Development and validation of a high performance liquid chromatographic method for *in vitro* mupirocin quantification in both skin layers and percutaneous penetration studies.

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Abstract

A simple, rapid and sensitive reversed-phase high-performance liquid chromatographic (HPLC) method for the measurement of mupirocin concentrations in both skin layers and percutaneous samples has been developed. Mupirocin was extracted from skin layers using PBS–acetonitrile (90:10, v/v). The method is sufficiently sensitive and repeatable to be used in percutaneous penetration studies. The samples were chromatographed on a 250x4 mm C_8 LiChrospher Select B (5 μm). The mobile phase composition was a mixture of acetonitrile-ammonium acetate 0.05 M (27.5:72.5, v/v) adjusted to pH 6.3 with acetic acid. The analyte was detected at 228 nm and the run time was 11 min. Linearity was confirmed in the concentration range 0.2-20 μg/ml and the limit of detection was 9.5 ng/ml.

Keywords: Mupirocin; HPLC; Percutaneous study; Skin recovery

Introduction

Mupirocin or pseudomonic acid (9-[[(2E)-4-[(2S,3R,4R,5S)-5-(2S,3S,4S,5S)-2,3-epoxy-5-hydroxy-4-methylhexyl]–3,4-dyhidroxy-3,4,5,6-tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid) (Figure 1) is an antibiotic produced by *Pseudomonas fluorescens* and has *in vitro* activity against a range of Gram-positive and some Gram-negative bacteria [1]. Mupirocin has excellent activity against staphylococci (including methicillin-resistant strains), streptococci, and gram-negative organisms such as *Haemophilus influenzae* , *Neisseria gonorrhoeae*, and *Moraxella catarrhalis* [2].

Mupirocin was found to inhibit bacterial isoleucyl-tRNA synthetase [3,4], blocking protein synthesis and indirectly inhibiting RNA synthesis [5]. The last part of the epoxy chain presents a structural analogy with isoleucyn and it interacts reversible and specifically with the aminoacid at the active site of the enzyme. The depletion of cellular levels of isoleucine-charged transfer RNA leads to the arrest of protein synthesis. The isoleucyl transfer RNA synthetase produced by *P. fluorescens* is resistant to the pseudomonic acid it produces which partly explains how the organism is able to survive the fermentation process [6]. This antibacterial activity is lost in human tissue, as the agent is rapidly broken down; thus mupirocin is therefore only available for topical use as a 2% ointment [1]

The mupirocin skin ointment with polyethylene glycol (Bactroban®, Smithkline Beecham and Plasimine®, Isdin) and/or nasal cream with soft paraffin (Bactroban nasal ointment®, Smithkline Beecham) are currently registered for use in more than 90 countries worldwide [7]. Mupirocin 2% ointment applied 2 or 3 times daily has demonstrable efficacy for the treatment of both primary and secondary skin infections [8]. The most common primary skin infection is impetigo, it is highly contagious and occurs mainly in children, while secondary skin infections may occur following damage

by accidental trauma, surgery, and burns or as a result of superinfection of an underlying skin disease [9].

No systemic absorption of mupirocin or its major metabolite, monic acid, has been detected in short courses of topical administration to healthy volunteers or to patients with primary skin infections after prolonged courses of therapy with Bactroban® ointment [10]. Penetration into the deeper epidermal and dermal layers is enhanced in traumatized skin and under occlusive dressings. Mupirocin is slowly metabolized by the skin to the antimicrobially inactive metabolite monic acid [11].

Use of mupirocin ointment has shown that it is extremely well tolerated and that side effects, such as itching, burning, rash, or dry skin, are minor [12, 13], mupirocin also lacks the potential to cause photosensitive irritant reactions and contact sensitization [14, 15].

Mupirocin 2% ointment has usually been applied topically 2 or 3 times daily for 5 to 14 days in patients with primary or secondary superficial skin infections. Patients with chronic or severe infection may require a prolonged therapy [11].

Topical ointment preparations are less acceptable to patients since ointments have high viscosities leading to difficulties in applications to skin lesions, and patients may report garment soiling from greasy residues [9]. To enhance patient acceptance and compliance could be developed new formulations such as a cream [9] or particulate drug carriers such as microparticles and nanoparticles, these carriers could protect mupirocin and release it in a controlled manner. Acyclovir-microparticles [16] and cidofovir-microparticles [17] have already been developed for topical therapy of Herpes virus simplex.

The analytical methods employed for the quantification of mupirocin from the samples obtained in permeation *in vitro* studies have to be specific, since these kind of samples were usually contaminated with skin endogenous compounds, as a large number of UV-absorbing nucleotides and nucleosides. Moreover, these methods also have to show enough sensitivity, due to the little volume of sample obtained from the receptor medium of Franz diffusion cells in the skin permeation studies, and the small size of the horizontal skin slices of whole stratum corneum, viable epidermis and dermis skin layers, obtained in *in vitro* permeation studies. None of the previously reported methods determined mupirocin both in skin layers and percutaneous penetration samples, furthermore some methods were developed for estimation of mupirocin purity and degradation by HPLC [18], ELSD-HPLC [19] and mass spectral and liquid chromatography [20]. Jagota *et al*. [18] studied the stability of Bactroban® mixed with various cream, lotion, ointment, gel, solution and liquid soap formulations with storage at 37ºC for 60 days. On the other hand, Porter and Chen [19] developed a gradient LC-MS compatible method using dual detection (UV and light scattering detectors) for the separation and identification of impurity and degradates in mupirocin in a complex matrix containing 98% (w/w) of polyethylene glycol (PEG) 400 and PEG 3350. Finally, McDowall *et al.* [20] developed a chromatographic method for the characterization of beta-lactam antibiotics and pseudomonic acids.

The HPLC method described in this paper was based on that developed by Jagota *et al*. [18]. This method has been successfully applied to determine the concentration of the analyte in the *in vitro* percutaneous penetration studies, through porcine skin, using Franz-type diffusion cells.

2. Experimental

2.1. Reagents

Mupirocin (purity 95.5%) was a gift from GlaxoSmithKline (West Sussex, England). Disodium hydrogenphosphate and potassium dihydrogenphosphate used for the preparation of Phosphate Buffer Saline (PBS) and ammonium acetate were obtained from Merck (Darmstadt, Germany). Acetonitrile, acetic acid and other solvents used were of HPLC analytical grade (Merck, Darmstadt, Germany).

2.2. Calibration standards

A stock solution of mupirocin with a concentration of 40 µg/ml was prepared by dissolving 4 mg of mupirocin in 100 ml of water. Thirteen standard solutions of 0.2, 0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 15 and 20 µg/ml of mupirocin were made by further dilution of the stock solution with appropriate volumes of acetonitrile and water (1:10 v/v). The concentration range of mupirocin for the standard curve samples was between 0.2 and 20 µg/ml.

The stock solutions of mupirocin were kept at 4ºC.

2.3. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Hewlett-Packard (Waldbronn, Germany) system equipped with a HP 1050 quaternary pump; a HP 1050 autosampler and a HP diode-array detector set at 228 nm. Data acquisition and treatment were performed with a Hewlett-Packard computer using ChemStation G2170 AA.

Separation was carried out at 45ºC on a reversed-phase, 250x4 mm C_8 LiChrospher Select B (5 µm) provided by Merck (Darmstad, Germany). A C_{18}, 10x4 mm precolumn provided by Teknokroma (Barcelona, Spain) was also used. The flow-rate was set to 1 ml/min and effluent was monitored with

UV detection at 228 nm. The mobile phase composition was 27.5:72.5 (v/v) acetonitrile and ammonium acetate (0.05M adjusted to pH 6.3 with acetic acid). The mobile phase was filtered through a 0.45 μm pore-size membrane filter. Under these experimental conditions the run time was 11 minutes and the injection volume was 100 μL.

2.4. Instrument calibration

Calibration curves were prepared using concentrations of 0.2, 0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 15 and 20 μg/ml of mupirocin. This calibration was determinated plotting a standard curve from the mupirocin peak areas versus the corresponding drug concentration in acetonitrile and water (1:10 v/v). Calibration curves were determined by least square linear regression analysis.

2.5. Sample preparation

Samples were obtained from the receptor at different times and from skin layers after *in vitro* percutaneous penetration studies.

2.5.1. Quantification of mupirocin in percutaneous penetration samples

This technical procedure was developed to determinate the *in vitro* percutaneous penetration of mupirocin through porcine skin using the Franz-type diffusion cells (FDC-400, Grown Glass Company, Somerville, NY). Porcine ears were obtained from the local slaughterhouse and after cleaning them with cold running water, the outer region of the ear was cut. The whole skin was dermatomed to 1.2 mm (GA 630, Aesculap®, Tuttlingen, Germany). The skin samples were clamped between the two chambers of Franz-diffusion cells, with the stratum corneum facing the donor compartment and the dermis facing the receptor one. The mupirocin solution was placed in the donor compartment and 0.4

ml samples were taken from the receptor side at different times (4, 6, 8, 22 and 24 h) and replaced by the same volume of PBS. Samples were filtered and analyzed immediately.

2.5.2. Quantification of mupirocin in skin layers

The quantitative determination of mupirocin at different depths from skin surface was performed on horizontal slices (40 μm) of the skin sample. After the permeation experiments, the skin was removed, cleaned three times with distilled water, included in O.C.T.™ Compound (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands) and frozen in liquid N_2. Ten slices of 40 μm (0.00015– 0.015 g) were cut at –19ºC in parallel to the skin surface (CM 1900-Cryostat, LEICA, Nussloch, Germany) and stored at –20ºC until analysis by HPLC. Mupirocin was extracted from the skin with 540 μl of PBS and 60 μl of acetonitrile for 20 minutes; during this time the tubes were vortexed twice for 30 seconds. The mixture was centrifuged at 6000 g for 10 minutes. The supernatant was filtered through a 0.45 μm nylon filter (Lida, U.S.A.) and analyzed by the HPLC method described in this work.

2.6 Validation

For ruggedness and robustness studies, a different phase columns such as Kromasil 15 x 0.4 (Teknokroma, Spain), Ultrabase 15 x 0.4 (Scharlau, Barcelona, Spain) and Spherisorb C_{18} (10x0. 46 cm; Teknokroma, Spain) were used. Similarly, the influences of mobile phase (percentage of acetonitrile ranged from 30% to 27%) and column temperature (20ºC) on the analytical procedure were also evaluated.

For the validation of mupirocin extraction from the skin, different known amounts of mupirocin in PBS and acetonitrile were directly added to a series of 40 μm slices of blank skin (which had not previously been in contact with mupirocin) and extracted as described above. The extraction recovery was

determined by computing the ratio of the amount of mupirocin extracted from spiked skin to the amount of mupirocin added. The skin slices were from different depths.

The linearity of the method was demonstrated by statistical comparison among the slopes obtained, the intercepts of calibration curves with zero and the correlation coefficients with 1. Furthermore a Student t-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones. Linearity was determined by plotting a standard curve from the mupirocin peak areas versus the corresponding drug concentrations in acetonitrile and water (1:10 v/v).

The selectivity of the assay was confirmed by the individual analysis of blank samples from skin layers and from the receptor side of the diffusion cells.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration, which can be determined with an accuracy and precision < 20%. Detection and quantification limits (LOD and LOQ) were determined by the analysis of the peak baseline noise in five blank samples.

Accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as standardized agreement between the measured value and the true value.

Precision of a method was expressed as the R.S.D. (relative standard deviation) of replicate measurements. In this work, precision of the method was tested on both within-day and between-day reproducibility in the assay. Within-day variability of the assay method was determined by repeated analysis of three concentrations (0.4, 1 and 10 μg/ml) of mupirocin in acetonitrile and water (1:10 v/v) in the same day. Similarly, between-day variability was determined by repeated analysis of the control samples in three different days [21, 22].

3. Results and discussion

The HPLC method described in this paper was based on that developed by Jagota *et al*. [18]. Although some modifications were made in order to obtain a better sensibility for the diluted samples in the permeation studies and avoid interferences with the skin compounds.

3.1. Validation of the method

The quantitative determination of mupirocin in skin layers at different depths and in the receptor side of diffusion cells required specific methods, since this kind of samples were usually contaminated with skin endogenous compounds. Moreover, the work diffusion cells provides little sample volumes and, therefore, the analytical method has to show enough sensitivity. In order to solve these problems we included ammonium acetate in the mobile phase, and used a slightly more polar encapped stationary phase; the Lichrospher C_8 60 RP-select B column. LiChrospher RP-Select B is a reversed-phase column packed with a monofunctional octylsilane phase bonded to extremely pure spherical porous silica particles with a 6-mm pore size. The silica material has been optimised in order to avoid any ionic interactions with more acidic and basic molecules, that are negatively or positively charged at pH range (3 to 8) usually employed in reversed phase liquid chromatography. Therefore, these compounds would be eluted as symmetric chromatographic peaks from these columns. Ammonium acetate is an amphoteric salt that minimises the basic solute interactions with residual silanol groups allocated in the stationary silica gel reversed-phases, which can cause tailing at mid-pH values. With the method described in this work, the analytical peaks of the mupirocin for samples obtained in permeation studies and from skin slices were well resolved (Figure 2 and 3). The percutaneous permeation experiment consisted on the topical application of a mupirocin solution (10 mg of mupirocin in 1 ml of water). The experiment was carried out during 24 hours. Figure 2 shows the chromatogram of a blank sample from

skin and the correspondent chromatogram of a skin slice after 24 h. The concentration determined was 0.85 μg/ml, above the limit of quantification of the developed HPLC method. The endogenous compounds eluted before 4 minutes, being the resolution with regard to mupirocin 11.54±0.43. The concentration of mupirocin in the receptor 9 hours after the topical application was 6.09 μg/ml (Figure 3). The retention of mupirocin in the stationary phase was 5.93±0.02 (k' value) and acceptable asymmetry coefficient was obtained (the asymmetry coefficient was 0.98±0.09), while the peak tailing factor obtained by Jagota *et al*. [18] was near to 1.5. Under the chromatographic conditions used, mupirocin has a retention time of 7.20±0.07 min. Jagota *et al.* [18] obtained a retention time of 5.5 ± 0.5 min using a octadecylsilane non-encapped stationary phase packed with 10 μm-silica particle size. Although flow rate was increased to 2 ml/min in order to assess good asymmetry coefficients for mupirocin, the selectivity of this method is still inadequate for the determination of this drug in samples coming from *in vitro* permeation studies. Indeed, using the chromatographic conditions described by Jagota *et al.* it is impossible to avoid chromatographic interferences between mupirocin and endogenous skin compounds.

3.1.1. Recovery

Previously to the mupirocin quantification in the skin layers, the drug was extracted from skin slices (with the method described above), and recoveries above 93% were obtained from all samples tested (Table I).

3.1.2. Selectivity of the assay

Under these chromatographic conditions, no peaks interfered with the detection of mupirocin and the resolution (Rs=11.54±0.43) was satisfactory in the analysis of samples from skin layers and receptor.

3.1.3. Sensitivity of the assay

The LOD and LOQ were 9.5 ng/ml and 30.6 ng/ml respectively.

3.1.4. Linearity of the assay

Standard curves were found to be linear on three different days over the range 0.2-20 µg/ml (table II). The curve for the solutions had a slope of 156.92 ± 0,89 SD and an intercept of -1.1172±1,3161. Linear regression analysis showed correlation coefficients greater than 0.999 in all curves (n=3). For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and intercept equal to 0 (Student t-test).

3.1.5. Accuracy of the assay

Accuracy values studies at low, medium and high mupirocin concentrations were always <3.8% (table III). These values were within acceptable limits (<15%) [21, 22]. Furthermore, the small percentage of difference between nominal and found concentrations of the standards showed that the assay is accurate enough for its application.

3.1.6. Precision of the method

The results for within-day and between-day precision are presented in table IV and the R.S.D. values were always below 4.81%. These data clearly indicate that the assay method is reproducible within the same day and between days [21, 22].

3.1.7. Robustness and ruggedness

For ruggedness studies, columns packed with Kromasil®, Ultrabase® and Spherisorb C_{18}® stationary phases were used and it resulted in a worse characterization of mupirocin peak due to its lower polarity (different asymmetry coefficients) but it did not change the elution time. If the temperature of the column was decreased to 25ºC, the symmetry of the peak also differed more from 1, so the temperature was adjusted to 45ºC with a consequent improvement of the asymmetry coefficient and no variations in elution time. Slight modification in the percentage of acetonitrile (3%) in the mobile phase did not significantly alter the mupirocin retention time.

3.1.8. Stability

Stability studies demonstrated that the samples could be well kept for three weeks when stored at 4ºC. After seven days the chromatographic response decreased slightly for the concentrations tested, after two weeks this response was lower and, finally, 21 days after the preparation of the samples there was a worse characterization of the peaks and the relative error was always over 4% at all the concentratioons tested.

3.2. Application of the method

The application of this method has been demonstrated for the mupirocin quantification in porcine skin layers and in the receptor compartment of the diffusion cells after *in vitro* percutaneous studies.

The amount of mupirocin found in the receptor compartment during a percutaneous permeation experiment after the topical application of a mupirocin solution, is represented in figure 4. During the experiment the amount of mupirocin in the receptor medium increased with time. The experiment was carried out over 24 h.

Figure 5 shows mupirocin concentration determined in porcine skin slices at different depths, after 24 h of percutaneous penetration experiment, using a mupirocin solution. Mupirocin could be quantified in all samples with the HPLC method described in this work. The accumulation of mupirocin in skin decreased from the stratum corneum to 200 μm, remaining nearly constant from 200 to 400 μm. Jenning *et al*. [23] reported that in the porcine skin the upper 100 μm represent mainly the stratum corneum and upper layers of viable epidermis, between 100 and 200 μm consists basically of viable epidermis and dermis was located from 200 to 500 μm. Figure 5 shows a higher concentration of mupirocin in the upper layers of the skin (stratum corneum and basal epidermis), site of herpes virus lesions. The decrease of the amount of the antibiotic in the deeper layers of the tissue is in accordance with the lower mupirocin penetration into the dermal layers and also, with the low mupirocin concentration found in the receptor side.

4. Conclusions

A simple chromatographic method has been developed for the rapid and precise determination of mupirocin in both skin layers and percutaneous permeation samples. The simplicity of the technique, the short analysis time and the high sensitivity makes this technique particularly attractive for this purpose. This method was sensitive, accurate and had a good level of precision. The results described in this paper showed that this assay is suitable for the determination of mupirocin in different strata of porcine skin and after skin permeation experiments.

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References

[1] I. Eltringham, J. Hosp. Infect., 35 (1997) 1.

[2] R. Sutherland, R.J. Boon, K.E. Griffin, P.J. Masters, B. Slocombe, and A.R. White, Antimicrob. Agents Chemother., 27 (1985) 495.

[3] J. Hughes and G. Mellows, J. Biochem., 176 (1978) 305.

[4] J. Hughes and G. Mellows, J. Biochem., 191 (1980) 209.

[5] J. Hughes and G. Mellows, J. Antibiot., 31 (1980) 330.

[6] J. Hughes, G. Mellows, S. Soughton, FEBS Lett., 122 (1980) 322.

[7] B. D. Cookson, J. Antimicrob. Chemother., 41 (1998) 11.

[8] Y.J. Lamb, J. Hosp. Infect., 19 Suppl. B (1991) 27.

[9] J. Gisby and J. Bryant, Antimicrob. Agents Chemother., 44-2 (2000) 255.

[10] K.A. Pappa, J. Am. Acad. Dermatol., 22- 5 Pt 1 (1990) 873.

[11] A. Ward and D.M. Campoli-Richards, Drugs, 32 (1986) 425.

[12] K. Bork, J. Brauers and M. Kresken, Br. J. Clin. Pract., 43 (1989) 284.

[13] G. Wainscott, in D.S. Wilkinson and J.D. Price (Editors), Mupirocin-a novel antibiotic, Royal Society of Medicine, London, 1984, p. 173.

[14] J.J. Leyden, Excerpta Medica Curr. Clin. Pract. Ser., 16 (1985) 68.

[15] T.C.G. Tasker and D. Jackson, in D.S. Wilkinson and J.D. Price (Editors), Mupirocin-a novel antibiotic, Royal Society of Medicine, London, 1984, p. 173.

[16] E. Gª de Jalón, M.J. Blanco-Príeto, P. Ygartua and S. Santoyo, J.Control. Release, 75 (2001) 191.

[17] S. Santoyo, E. Gª de Jalón, P. Ygartua, M.J. Renedo and M.J. Blanco-Príeto, Int. J. Pharm., 242 (2002) 107.

[18] N.K. Jagota, J.T. Stewart, F.W. Warren, and P.M. John, J. Clin. Pharm. Ther., 17 (1992) 181.

[19] R.S. Porter and T.K. Chen, J. Chromatogr. A, 732(2) (1996) 399.

[20] M.A. McDowall, D.E. Games and J.L. Gower, Int. J. Mass Spectrum Ion Process, (1983) 48157.

[21] D.R. Jenke, J. Liq. Chrom. & Rel. Technol., 19 (1996) 719.

[22] D.R. Jenke, J. Liq. Chrom. & Rel. Technol., 19 (1996) 737.

[23] V. Jenning, A. Gysler and M. Schäfer-Korting, S.H. Gohla, Eur. J. Pharm. Biopharm., 49 (2000) 211.

Table I**.** Recovery of mupirocin from skin slices.

MUP added (μg/ml)	MUP extracted (μg/ml)	n	Recovery (%)
0.85	0.83	4	98.14
0.95	0.93	3	98.22
6.66	6.51	3	97.72
8.15	7.64	3	93.78

Table II. Statistical evaluation of the standard curves of mupirocin along three days (results expressed as relative error in %).

Concentration (μg/ml)	Day 1	Day 2	Day 3
0.2	-5,04	-19,60	-0,10
0.4	-12,04	0,51	—
0.5	-2,99	5,79	-11,88
0,6	-1,15	-3,50	0,25
0,8	-13,03	-5,50	-3,96
1	—	-4,31	-2,58
2	0,12	3,05	-2,22
4	1,97	2,77	0,28
6	3,19	-1,05	0,73
8	2,04	-0,15	1,40
10	3,23	-0,16	1,12
15	-2,43	0,75	1,73
20	-0,06	-0,38	-1,51

Table III. Accuracy of the method for determining mupirocin concentrations (expressed as relative error in %) (n= 5).

Concentration added (μg/ml)	Concentration found (mean ± S.D.)(μg/ml)	Relative error (%)
0.4	0.388 ± 0.018	-3.00
1	0.962 ± 0.013	-3.80
10	10.002 ± 0.038	0.02

Table IV. Between-day and within-day variability of the HPLC method for determining mupirocin concentrations.

	Between-day variability (n = 5)		Within-day variability (n = 5)	
Concentration added (μg/ml)	Concentration found (Mean ± S.D.) (μg/ml)	R.S.D. (%)	Concentration found (Mean ± S.D.) (μg/ml)	R.S.D. (%)
0.4	0.420 ± 0.016	3.8	0.388 ± 0.018	4.64
1	1.088 ± 0.052	4.81	0.962 ± 0.013	1.351
10	10.253 ± 0.492	4.80	10.002 ± 0.038	0.379