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Determination of fluconazole in human plasma by micellar electrokinetic capillary chromatography with detection at 190 nm

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Abstract

The determination of fluconazole (Diflucan) in human plasma by micellar electrokinetic capillary chromatography (MECC) with on-column UV absorption detection at 190 nm from primary, deproteinized and extracted plasma samples is discussed. Direct injection of plain plasma or of the supernatant after protein precipitation with acetonitrile is shown to permit the determination of fluconazole drug levels of >5 μg/ml only. With liquid–liquid extraction employing dichloromethane, the detection limit is about 1 μg/ml. After extraction using disposable solid-phase C_{18} cartridges and 1 ml of plasma, however, drug levels as low as 100 ng/ml can be determined unambiguously. Calibration graphs between 0.125–25.0 μg/ml (seven data points) are shown to be linear, with a regression coefficient $r>0.999$. For fluconazole plasma levels of 5 μg/ml, intra-day and inter-day imprecisions ($n=10$) are about 2 and 5%, respectively. Using the same solid-phase extraction procedure, 44 fluconazole plasma levels that were determined by MECC are shown to agree well with those obtained by HPLC and elucidated pharmacokinetic data compare well with those found in the literature. The advantages of using MECC instead of HPLC for the determination of fluconazole plasma levels and pharmacokinetics are the high resolution efficiency, low-cost capillary columns and the small consumption of inexpensive and environmentally friendly chemicals.

Keywords: Fluconazole; Micellar electrokinetic capillary chromatography; Therapeutic drug monitoring

1. Introduction

Fluconazole is a highly water-soluble antifungal agent with the chemical structure shown in Fig. 1. It is a broad spectrum bis-triazole derivative that has been found useful in the treatment of mucocutaneous candidiasis, cryptococcal meningitis and aspergillosis [1–3]. The in vitro antifungal activity (IC_{99}) of fluconazole was 0.39 μg/ml or less against *Candida albicans* and 12.5 μg/ml or less against *Cryptococcus neoformans* [3]. Equivalent plasma levels are obtained after ingestion of 10 and 200 mg, respectively, of fluconazole per day. Pharmacokinetic studies have revealed that the bioavailability of oral doses of fluconazole is over 90% [3,4], and that the half life ($t_{1/2}$) in blood lies between 22 and 37 h [1,3–5]. Fluconazole exhibits low toxicity. It is found in high concentrations in organs, tissues and

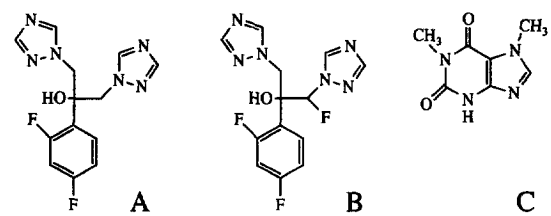

Fig. 1. Chemical structures of (A) fluconazole, (B) UK-54373 and (C) 1,7-dimethylxanthine (1,7-DMX).

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fluids throughout the body [1,4,6], including the eye [6], cerebrospinal fluid (60–80%, compared to plasma levels; 100% for patients with cryptococcal meningitis) and saliva (~100%) [4]. Urine concentrations correspond to about ten times the plasma level [4]. Fluconazole is metabolically stable, with a renal excretion of about 80% as unchanged drug [1,3,4,6]. The distribution volume is about 0.75 l/kg, corresponding to about the total body fluid [1,3,4,6]. While it is generally not necessary to measure fluconazole blood levels [2], the dose of fluconazole must be adjusted in patients with renal insufficiency [4] and may require adjustment in patients taking drugs that influence fluconazole pharmacokinetics, such as rifampicine [2,7].

Currently available assays for fluconazole comprise a bioassay [8], gas chromatography (GC) using nitrogen-selective [9] or electron-capture [10] detection and high-performance liquid chromatography (HPLC) [11]. The bioassay for fluconazole is not very reliable, because antifungal triazoles and most imidazoles often show a discrepancy between in vitro and in vivo activity [2]. Non-biological assays offer greater precision and permit determination of concentrations of individual drugs in patients receiving combination therapy [2]. Recently, micellar electrokinetic capillary chromatography (MECC) was reported to be an attractive method for the analysis of drugs and metabolites in body fluids, including therapeutic drug monitoring [12–16], pharmacokinetic and pharmacogenetic studies [17], forensic medicine [18] and confirmation of illicit drugs in urine of potential drug abusers [19]. MECC combines the separation capabilities of electrophoresis and chromatography [20]. Under the applied electric field, two distinct phases, an aqueous and a micellar or pseudostationary phase, are pumped electroosmotically through an open tubular fused-silica capillary. Separation of solutes which are applied in small quantities at one end of the capillary can be based solely on differential partitioning (non-ionic solutes) and also on differences in electrophoretic behavior and ion pair formation (charged solutes) [20]. Due to the capability of dodecyl sulfate to solubilize proteins, proteinaceous samples (including plasma and serum) can directly be injected into an untreated fused-silica capillary containing a buffer with dodecyl sulfate micelles and drugs can be analyzed, provided they elute before, or after, the proteins [12–15].

Fluconazole is a poorly absorbing compound that can only be detected at wavelengths <220 nm. In this paper, analysis of fluconazole from primary, deproteinized and extracted plasma samples is assessed and a complete MECC assay for the determination of fluconazole in plasma is presented. Blood levels obtained by MECC are compared with those determined by HPLC.

2. Materials and methods

2.1. Chemicals, blank matrix and origin of plasma samples

All chemicals were of analytical reagent or research grade. Sodium dodecyl sulfate (SDS) was from Sigma (St. Louis, MO, USA). Na_2HPO_4, NaH_2PO_4, $Na_2B_4O_7$, tris(hydroxymethyl)aminomethane (Tris) and dichloromethane were from Merck (Darmstadt, Germany). 1,7-Dimethylxanthine (1,7-DMX, Fig. 1) and 2-propanol were from Fluka (Buchs, Switzerland). Methanol and acetonitrile (HPLC-grade) were from Biosolve (Amsterdam, Netherlands). Fluconazole and UK-54373 (Fig. 1) were kindly provided by Pfizer (Zürich, Switzerland). Bovine plasma (from the local slaughter house) was used to prepare calibrators and controls. Plasma samples from healthy volunteers (who gave their consent prior to fluconazole ingestion) and of patients under fluconazole pharmacotherapy were drawn at selected time intervals after a single oral administration of 50, 100, 200 or 400 mg fluconazole capsules (Diflucan, Pfizer). Plasma samples were prepared by centrifugation (1500 *g* for 10 min) and stored at −20°C until analysis.

2.2. Instrumentation and running conditions for MECC

MECC runs were performed using the model ABI 270A-HT capillary electrophoresis system (Perkin-Elmer, Foster City, CA, USA). This apparatus features automated capillary rinsing, sampling and execution of the electrophoretic run. If not stated otherwise, it was equipped with a 75-μm I.D. fused-

silica capillary of 100 cm total length (70 cm to the detector) (Polymicro Technologies, Phoenix, AZ, USA). Injection of sample occurred via vacuum suction for 0.6 s (application of 5 in.Hg vacuum; 1 in.Hg=3386.38 Pa). A constant voltage of 25 kV (250 V/cm, current 76 μA) was applied, the temperature was set at 35°C and detection was effected by UV absorbance at 190 nm. A PC integration pack (version 3.0; Kontron Instruments, Zürich, Switzerland), together with a Mandax AT 286 computer system, was used for data acquisition, raw data storage and data evaluation. 1,7-DMX (Fig. 1) was used as the internal standard (IST) and quantitation was based upon multilevel internal calibration using peak heights. If not stated otherwise, a buffer composed of 75 m*M* SDS, 10 m*M* Na_2HPO_4 and 6 m*M* $Na_2B_4O_7$ (pH 9.2) was employed. In MECC with direct sample injection, no IST was employed and the separation buffer was modified by adding 5% (v/v) 2-propanol and increasing the SDS concentration to 100 m*M*. Between runs, the capillary was equilibrated by sequential rinsing with 0.1 *M* NaOH and running buffer for 2 min each. For the sake of constant running conditions, the anodic buffer vial was replenished with fresh buffer after each set of ten runs.

2.3. Instrumentation and running conditions for HPLC

An HPLC assay similar to that described by Inagaki et al. [11] was employed. The system consisted of a model 501 HPLC pump (Waters, Milford, MA, USA), a model 712 WISP autosampler (Waters) and a Spectroflow 757 absorbance detector (Kratos Analytical, Ramsey, NJ, USA). Chromatography was performed at ambient temperature using a 250/8/4 Nucleosil 5 μm C_{18} column (Macherey-Nagel, Oensingen, Switzerland). A 15-μl volume of pretreated sample was injected. The mobile-phase was composed of 25 m*M* Tris (pH 7.0) and acetonitrile (75:25, v/v) and the flow-rate was 1.0 ml/min. Detection was effected by UV absorbance at 210 nm. A Spectraphysics datajet integrator (Thermo Separation Products, Allschwil, Switzerland) and a PC integration pack (version 3.0; Kontron Instruments), together with a Mandax AT 286 computer system, were used for data acquisition, raw data storage and data evaluation. UK-54373 (Fig. 1) was used as the IST and quantitation was based upon multilevel internal calibration using peak areas.

2.4. Preparation of calibrators and controls

Seven calibrators containing 0.125, 0.25, 0.5, 1.0, 5.0, 10.0 and 25.0 μg/ml of fluconazole were prepared by drying proper aliquots of a fluconazole stock solution [100 μg/ml in methanol–water (10:90, v/v)] at 40°C, under a gentle stream of nitrogen, prior to reconstitution of the residues with drug-free bovine plasma. Aliquots containing 1 ml of the calibrators were frozen at −20°C. For the preparation of controls containing 5.0 μg/ml of fluconazole, a stock solution with 250 μg/ml of fluconazole in methanol–water (10:90, v/v) was used. A 1-ml volume of this solution was added to 49 ml of drug-free bovine plasma and was divided into aliquots of 1 ml that were frozen at −20°C.

2.5. Sample pretreatments

For direct sample injection in MECC, plasma samples were centrifuged at 1500 *g* for 10 min prior to injection of the supernatant. In another effort, proteins were removed using 100 μl of acetonitrile which was added to 200 μl of plasma that contained 40 μg/ml of IST. The mixture was vortex-mixed for 30 s, was kept for 5 min at room temperature and was then centrifuged at 9500 *g* for 3 min. The supernatant was injected for analysis by MECC and HPLC. For liquid–liquid extraction with dichloromethane, 200 μl of plasma, 100 μl of IST solution (20 μg/ml in water–methanol, 90:10) and 2.0 ml of dichloromethane were pipetted into a glass tube and vortex-mixed for 20 s. After centrifugation (10 min at 1500 *g*), the aqueous (upper) phase was discarded and the organic phase was dried at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of running buffer (MECC) or 200 μl of methanol (HPLC). For routine purposes, extraction of fluconazole and IST was achieved using Bakerbond C_{18} cartridges (No. 7020-6 with 500 mg of sorbent and a 6-ml volume, Baker Chemikalien, Gross-Gerau, Germany) in a similar way as was previously described by Inagaki et al. [11]. The columns were placed onto a vacuum manifold (Vac-

Elut, Analytichem International, Harbor City, CA, USA). Each column was preconditioned by sequential washes with two column volumes of methanol, followed by two column volumes of 0.1 *M* sodium phosphate buffer, pH 6.0. A mixture composed of 1 ml of plasma (calibrator, control or patient's sample), 100 μl of IST solution (100 μg/ml in 0.1 *M* sodium phosphate buffer, pH 6.0) and 2 ml of sodium phosphate buffer, pH 6.0, was applied onto the column under a light vacuum (3 to 4 in.Hg). The column was washed with 1 ml of sodium phosphate buffer, pH 6.0, and with 1 ml of 15% (v/v) methanol in sodium phosphate buffer, pH 6.0. Then, the column was dried under a stronger vacuum (8 to 9 in.Hg; 1 min). Fluconazole and IST were first eluted with 500 μl of methanol and without vacuum and then again with 500 μl of methanol under a vacuum of 3 to 4 in.Hg. The combined eluates were dried at 40°C under a gentle N_2-stream and then reconstituted with 50 μl of running buffer (MECC) or with 200 μl of the mobile-phase (HPLC).

2.6. Recovery

The recovery after sample pretreatment was determined by comparing MECC peak heights or HPLC peak areas after extraction with the corresponding peak properties obtained by injection of equal amounts of drugs dissolved in the separation buffer.

3. Results and discussion

The data presented in Fig. 2 represent MECC electropherograms of plasma samples obtained after different pretreatments. Panel A depicts an electropherogram of a directly injected plasma sample of a patient receiving 400 mg of fluconazole per day. Although the separation buffer was modified for better resolution, fluconazole eluted within the proteins and drug levels lower than 5 μg/ml could not be detected. To obtain a cleaner electropherogram, plasma proteins were removed by acetonitrile precipitation. A typical electropherogram is presented in panel B of Fig. 2. Using this approach, which is characterized by an excellent recovery of about 93% for fluconazole and 99% for UK-54373 at the 10

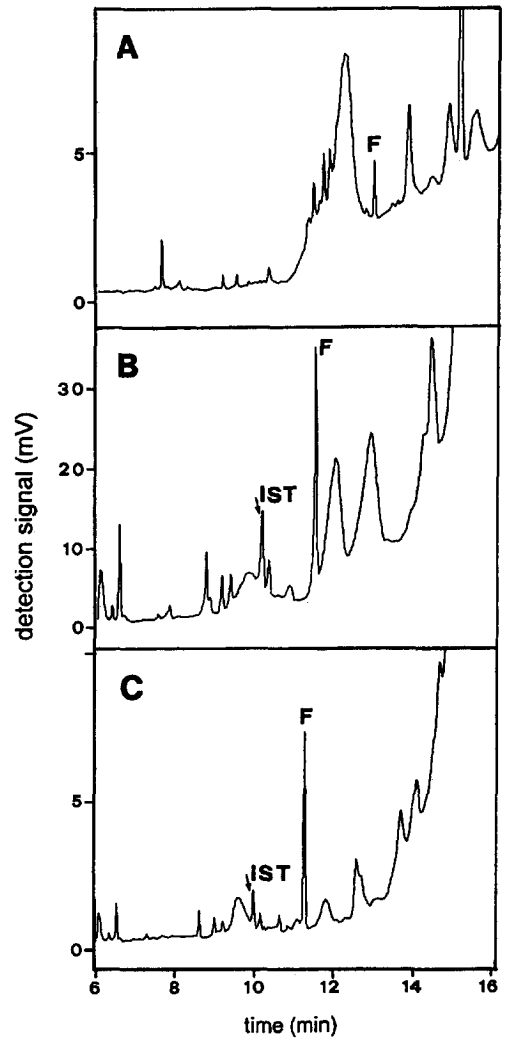

Fig. 2. MECC data obtained after different sample pretreatments having (A) a patient plasma sample containing 22.1 μg/ml of fluconazole which was directly injected into the capillary, (B) bovine plasma containing 100 μg/ml of fluconazole and 40 μg/ml of 1,7-DMX after protein precipitation with acetonitrile and (C) bovine plasma containing 25 μg/ml of fluconazole and 10 μg/ml of 1,7-DMX after extraction with dichloromethane. For (A) a capillary of 64 cm separation length and an electric field of 260 V/cm (current 79 μA) were employed and detection was effected at 200 nm. For (B) and (C) a 70-cm separation length with an electric field of 250 V/cm (current 76 μA) and detection at 190 nm were used. F refers to fluconazole.

μg/ml level, insufficient removal of endogenous substances also prevented baseline resolution for both fluconazole and 1,7-DMX (IST). Furthermore,

fluconazole became diluted and could not be detected at levels <5 μg/ml. Using HPLC, this sample clean-up was found to be applicable for concentrations ≥1 μg/ml (data not shown). However, unacceptable inter-day imprecision of 24% (n=3) for a 5 μg/ml drug concentration was encountered. Employing the procedure based upon liquid–liquid extraction with dichloromethane resulted in much cleaner MECC electropherograms compared to those obtained after acetonitrile protein precipitation. A typical electropherogram of a bovine plasma sample containing 25 μg/ml of fluconazole and 10 μg/ml of IST (1,7-DMX) is presented in panel C of Fig. 2. Fluconazole was concentrated and baseline separated. Unfortunately, UK-54373 (data not shown) and 1,7-DMX (Fig. 2C) co-eluted with endogenous substances. In HPLC, however, fluconazole and UK-54373 were found to elute interference free so that linear calibration curves (six data points) could be constructed between 0.25 and 25.0 μg/ml. The day-to-day imprecision of a 5 μg/ml control sample had a R.S.D. of 9.4% (n=8) and the recoveries for fluconazole and UK-54373 were determined to be 70 and 60%, respectively. No further efforts were undertaken to modify the MECC conditions to allow an electrokinetic analysis of such extracts prepared with dichloromethane.

The data presented in Figs. 3 and 4 depict typical MECC electropherograms and HPLC chromatograms, respectively, that were obtained after solid-phase extraction of fluconazole and IST with a C_{18} cartridge. Using this extraction procedure, clean electropherograms and chromatograms could be obtained (panel B of Figs. 3 and 4). For HPLC, UK-54373 served as the IST. In MECC, however, this compound was found to co-elute with an endogenous substance and could therefore not be used (data not shown). Instead, 1,7-DMX behaved satisfactorily (Fig. 3A) and was determined to separate well from related substituted purines, including caffeine, theophylline and theobromine (data not shown and [16]). Extraction recoveries for fluconazole (5 and 10 μg/ml sample) and 1,7-DMX (10 μg/ml sample) were determined to be 65 and 75%, respectively (n=5). MECC is shown to provide a higher separation performance than HPLC. For a sample containing about 5 μg/ml of fluconazole 130 000 theoretical plates (plate height of about 5.4 μm, Fig. 3C) are attained in MECC. Corresponding values for HPLC are about 2500 and 98 μm, respectively. Furthermore, detection times in MECC (Fig. 3) are slightly higher than those in HPLC (Fig. 4). They are reproducible in both methods (R.S.D.≤ 2%). It is important to note that with human serum instead of plasma, the baseline in the MECC electropherograms were not as clean, which prevented unambiguous detection of fluconazole at low concentrations (≤1 μg/ml; data not shown)

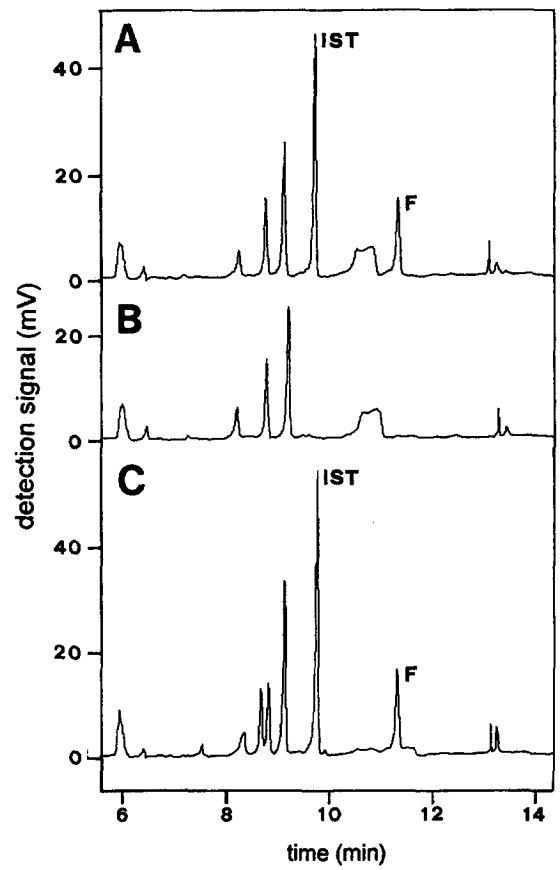

Fig. 3. Typical MECC electropherograms obtained after solid-phase extraction of (A) bovine plasma spiked with 5 μg/ml of fluconazole and 10 μg/ml of IST, (B) blank bovine plasma and (C) plasma of a healthy volunteer which was drawn 45 min after oral administration of 200 mg of fluconazole (drug level: 4.48 μg/ml). Other conditions are as for Fig. 2B.

Using 1,7-DMX and UK-54373 (10 μg/ml each) as I.S. in MECC and HPLC, respectively, and seven-level internal calibration based upon peak heights (MECC) and areas (HPLC), linear calibration graphs

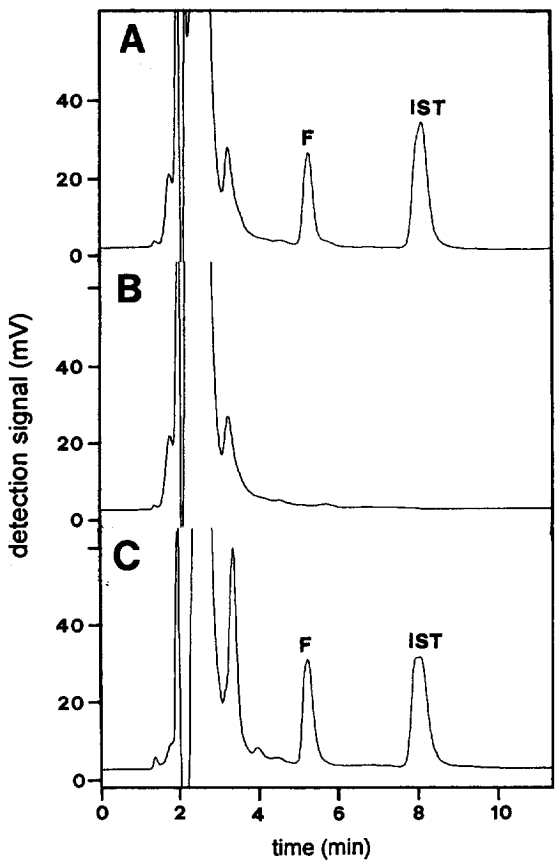

Fig. 4. Typical HPLC chromatograms obtained after solid-phase extraction of (A) a bovine plasma calibrator containing 5 μg/ml of fluconazole, (B) blank bovine plasma and (C) plasma of a healthy volunteer which was drawn 45 min after ingestion of 400 mg of fluconazole (drug level: 6.14 μg/ml).

were obtained. Linear regression analysis revealed coefficients $r=1.0000$ and $r=0.9999$, respectively, and very small (<0.05 μg/ml) y-intercepts. Both assays were determined to be reproducible (Table 1)

Table 1
Imprecision data assessed with a control sample containing 5 μg/ml of fluconazole and solid-phase extraction

			MECC	HPLC
Intra-day	R.S.D.	%	2.05	1.98
	Mean value	μg/ml	5.11	5.06
	n		10	10
Inter-day	R.S.D.	%	5.85	9.17
	Mean value	μg/ml	4.87	4.84
	n		10	10

and detection limits were determined to be about 0.1 μg/ml. Comparable drug levels of 44 samples obtained by MECC and HPLC are presented in Fig. 5. Based upon linear regression analysis (panel A), the data are shown to be linearly dependent, with a regression coefficient $r=0.985$. The slope and y-intercept were determined to be 0.858 and 0.30 μg/ml, respectively. Furthermore, a graphical presentation of the differences vs. mean of the 44 data pairs according to the suggestion of Bland and Altman [21] is depicted in panel B of Fig. 5. As expected, 42 of the 44 samples are shown to be within the range defined by the mean of the differences ±2 S.D. The data reveal that drug levels determined by HPLC are slightly higher compared to those monitored by MECC. The bias of 0.24 μg/ml corresponds to 4.8% with regard to a fluconazole level of 5 μg/ml (center of concentration range

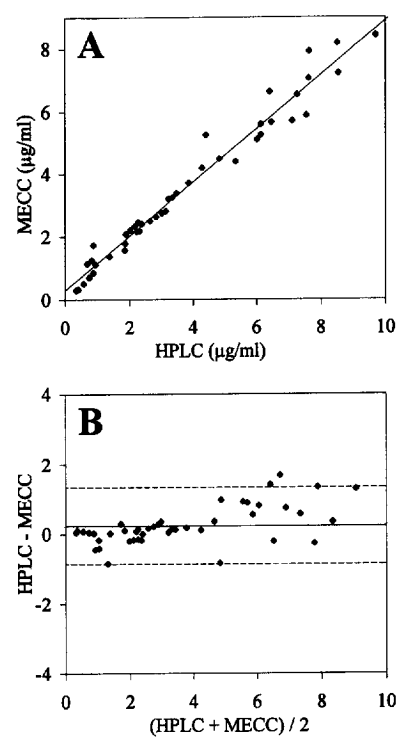

Fig. 5. Comparison of MECC- and HPLC-plasma levels of 44 samples. (A) MECC versus HPLC with linear regression analysis (slope=0.858, intercept=0.30 μg/ml, $r=0.985$). (B) Difference versus mean for comparative fluconazole plasma levels. The solid line represents the mean of the differences (0.24 μg/ml) and the broken lines the mean ±2 S.D. (+1.337/−0.864 μg/ml).

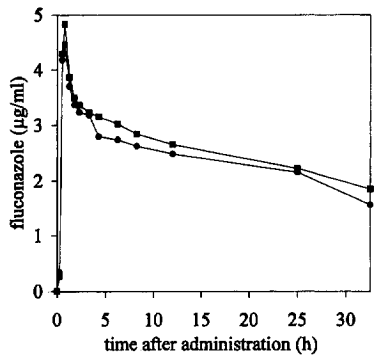

Fig. 6. Plasma levels monitored after administration of 200 mg of fluconazole to a healthy volunteer. ●=MECC, ■=HPLC.

given in Fig. 5), a difference that is insignificant for clinical purposes. Thus, MECC instead of HPLC can be employed for drug monitoring of fluconazole.

Pharmacokinetic data obtained after a single oral dose of 200 mg of fluconazole are presented in Fig. 6. Following rapid absorption, maximal plasma levels were determined about 45 min after drug administration. The half life was calculated to be 33 h for the beta phase. With single oral doses of 100 and 400 mg of fluconazole, half lives of 25 and 19 h were obtained, respectively (Table 2). The data presented in Table 2 further reveal the linear relationship between fluconazole dose and plasma level observed after a single oral administration of this drug. Values obtained via MECC drug determination compare well with those obtained by HPLC and with those reported in the literature [1,3,4,6]. The data presented in Fig. 6 also reveal fluconazole plasma levels measured by HPLC to be slightly higher than those obtained by MECC. The selection of 1,7-DMX as the I.S. could be the reason for this difference. 1,7-DMX (paraxanthine) is an intermediate in the caffeine metabolism and thus is present in small concentrations in the plasma of individuals consuming beverages which contain caffeine, such as coffee [16,22]. The individual whose fluconazole plasma data are depicted in Fig. 6 did indeed consume coffee about 2 h after fluconazole ingestion. Based upon this interference, the MECC assay described cannot be employed to assess accurate fluconazole plasma levels in patients under caffeine pharmacotherapy. This restriction mainly applies to the use of caffeine pharmacotherapy to treat apnoea in prematurely born infants. 1,7-DMX is not a metabolite of theophylline and theobromine.

Table 2
Pharmacokinetic data of fluconazole obtained by MECC and HPLC

Expected plasma levels		Literature[c]	MECC	HPLC
Single dose	50 mg	0.95		
maximum levels[a]	100 mg	2.0	2.5	2.4
(µg/ml)	200 mg		4.5	4.8
Trough steady-state	100 mg/d	6.25		
level[b]	200 mg/d		14.9	
(µg/ml)	400 mg/d		25.4	25.6
Half life (h)		22–37	19–33	19–35

[a] Values from pharmacokinetic experiments.
[b] Patients' samples
[c] See references [1,3,4,7].

4. Conclusions

MECC is shown to permit unambiguous analysis of fluconazole in human plasma when detection is effected at 190 nm. The detection limit for a directly injected plasma sample or for analysis of the supernatant after protein precipitation with acetonitrile was determined to be 5 µg/ml, this being too high for therapeutic drug monitoring and pharmacokinetic studies with expected maximum plasma levels of 1 and 8 µg/ml for a single dose of 50 and 400 mg, respectively. Furthermore, employing liquid–liquid extraction with dichloromethane, fluconazole levels of ≥1 µg/ml could be determined. After C_{18} solid-phase extraction, however, the detection limit was 100 ng/ml. Using that assay, intra-day and day-to-day imprecisions for a 5 µg/ml fluconazole level were determined to be around 2 and 6%, respectively. MECC plasma levels compared well with those obtained by HPLC. Pharmacokinetic data obtained by MECC agreed well with those given in the literature. Thus, MECC can be employed for drug monitoring of fluconazole. Compared to HPLC, MECC is an attractive, low-cost analytical method that does not require large amounts of potentially polluting solvents and chemicals. It provides a higher efficiency than HPLC and permits solute detection at 190 nm.

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