ELSEVIER

Journal of Chromatography B, 672 (1995) 300–304

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of metronidazole in vaginal tissue by high-performance liquid chromatography using solid-phase extraction

T.G. Venkateshwaran, J.T. Stewart*

Department of Medicinal Chemistry, College of Pharmacy, University of Georgia, Athens, GA 30602-2352, USA

First received 13 February 1995; revised manuscript received 12 May 1995; accepted 12 May 1995

Abstract

A sensitive high-performance liquid chromatographic (HPLC) method for the determination of metronidazole in vaginal tissue is reported. The method uses a Zorbax SB phenyl column with a 0.01 *M* aqueous monobasic potassium phosphate buffer (pH 4.0)–absolute methanol (85:15, v/v) as mobile phase at a flow-rate of 1.0 ml/min and detection at 313 nm. Tinidazole was used as the internal standard. The method employed homogenization of tissue followed by solid-phase extraction. The quantitation was achieved within 30 min with sensitivity in the ng/g range. Metronidazole was linear in the 100–2000 ng/g range. The accuracy and precision were in the 1–4% range for the drug and the limit of detection was approximately 100 ng/g based on a signal-to-noise ratio of 3 and a 100-μl injection.

1. Introduction

Metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, is widely used for the treatment of a number of protozoal infections and in the prevention and treatment of parasitic and bacterial infections. Intravenous antibiotics are used during surgery to decrease post-operative infections, but they are costly and surgeons are looking for alternative methods such as intravaginal gels to prevent infection. Metrogel, a gel dosage form of metronidazole, is currently being used to treat bacterial vaginosis since it is easy to apply and has fewer side-effects than oral forms of the medication [1]. A study was recently conducted to explore the possibility of using metrogel intravaginally for surgical prophylaxis. There have been no studies to calculate the absorption of metrogel by vaginal tissue. Hence, there was a need to develop an HPLC method to analyse tissue levels of metronidazole.

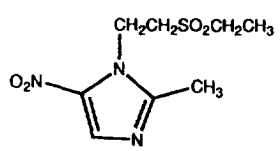
Metronidazole and nitroimidazole derivatives have been measured by various assay methods including microbiological techniques [2], spectrophotometry [3], thin-layer chromatography (TLC) [4], gas chromatography (GC) [5] and HPLC [6–14]. The microbiological assay was used to measure μg/ml levels of metronidazole in serum in the presence of gentamicin and penicillin. High-performance thin-layer chromatographic methods reported detection levels of 0.5 μg/ml for metronidazole in serum. The sample was deproteinized using acetone, centrifuged and the supernatant evaporated to give a

* Corresponding author.

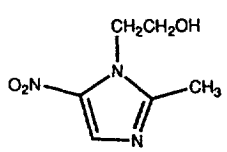
0378-4347/95/$09.50
SSDI 0378-4347(95)00217-0

residue, which was reconstituted in acetone and applied to the TLC plates. The developed plate was then scanned with a densitometer. Plasma levels of metronidazole were reported by GC in which the metronidazole was extracted from plasma with chloroform and determined as the trimethylsilyl derivative using a flame-ionisation detector. One of the HPLC procedures reported the determination of metronidazole in human serum with reasonable accuracy based on a separation with an octylsilane column and an absolute methanol–phosphate buffer mobile phase [12]. The sample preparation involved extraction of the drug from serum using a small gravity column. Another HPLC procedure described the extraction of the drug from biological fluids such as plasma, saliva, urine, serum and whole blood [13]. Chacko et al. [14] reported an HPLC method to determine μg/g levels of the drug from liver and ovaries after intraperitoneal administration. The method had a low and variable recovery (ranging from 55 to 75%) of metronidazole and would not detect ng/g levels of the drug.

In this paper, an HPLC method is reported for vaginal tissue levels of metronidazole. The method employs homogenisation of tissue followed by solid-phase extraction (SPE) to concentrate the sample and minimize interferences from endogenous substances present in the tissue. The method provides reasonable accuracy and precision and is sensitive to low ng/g levels. The structural formulae of metronidazole and tinidazole (internal standard) are shown in Fig. 1.



Tinidazole



Metronidazole

Fig. 1. Chemical structures of metronidazole and tinidazole (internal standard).

2. Experimental

2.1. Reagents, chemicals and tissue

Metronidazole and tinidazole were purchased as the free base from Sigma (St. Louis, MO, USA, Lot Nos. 97F0147 and 32H0386, respectively). Absolute methanol (J.T. Baker, Phillipsburg, NJ, USA) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA, USA). Monobasic potassium phosphate and phosphoric acid were Baker-analysed reagents.

Drug-free dog vaginal tissue was obtained from the College of Veterinary Medicine, University of Georgia (Athens, GA, USA). Human vaginal tissue (drug-free and patient-dosed) was obtained from Georgia Baptist Hospital (Atlanta, GA, USA).

2.2. Instrumentation

The chromatographic separation was performed on a HPLC system consisting of a Micromeritics Model 760 pump (Norcross, GA, USA), a Spectra-Physics Model SP8780XR autosampler equipped with a 100-μl loop (San Jose, CA, USA), and a Waters Model 990 plus photodiode-array detector (Milford, MA, USA) was used to detect the analytes. The data was collected using a NEC powermate 2 computer with the aid of the 990 plus software. A Tekmar tissumiser (Cincinatti, OH, USA) was used to homogenise the tissue samples, and Accubond C_{18} cartridges (J&W Scientific, Folsom, CA, USA) were used for solid-phase extraction. Separation of the analyte from the tissue components and internal standard was achieved on a phenyl column (150 mm $\times$ 4.6 mm I.D., 5 μm, Zorbax SB-phenyl, Chaddsford, PA, USA) using 0.01 *M*

aqueous monobasic potassium phosphate, pH 4.0 (adjusted with 10% phosphoric acid)–absolute methanol (85:15, v/v). The run-time for a sample was about 30 min. The mobile phase was filtered through a 0.45-μm Nylon-66 filter (MSI, Westborough, MA, USA) and degassed by sonication prior to use. The flow-rate was 1.0 ml/min and the diode-array detector was set at 313 nm.

2.3. Preparation of stock and standard solutions

Individual stock solutions of metronidazole (1 μg/ml) and tinidazole (400 ng/ml) were prepared by weighing appropriate amounts of each compound into volumetric flasks and additional dilutions performed as needed with deionized water. Additional solutions of metronidazole containing 25, 100, 200, 300, 400 and 500 ng/ml were prepared in water from the 1 μg/ml metronidazole stock solution and were used to spike blank tissue samples for preparation of the calibration curve and for accuracy and precision studies.

2.4. Preparation of calibration curve

Blank vaginal tissue (0.25 g) was accurately weighed and placed in individual 10-ml beakers. Aliquots of 1 ml of each of the 25, 200, 300 and 500 ng/ml metronidazole solutions were added to the beakers followed by 1 ml of tinidazole internal standard solution (400 ng/ml). Water (2 ml) was added to one of the beakers as a control sample. The 100 and 400 ng/ml metronidazole solutions were used to prepare spiked tissue samples in two other beakers for determination of accuracy and precision of the method. Each tissue sample was homogenised for 30 s while cooled with ice. Care was taken to wash the homogeniser to get maximum recovery of the drug. The homogenates and washes were transferred to 10-ml culture tubes and trichloroacetic acid (100 μl of 5%, w/v solution) was added to each homogenate to precipitate the proteins and free any bound drug. The homogenates in the culture tubes were then centrifuged at 1500 *g* for 30 min and transferred to another set of 10-ml culture tubes. Care was taken to wash the precipitate with 1 ml of water to remove any metronidazole left in the tube. Octadecylsilane (C_{18}) solid-phase extraction cartridges (1 ml size, J&W Scientific) were placed on the vacuum manifold (Vac Elut, Varian). They were conditioned with three 1-ml aliquots of absolute methanol followed by three 1-ml aliquots of deionized water. Each homogenate was passed through the cartridge and washed with two 1-ml aliquots of water. The cartridge was placed under vacuum for an additional 10 min. The drug and internal standard were eluted with 4×250 μl of absolute methanol into 1-ml volumetric tubes. The methanol was evaporated with the aid of a nitrogen stream and mobile phase added to volume. After being vortex-mixed for 30 s, a 100-μl injection was made into the liquid chromatograph.

Human vaginal tissue was prepared for assay in the same manner as described above except that the tissue was homogenized with 1 ml of deionized water and 1 ml of the 400 ng/ml internal standard solution.

Quantitation was based on linear regression analysis of drug/internal standard (D/I.S.) peak-height ratio versus metronidazole concentration normalized to ng/g of tissue. The slope and intercept data were used to calculate the metronidazole concentration in tissue samples using the formula: D/I.S. = (slope)(concentration) + intercept.

3. Results and discussion

The goal of this study was to develop an HPLC method to determine ng/g levels of metronidazole in vaginal tissue. Homogenization of the tissue was a very important step in the method since it allowed the use of solid-phase extraction to clean up and concentrate the sample prior to assay. In the development of the procedures, human vaginal tissue was in short supply. It thus became necessary to use dog vaginal tissue for much of our assay development. A chromatographic comparison of dog

versus human vaginal tissue indicated that they were essentially equivalent.

Initially an octadecylsilane HPLC column (Brownlee RP-18, 220 mm × 4.6 mm I.D., 10 μm) with a mobile phase of 0.01 *M* aqueous monobasic potassium phosphate, pH 4.0–absolute methanol (85:15, v/v) was used to separate the analytes. It was found during the course of the study that, in certain tissue samples, cephalosporins might interfere with the assay since they are also used as anti-infective agents. To avoid this interference, other columns and mobile phases were investigated. A phenyl column was finally selected for the assay since it provided minimum interference of cephalosporins. The retention times of cephalosporins such as cefmetazole, cefazolin and cephalexin on the phenyl column were 35.5, 8.1 and 34.4 min, respectively, and did not interfere with the retention times of metronidazole or tinidazole at 9.2 and 23.2 min, respectively. Other possible drug interferences would need to be checked prior to assay. Furthermore, the peaks were sharper on the phenyl column compared to the octadecylsilane column, and the mobile phase which had previously been used with the octadecylsilane column was also useful with the phenyl column. A typical chromatogram of metronidazole and internal standard extracted from vaginal tissue is shown in Fig. 2. A blank vaginal tissue sample was prepared in the same manner as above and chromatographed in the system. It showed no interference with the drug or internal standard. The detector wavelength was set at 313 nm since metronidazole showed a maximum at that wavelength in the mobile phase.

Despite the good solubility of the drug in methanol, water was used during the tissue homogenisation step to extract the drug from the tissue samples. Water was also important for the SPE clean-up since metronidazole was not retained on the C_{18} SPE cartridge when the sample was largely methanol. The percentage extraction of the internal standard was 88%. The recovery of metronidazole in the 100–2000 ng/g range was 87.0 ± 4.5% ($n = 9$). A correlation coefficient (r^2) of 0.9900 was obtained based on linear regression analysis of D/I.S. peak-height ratios versus drug concentration in ng/g. The accuracy and precision of the method were evaluated using spiked tissue samples. The results shown in Table 1 indicate acceptable values in the 1–4% range. Inter- and intra-day variabilities expressed as R.S.D. for the drug were in the 1.03–3.3% ($n = 9$) and 0.76–5.4% ($n = 3$) ranges for 2000 and 200 ng/g metronidazole concentrations, respectively. The levels of metronidazole found in actual human vaginal tissue samples obtained from three patients dosed with 3.75 mg of metronidazole contained in 5 g of gel were 105, 135 and 270 ng/g. No information was available to the analyst on sample collection time and storage.

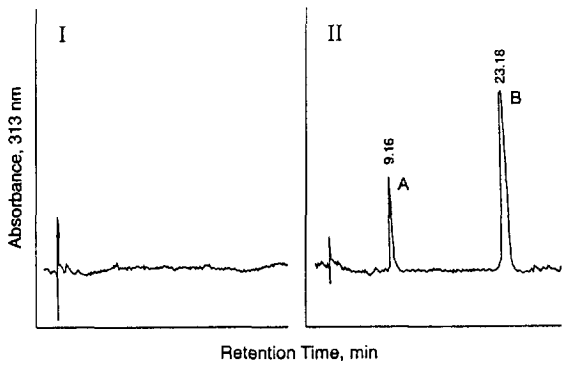


Fig. 2. Typical chromatograms of (I) a drug-free tissue extract and (II) 200 ng/g metronidazole (A) and 1600 ng/g internal standard tinidazole (B) on a phenyl column using 0.01 *M* aqueous monobasic potassium phosphate buffer (pH 4)–methanol (85:15, v/v) at a flow-rate of 1 ml/min and detection at 313 nm.

Table 1
Accuracy and precision data for metronidazole added to vaginal tissue

Concentration added (ng/g)	Concentration found (ng/g)[a]	Percent error	R.S.D. (%)
165.3	175.7	5.98	1.32
424.0	438.0	3.3	0.68
1364.5	1414.0	3.6	1.20

[a] Based on $n = 3$.

4. Conclusion

The HPLC method described herein can be used to determine ng/g levels of metronidazole in vaginal tissue with acceptable accuracy and precision. The method is free of interference from selected cephalosporins which may be co-administered with the drug.

References

[1] L. Franklin, Georgia Baptist Hospital, Atlanta, GA, 1994, personal communication.
[2] M.E. Lavison, Antimicrob. Agents Chemother., 5 (1974) 466.
[3] P. Populaire, B. Decouveleare, G. Lebreton and S. Pascal, Ann. Pharm. Fr., 26 (1968) 549.
[4] E. Gattavechia, D. Tonneli and A. Breccia, J. Chromatogr., 224 (1981) 465.
[5] N.F. Wood, J. Pharm. Sci., 64 (1975) 1048.
[6] R.A. Marques, B. Stafford, N. Flynn and W. Sadee, J. Chromatogr., 146 (1978) 163.
[7] H. Merdjan, C. Bonnat, E. Singlas and B. Diquet, J. Chromatogr., 273 (1989) 475.
[8] J.C. Jensen and R. Gugler, J. Chromatogr., 277 (1983) 381.
[9] G.A. Wollard, J. Chromatogr., 303 (1984) 222.
[10] J. Adamnovics, J. Chromatogr., 309 (1984) 436.
[11] A. Groppi, P. Papa, M. Montagna and G. Carosi, J. Chromatogr., 380 (1986) 437.
[12] K. Rona and B. Gechalyi, J. Chromatogr. B, 420 (1987) 228.
[13] C.M. Kaye, M.G. Sankey and L.A. Thomas, Br. J. Clin. Pharmacol., 9 (1980) 528.
[14] M. Chacko, J. Nair and S.V. Bhide, Ind. J. Biochem. Biophys., 23 (1986) 220.