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HPLC Detection of miltefosine using an evaporative light scattering detector

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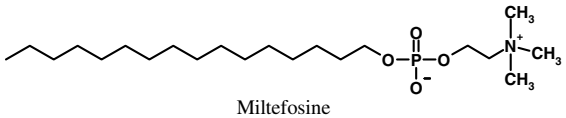
Miltefosine has recently been introduced as leishmanicidal drug for oral administration (Impavido®). Previous communications report about the use of liquid chromatography coupled with mass spectrometry detection to detect miltefosine in pharmaceutical preparations and biological fluids. We report about a new method to detect miltefosine using an evaporative light scattering detector (ELSD). The absolute recovery of the analyte was greater than 98.0%. The limit of quantification for miltefosine in plasma at a signal-to-noise ratio of 7.3 was 0.34 μg/ml. The precision of the assay yielded coefficients of variation ranging from 1.8 to 4.5% and an accuracy of 97–107%. Our method advances the qualitative and quantitative detection of miltefosine by combining rapid and efficient solid phase extraction and analysis with an evaporative light-scattering detector.

1. Introduction

Miltefosine is the first leishmanicidal drug for oral use (Sundar et al. 1998; Kaminsky 2002). Formerly developed as anticancer drug (Unger et al. 1990), it became important as a highly efficient antiparasitic drug for the treatment of leishmaniasis. Miltefosine has been registered under the brand name Impavido® in India and has recently received orphan drug status in Europe and has been approved 2004 by the German Food and Drug Agency (BfArM) for the treatment of visceral and cutaneous leishmaniasis (Unger and Eibl 2001; Sindermann et al. 2004). Analytical detection of miltefosine is limited because of the absence of chromophors in the core structure. Only liquid chromatography coupled with mass spectrometry (LC-MS) is reported in literature for the detection of the drug (Knebel et al. 1999). For laboratory use and quality control the use of LC-MS is more or less time consuming and requires a complicated purification step for drug extraction. Therefore, most of the reported methods suggest an expensive sample extraction by switching devices such as liquid-liquid extraction.



Miltefosine

To carry out pharmacokinetic analyses involving miltefosine and its metabolites, the development of a precise analytical method is of interest that is less time consuming, allows easier sample preparation and shows sufficient recovery of miltefosine. In this communication we report about light scattering as a new principle to detect miltefosine in pharmaceutical preparations and biological samples. The use of light scattering as a detection principle in HPLC has previously been described by Stolyhwo et al. (1983, 1984) and Webster et al. (2004). This type of detector works by measuring the light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase (Righezza and Guiochon 1988). Main advantage is the detection of chemical compounds and drugs without chromophors like lipids, fatty acids and steroids (Asmus 1993; Carraud et al. 1987; Lafosse et al. 1990). Separation and analysis have also been described for phospholipids (Caboni et al. 1996; Bunger and Pison 1995; Breton et al. 1989), but common methods cannot be easily transferred to miltefosine because of its ether-lipid character. Also liquid-liquid extraction with water and various organic solvents is tedious and sensitivity is constrained by high interference of miltefosine with plasma proteins. An alternative to liquid-liquid extraction is solid phase extraction (SPE) with normal and reverse phase cartridges. But SPE is a technique that is limited by incomplete removal of plasma constituents. As a consequence a low recovery rate and variable reproducibility will limit precise analytics.

In this paper we report about a novel HPLC-ELSD method to propose an alternative for detection and quantification of miltefosine from pharmaceutical preparations and biological samples after protein precipitation and extraction without internal standard. Plasma and fetal calf serum samples spiked with different miltefosine concentrations were analyzed. Sample extraction was performed on four different types of SPE cartridges (non-polar, polar, polar Cyano (CN-U), and polar anion exchange) and will be intensively discussed.

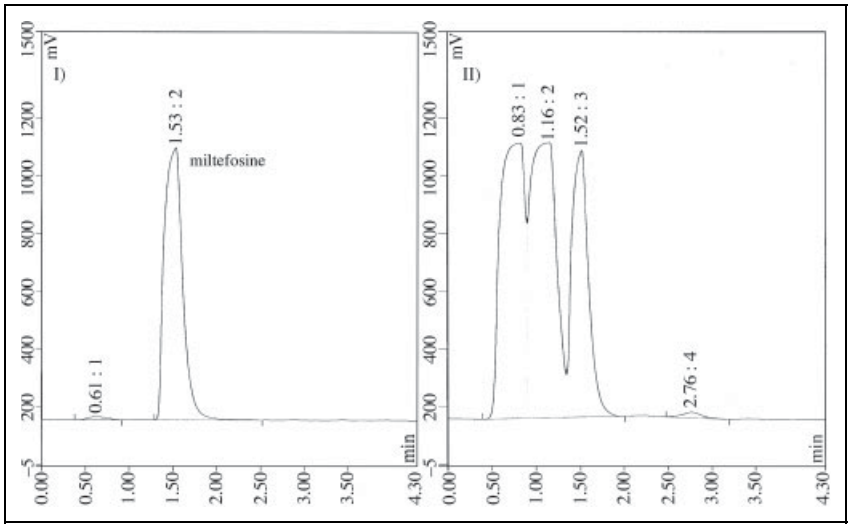


Fig.:
HPLC-ELSD chromatograms of I) miltefosine standard stock solution and II) miltefosine standard stock solution spiked fetal calf serum (5%)

Table 1: Absolute recovery values of miltefosine after solid phase extraction with non-polar Bond Elut (PH), polar Bond Elut (Si), polar Bond Elut Cyano (CN-U) and polar anion exchange Bond Elut Aminopropyl (NH_2)

Spiked conc. (μg/mL)	Cartridge (Bond Elut)	Measured conc. (μg/mL)	CV (%)	Recovery (%)
40.0	PH	0.6 ± 0.03	4.2	1.5
40.0	Si	30.2 ± 0.84	2.8	75.5
40.0	CN-U	35.1 ± 0.74	2.1	87.75
40.0	NH_2	38.9 ± 0.71	1.9	97.25

Data are mean ± S.D. of triplicate determinations. CV: coefficient of variation

Table 2: Recovery values for miltefosine for three injections

Medium	Recovery (%)	CV (%)
Water	99.84 ± 0.2	1.8
FCS (5%)	95.41 ± 0.4	2.7
FCS (10%)	84.47 ± 2.3	4.5
Murine plasma	91.12 ± 1.6	2.1

Data are mean ± S.D. of triplicate determinations. CV: coefficient of variation

2. Investigations, results and discussion

2.1. Detection and quantification of miltefosine

In a first step the detection of miltefosine and constant peak geometry from standard stocks were analyzed. Using a water/acetonitrile (1 : 1, V/V) mixture and a normal phase column miltefosine showed a retention time of Rt = 1.53 min with a cycle time of 5 min. These results are consistent with published data indicating retention times of Rt = 2.14 min under similar conditions (Sundar et al. 1998). Experiments to achieve acceptable chromatographic peaks on commonly applied reversed phase columns failed or revealed unsatisfactory results and are not discussed here. Main reason for poor retention might be the presence of quaternary ammonium molecules from the packing material. The detector response was linear in the range of 300 to 2500 ng/mL. The detection limit for miltefosine was 300 ng/mL in water and 341 ng/mL in samples spiked with 5% fetal calf serum. The chromatographic peak shape (Fig. I) was accepted and no further attempts were made to reduce peak tailing.

2.2. Recovery of miltefosine

An important reason why miltefosine should be separated from biological constituents like proteins or cell debris is that the miltefosine peak may be depleted when sample and large excess of lipids and other components coelute without any purification. Recovery of miltefosine standard stocks from different SPE cartridge types shows that the absolute recovery rate from a non polar SPE-cartridge (Bond Elut PH) with 1.5% were significantly increased to polar Bond Elut Cynao (CN-U) or Bond Elut Aminopropyl (NH_2) cartridge with 87.75 and 97.25%, respectively (Table 1). According to the amphoteric character of the drug and its high solubility in polar media we did not expect such data. To explain this behavior we expect interaction of the saturated ether chain with the reverse phased packing material. Using polar packing material in the purification process is correlated with an increased wash out of unwanted constituents. A complete removal is not possible, using only Elut Aminopropyl (NH_2) cartridges allows a balanced compromise of sensitivity and resolution as demonstrated for spiked samples with 5% fetal calf serum (Fig. II). Additional peaks at approximately 0.83 and 1.16 min can be explained due to agglomeration of soluble serum proteins associated with the drug. The concentration of plasma protein is important for the recovery rate (Table 2). Protein binding of miltefosine is approximately 90% explaining why increased plasma concentrations from 0%, 5%, and 10% are correlated with reduced recovery rates of 99.84%, 95,41%, and 84.47%, respectively. The absolute recovery of miltefosine in murine plasma ranged between 87.12% and 94.27% with a CV of 1.8–2.1%.

2.3. Analysis of Impavido®

The recovery of miltefosine from commercial products was performed to evaluate the accuracy of our HPLC system. Both commercial products (Impavido 10 mg and 50 mg) were analyzed according to ICH Q2B guidelines (ICH 1997). The composition of these samples was determined by normalization on a 1.00 mg/mL stock solution. Results are summarized in Table 3. From the results the concentration of miltefosine is in both commercial products constant and matches with the claimed concentra-

Table 3: Recovery values of miltefosine from Impavido® 10 and 50

Sample	Concentration (μg/mL)	Measured conc. (μg/mL)	CV (%)	Accuracy (%)
Commercial Product				
Impavido® 10	10.0	10.03 ± 0.12	1.2	100.30
Impavido® 50	50.0	50.07 ± 0.25	0.5	100.14
Stock Solution 10	10.0	10.27 ± 0.06	0.6	102.70
Stock Solution 50	50.0	50.23 ± 0.15	0.3	100.46

Data are mean ± S.D. of triplicate determinations. CV: coefficient of variation

tions. Therefore, each sample composition was in good agreement with specifications required by the pharmaceutical company Zentaris.

2.4. Conclusion

In conclusion, our HPLC-ELSD method is a suitable analytical tool for the detection of miltefosine in biological fluids and commercial products. In comparison to HPLC-MS (Sundar et al. 1998) it allows high sample throughput and requires short retention times. It is a fast, sensitive, selective method and it is suitable for analyzing miltefosine samples in preclinical studies and biological fluids. The use of ELSD is shown to be suitable to describe a complex pharmaceutical product as well as its related substances without any derivatization in a short time.

3. Experimental

3.1. Drugs and chemicals

Miltefosine (Hexadecylphosphocholine) and the pharmaceutical preparation Impavido® containing Miltefosine 10 and 50 mg was obtained from Zentaris GmbH, Frankfurt, Germany. The batch number of Impavido® 10 mg was ID0434 and that of Impavido® 50 mg was ID0414. HPLC grade acetonitrile and methanol was obtained from Merck KG, Darmstadt. Bidistilled water was obtained in-house by distillation. The mobile phase was passed through a 0.22 μm filter (Millipore, Germany) and degassed by sonication for 5 min. Cartridges were a gift from Varian Inc. Drug free fetal calf serum was obtained from GIBCO, Germany. Drug free murine plasma was a gift from the Robert-Koch-Institute, Berlin, Germany. All other chemicals were purchased HPLC grade from Sigma Aldrich, Germany. All samples were stored at 4 °C until use.

3.2. Standard solution and calibration standards

Stock solutions (1.0 mg/mL) were prepared by dissolving 25 mg miltefosine in 25 mL water and stored at 4 °C. The solution was sonicated for 5 min in a Bandelin Sonorex® RK100 H ultrasonic bath. Standard solutions (20 μg/mL) were produced by dilution of the stock solution in 50 mL bidest. water. Plasma standards were prepared by using 0.1 mL of fresh prepared standard solution to spike 0.9 ml murine plasma or fetal calf serum (FCS). The concentrations of the plasma standards ranged between 0.1 and 3.1 μg miltefosine.

3.3. Extraction procedure

For miltefosine sample preparation the sample was subjected to solid phase extraction. Extraction was performed on four different types of Bond Elut-SPE-cartridge under vacuum. SPE cartridges were activated with 1 mL aqueous 50% MeOH and 1.5 mL 99% MeOH with 1% triethylamine. 1 mL sample was given at the activated cartridge and extraction was performed under vacuum. The eluent was injected directly into the HPLC system.

3.4. Equipment

The HPLC system was a Kontron 560 (Kontron Inst., Neufahrn, Germany) consisting of a Kontron 525 pump, a Sedex 75 ELS-detector (Sedere, France), and a column heater. Solid phase extraction was performed with 4 different types of Bond Elut-SPE-cartridges from Varian Inc. which were the non-polar Bond Elut PH, the polar Bond Elut Si, the polar Bond Elut Cyano (CN-U) and the polar anion exchange Bond Elut Aminopropyl (NH_2) cartridges. Each cartridge had a maximum loading capacity of 100 mg/mL.

3.5. HPLC conditions

The detector was operated with compressed air at 3.5 bars and 40 °C. Data were analysed with Kroma 2000 Software. Chromatographic separation achieved in 4 min at 40 °C with a LiChrospher 60 SI 5 μm 12.5 × 4 mm column. The mobile phase consisted of a water/acetonitrile (1 : 1, V/V) mixture. The pH 6.5 was adjusted by a 0.008 M ammonium acetate buffer. The HPLC pump delivered the mobile phase at 1.5 mL/ min up to 5 min. The injection volume for each sample was 25 μl.

3.6. Statistical analysis

A calibration curve was constructed by plotting the peak area as a function of detected miltefosine concentration. Data were fitted with an unweighted least squares regression analysis to the equation $y = ax + b$. The mean calibration curve was $y = 2.5164x - 4.2024$ with a correlation coefficient of 0.9988. No weight factors were applied and the residuals were within ± 7%. The limit of quantification has been detected at 341 ng/mL after solid phase extraction. The data are expressed as mean value ± S.D.

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