eluted as a sharp single peak at 8.40 min. In contrast, polysulfone filters gave several additional peaks partially overlapping with the FLC peak. These ghost peaks were observed also after ultra-filtration of water with polysulfone filters suggesting the release of filter components into the filtrate (data not shown). The recovery rates of FLC in ultra-filtered plasma using both types of filters are shown in Fig. 2. These ranged

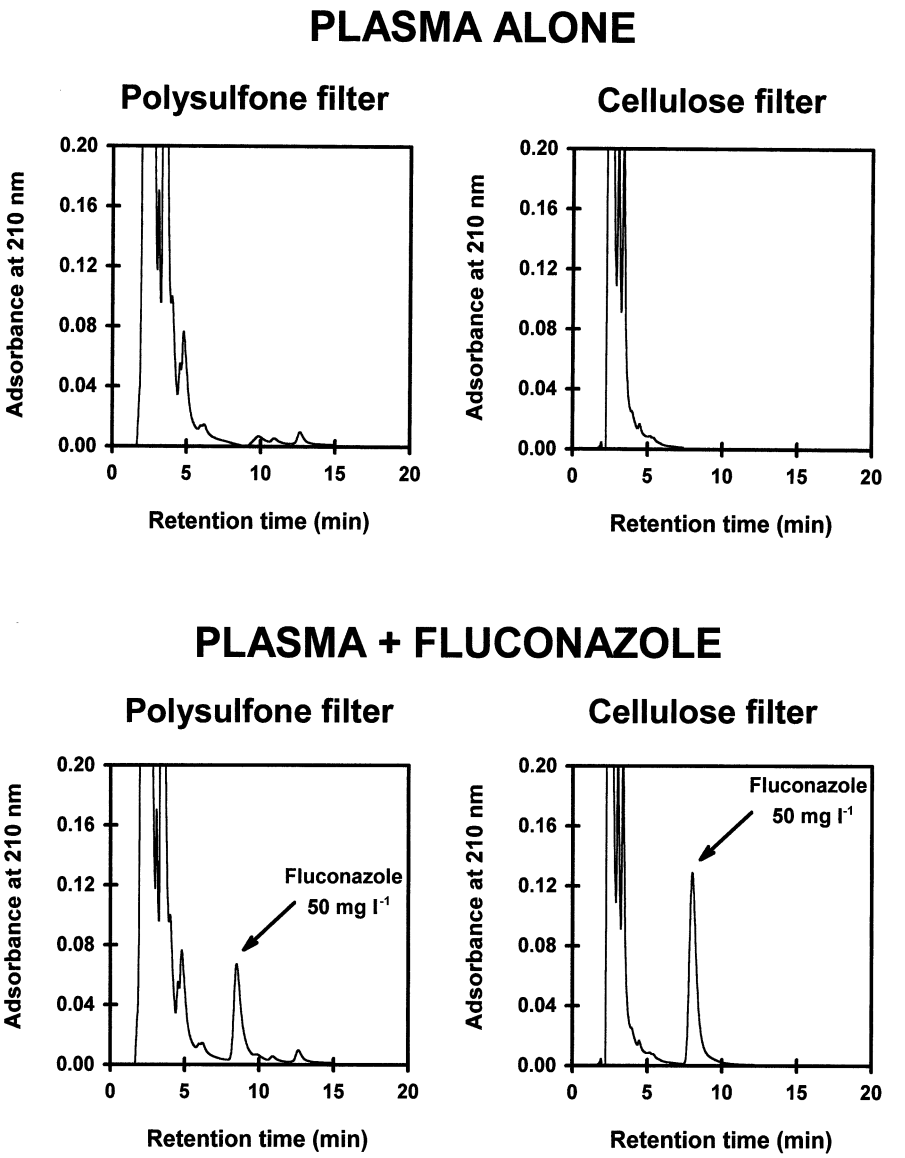

Fig. 1. Chromatograms obtained following injection of human plasma alone (upper panel) and human plasma containing 50 mg l^{-1} FLC (lower panel) after ultra-filtration using polysulfone (left figures) and cellulose filters (right figures). FLC peaks are marked with an arrow.

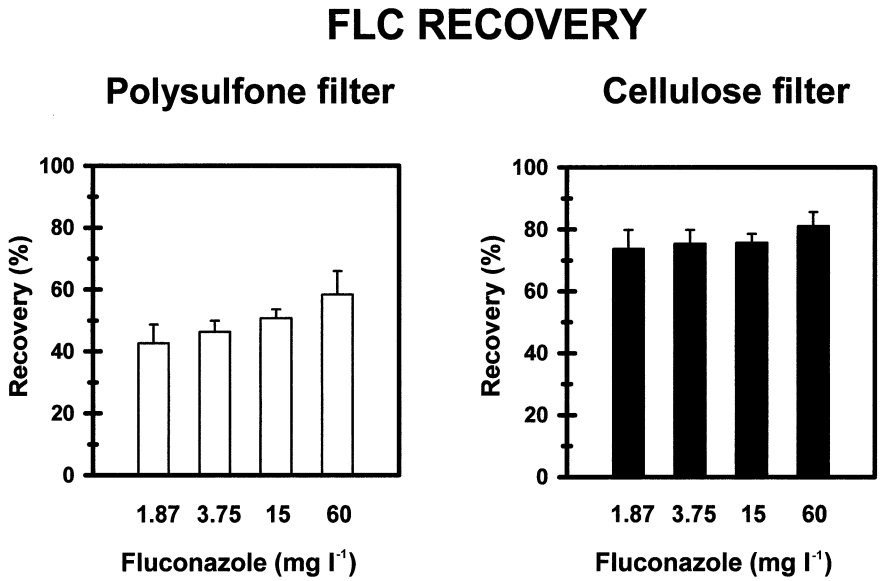

Fig. 2. Recovery of FLC after ultra-filtration using polysulfone (left panel) or cellulose filters (right panel) of human plasma containing the following FLC concentrations: 1.87, 3.75, 15 and 60 mg l^{-1}. The recovery rates for each tested concentration were calculated according to the formula: FLC peak area in the plasma ultra-filtrate/FLC peak area in unfiltered water × 100. Mean values and standard deviations of triplicate experiments are shown.

between $42.7 \pm 6\%$ for 1.875 mg l^{-1} and $58.3 \pm 7.6\%$ for 60 mg l^{-1} ($49.5 \pm 6.7\%$; CV = 13.5%) using polysulfone filters and between $73.7 \pm 6.1\%$ for 1.875 mg l^{-1} and $81 \pm 4.6\%$ for 60 mg l^{-1} ($76.4 \pm 3.2\%$, $P < 0.001$ when compared to polysulfone filters; CV = 4.2%) using cellulose filters, respectively. As the protein bound fraction contributes approximately to 10% loss, the remaining loss during ultra-filtration might be due to a filter-dependent retention of free circulating FLC in the filtration unit.

Cellulose filters were chosen for further validation studies due to both, the lack of peaks interfering with that of FLC and the significantly higher and better reproducible recovery rates, when compared to those observed with polysulfone filters.

3.2. Validation of the method using cellulose filters

The quantification limit was 0.195 mg l^{-1} corresponding to 0.024 μg FLC for a 60 μl injection volume. Standard curves over the 0.195–100 mg l^{-1} range had a quadratic regression coefficient $\geqslant 0.9999$ and the following parameters (mean ± standard error): $b_0 = 0.3817 \pm 0.0205$, $b_1 = 3.0371e^{-5} \pm 0.0747e^{-5}$, $b_2 = -8.2258e^{-13} \pm 0.6193e^{-13}$. These results were comparable with those obtained with previously published methods, that needed more complex sample preparation [1–3,6,7]. For example, Rex et al. reported that over 4 h were required to prepare 40 samples [1]. Using this simplified single-step method, this time was reduced to 75 min (60 min for ultra-filtration and 15 min for the different manipulations before and after ultra-filtration) for an analytical run comprising 50 samples (eight standards, four quality controls and 38 samples) [5]. Table 1 summarizes the results of the intra- and inter-run validation procedure over the clinically relevant concentration range of 1.875–60 mg l^{-1}. All deviations and coefficients of variation lay well within the recommended ±15% limits [12]. An identical validation procedure was run in rat plasma and gave similar results (data not shown). Finally, the retention times of commonly used antibiotics, antifungals,

Table 1
Intra and inter-run validation of the determination of FLC concentrations in human plasma by HPLC after single-step ultra-filtration with cellulose filters

Nominal FLC concentration ($mg\,l^{-1}$)	Intra-run validation ($n = 5$)			Inter-run validation ($n = 5$)		
	Measured FLC concentration ($mg\,l^{-1}$)[a]	Deviation[a,b] (%)	Coefficient of variation[c] (%)	Measured FLC concentration ($mg\,l^{-1}$)[a]	Deviation[a,b] (%)	Coefficient of variation[c] (%)
1.875	1.813 ± 0.102	-3.32 ± 5.45	5.5	1.820 ± 0.127	-2.93 ± 6.79	7.73
3.75	3.628 ± 0.081	-3.25 ± 2.16	2.2	3.595 ± 0.049	-4.14 ± 1.32	1.39
15	15.144 ± 0.319	0.96 ± 2.13	1.5	14.944 ± 0.234	-0.37 ± 1.56	1.54
60	59.742 ± 2.232	-0.43 ± 3.72	3.7	60.690 ± 1.290	1.15 ± 2.15	2.13

[a] Mean $\pm$ standard deviation.

[b] The accuracy is expressed as the deviation between measured and nominal FLC levels of five different determinations of the same plasma sample calculated according to the formula described in Section 2.3.

[c] The precision is expressed by the coefficient of variation of five different determinations of the same plasma sample calculated according to the formula described in Section 2.3.

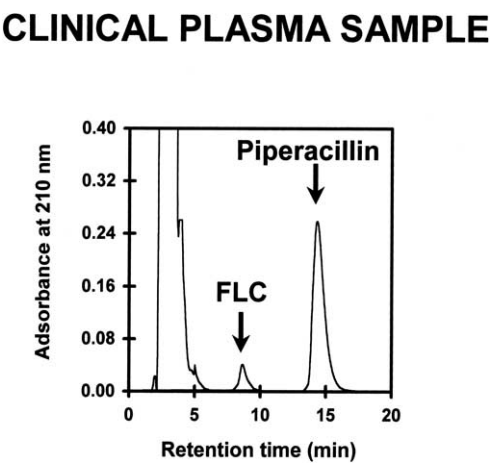

Fig. 3. Chromatogram of the ultra-filtered plasma of a patient treated with FLC and piperacillin/tazobactam. Blood was drawn for the determination of the FLC 24 h-trough level in the setting of rapid declining renal function. The FLC concentration was 14.5 mg l^{-1}. The piperacillin peak is marked with an arrow. Tazobactam was not detected.

antivirals and immunosuppressive drugs and of FLC, all diluted in water and directly injected in the HPLC, were measured. None of the tested compounds co-eluted with FLC. The results of this screening show the selectivity of this simplified HPLC method for measuring FLC concentrations. An example of the chromatogram of the ultra-filtrated plasma drawn to determine the FLC 24-h trough level in a patient treated with FLC is shown in Fig. 3. The patient had multiple myeloma, chemotherapy-induced neutropenia and chronically impaired renal function. FLC 200 mg was given once daily i.v. for *Candida albicans* bloodstream infection and piperacillin/tazobactam 4.5 g twice daily i.v. for *Escherichia coli* sepsis. FLC 24-h trough level was measured after a 7-day treatment in the setting of a rapid decline in renal function attributed to infection. FLC eluted as a sharp single peak and no overlap occurred with the co-administered piperacillin/tazobactam. The FLC 24-h trough level was 14.5 mg l^{-1}.

4. Conclusions

In conclusion, ultra-filtration with cellulose filters with a 5 kDa cut-off allows the extraction of FLC from volumes of plasma as low as 240 μl. The determination of the FLC concentrations by HPLC after this simplified single-step procedure is simple, selective, precise and accurate.

Acknowledgements

We are thankful to José Entenza, Marlyse Giddey, Françoise Ischer and Jacques Vouillamoz for their helpful technical assistance.

References

[1] J.H. Rex, L.H. Hanson, M.A. Amantea, D.A. Stevens, J.E. Bennett, Antimicrob. Agents Chemother. 35 (1991) 846–850.
[2] J.W. Hulsewede, H. Dermoumi, Zentralbl. Bakteriol. 283 (1996) 492–496.
[3] J.E. Wallace, S.C. Harris, J. Gallegos, G. Foulds, T.J.H. Chen, M.G. Rinaldi, Antimicrob. Agents Chemother. 36 (1992) 603–606.
[4] M.K. Nishihima, T. Okuda, T. Taenaka, I. Yoshiya, J. Chromatogr. B: Biomed. Appl. 529 (1990) 223–228.
[5] O. Marchetti, P.A. Majcherczyk, M.P. Glauser, J. Bille, P. Moreillon, D. Sanglard, Antimicrob. Agents Chemother. 45 (2001) 696–700.
[6] C.H. Koks, H. Rosing, P.L. Meenhorst, A. Bult, J.H. Beijnen, J. Chromatogr. B: Biomed. Appl. 663 (1995) 345–351.
[7] K. Inagaki, J. Takagi, E. Lor, M.P. Okamoto, M.A. Gill, Ther. Drug. Monit. 14 (1992) 306–311.
[8] D. Breilh, C. Lavallee, A. Fratta, D. Ducint, P. Cony-Makhoul, M.C. Saux, J. Chromatogr. B: Biomed. Appl. 734 (1999) 121–127.
[9] L. Garcia-Capdevila, C. Lopez-Calull, C. Arroyo, M.A. Moral, M.A. Mangues, J. Bonal, J. Chromatogr. B: Biomed. Appl. 692 (1997) 127–132.
[10] K.B. Kenney, S.A. Wring, R.M. Carr, G.N. Wells, J.A. Dunn, J. Pharm. Biomed. Anal. 22 (2000) 967–983.
[11] S.M. Grant, S.P. Clissold, Drugs 39 (1990) 877–916.
[12] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249–255.