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DETERMINATION OF RIFAMPICIN, DESACETYLRIFAMPICIN, ISONIAZID AND ACETYLISONIAZID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO HUMAN SERUM EXTRACTS, POLYMORPHONUCLEOCYTES AND ALVEOLAR MACROPHAGES

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SUMMARY

A method for the determination of rifampicin, desacetylrifampicin, isoniazid, and acetylisoniazid by high-performance liquid chromatography and using the same extract of the same sample is reported. After protein precipitation and extraction of these antituberculous drugs, two reversed-phase chromatographies were necessary. The technique was applied to serum extracts, polymorphonucleocytes and alveolar macrophages from patients treated for tuberculosis.

INTRODUCTION

Rifampicin (RFP) and isoniazid (INH), two of the most widely used antituberculous drugs, present very interesting pharmacologic properties: on the one hand liver degradation of INH into inactive acetylisoniazid (AC-INH) by an acetyltransferase whose activity is genetically determined; on the other hand a powerful induction of RFP on liver metabolism occurs; the interaction of both mechanisms is responsible for actual toxicity [1].

There has been increasing interest in the determination of serum levels of the main antituberculous drugs but, as little knowledge of the action of these drugs in the organs involved has yet been attained, we sought to develop a method of

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assay capable of meeting the following criteria: the development of a technique sensitive enough to allow intracellular assays, notably in the case of polymorphonuclear cells and alveolar macrophages; the simultaneous determination of RFP and INH and their corresponding metabolites desacetylrifampicin (DS-RFP) and AC-INH.

The assay of the two antituberculous drugs in biological fluids can be effected according to various methods. The usual methods for RFP assay are colorimetry, microbiology and high-performance liquid chromatography (HPLC) [2—4]. The methods for INH assay are colorimetry [5, 6], spectroscopy [7], fluorometry [8] and HPLC [9]. Our objective was to use reversed-phase HPLC to assay both antituberculous drugs from one single sample. The determination of serum and intracellular levels of the drugs and their metabolites should help understand the way they diffuse through the body. Also, one single extraction allows the use of smaller sample amounts, which is an invaluable advantage.

MATERIAL AND METHODS

INH and AC-INH were kindly offered by Laboratoires Roche (France). RFP and DS-RFP were kindly offered by Laboratoires Lepetit (Milan, Italy). Evaporation was performed in a Brinkmann SC 48R evaporator.

A Waters HPLC chromatograph was used, equipped with a pump Model 6000 A, a universal injector Model U6K and a two wavelength ultraviolet (UV) detector Model 440 (254—280 nm) coupled with a two-channel Houston Omniscribe recorder.

A stainless-steel column was used (30 cm × 3.9 mm) filled with Waters μBondapak C_{18} (particle size 10 μm) non-polar phase. The top of the column was protected by a Waters prefilter No. 84560 to prevent obstruction by minute particles.

RFP and DS-RFP were eluted by methanol—0.05 *M* ammonium formate (65:35); the pH was adjusted to 7.3; the flow-rate was 2 ml/min (mobile phase I). Samples (20 μl) were injected on top of the column and detection was read at 254 nm.

INH and AC-INH were eluted by methanol—water (5:95) containing 5 mmoles/l *n*-heptanesulfonic acid (Pic-Reagent B7, Waters Associates); the flow-rate was 2 ml/min (mobile phase II). Samples (90 μl) were injected and detection was read at 280 nm.

Sample preparation

Standard solution. A 10 mg/ml stock solution was prepared by dissolving RFP and DS-RFP in methanol. The two stock solutions obtained were diluted at a concentration of 1/1000 in mobile phase I and made to form one single 10 μg/ml solution. The standard solution of INH and AC-INH was prepared in the same manner and the solutions were stored at 4°C.

Serum. Sampling of the sera required dry tubes. The sera were obtained from patients treated for pulmonary tuberculosis by a combination of INH and RFP. Assays were performed 3 h after oral administration of the usually prescribed doses of INH (250—300 mg) and RFP (600 mg).

Polymorphonuclear cells. Separation of polymorphonucleocytes was performed according to the method of Losito and Lorusso [10]. Polymorphonucleocytes were counted and placed in suspension in 1 ml of a 9 g/l sodium chloride solution.

Alveolar macrophages. These were obtained after centrifugation of the broncho-alveolar wash fluid. The cells were counted and placed in suspension in saline.

Cell lysis

The cells in suspension in saline (polymorphonucleocytes and alveolar macrophages) were treated for 1 min by an ultrasonic cell disintegrator Sonifier RB 30. A standard microprobe was used, screwed onto a disruptive sonotrode.

Extraction of antituberculous drugs

Mix 1 ml of sample and 2.5 ml of acetone in a vortex mixer. After centrifuging for 10 min at 200 *g*, 2 ml of the supernatant are transferred to a conical glass tube and evaporated at 45°C. The residue is taken up in 0.5 ml of mobile phase I and 1.5 ml of butanol—chloroform (30:70) are added. Mix and centrifuge for 5 min at 200 *g* 0.5 ml of organic phase and evaporate at 45°C in two separate tubes. The residue of the first tube is taken up in 0.2 ml of mobile phase I. The residue of the second tube is taken up in 0.2 ml of mobile phase II. Both tubes are then mixed in the vortex mixer and centrifuged for 5 min at 200 *g*.

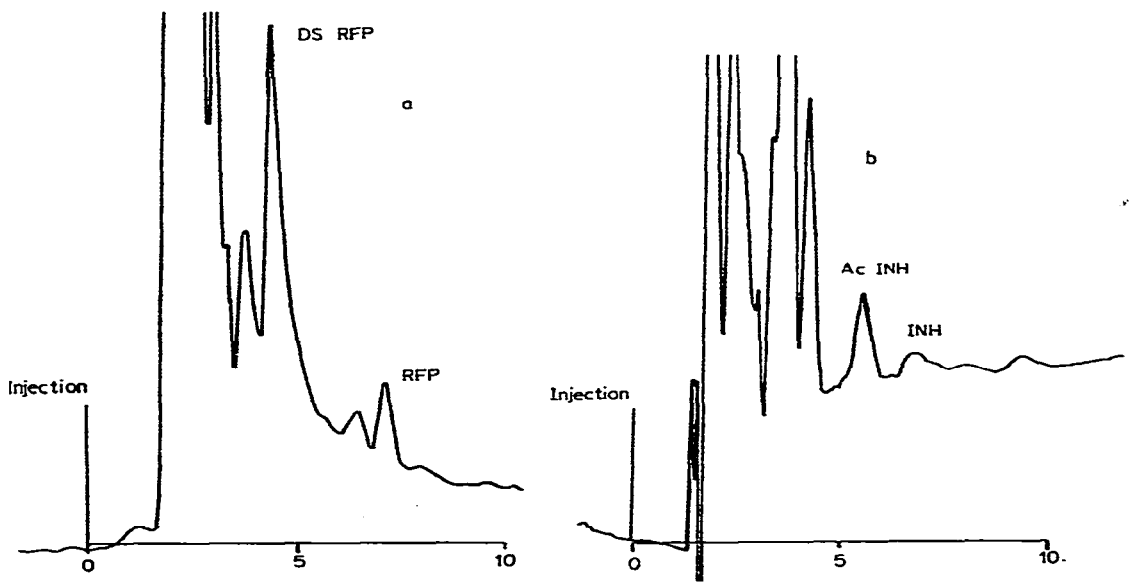

Fig. 1. (a) Chromatogram of a serum extract. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 2 ml/min. Injection volume: 20 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of a serum extract. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. Detector: 280 nm, 0.01 a.u.f.s. Chart speed: 1 cm/min.

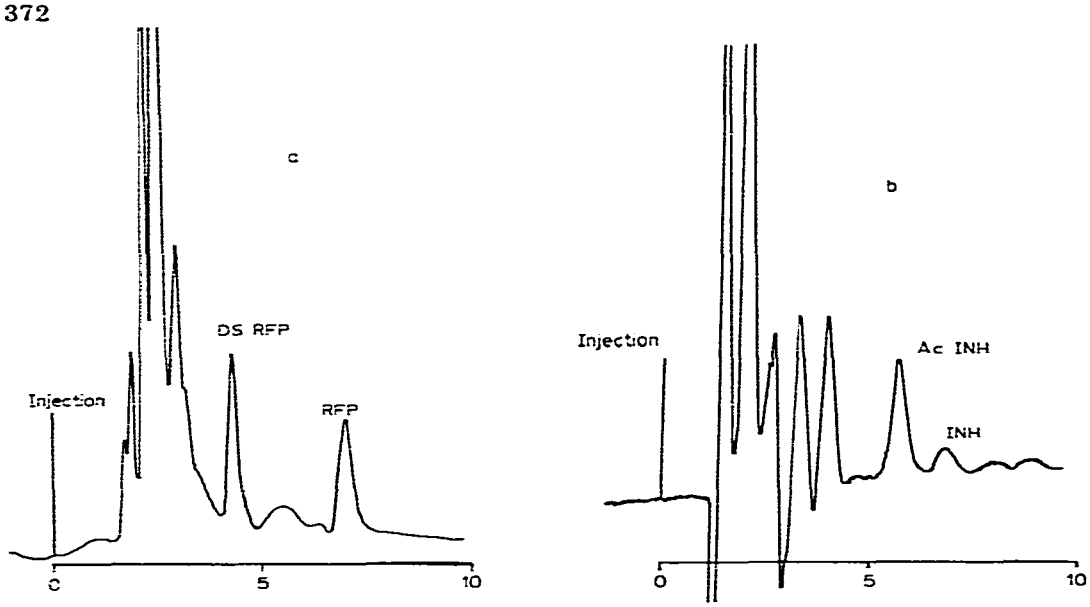

Fig. 2. (a) Chromatogram of an extract of polymorphonuclear cells. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 1 ml/min. Injection volume: 40 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of an extract of polymorphonuclear cells. Column: 30 × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. UV detector: 280 nm, 0.05 a.u.f.s. Chart speed: 1 cm/min.

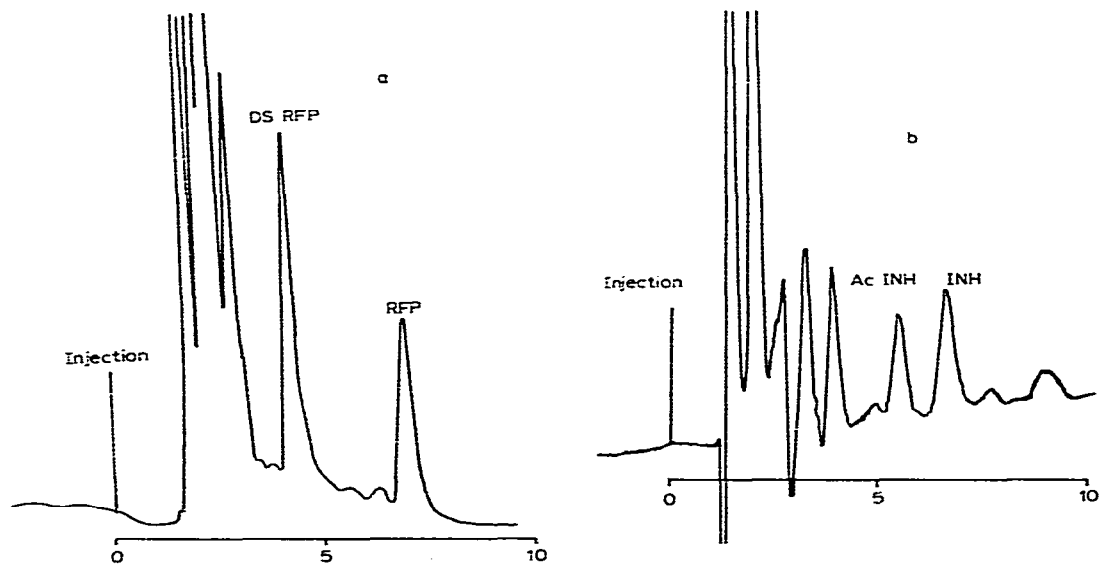

Fig. 3. (a) Chromatogram of an extract of alveolar macrophages. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol—0.05 *M* ammonium formate (65 : 35, v/v), pH 7.30. Flow-rate: 2 ml/min. Injection volume: 40 μl. UV detector: 254 nm, 0.02 a.u.f.s. Chart speed: 1 cm/min. (b) Chromatogram of an extract of alveolar macrophages. Column: 30 cm × 3.9 mm; particle size 10 μm; μBondapak C_{18}. Mobile phase: methanol Pic B_7—water Pic B_7 (5 : 95, v/v). Flow-rate: 2 ml/min. Injection volume: 90 μl. UV detector: 280 nm, 0.005 a.u.f.s. Chart speed: 1 cm/min.

RESULTS

During the chromatography using mobile phase I, DS-RFP and RFP were eluted at retention times of 4.20 and 7 min, respectively. When using mobile phase II, retention times of AC-INH and INH were 5.20 and 6.30 min, respectively.

Fig. 1a and b represent the chromatograms obtained with mobile phases I and II, respectively, from the serum extract of a treated patient. Fig. 2a and b represent the same chromatograms obtained from a polymorphonuclear extract of the same patient.

Fig. 3a and b represent the chromatograms obtained from an extract of alveolar macrophages.

On each of these chromatograms the peaks of RFP, DS-RFP, INH and AC-INH are all sharply distinguished, which allowed their identification and their quantitative determination.

Linearity of response

Sample concentrations were calculated by measuring the peak heights with reference to the peak heights of increasing amounts of the standard solution. We found a linear relationship between peak heights and the concentrations: rifampicin, $r = 0.9994$; desacetylrifampicin, $r = 0.9847$; isoniazid, $r = 0.9403$; acetylisoniazid, $r = 0.9996$.

Overloading tests

These tests were performed by adding known amounts of antituberculous drugs to the serum of an untreated person. After extraction of the molecules, the concentrations and the recoveries were calculated (Table I).

Lower detection limits

In the operating conditions described above and for 1 ml of serum sample, the detection limits were: rifampicin 17 μg/l, desacetylrifampicin 10 μg/l, isoniazid 95 μg/l, acetylisoniazid 85 μg/l.

For polymorphonucleocytes and macrophages, the detection limits were found to be variable, depending on the number of cells contained in the original (initial) sample. In order to obtain reproducible results it is preferable to work with samples containing a constant number of cells. With a cell concentration of $5 \cdot 10^6$ cells/ml the detection limits were: rifampicin 3.4 ng per 10^6 cells, desacetylrifampicin 2 ng per 10^6 cells, isoniazid 19 ng per 10^6 cells, acetylisoniazid 17 ng per 10^6 cells.

DISCUSSION

Initially, our aim was to determine the serum or intracellular levels of the two antituberculous drugs by using one single chromatographic procedure.

The chromatography using mobile phase I does not allow the separation of INH from AC-INH without interference. The separation could be achieved theoretically by increasing the polarity of the mobile phase, but the retention of RFP and DS-RFP was enhanced and the separation of INH from AC-INH re-

TABLE I

EXTRACTION RECOVERIES

DS-RFP			RFP			AC-INH			INH		
Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)	Added (μg)	Measured (μg)	Recovery (%)
0.52	0.47	92	0.52	0.48	93	0.61	0.57	93	0.61	0.51	94
1.04	1.03	98	1.04	0.93	92	1.22	1.09	90	1.22	1.04	86
2.08	1.99	96	2.08	1.89	90	2.44	2.26	92	2.44	2.10	86
5.20	5.13	99	5.20	5.14	99	6.10	5.08	83	6.10	5.11	84
10.40	9.86	95	10.40	9.38	90	12.20	11.24	92	12.20	10.76	88
Mean		96			93			90			88
Standard deviation		2.45			3.70			4.06			3.85

mained unsatisfactory. Therefore, we kept mobile phase I for assay and identification of RFP and DS-RFP, while for INH and AC-INH we had to revert to another chromatographic technique using ion-pair chromatography.

In acid medium INH and AC-INH can become ionized and can then be submitted to ion-pair chromatography with *n*-heptanesulfonic acid. The then nonpolar ion—counterion complex can be eluted by reversed-phase liquid chromattography. The use of mobile phase II allowed satisfactory separation of INH from AC-INH (Figs. 1b, 2b and 3b).

However, in order to assay the drugs and their metabolites, two successive chromatographic procedures were necessary. This is due to the basic difference in chemical structure of the four antituberculous drugs. Nevertheless, the advantage of one single extraction must be retained, as extraction represents the longest procedure of analysis. Besides, as pointed out before, the sample volume is reduced, which is quite an advantage when cells are to be obtained, particularly polymorphonucleocytes, as only 20 ml of blood are required for this operation.

The high sensitivity of the method allowed the determination of residual levels of antituberculous drugs: the comparison between those daily levels is more reliable than the comparison between maximum levels observed shortly after absorption of those drugs.

Being more commonly used, reversed-phase C_{18} columns, such as were used by Saxena et al. for isoniazid determination [9], prove to be more reliable than silica columns [3].

CONCLUSION

The development of the technique described allows the assay of the two antituberculous drugs and their metabolites when using one single test sample. The technique is also sensitive enough to ensure the determination of intracellular levels.

The use of the technique would therefore allow study of the comparison between levels in sera and in phagocytic cells (polymorphonucleocytes and macrophages), thus allowing a better understanding of the diffusion and mechanisms of action of those antituberculous drugs.

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