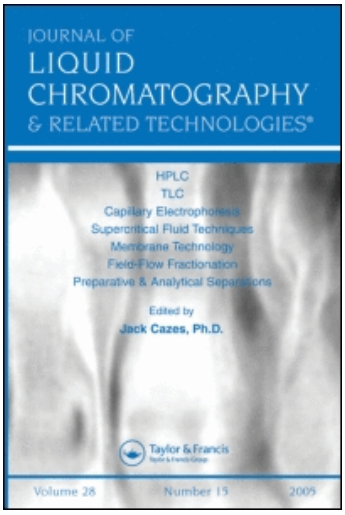

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Determination of Ofloxacin, A New Oxazine Derivative in Human Serum, Urine, and Bile by High-Performance Liquid Chromatography

G. Carlucci[a]; S. Guadagni[b]; G. Palumbo[c]

[a] Department of Chemistry Chemical Engineering and Materials, Chair of Pharmacology University of L'Aquila Via Assergi, L'Aquila, Italy [b] Department of Surgery Clinical, Chair of Pharmacology University of L'Aquila Via Assergi, L'Aquila, Italy [c] Department of Internal Medicine and Public Health, Chair of Pharmacology University of L'Aquila Via Assergi, L'Aquila, Italy

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DETERMINATION OF OFLOXACIN, A NEW OXAZINE DERIVATIVE IN HUMAN SERUM, URINE, AND BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

G. Carlucci[1], S. Guadagni[2], and G. Palumbo[3]

[1]*Department of Chemistry*
Chemical Engineering and Materials
[2]*Department of Surgery Clinical*
[3]*Department of Internal Medicine and Public Health*
Chair of Pharmacology
University of L'Aquila
Via Assergi, 4-67100
L'Aquila, Italy

ABSTRACT

A simple and precise high-performance liquid procedure has been developed for the determination of Ofloxacin (DL-8280), a new oxazine derivative in human serum, urine and bile using Norfloxacin as internal standard. The work-up procedure involves a chemical extraction step followed by isocratic chromatography on a anion-exchange analytical column, with ultraviolet detection. The run time for the assay was 11.5 minutes.

Send reprint requests to: Dr.G.Carlucci- Department of Chemistry University of L'Aquila- via Assergi, 4-67100 L'Aquila (Italy).

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INTRODUCTION

Ofloxacin DL-8280,(+-)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido-(1,2,3-de)1,4-benzoxazine-6-carboxylic acid, is a new pyridone carboxylic acid derivative.

Drugs of this group include Nalidixic acid, Pipemidic acid, Cinoxacin, Ciprofloxacin and Norfloxacin. However, Ofloxacin acid differs from these in that it is a tricyclic compound in which the oxazine is bound to the quinoline ring (Figure 1).

Experimental studies performed on animals demonstrated that the distribution of drug in both tissues and organic fluids is rapid, and the reached concentrations are higher than the corresponding serum levels (1). The in vitro and in vivo activity of Ofloxacin was compared with that of other antibacterial compounds (2-3-4-5-6). Ofloxacin was found to have a broad antibacterial spectrum which includes both Gram + and Gram- aerobic and anaerobic species (7). The report of metabolic disposition of DL-8280 with C(14)-DL-8280 in various animal species identified four metabolites as follows: M-1 ester glucuronide of DL-8280, M-2 unchanged form, M-3 N-desmethylated DL-8280, M-4 DL-8280 N-oxide (8).

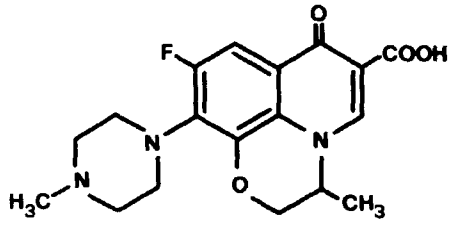

FIGURE 1: Chemical structure of Ofloxacin

In the present communication, we describe an extraction and quantitation procedure for measuring Ofloxacin concentrations in human serum, urine and bile after oral administration of the drug.

MATERIALS AND METHODS

Ofloxacin was supplied by Sigma-Tau (Pomezia - Roma-Italy) and 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid, used as the internal standard, was obtained from Merck-Sharp & Dohme. Methylene chloride, acetonitrile (HPLC grade), monobasic and dibasic phosphate were all from Fluka (Fluka AG.- Switzerland). Water (HPLC grade) was obtained from double distillation in glass and purification through a "Milli-Q Water Purification System" (18 MΩ cm resistivity; cartridge sequence: Super-C, Ion-EX, Ion-EX, Twin-90) (Millipore, Bedford, MA, U.S.A.).

Chromatography

We used a Waters liquid chromatograph equipped with a Model 440 absorbance ultraviolet detector, a M6000 A pump and a U6K injector (Waters Assoc., Milford, MA, U.S.A.), a Leeds- Northrup Italiana Speedomax XL-682 linear recorder and a Hewlett-Packard HP 18850 A integrator. The chromatographic separation was achieved in a Vydac anion - exchange 10 μm column (25 cm x 4.5 mm; Separation Group, Hesperia, CA, U.S.A.), connected to an AXGU 10 μm anion - exchange precolumn (Brownlee Labs Inc. Santa Clara, CA, U.S.A.). The mobile phase used was a mixture of acetonitrile and 0.05 M phosphate buffer pH 7 in HPLC grade water (20:80, V/V) at a flow-rate of 2.0 ml/min.. The mobile phase was prepared daily. The phosphate buffer was filtered through an HA 0.45 μm filter, while the acetonitrile was filtered through an FA 0.5 μm filter (Millipore, Bedford, MA, U.S.A.). The mixture was degassed by ultrasonication prior to use. The wavelength used was 280 nm, chart speed 0.2 cm per minute. All chromatography was carried out at room temperature.

Preparation of Standard Curve

Stock solutions of Ofloxacin and the internal standard Norfloxacin, were prepared by dissolving the

compounds in 0.1 M sodium hydroxide. The serum Ofloxacin standard curve was prepared by diluting the above standard (10.0 mg/ml) with drug-free human sera so that 100 µl of serum would correspond to a concentrations range of 0.5 - 6.0 µg/ml. The urine Ofloxacin standard curve was prepared similarly, yielding a concentration range of 10 - 600 µg/ml. Standard curves for Ofloxacin were constructed using peak area ratios obtained for the drug to the internal standard versus the concentration of the drug.

Extraction Procedure

Serum (1 ml), bile (1 ml), and urine (200 µl) samples were mixed with 100 µl of 0.05 M sodium hydroxide, and then added to norfloxacin as internal standard in a 50 ml plastic centrifuge tube. Methylene chloride (8 ml) and 0.5 M sodium phosphate buffer pH 7.5 (0.5 ml) were added to all the samples and the tubes were shaken for 10 minutes in a Dubnoff mechanical shaker (150 cycles/ min). Separation of the two phases was achieved by centrifugation at 1500 g for 10 minutes and 7 ml of the organic phase (lower layer) were transferred into a second tube. Fresh methylene chloride (8 ml) was added to a second tube

and the same extraction procedure was repeated twice. The organic phases collected from the extractions of the same sample were pooled (21 ml). Sodium hydroxide (0.3 M) was then added to the serum (250 µl), to the bile extract (250 µl) and to the urine (500 µl) samples. The tubes were shaken for 10 minutes and then centrifuged at 1500 g for 10 minutes. The aqueous phase (upper layer) was collected in Eppendorf tubes and frozen (-20 °C) until the HPLC assay; 25 µl were used for chemical analysis.

RESULTS AND DISCUSSION

Figure 2, shows the separation of Ofloxacin (marked with a star) using internal standard Norfloxacin in serum, bile and urine respectively. Ofloxacin and the internal standard are well resolved their retention times being 10.40 and 6.70 minutes respectively. Concentration of Ofloxacin in samples were determined by measuring the ratio of the peak area for the drug to that for the internal standard, then comparing with the standard curves obtained after linear regression analysis of the calibration samples. No interference was observed during the assay between Ofloxacin and endogenous substances in the clinical samples.

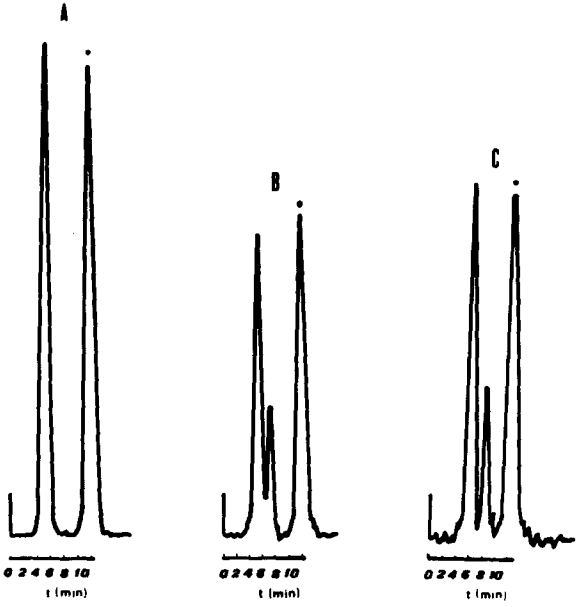

FIGURE 2 : HPLC chromatograms from biological samples of patient G.P. after 400 mg dose of Ofloxacin. Vertical axis: UV detector response (280 nm); horizzontal axis: retention time (min.). (A) urine sample, 583,4 µg/ml; sensitivity 1.0. (B) bile sample, 18,5 µg/ml; sensitivity 0.5. (C) serum sample, 2,4 µg/ml; sensitivity 0.05.

Analitycal recovery was determined by adding Ofloxacin to fresh serum and to a solution of mobile phase, to yield concentrations of 0.5 µg/ml and 6.0 µg/ml, then analyzing. The serum standards were treated as described above, but with no internal standard added. Recovery was calculated by dividing the average Ofloxacin area for prepared serum-based standards by that obtained from direct injection of

volume-adjusted standards in mobile phase. Five determinations were made at each concentration. The standard curve for serum was linear (range 0.5 to 6 μg/ml) with r=0.998 (y=0.4332x-0.0154 μg/ml). For 6.0 μg/ml and 0.5 μg/ml concentrations, the mean analytical recovery was 66% and 53.4% respectively. The standard curve for Ofloxacin in urine and bile, over a concentration range of 10 to 600 μg/ml, was linear (r=0.999, y=0.515x-0.0467 μg/ml).

The assay was evaluated in eighteen biliopathic hospitalized patients who were administered Ofloxacin (400 mg per os) 3 hours before surgery. Serum, bile and urine samples were obtained from patients during surgery.

A sensitive and specific HPLC method was developed utilizzing the natural U.V. absorbing properties of Ofloxacin. Using the chromatographic conditions described for this assay, Ofloxacin was completely resolved from the internal standard.

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