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Journal of Chromatography A, 846 (1999) 181–184

JOURNAL OF CHROMATOGRAPHY A

Short communication

Microcolumn high-performance liquid chromatographic assay for doxycycline in isolated alveolar macrophages

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Abstract

A procedure for the isolation of doxycycline from the alveolar macrophages is described. Due to the minimal amount of the sample, it was necessary to employ microcolumn HPLC. A great advantage of the methodology developed in the present paper consists in the direct injection of an aqueous supernatant over disintegrated macrophages without the use of liquid–liquid or solid–liquid extraction. Another advantage of the method is the fact that the injection of an aqueous solution on a microcolumn with a lipophilic stationary phase makes the analyzed substances concentrate, which increases the sensitivity of assay. Separation was performed on a Separon SGX C_{18} microcolumn, 150×1 mm, detection at 254 nm by UV, 1 μl flow cell. Rabbits were treated intravenously with Vibramycin inj. sic. in a dose of 13 mg/kg body weight doxycycline. A procedure for washing macrophages from rabbit lungs was developed. Dry matter of macrophages ranged from 5.9 to 11.4 mg. In the samples of three rabbits the levels of doxycycline ranged from 110 to 270 ng per 1 mg of macrophages dry matter.

Keywords: Doxycycline; Tetracycline; Antibiotics; Tetracyclines

1. Introduction

Macrophages are a group of cells of the immunological system which rank among mononuclear phagocytes. Their localisation and functions are varied but they have common precursors in the bone marrow. From there they are transported as monocytes by the peripheral blood into the target tissue where they are transformed into some type of macrophages, e.g. peritoneal, alveolar macrophages, macrophages in the spleen or lymphatic nodes. Alveolar macrophages in the lungs are most accessible for study.

This first analytical study of drug levels in isolated alveolar macrophages is devoted to doxycycline (DX) as a representative of the tetracycline antibiotics which are widely used in therapy.

High-performance liquid chromatography has been used to determine tetracycline antibiotics in biological fluids, primarily in plasma, serum, urine or blood. Tetracyclines were also determined in fish [1], in bovine and porcine muscle [2] and in kidney [3]. Tetracycline antibiotics were determined in serum using RP-HPLC with fluorescence detection [4]. White et al. [5] focus their paper on a comparative study of HPLC methods for tetracycline determination. Fifteen tetracycline derivatives were assayed in small blood and plasma samples [6].

The aim of the present study was to develop a microcolumn HPLC methodology to determine DX in isolated alveolar macrophages.

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00148-X

2. Experimental

2.1. Chemicals and reagents

Vibramycin inj. sic. (Pfizer), tetracycline and DX (VÚAB Prague, Czech Republic), and acetonitrile, oxalic acid, dimethylformamide (Lachema, Brno, Czech Republic) were used. All these reagents were analytical-reagent grade. Double-distilled water was used.

2.2. Instrumentation

A liquid chromatograph consisted of a solvent delivery system SP 7800 (Spectra-Physics), a Hewlett-Packard UV detector at 254 nm, a 1 μl flow cell, and an integrator HP 3394A. Tetracycline proved to be the best internal standard (I.S.). Separation was performed on Separon SGX C_{18}, particle size 5 μm, microcolumn 150×1 mm (Tessek, Prague, Czech Republic). The injection valve had a 20 μl loop, the mobile phase was a mixture of acetonitrile–aqueous solution of oxalic acid (0.03 *M*)–dimethylformamide (30:60:6, v/v), the flow-rate was 60 μl/min.

2.3. Biological samples

Rabbits were treated intravenously with Vibramycin inj. sic. in a dose of 13 mg/kg body weight DX. The dose was repeated for 3 days in an interval of 12 h, the last administration taking place 3 h before the withdrawal. The rabbits were anaesthesized and after exsanquination the lungs were washed with 50 ml of Ringer's solution. After centrifugation the mass of macrophages was analyzed.

2.4. Adjustment of macrophages samples

Centrifugation (1950 *g*) of lavage liquid yielded a sediment of macrophages to which in a small-volume test-tube 90 μl of redistilled water and 10 μl of an aqueous solution of I.S. are added. Repeated freezing and defreezing in a freezing box produced disintegration of cells. In order to effectively release their content, an ultrasonic bath was subsequently used. After final centrifugation, the microcolumn was injected with 20 μl of clear supernatant over the disintegrated macrophages.

2.5. Standard and sample preparation, calculation

Calculation of the DX level in the macrophages of premedicated rabbits was performed from the ratio of the peak areas of the supernatant of the blank of macrophages with a defined addition of DX (5 μl of aqueous solution of a concentration of 200 μg/ml) and of I.S. (5 μl of aqueous solution of a concentration of 140 μg/ml), and the supernatant of a sample of the analyzed macrophages with a defined addition of I.S. (10 μl of the solution with a concentration 70 μg/ml). As follows from the scheme, 90 μl of redistilled water was always added to the separated sediment of macrophages, so the total volume of the aqueous phase made 100 μl in all cases. For the evaluation of the results with unequal amounts of macrophages in the individual withdrawals it was necessary to determine their dry matter. The mass of macrophages which remained after supernatant analysis in a test-tube in the aqueous phase, was dried at 40°C in a drier to a constant mass. The test-tubes were immersed into chromosulfuric acid, thoroughly washed, dried to a constant mass and weighed. The difference served for the calculation of the mass of the dry matter, which ranged in an interval of 5.9–11.4 mg. The values of DX levels in the macrophages complex of three rabbits are listed in Table 1.

Table 1
Levels of doxycycline in isolated alveolar macrophages

Rabbit no.	Macrophages dry matter (mg)	Total amount DX in sample (ng)	DX (ng) per mg of dry matter
1	7.3	949	130
2	11.4	1254	110
3	5.9	1593	270

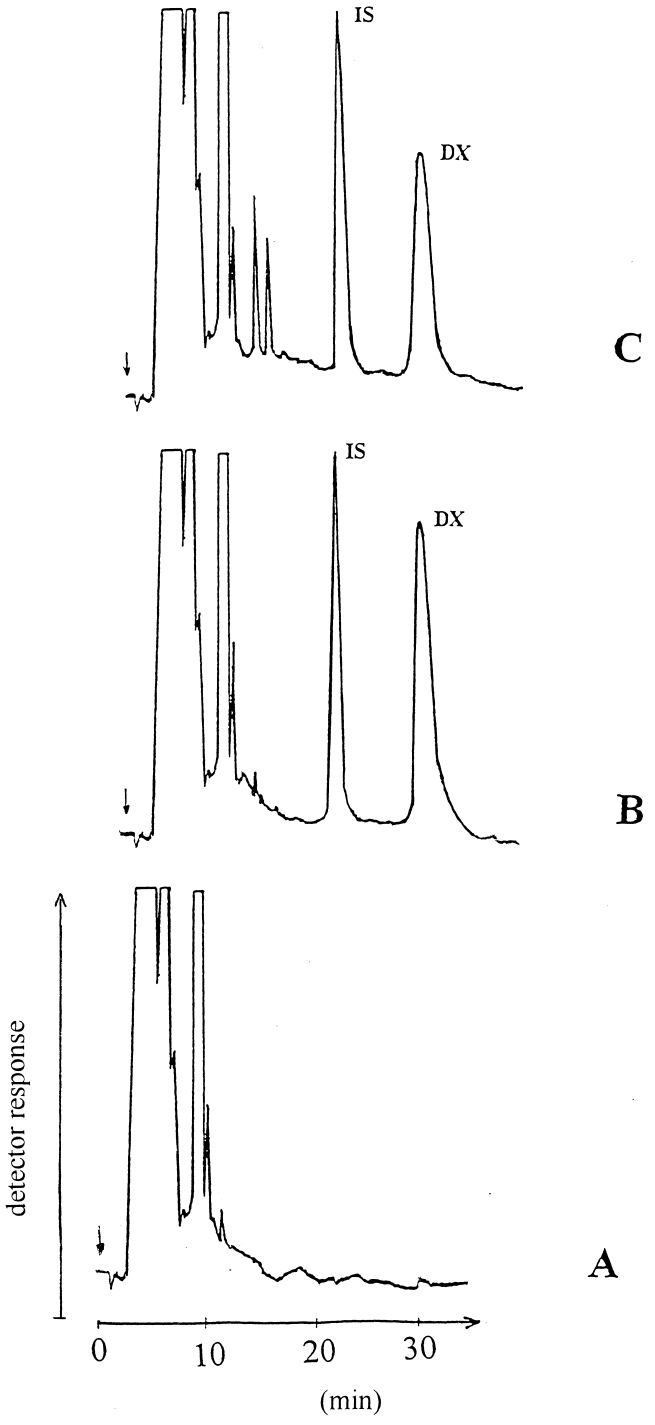

Fig. 1. Typical chromatograms for DX and I.S. in rabbit macrophages. (A) Blank sample; (B) control sample spiked with the standard solution of DX (10 μg/ml) and I.S. (tetracycline, 7 μg/ml); (C) sample macrophages of a premedicated rabbit.

3. Results and discussion

The present study aimed to develop an analytical methodology to determine drug levels in isolated pulmonary macrophages. The first study employed DX. Due to the minimal amount of the sample, it was necessary to use a microcolumn HPLC. The procedure for the isolation of DX from the alveolar macrophages achieved recovery 83%±3.7% ($n=5$). The limit of quantification, 60 ng/ml, was the lowest standard concentration of DX which could be determined with acceptable accuracy and precision. The relative standard deviation was less then 13% ($n=5$). The limit of detection was 15 ng/ml DX.

In the samples of three rabbits the levels of DX ranged from 110 to 270 ng per mg of macrophages dry matter (Table 1). Peaks of residues from biological matrix did not interfere with the peaks of DX and I.S.. The chromatograms obtained from a blank macrophages sample, a control spiked sample and a sample from the rabbit are shown Fig. 1. A great advantage of the methodology developed in the present paper consists in the direct injection of an aqueous supernatant over disintegrated macrophages without the use of a liquid–liquid or a solid–liquid extraction. Another advantage of the method is the fact that the injection of an aqueous solution on a microcolumn with a lipophilic stationary phase makes the analyzed substances (DX and tetracycline) concentrate, which increases the sensitivity of the assay.

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