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Ion-pair high-performance liquid chromatographic assay method for the assessment of clarithromycin stability in aqueous solution and in gastric juice

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Abstract

A simple and selective ion-pair HPLC method has been developed for the analysis of clarithromycin in aqueous solutions and in gastric juice. A Hypersil ODS 5-μm (150×4.6 mm I.D.) column was used with a mobile phase consisting of acetonitrile–aqueous 0.05 *M* phosphate buffer (pH 4.6) containing 5 m*M* 1-octanesulphonic acid (50:50, v/v). The column temperature was 50°C and detection was by UV absorption (210 nm). The limits of detection of 50-μl samples were 0.4 μg/ml (aqueous) and 0.78 μg/ml (0.5 ml gastric juice) or better. The assay was linear in the range of 1.56 to 100 μg/ml with r^2 values greater than 0.99. The recovery from the gastric juice samples was 98.5±2.9%. The method was applied successfully to determine the stability of clarithromycin in 0.01 *M* HCl and gastric juice.

Keywords: Clarithromycin

1. Introduction

Clarithromycin is a 14-membered macrolide antibiotic having a structure in which the hydroxy group of erythromycin is methylated at the C-6 position of the lactone ring [1]. It is insoluble in water and poorly soluble in alcohol, methanol and acetonitrile [2]. At very low pH, it degrades rapidly to 5-O-desosaminyl-6-O-methylerythronolide and cladinose (Fig. 1). When used for antibacterial therapy in the treatment of *Helicobacter pylori* infections, clarithromycin is most frequently given orally and will reside in gastric juice, under conditions of low pH, for a variable period of time. In order to characterise the gastric stability of clarithromycin it is necessary to be able to assay the drug in such a biological matrix.

Analytical methods reported previously include high-performance liquid chromatography (HPLC) using fluorescence, electrochemical or ultraviolet (UV) detection and microbiological assay [1–3]. The specificity of microbiological assay when used in the analysis of biological samples is limited because of the presence of the 14-hydroxy metabolite of clarithromycin which may have antimicrobial activity that could be additive or synergistic to that of the parent compound [4]. The limitations of the existing chromatographic assay methods include poor peak shape and low sensitivity, both of which have the potential to be improved by the use of ion-pair chromatography.

We describe herein an HPLC assay method using

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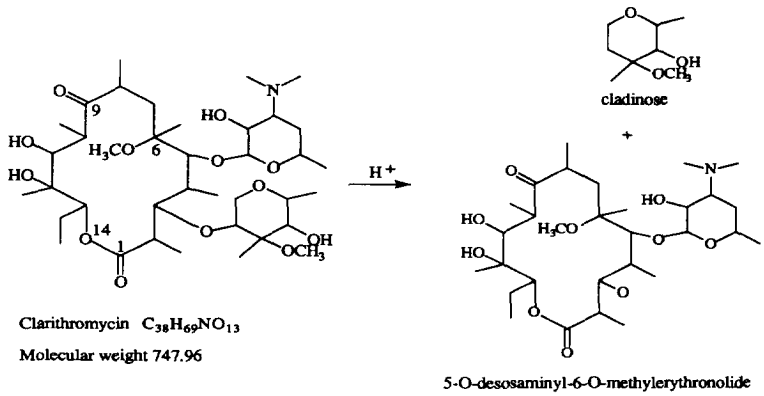

Fig. 1. Structure of clarithromycin and its major decomposition products.

ion-pairing chromatography and UV detection which permits the rapid analysis of clarithromycin in aqueous solutions and gastric juice.

2. Experimental

2.1. Chemicals

Clarithromycin was obtained from Abbott Laboratories (Kent, UK). Potassium dihydrogenphosphate, orthophosphoric acid (85%), anhydrous potassium carbonate and triethylamine were purchased from Fluka (Poole, UK). Sodium carbonate was obtained from Sigma (Poole, UK). HPLC-grade acetonitrile, concentrated HCl, sodium dodecyl sulphate (SDS), cetyltrimethylammonium bromide (CTAB) and 1-octanesulphonic acid (OCTS) were purchased from Fisons (Loughborough, UK) while the standard solution of HCl (0.1 *M*) was purchased from Aldrich (Poole, UK). Perchloric acid (70% v/v) and sodium hydroxide were obtained from BDH (Poole, UK). All chemicals were of analytical or HPLC grades.

2.2. Chromatographic systems

The HPLC system consisted of an automatic sample injector 231, dilutor 401, 302 solvent pump, pressure measurement unit 802C and computer software 714 (Gilson Medical Electronics, Villiers le Bel, France), an Applied Biosystems (Foster City, CA, USA) 759A UV absorbance detector set at a wavelength of 210 nm. The analytical column used was a Hypersil ODS 5 μm (150×4.6 mm I.D.) purchased from Hypersil (Runcorn, UK). A Waters 625 LC system was also used with a photodiode-array detector and computer software, Millennium 2010 (Waters, New England, USA). A guard column (20×2 mm I.D.) packed with Hypersil ODS 5-μm material was placed between the injector and the analytical column. The mobile phase consisted of acetonitrile–aqueous 0.05 *M* phosphate buffer containing 5 m*M* OCTS (50:50, v/v). The mobile phase was filtered through a 0.2-μm membrane filter and degassed for 30 min with helium before use. The flow-rate of the mobile phase was 1 ml/min and the sample injection volume was 50 μl. The samples were analysed with the column at a temperature of 50°C.

2.3. Preparation of gastric juice samples

The present study, which necessitated the removal of gastric juice samples from three healthy adult male volunteers was approved by the Ethical Committee of University Hospital, Nottingham. A simple, rapid and reproducible sample preparation method was required for the analysis of the gastric juice samples. Several different procedures were evaluated (see Section 3.2) and the following method was selected as the most suitable. Each gastric juice sample (0.5 ml) was spiked with 50 μl of acetonitrile containing clarithromycin to produce the required concentration (0.4 to 100 μg/ml). The sample was diluted to 2.5 ml using deionised water and

vortex-mixed for 1 min. It was then filtered through a nylon membrane filter (0.45-μm pore size) and the filtrate was analysed by HPLC.

2.4. Calibration standards

Calibration samples of clarithromycin were prepared in the HPLC mobile phase, 0.01 *M* HCl (pH 2) and gastric juice samples. The stock solution of clarithromycin (100 μg/ml) that was serially diluted was prepared by dissolving 10 mg of clarithromycin in 2 ml of acetonitrile and diluting the sample to 100 ml with the mobile phase or 0.01 *M* HCl. Gastric juice samples were prepared by spiking the sample (0.5 ml) with the appropriate amount of the compound in aqueous solution. The calibration lines were performed over concentration ranges of 1.56 to 100 μg/ml (aqueous solutions) and 3.125 to 100 μg/ml (gastric juice samples).

2.5. Validation of method

2.5.1. Linearity

Calibration lines of HPLC peak areas were constructed against the drug concentrations. Linear regression analysis was used to calculate the slope, intercept and the correlation coefficient (r^2) of each calibration line. The assay precision (C.V., %) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. Intra-day precision was estimated from replicates of measured peaks. Inter-day precision was estimated from the analysis of freshly prepared control samples on separate days.

2.5.2. Specificity

A photodiode-array detector was used to assess the purity of the peaks. Specificity was assessed by the lack of interference at the retention time of the peak of clarithromycin by its degradation product or endogenous peaks.

2.5.3. Accuracy and bias

Accuracy was estimated from the concentration of the measured peak calculated from the calibration line, while sample bias was estimated as the percentage deviation of the measured concentration from the nominal (added) concentration.

2.5.4. Detection limit

This was estimated as the minimum detectable quantity of clarithromycin (signal-to-noise ratio of 3:1) which could be detected without interference from the baseline noise.

2.5.5. Recovery

The recoveries of clarithromycin from the gastric juice samples were estimated by expressing the peak areas of samples in gastric juice as percentages of the peak areas measured following HPLC analyses of amounts equivalent to 100% of the respective solutions of the compound in the mobile phase.

2.6. Application in stability studies

2.6.1. Aqueous solutions

Clarithromycin solutions at a concentration of 100 μg/ml were prepared in 0.01 *M* HCl (in triplicates) preheated to 37°C by dissolving 10 mg of clarithromycin in 2 ml of acetonitrile and making the solution up to a 100-ml volume with the HCl solution. The solutions were immediately stored in "jacketed beakers" maintained at 37°C and sealed with Parafilm (American National Can, USA). Samples were withdrawn every hour by an autosampler and were analysed by HPLC.

2.6.2. Gastric juice

The gastric juice samples from three volunteers were adjusted to pH 2 using 1 *M* NaOH or HCl. They were centrifuged at 600 *g* for 10 min to remove any solid materials. A 19-ml volume of each sample was preheated to 37°C and 1 ml of 2 mg/ml clarithromycin in solution of acetonitrile–water (40:60, v/v) was then added to produce a final concentration of 100 μg/ml. The mixture was vortex-mixed for 2 min and the samples (1 ml each), dispensed into 2-ml HPLC vials (Fisons), were incubated at 37°C for 28 h. At intervals, samples withdrawn from the vials were diluted five-fold and analysed after filtration through a nylon membrane filter (0.45-μm pore size).

2.6.3. Analysis of data

The unknown concentrations of clarithromycin remaining in the incubated samples were calculated from the slopes and intercepts of the calibration

lines. The order of degradation reaction, degradation rate constants and half-lives in the different samples were determined using the degradation rate equations [5] and linear regression analysis. Statistical analysis was carried out with the one-way analysis of variance using a computer software Instat version 2.03 (Graph Pad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Chromatographic conditions

Initial investigations to select the appropriate chromatographic conditions for the analysis of clarithromycin in gastric juice centred on the use of a base-deactivated reversed-phase ODS silica column [Hypersil BDS 5 μm (150×4.6 mm I.D.)]. This approach was unsuccessful and resulted in broad peaks indicating poor efficiency. It was therefore decided to investigate the effects of mobile phase pH, the addition of triethylamine and the use of ion-pairing agents using a standard ODS silica column [Hypersil ODS 5 μm (150×4.6 mm I.D.)]. The phosphate buffer solutions (at pH 3, 4.6 and 7) used in the mobile phase were prepared with 0.1% (v/v) triethylamine, 1.75 m*M* SDS, 1.75 m*M* CTAB or 1.75 m*M* OCTS. The mobile phases both with or without triethylamine, or containing SDS, CTAB and 1.75 m*M* OCTS produced broad asymmetrical peaks with substantial fronting. The change in the pH of the phosphate buffers did not produce a better peak shape for clarithromycin. An improved peak shape with retention time of 11.5 min was produced when the OCTS concentration was raised to 5 m*M* and 40% acetonitrile was used in the mobile phase at an ambient column temperature. At a column temperature of 40°C using 50% acetonitrile and 5 m*M* OCTS in the mobile phase, a sharp and symmetrical peak for clarithromycin was obtained with a retention time of 5.8 min. Increasing the column temperature to 50°C further sharpened the peak. Therefore, the optimised mobile phase conditions which were developed for the analysis of clarithromycin in aqueous solutions were as follows: acetonitrile–aqueous 0.05 *M* phosphate buffer (pH 4.6) containing 5 m*M* OCTS (50:50, v/v), at a column temperature of 50°C. These conditions were selected because they gave the best peak symmetry, provided minimal interference from endogenous peaks and the lowest detection limit of clarithromycin. The same chromatographic conditions were also used to analyse the gastric juice samples. Representative chromatograms of clarithromycin in the mobile phase, drug-free gastric juice sample and the incubated 0.01 *M* HCl solution and gastric juice are given in Fig. 2 and Fig. 3. No interfering peaks were noticeable in the chromatograms.

3.2. Preparation of gastric juice samples

An attempt was made to extract clarithromycin from the gastric juice samples into acetonitrile by adding 1 g of solid potassium carbonate (to adjust the sample pH above the pK_a of clarithromycin) and 0.5 ml of acetonitrile to 0.5 ml of the gastric juice sample containing clarithromycin, mixing for 1 min and centrifuging at 11 600 *g* for 5 min. This extracted sample produced chromatograms for clarithromycin with two peaks appearing at 2 min and 4

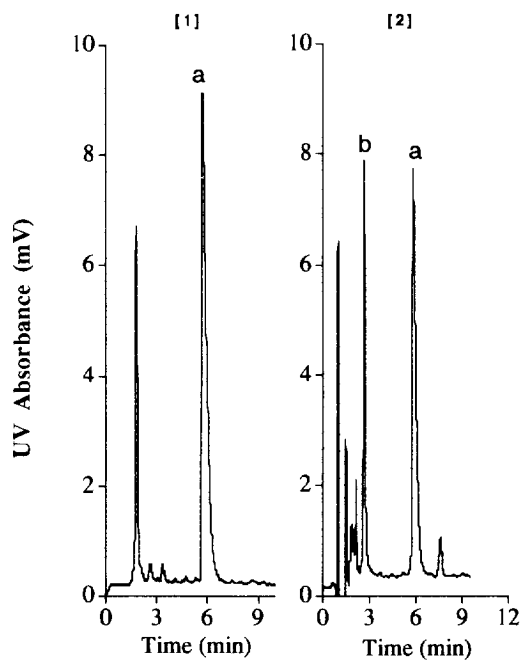

Fig. 2. Chromatograms of [1] clarithromycin (a) in mobile phase and [2] clarithromycin (a) and its major degradation product (b) after incubation in 0.01 *M* HCl (37°C, 1 h).

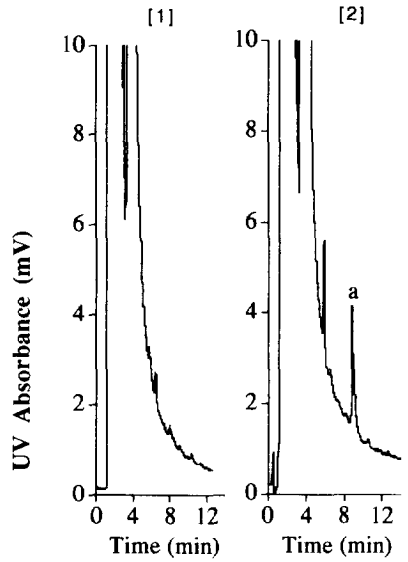

Fig. 3. Chromatograms of [1] drug-free gastric juice and [2] clarithromycin (a) (0.75 μg on column) remaining after incubation in gastric juice at pH 2 (37°C, 1 h).

min and was not considered suitable for further development. Extraction of the clarithromycin from the gastric juice samples was also attempted by vortex mixing and then centrifuging 0.1 ml of 0.2 *M* sodium carbonate, 0.3 ml of hexane–ethyl acetate mixture (50:50, v/v) and 0.5 ml of gastric juice sample, evaporating the organic layer and redissolving the solid materials obtained into acetonitrile. The chromatograms obtained from the samples prepared in this way were similar to those described above. Samples were also prepared by adding 50 μl of perchloric acid (50%, v/v) to precipitate proteinaceous material prior to the addition of 1 g of solid potassium carbonate. It was not possible to analyse these samples by HPLC due to the formation of an emulsion following centrifugation of the samples. The filtration method of sample preparation (see Section 2.3) produced chromatograms which showed no significant interference from endogenous peaks as confirmed by the photodiode array analysis. The filtration method of sample preparation was chosen in the analysis of the samples because it was the simplest to perform and produced a valid method of analysis at least as good as the more complex methods which were evaluated.

Table 1
Precision and accuracy for the assay of clarithromycin in aqueous solutions

Nominal concentration (μg/ml)	Measured concentration (mean±S.D.) (μg/ml)	Bias (%)	C.V. (%)
Within-day ($n=6$)			
100	100.5±1.2	0.8	1.2
12.5	12.3±0.2	−3.2	4.1
1.56	1.9±0.1	14.5	4.6
Inter-day ($n=6$)			
100	100.7±1.1	0.9	1.1
12.5	12.3±0.2	1.4	1.5
1.56	1.9±0.2	18.4	9.4

3.3. Validation of method

The assay was validated using seven-point calibration lines of clarithromycin in the mobile phase, 0.01 *M* HCl and gastric juice samples. The lines were all linear with r^2 values greater than 0.99. The within-day and inter-day accuracy and precision at a range of concentrations are given in Table 1 and Table 2. The lower limits of detection of clarithromycin were found to be 0.4 μg/ml (aqueous solutions) and 0.78 μg/ml (0.5 ml gastric juice samples). The recovery of clarithromycin from the gastric juice samples was 98.5±2.9% ($n=9$). The validation data obtained indicate that the method will prove suitable for the analysis of clarithromycin in aqueous solutions and gastric juice samples where

Table 2
Precision and accuracy for the assay of clarithromycin in gastric juice samples

Nominal concentration (μg/ml)	Measured concentration (mean±S.D.) (μg/ml)	Bias (%)	C.V. (%)
Within-day ($n=6$)			
100	99.3±2.0	−0.8	2.0
25	24.3±1.3	−2.6	5.3
3.125	2.9±0.4	−8.0	13.2
Inter-day ($n=3$)			
100	98.4±1.1	−1.6	1.0
25	25.1±0.7	0.5	2.9
3.125	2.7±0.4	−12.9	15.7

the minimum quantities of clarithromycin required to be detected are in the microgram range.

3.4. Application in stability studies

The order of degradation of clarithromycin in aqueous solutions and gastric juice was found to be pseudo-first order. This agrees with the order of degradation earlier reported [1]. The pseudo-first order plot of the percentage of clarithromycin remaining in the samples against time (Fig. 4) was linear with r^2 values greater than 0.99. The calculated half-lives in the 0.01 *M* HCl and gastric juice samples at pH 2 were 1.3 h and 1.0 h, respectively. There was a significant difference between the degradation rate constants of clarithromycin in gastric juice samples when compared with the rate constants in the HCl solutions ($p<0.001$).

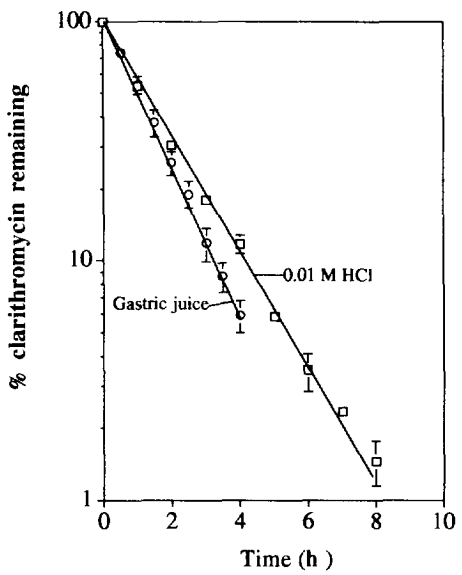

Fig. 4. Pseudo-first order plot of the degradation of clarithromycin in 0.01 *M* HCl and gastric juice samples of pH 2 at 37°C against time (h) ($n=3$).

4. Conclusions

An ion-pair HPLC assay method has been developed for the rapid analysis of clarithromycin in aqueous solutions and in gastric juice. The method is simple, selective, reproducible and useful for the analysis of the compound in presence of its degradation products. It provides adequate sensitivity and specificity for application in stability studies.

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