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Determination of clotrimazole in mice plasma by capillary electrophoresis

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

In addition to its antifungal activity, clotrimazole attracts interest as an anti-inflammatory drug. In order to correlate this effect with plasma concentrations in mice, a capillary electrophoretic method was developed. Sample preparation was carried out by protein precipitation using methanol. Quantification of clotrimazole was achieved by means of capillary electrophoresis using ketoconazole as an internal standard (IS). The background electrolyte (BGE) composed of a Tris buffer solution (100 mM, pH 3.0, adjusted with acetic acid) and methanol (8:2, v/v). Injection was carried out electrokinetically with 10 kV over a time period of 20 s. A special rinsing procedure utilizing a sequence of a SDS/methanol solution, a sodium hydroxide solution, water and BGE, was applied to enhance the reproducibility. With this procedure, an intermediate precision (day-to-day precision) of the area ratios of clotrimazole and IS of 5.0% for 0.5 μg ml^{-1} and 2.6% for 10 μg ml^{-1} was obtained. In summary, with the described capillary zone electrophoresis (CZE) method it is possible to handle small sample volumes of 60 μl, to detect clotrimazole concentrations of 0.3 μg ml^{-1} (limit of detection), and to quantify clotrimazole down to concentrations of 0.5 μg ml^{-1} (limit of quantification).

Keywords: CZE; Clotrimazole; Plasma

1. Introduction

Clotrimazole (cf. Fig. 1) is known to be an efficient drug acting against a broad spectrum of fungi. In addition, clotrimazole is a potent inhibitor of calcium-sensitive potassium channels of the IK1- or SK4-type [1] which are, among others, expressed in many secretory epithelial cells [2] and in migrating cells like those of the immune system, e.g. lymphocytes, granulocytes, or macrophages [3]. Their importance for the cellular immune response gained particular interest. They are involved in cell migration [4], in proliferation of

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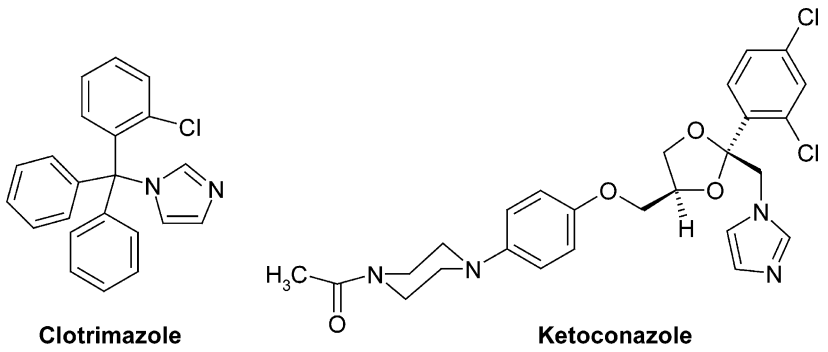

Fig. 1. Chemical structures of clotrimazole and ketoconazole (IS).

activated T-lymphocytes [5], and in the generation of reactive oxygen intermediates [6]. IK1/SK4 channel may therefore be potential therapeutic target in inflammatory diseases when a modulation of the immune responses has to be achieved. Such an anti-inflammatory effect was already observed in a study in which clotrimazole was given to patients with rheumatoid arthritis [7].

The functional significance of IK1/SK4 channel is pharmacologically assessed by applying channel inhibitors like clotrimazole. The IC_{50}-values for cloned channels were reported to be in a range between 24 nmol/l [8] and 387 nmol/l [9]. While the drug concentration is easily controlled in in vitro experiments, the situation is more complex in in vivo experiments. However, it is crucial to correlate a biological effect of the IK1/SK4 channel inhibitor clotrimazole in in vivo experiments with its plasma concentration. Therefore, we developed a technique to determine the concentration in mice plasma. Bearing the pharmacological background in mind, it was attempted to develop a capillary electrophoresis (CE) method, which can be used to determine clotrimazole simply, rapidly, and reproducibly in the small volumes of blood available from mice.

Usually, HPLC represents the standard instrumentation in industry. Thus, several HPLC methods for the determination of clotrimazole and other azole antifungal drugs were reported [10–13]. In addition, the development of different methods for the quantification of mixtures of azole antifungal drugs [14,15] and the quantification of clotrimazole in certain pharmaceutical formulations [16] by HPLC were described. Since 1990s CE attracts more and more attention as an analytical separation technique. The direct comparison between HPLC and CE (MEKC) was evaluated in Ref. [16]. Moreover, a CE method for the determination of fluconazole from plasma was also described [17]. However, this method was developed for human plasma and needs 200 μl of plasma. Only a small amount of plasma (approximately 300–600 μl) can be obtained from the blood of mice. Consequently, this method cannot be used without major modification if it is intended to measure several replicates of the mice plasma samples. Another important difference with respect to method development between fluconazole and clotrimazole is plasma binding. Whereas fluconazole shows a plasma binding of about 20% only, clotrimazole exhibits a plasma binding of 98% [18]. Since CE methods need only small sample volumes, and since plasma samples can be used even without sample preparation [19], our goal was to develop a method which is able to determine the amount of clotrimazole in mice plasma by means of CE from approximately 100 μl of plasma or less. During the course of method development, two methods for sample pretreatment were compared: protein precipitation (PP) and liquid–liquid extraction (LLE).

2. Experimental

2.1. Reagents and materials

Clotrimazole CRS was purchased from Promochem (Wesel, Germany); ketoconazole CRS was a gift from Janssen-Cilag GmbH (Neuss, Germany). Tris(hydroxymethyl)aminomethane (Tris), acetic acid (p.a.), methanol (HPLC grade) and 2-propanol were purchased from Merck Eurolab (Darmstadt, Germany); sodium dodecyl sulfate (SDS) and dichloromethane from Fluka/Riedel-de-Haen (Seelze, Germany) and acetonitrile (HPLC grade) from Carl Roth (Karlsruhe, Germany). Commercial samples of clotrimazole were gifts from Bayer AG (Leverkusen, Germany) and Hexal AG (Holzkirchen, Germany) and commercial samples of ketoconazole were purchased from Fährhaus Pharma (Hamburg, Germany). The capillaries were purchased from Polymicro (Optronis GmbH, Kehl, Germany). All samples and buffers were prepared using ultrapure Milli-Q water (Millipore, Milford, MA).

2.2. Instrumentation

The measurements of the samples obtained by PP were performed on a P/ACE MDQ system (Beckman, Fullerton, CA) equipped with a diode array detector (DAD) using an uncoated fused-silica capillary with a total length of 31.2 cm, a detection length of 21.0 cm and an internal diameter of 75 μm (375 μm OD). Additionally, this method was performed on an HP^{3D}-CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a DAD using an uncoated fused-silica capillary with a total length of 33.0 cm, a detection length of 24.5 cm and an internal diameter of 75 μm (375 μm OD). The measurement of the samples obtained by LLE was also performed on the HP^{3D}-CE.

All samples were centrifuged with a Heraeus Christ centrifuge (Hanau, Germany), model Biofuge A, type 1230. The pH-value of all solutions was measured with a PHM 220 Lab-pH-meter build by Radiometer Copenhagen (Lyon, France) using an electrode suited for small volumes (pHC3359).

2.3. Buffers and solutions

Tris buffer solution (100 mM, pH 3.0)/methanol (8:2, v/v) was prepared by mixing an aqueous Tris stock solution (125 mM, pH 3.0, adjusted with acetic acid) with an appropriate volume of methanol. The SDS/methanol rinse solution was prepared by mixing an aqueous SDS stock solution (200 mM) with appropriate volume of methanol to give a concentration of 100 mM.

For PP clotrimazole, calibration stock solutions (PP-SS_c) were prepared by dissolving 60.0 mg of clotrimazole in a 10.0 ml flask in methanol. One-hundred-microliter of the solution was diluted to 10.0 ml with methanol (PP-DS_c1). For working solutions, an aliquot volume was taken from PP-DS_c1 and diluted with methanol to 10.0 ml to prepare solutions ranging from 0.3 to 10 μg ml^{-1}. The ketoconazole internal standard (IS) stock solution (PP-SS_k) was prepared by dissolving 30.0 mg of ketoconazole in a 10.0 ml flask in methanol. One-thousand-microliter of this solution was diluted with methanol to 10.0 ml (PP-DS_k1). For working solutions, 100 μl was taken and diluted to 10.0 ml with methanol (PP-SD_k2).

For LLE clotrimazole calibration stock solutions (LLE-SS_c) were prepared (0.5–3.0 mg ml^{-1}) by dissolving 30.0, 60.0, 120.0 and 180.0 mg of clotrimazole in dichloromethane, respectively. One-hundred-microliter of each solution was diluted to 10.0 ml with dichloromethane (LLE-DS_c1). Again, 100 μl of these solutions was diluted to 10.0 ml with dichloromethane (LLE-DS_c2). The ketoconazole IS stock solution (LLE-SS_k) was prepared by dissolving 30.0 mg in a 10.0 ml flask in dichloromethane. One-thousand-microliter of this solution was diluted to 10.0 ml with dichloromethane (LLE-DS_k1), and 100 μl of this solution was diluted to 10.0 ml with dichloromethane (LLE-DS_k2). Additionally, an aqueous solution (pH 2.0, adjusted with acetic acid)/methanol (1:1, v/v) was prepared.

With exception of the sample and calibration solutions, all solutions were filtered (polyvinyidenefluoride membrane, PVDF, 22 μm pore size) before use.

2.4. Electrophoretic conditions

The background electrolyte (BGE) for all measurements composed of a Tris solution (100 mM, pH 3.0)/methanol (8:2, v/v). Before starting the measurements, the capillary was first rinsed with 0.1 M NaOH, water and the appropriate buffer for 5 min each. Between runs, the capillary was rinsed for 3 min with the SDS/methanol solution, 2 min with an aqueous NaOH solution (0.1 M), 1 min with water and 2 min with the appropriate buffer. Using the PP method, samples were injected applying a voltage of 10 kV for 20 s. When using the LLE method, samples were injected applying 50 mbar (= 0.03447 psi) for 10 s. While measuring the samples were stored at 15 °C in the Beckman system. The Agilent system is not able to control the temperature in the sample storage. Measurements with both the systems were carried out at 25 °C using a constant voltage of 18 kV. Analytes were detected with an UV-DAD at 196 nm.

2.5. Sample preparation by PP

For sample preparation, exactly 60 μl of plasma was taken; next 100 μl of clotrimazole solution PP-DS_c2 (for calibration solutions, otherwise 100 μl of methanol) and 100 μl of ketoconazole solution PP-DS_k2 were added. In order to precipitate the proteins, this mixture was diluted to 0.50 ml with methanol. Afterwards, the samples were vortexed, 15 min sonicated and 10 min centrifuged at 10,000 rpm. The entire volume of the clear supernatant was taken and reduced for approximately 1.5 h in a water bath at 60 °C. Afterwards, a drop of acetic acid was added to achieve a pH of about 2.0. The sample was filled in a vial, which is specifically suited for sample volumes less than 200 μl.

2.6. Sample preparation by LLE

Sixty-milliliter of plasma was taken, then 100 μl of clotrimazole solution LLE-DS_c2 and 100 μl of ketoconazole solution LLE-DS_k2 were added. The solution was vortexed for 10 s and the organic layer was collected. The procedure was repeated twice (i.e. three extractions were carried out in total), the organic phases were collected and reduced in a water bath (50 °C) to dryness. Sixty-milliliter of the aqueous acetic acid solution (pH 2.0)/methanol (1:1, v/v) was added and poured in a vial, which is specifically suited for sample volumes less than 200 μl.

3. Results and discussion

The goal of this study was to develop a simple method with short analysis time and low detection limit. In order to eliminate the proteins, two methods, the PP method and the LLE method were evaluated. An electropherogram of the optimized PP method is shown in Fig. 2.

3.1. Variations in sample preparation: PP versus LLE

The PP method which was already used by von Heeren et al. [17] was evaluated first. One major difference to von Heeren's method is the smaller sample volume needed by the method described here (60 μl instead of 200 μl, see Section 2.5). During optimization of the method three precipitation reagents, methanol, acetonitrile and trichloro acetic acid, were tested. Trichloro acetic acid was found to decompose clotrimazole. In comparison with methanol, the use of acetonitrile as precipitation reagent did not result in a better yield. However, methanol turned out to be the better organic modifier in CE; so, further PPs were done with methanol. In addition, different buffers, i.e. phosphate buffers, acetate buffers and Tris buffers were tested. The phosphate buffer resulted in a bad peak shape. Acetate buffer within a pH range 4.0–5.18 gave relatively stable runs but the calibration was not feasible. Measurements in Tris buffer, pH 3.0, achieved satisfactory reproducibility and an acceptable limit of detection (LOD). Bifonazole as IS was tried in the first place but was found to co-elute with clotrimazole. Ketoconazole turned out to be suitable. Owing to the excessive plasma binding of clotrimazole, recovery from plasma for the PP method was assessed by the following experiment. First, sample preparation was done as described in Section 2.5 (i.e. clotrimazole and ketoconazole were added to plasma

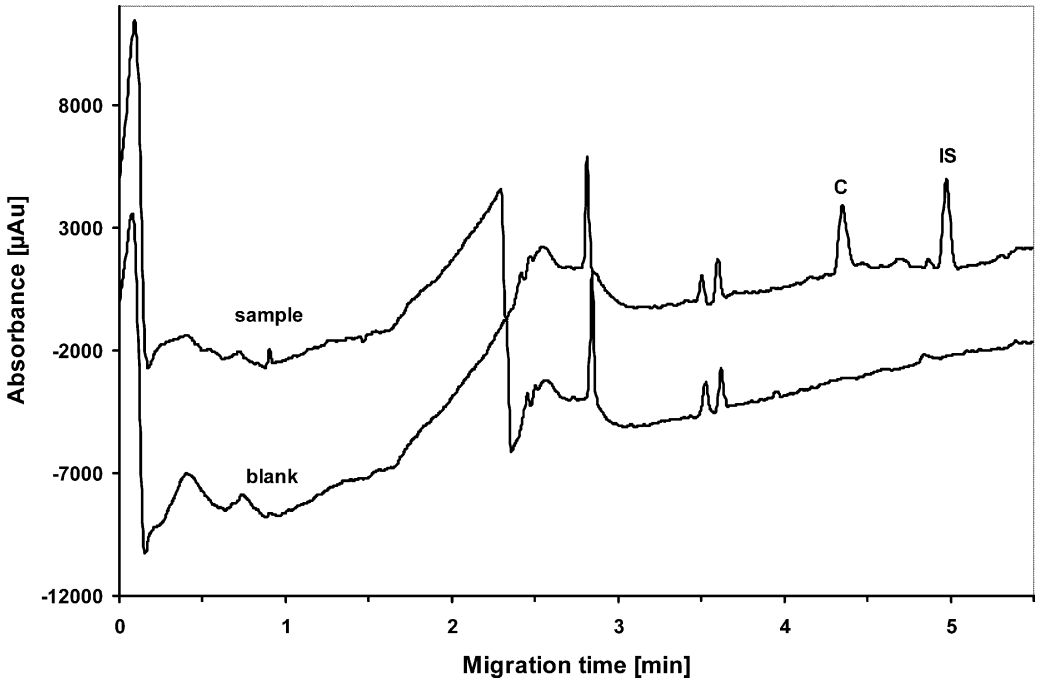

Fig. 2. Electropherogram of clotrimazole (C, 1.0 μg ml^{-1}) and ketoconazole (IS, 60 μg ml^{-1}) in plasma and a plasma blank. Conditions: BGE—Tris (100 mM, pH 3.0)/methanol (8:2, v/v); injection—10 kV, 20 s; run voltage—18 kV; $\lambda = 196$ nm.

from the beginning). Second, clotrimazole and ketoconazole solutions were added only after the proteins were already precipitated. From these experiments, which were carried out at clotrimazole concentrations of 1.0 and 10.0 μg ml^{-1} (five replicates each), recovery was computed to be 73 and 70% for 1.0 and 10.0 μg ml^{-1}, respectively. The recovery is considered to be acceptable in light of the 98% plasma binding of clotrimazole.

For the LLE method, dichloromethane and tetrachloromethane were used as the extraction solvent. Since similar results were obtained with both solvents, dichloromethane was used subsequently owing to its lower toxicity. The sensitivity of the LLE method as compared with the PP method was far better (cf. Fig. 3). However, it was impossible to calibrate the LLE method. It can be seen in Fig. 3 that clotrimazole co-elutes with some unknown compound from plasma (shoulder). Probably too many plasma constituents were dissolved in dichloromethane which adhere to the capillary wall over the runs and make a reproducible separation difficult. Even a repeated exchange of the capillary and adaptation of the rinsing procedures did not improve the results. Moreover, it was found that with the LLE method recovery of clotrimazole from plasma depends on the equilibration time. Put differently, if clotrimazole is vortexed immediately (as described in Section 2.6), recovery is greater as if clotrimazole is exposed to plasma over a period of 10 min and then vortexed. Recovery also decreases if clotrimazole is vortexed immediately and the sample pretreatment is interrupted afterwards for some time. Both findings, the unsatisfactory separation of clotrimazole from plasma constituents (cf. Fig. 3) and the equilibrium time dependence, led us to prefer the PP method over the LLE method. Therefore, solely the PP method was applied in the following.

3.2. CE condition variations

In order to optimize the CE separation, the following parameters were varied: voltages from 15 to 18 kV proved to be the best. Shorter runs

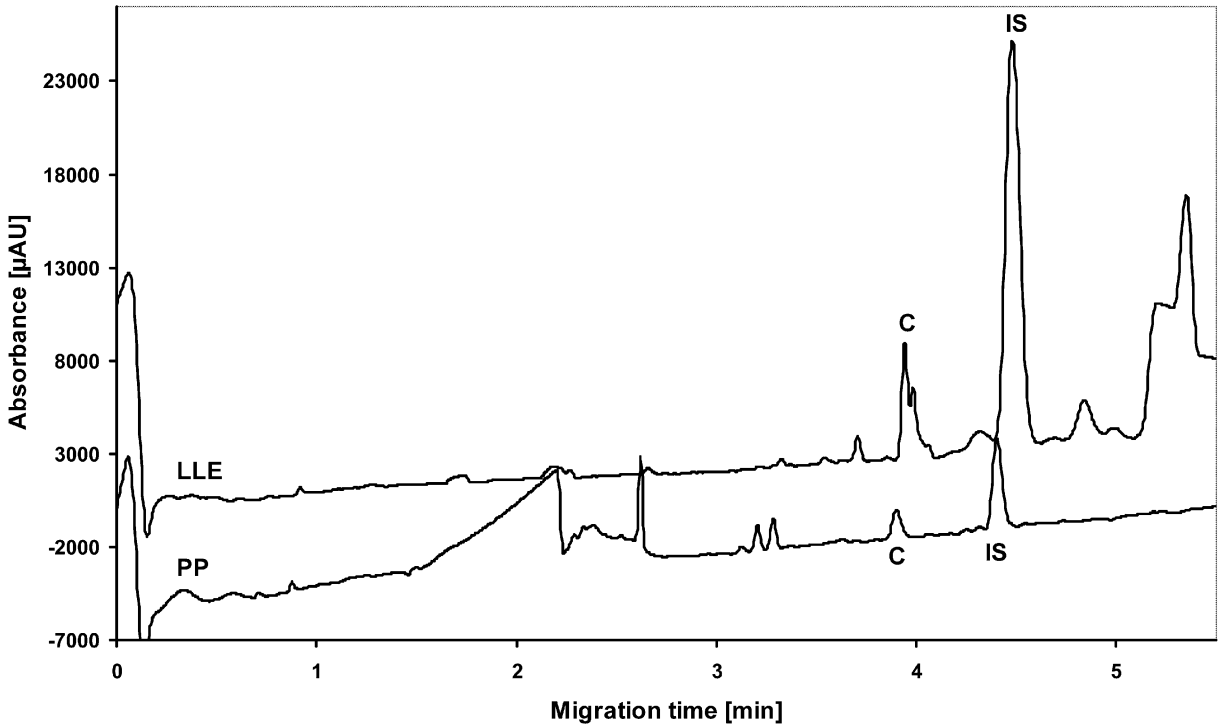

Fig. 3. Comparison of the electropherograms (for experimental conditions, see Fig. 2) obtained after PP and LLE. Concentration of clotrimazole in both cases was 1.0 µg ml^{-1}. Concentration of ketoconazole was 6.0 µg ml^{-1} for both methods.

could be obtained by optimizing capillary length and voltage. With a capillary as short as 24.5 cm/33.0 cm (detection length/effective length), reproducible runs of good separations were obtained within 6 min. In order to enhance the sensitivity, a single-wavelength UV detector was tested, but differences concerning LOD and limit of quantification (LOQ) were negligible as compared with DAD. Thus, DAD was used for all experiments.

3.3. The influence of the rinsing procedure

Furthermore, the rinsing procedures were optimized. The use of SDS in methanol or acetonitrile solution was shown to be advantageous for measurements of biological samples by Kunkel et al. [19]. This finding can be supported with our study. As soon as the rinsing step was omitted, the relative standard deviation (RSD) of the area ratio (clotrimazole/IS) worsened markedly. The rinsing procedure uses, among other steps, rinsing with NaOH solution for 2 min (see Section 2.4). Despite the large pH shift between rinsing and the separation conditions (Tris buffer, pH 3.0), applying NaOH solution for rinsing lowered baseline noise considerably and was thus found to be valuable.

4. Validation of the method

4.1. Statistical validation of the calibration data

Linear least-squares fitting was used for computing the calibration line. Statistical validation of the calibration data should always be carried out to check whether the data could be used for the intended purpose [20]. In particular, when deriving figures of merit from the calibration line, such as LOQ, a statistical validation becomes important. Linearity, homoscedasticity, and normality of the data are the key topics that should be checked for the present method. Linearity is often proved with

the help of the correlation coefficient, which is 0.9999 for the calibration line shown in Fig. 4. Since the correlation coefficient alone is not a reliable indicator for linearity (it depends on the residual sum of squares and the slope of the calibration line) [21], an additional statistical test was carried out. The data were fit by a second-order polynomial and the quadratic coefficient determining the curvature of the resulting fit was tested for statistical significance. Since the quadratic coefficient was found to be not significant (t-test, error of the first kind: 5%, two-sided), it can be concluded that the calibration data are better represented by a straight line than a second-order polynomial. Consequently, there is no evidence for deviations from linearity. Computing LOQ can be based on the standard deviation of the response and the slope of the calibration line [22]. In this case it is calculated as follows:

$$\mathrm{LOQ} = \frac{10\hat{\sigma}}{S}, \tag{1}$$

where $\hat{\sigma}$ is an estimator of the standard deviation of the response and S is the slope of the calibration curve. When $\hat{\sigma}$ is estimated by the residual standard deviation of the calibration line (as is usually done for calibration lines), it is important for the calibration data to be homoscedastic. Homoscedasticity means that the residual standard deviation is constant across the entire calibration range. If this was not the case, Eq. (1) could not be used to compute LOQ without modification. Since we have computed LOQ using Eq. (1) and the residual standard deviation of the calibration line as an estimator for $\hat{\sigma}$, the data were checked for homoscedasticity with the Cook-Weisberg test. The exact algorithm for computing the test statistic can be found elsewhere [20,23].

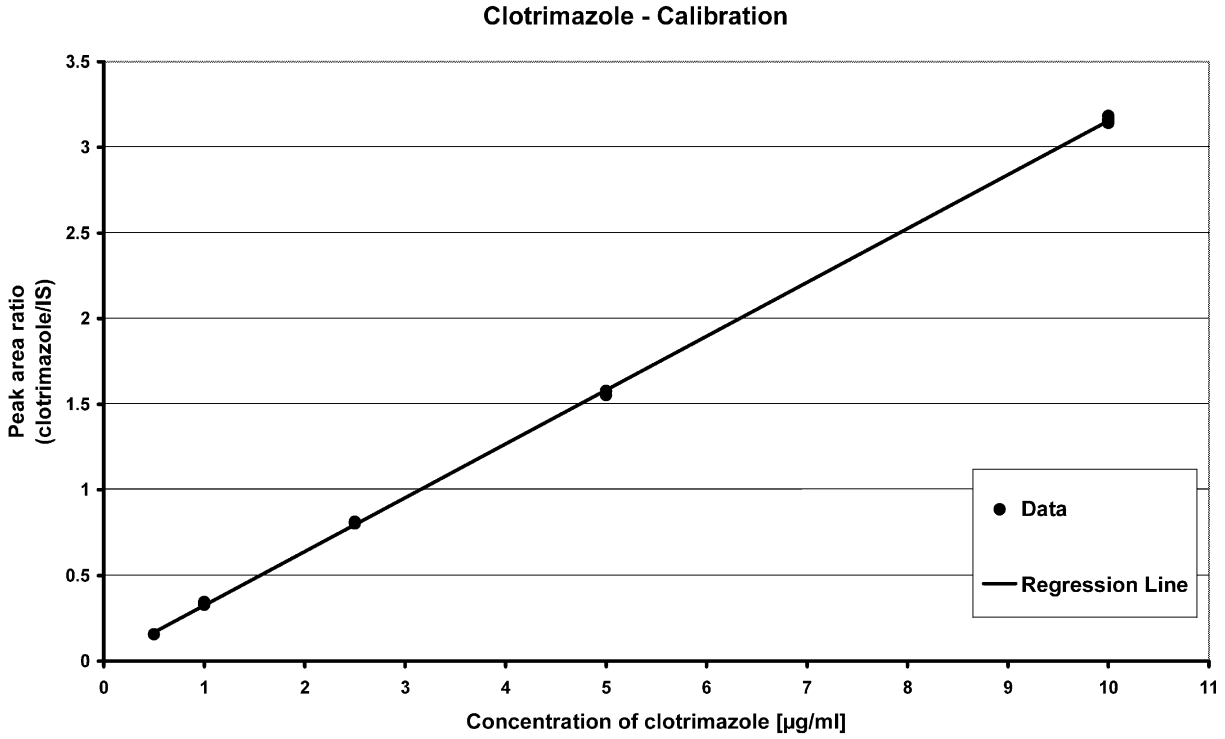

Fig. 4. Diagram showing the ratios of the peak areas (clotrimazole/IS) versus the concentration for the calibration of clotrimazole (ketoconazole = IS) in plasma. Figures of merit of the calibration line can be found in Table 1.

The Cook-Weisberg test indicated that the data are homoscedastic (error of the first kind: 5%). Finally, the data were checked for normality. Standard deviations (as well as confidence intervals) derived from the calibration line are only valid estimators of the error if the data are normally distributed. Hence, the D'Agostino test and the *R*-test for checking the normality of the data were carried out. Both tests were chosen since they performed well in detecting deviations from normality for regression data [24]. Algorithms for computing the test statistics are available [23,24]. Both tests indicated no deviation from normality for the calibration data obtained by the PP method presented above (error of the first kind: 5%). Since the data are linear, homoscedastic, and normally distributed, Eq. (1) can be used to calculate a reliable estimate of LOQ.

4.2. Method validation according to ICH guidelines

After the statistical validation of the calibration data, a method validation was carried out according to ICH guidelines [22]. The following figures of merit were determined and are presented in Table 1: linearity, range, precision, LOD, and LOQ were determined. Recall that all figures were computed using the ratio of the peak areas of clotrimazole and ketoconazole (IS). Linearity was part of the statistical validation (see above). The PP method was demonstrated to yield a linear calibration curve in the range from 0.5 to 10.0 μg mg^{-1}. Two measures of precision were determined. First, the repeatability (intra-day precision) was assessed at the highest and the lowest concentration of the range using five replications each. Second, the intermediate precision was determined as day-to-day precision. Measurements were carried out on three different days at the highest and lowest concentration of the range using five replications each. RSDs of the area ratios clotrimazole/IS for repeatability (0.5 μg mg^{-1}: 0.4%, 10.0 μg mg^{-1}: 0.6%) and for intermediate precision (0.5 μg mg^{-1}: 5.0%, 10.0 μg mg^{-1}: 2.6%) show that the method performs quite well (cf. Table 1). LOD was determined as the lowest concentration measured for which the clotrimazole peak exhibited a signal-to-noise ratio (SNR) greater than 3. This requirement was fulfilled by 0.3 μg ml^{-1}. SNR was determined according to the European pharmacopoeia [25]. Baseline regions before and after the clotrimazole peak were used to assess the baseline noise (peak-to-peak noise). Two times the peak height resulting from the integration of the peak was then divided by the peak-to-peak noise to obtain SNR. Once more, five replications were carried out. SNR varied from 3.6 to 5.4 with a mean value of 4.4. LOQ was determined using Eq. (1). $\hat{\sigma}$ was estimated by the residual standard deviation of the calibration line and S by the slope of the calibration line. This yields an LOQ of 0.49 μg ml^{-1}. Consequently, LOQ was set to 0.5 μg ml^{-1}, which is a concentration that was actually measured. In addition to the aforementioned figures of merit, the migration time ratio of clotrimazole and ketoconazole which reflects the reproducibility of the migration times of both analytes relative to each other was determined across the entire range of the calibration. RSDs ranging from 0.07 to 0.25% were obtained showing very good reproducibility of the migration times. The values can be found in Table 2.

Table 1
Method validation based on the ICH guideline

Linearity			
Correlation coefficient	0.9999		
y-intercept	0.0116		
Slope	0.3143		
Residual sum of squares	0.0054		
Range	0.5–10.0 μg ml^{-1}		
		S.D.	RSD (%)
Precision			
Repeatability	0.5 μg ml^{-1}	0.002	0.4
($n = 5$) [a]	10.0 μg ml^{-1}	0.058	0.6
Intermediate precision	0.5 μg ml^{-1}	0.008	5.0
($n = 15$; 3 days) [a]	10.0 μg ml^{-1}	0.067	2.6
LOD (S/N > 3)	0.3 μg ml^{-1}		
LOQ (Eq. (1))	0.5 μg ml^{-1}		

Figures of merit for linearity and precision are based on peak area ratios of clotrimazole and IS.
[a] Number of replicates.

Table 2
Precision of migration time ratios (clotrimazole/IS)

Concentration (μg ml^{-1})	RSD (%)	*n*
0.5	0.07	8
1.0	0.25	8
2.5	0.09	8
5.0	0.13	8
10.0	0.04	8
All samples	0.55	40

5. Conclusions

Applying the PP method, small sample volumes of 60 μl can be handled. With the presented CZE method using electrokinetic injection, it is possible to detect clotrimazole down to 0.3 μg ml^{-1}. Plasma concentrations of clotrimazole down to 0.5 μg ml^{-1} can be quantified. For drug monitoring and pharmacokinetic studies, this LOQ is still a little high, since concentrations even less than 0.5 μg ml^{-1} may be expected. The small recovery from plasma is attributed to the strong plasma binding of clotrimazole which is about 98%. As a result, sample amounts of free clotrimazole are very small. A solid-phase extraction (SPE) may result in a lower LOD. Studies utilizing SPE are in progress. Preliminary results show that LOD and LOQ are at least as good as those obtained by the PP method. More detailed studies will show whether LOD and LOQ can significantly be decreased.

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