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Short communication

A new on-line, in-tube pre-column derivatization technique for high performance liquid chromatographic determination of azithromycin in human serum

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Abstract

Pre-column derivatization methods for high performance liquid chromatographic assay of specific pharmaceutical agents using 9-fluorenylmethyl chloroformate (FMOC-Cl) have received special attention because highly fluorescent and stable adducts are provided by these methods. However, unlike the post-column on-line techniques, long derivatization time is needed and the reaction cannot be well controlled. A new, sensitive and fast pre-column on-line derivatization technique coupled with high-performance liquid chromatography using FMOC-Cl as labeling agent is described and validated for determination of azithromycin in human serum. After extraction of the drug from serum, the residue was reconstituted in mixture of acetonitrile–phosphate buffer (3:1, v/v; pH 8.5) and directly injected onto the chromatographic system. Continuous on-line derivatization and analysis of the compounds were successfully performed using in-tube elution of FMOC-Cl. The total time needed for derivatization and chromatographic analysis of the drug was 13 min. The assay was reliable and reproducible, with limit of quantification of 10 ng/ml. The described technique may offer significant advantages over existing off-line derivatization methods using FMOC-Cl.

Keywords: On-line derivatization method; Reverse phase chromatography; HPLC; Azithromycin; Serum; Macrolide antibiotics; FMOC-Cl

1. Introduction

9-Fluorenylmethyl chloroformate (FMOC-Cl) is a suitable reagent for the fluorescent labeling of both primary and secondary amines [1]; also it has been used to form stable ester-bonded derivatives with hydroxyl groups. The reagent is widely used to enhance sensitivity and selectivity for determination of amino acids as well as specific pharmaceutical agents using off-line pre-column derivatization methods. However, the derivatization conditions, such as temperature and time of the reaction, as well as amounts of the labeling agent are critical for high efficient and reproducible derivatization reaction. Furthermore derivatization procedures are complicated and multi steps additions of the reagents are time consuming. In the post-column on-line derivatization methods, however, the reaction can be well controlled by flow rates of eluate and reagents. On-line pre-column derivatization methods with FMOC-Cl using auto sampler for specific pharmaceutical agents have frequently been reported [2–7]. In the present paper, however, a novel on-line in-tube method for pre-column derivatization technique using FMOC-Cl as the fluorescence labeling agent is described and validated for analysis of azithromycin in human serum [8]. By this method pre column on-line derivatization of azithromycin is achieved using in- tube elution of the labeling agent. Compared to the conventional pre-column derivatization methods with FMOC-Cl, the new technique allows continuous derivatization and analysis of the drug in short time periods. Also the chemical reaction can be well controlled by the flow rate and concentration of the reagents as well as by adjustment of the oven temperature.

2. Experimental

2.1. HPLC method

Our previously published HPLC method for analysis of azithromycin was used in this study with some modifications [8]. Briefly to the 1 ml serum samples (calibration or unknown)

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100 μl of amantadine (10 μg/ml in water) as internal standard (I.S.), and 5 ml diethyl ether were added. Following vortex mixing (30 s), centrifugation (5 min; 6000 × *g*), and evaporation of the organic phase, the residue was reconstituted in 100 μl mixture of the phosphate buffer (0.5 M; pH 8.5) and acetonitrile (1:3, v/v) and a volume of 50 μl was injected on to the chromatograph. The chromatographic system used consisted of three high pressure pumps (LC-10AD), a column oven (CTO-10A), a spectroflurometric detector (RF-551; Ex 260, Em 315 nm), a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. A 7125i Rheodyne sample injector valve (valve 1) and a six-port switching valve (valve 2) with 50 and 100 μl filling loops, respectively (Berkeley, CA, USA) were used. The analytical column was a Shimpack CLC-C8 (Shimadzu, Kyoto, Japan), 150 mm × 4.6 mm I.D., 5 μm particle size and the mobile phase consisted of methanol–0.05 M sodium phosphate buffer (70/30, v/v) containing 0.1% triethylamine (v/v; pH 4.3).

2.2. On-line in-tube derivatization procedure

Fig. 1 shows the analytical setup used for continuous derivatization and analysis of the samples and as it has been shown temperature of the system was kept at 62 °C throughout of the analysis and this temperature was necessary for maximize the performance of the analytical method. The six-port switching valve (valve 2) was initially set at Prep. position, and the mobile phase was eluted by pumps A and B directly through the analytical column to obtain a stable baseline for chromatographic separation. Before analysis the Rheodyne sample injection valve (valve 1) was set at load position and the FMOC-Cl solution (500 μg/ml in acetonitrile) was introduced by pump C through stainless steel pipe (SUS, O.D. 1.6 mm × I.D. 0.8 mm × length 500 mm; Shimadzu Kyoto, Japan) at a flow rate of 0.1 ml/min. At load position a volume of 50 μl of each samples was injected onto the system (valve 1). When analysis began, valve 1 was switched to inject position for a given time interval (270 s). During this time which was enough for derivatization of the drugs and filling of the 100 μl loop in valve 2, the FMOC-Cl solution should be held at constant flow. The valve 1 should be maintain at the inject position for at least 1 min. After this time (270 s), the 100 μl loop was automatically filled and by switching of the valve 2 to inject position the reaction products were inserted into the mobile phase carrier stream and driven to the analytical column.

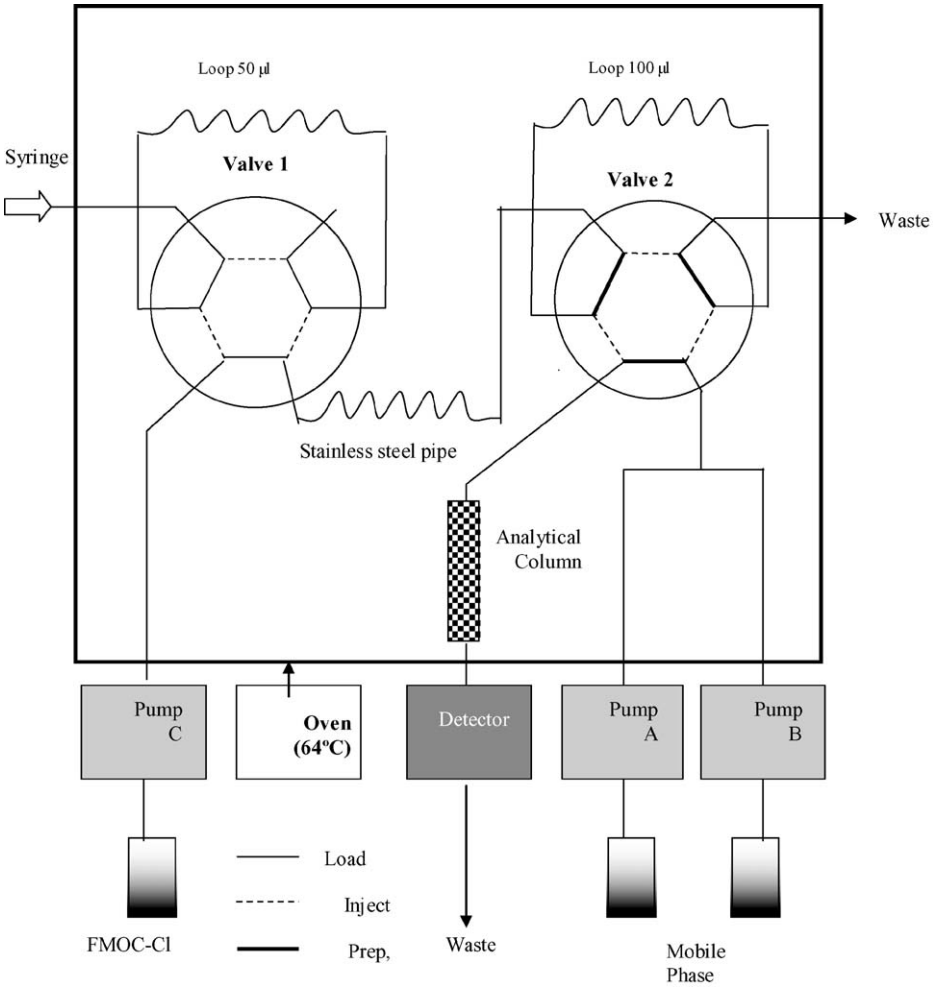

Fig. 1. Schematic construction of the system used for on-line in-tube derivatization of azithromycin by FMOC-Cl.

2.3. Optimization of the on-line in-tube derivatization and method validation

The derivatization of azithromycin with FMOC-Cl was optimized using solutions of 1, 4 and 10 μg/ml of the drug in acetonitrile while, amantadine was reacted with the reagent at the concentration of 25 μg/ml. Concentrations of the FMOC-Cl solutions ranging from 100 to 1000 μg/ml, pH of buffer solutions ranging from 6 to 11, reaction temperatures ranging from 45 to 70 °C (the maximum tolerable temperature which has been recommended by the column manufacture) and pH of the mobile phase ranging from 3 to 7 were tested to obtain desired values. After extraction of azithromycin and the I.S., the residue was reconstituted in various acetonitrile–buffer proportions, ranging from 1:1 to 10:1 to optimize the polarity of the reaction solution. The derivatization time was optimized by using different flow rate of FMOC-Cl ranging from 0.05 to 0.2 ml/min as well as by using different length of stainless steel pipes. The resulting peak areas of the analytes were compared with those obtained by injections of standards, containing equivalent amounts of the drug derivatized using the off-line derivatization reaction.

The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3:1. The lower limit of quantification was obtained as the lowest serum concentration of azithromycin quantified with a coefficient of variation of less than 20%. Within-day variation was measured by assessing six replicates at each quality control concentration. Between-days variation was based on repeated analysis of the same concentration controls in ten analytical run performed on different days.

3. Results and discussion

3.1. Development of the derivatization method

In the on-line derivatization system, the exposure time is determined by the flow rate of labeling agent, as well as by the reactor volume. In the method described above a stream of labeling agent with flow rate of 0.1 ml/min and a stainless steel pipe of 500 mm length and 0.8 mm internal diameter and filling loops with capacities of 50 and 100 μl were found to provide suitable response for both azithromycin and the I.S. Due to dispersion of the analytes in the reactor the peak heights were reduced when stainless steel pipes with a length of more than 500 mm were used. Also increase of the flow rate of the labeling agent reduced the exposure time and consequently derivatization yields. Using abovementioned setup an exposure time of 270 s was obtained and this reaction time was sufficient to achieve derivatization and analysis of the drug. In this study, reagent concentrations ranging from 100 to 1000 μg/ml were tested. Although the responses were magnified by increasing the concentration of FMOC-Cl but from a practical point view due to deleterious effect of the reagent excess (in the concentration of more than 500 μg/ml) on the determination of low level of the drug, the concentration of 500 μg/ml was selected.

3.2. Analytical conditions

In our previously published method for analysis of azithromycin, a mobile phase with pH 5.9 and clarithromycin as the I.S. were used and excess of the labeling reagent was removed by addition of glycine to the reaction mixture. In the present technique, however, removing of the reagent excess was not possible. As a result clarithromycin was eluted in the first part of the chromatogram where excess of the reagent was appeared. Therefore attempts were made to find another I.S. Unlike clarithromycin and amantadine the retention behavior of azithromycin was highly pH dependent and its retention time was found to increase proportionally with the pH of the mobile phase. Thus, a mobile phase with pH 4.3 was used and among a number of drugs with hydroxyl or amine groups (erythromycin, spiramycin, gentamicin, amikacin, alendronate, ethidronate, cephalexin, topiramate and amantadine) amantadine was selected as the I.S.

3.2.1. Validation of the method

Representative chromatograms obtained from human blank serum, human blank serum containing the I.S. and human blank serum spiked with azithromycin (10 ng/ml) and the I.S. are shown in Fig. 2A–C, respectively. Endogenous components and excess of the reagent chromatographed within 3.2 min and the peaks of interest were well resolved from each other. The retention times for the I.S. and azithromycin were 4.2 and 5.8 min, respectively. Fig. 2D and E show the chromatograms of serum samples obtained at 8 and 48 h after a single oral dose of 400 mg azithromycin (zithromax suspension; 200 mg/5 ml) from a healthy adult volunteer containing 165 and 38 ng/ml of azithromycin, respectively. In our previously published paper *n*-hexane was used as the extracting solvent. In the present method, however, due to inadequate recovery of amantadine, diethyl ether was selected as extracting solvent and the recoveries of the drug and I.S. were calculated to be $98 \pm 4\%$ and $65 \pm 5\%$, respectively ($n = 6$). Efficiency of the derivatization method for the drug and I.S. were calculated using blank serum samples spiked with known amounts of the agents at different concentrations. These samples were analyzed in replicate as described and peak areas were compared with those obtained from the off-line derivatization of the same amounts. The efficiency of the method for azithromycin and the I.S. were determined to be 75% and 90%, respectively.

The coefficient of variation values were 1.5–14.1% for inter-day assay and 1.8–16.5% for intra-day assay, respectively and accuracy never deviated from 100% by more than 9.2%. The limits of detection and quantification were estimated to be 5 and 10 ng/ml, respectively using 50 μl injections. The calibration curves were linear over the concentration ranges of 10–1000 ng/ml using line-fit plot in regression analysis. Intra and inter days reproducibility were studied for calibration curves prepared at the same day ($n = 4$) and different days ($n = 10$) in replicate, respectively, A slope of 0.3645 ± 0.0155 (C.V. = 6.2%) with an intercept of 2.9410 ± 0.2741 (C.V. = 14.1%) and a correlation coefficient of 0.9975 ± 0.0021 (C.V. = 0.23%) were obtained for inter-day assay and a slope of 0.3586

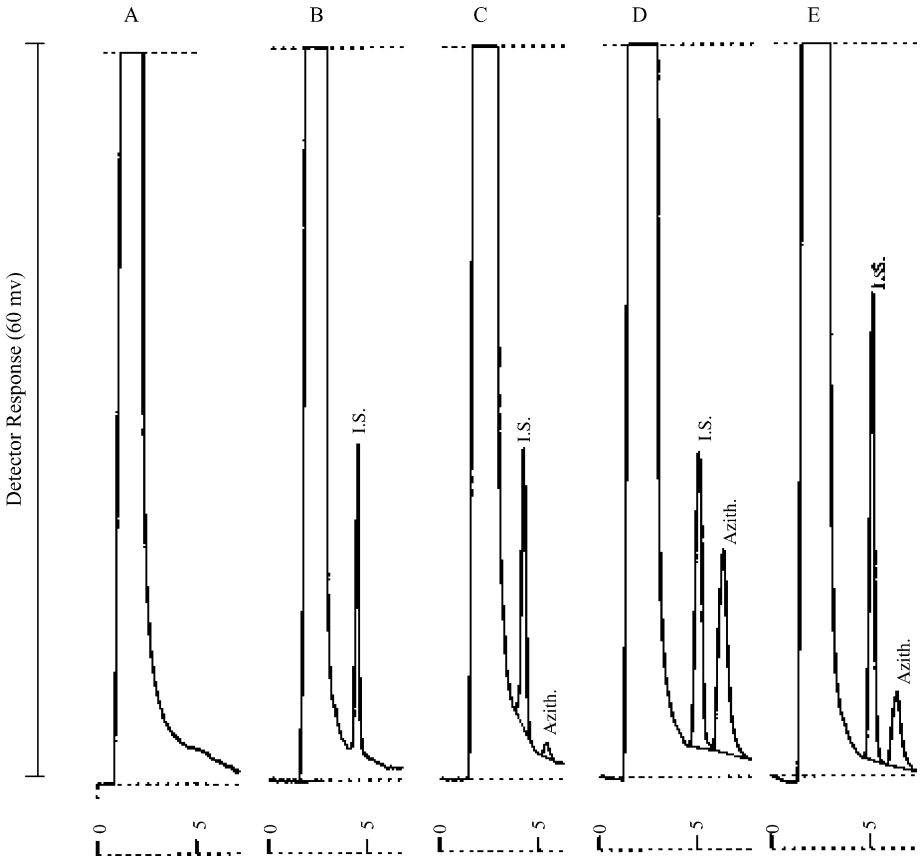

Fig. 2. Reprehensive chromatograms of extracts of (A) human blank serum; (B) human blank serum spiked with amantadine; (C) human blank serum spiked with 10 ng/ml azithromycin and amantadine; (D and E) serum samples from a volunteer 8 and 48 h after a single oral dose of 400 mg drug containing 165 and 38 ng/ml of azithromycin, respectively.

$\pm$ 0.0185 (C.V. = 7.1%) with an intercept of 2.8850 $\pm$ 0.3348 (C.V. = 14.8%) and a correlation coefficient of 0.9961 $\pm$ 0.0024 (C.V. = 0.27%) were obtained for intra-day assay.

The presented method is simple and fast in which the samples were directly injected on to the system. In contrast to the conventional pre-column methods, the reaction can be well controlled by the flow rate and concentration of the reagents as well as by adjustment of the oven temperature. This technique may be used for analysis of other drugs which react with the FMOC-Cl.

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