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- In Memory of Prof. J. G. Yudelevich

DETERMINATION OF ANTI-TUBERCULOSIS DRUGS IN HUMAN SERUM BY HPLC

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Tuberculosis is one of the major communicable diseases in developing countries. The drugs used in treatment of tuberculosis are generally assigned to two major categories. The "first-line" drugs, that combine good efficiency with acceptable level of toxic side-effects, include isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF). In the presence of microbial resistance or human immunodeficiency virus (HIV) infection, "second-line" drugs, such as protionamide (PNA), must also be used. The molecular structures of above-mentioned drugs are shown in Fig. 1.

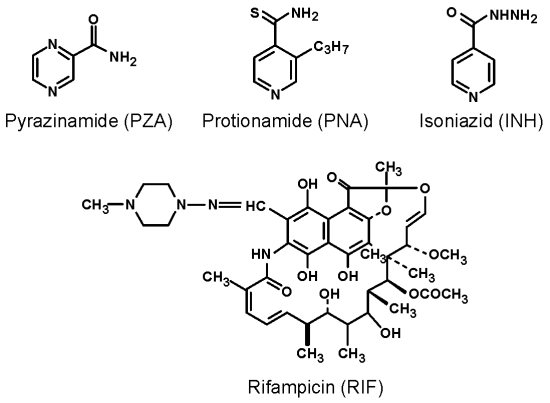

Fig. 1 Molecular structure of drugs

Either the threat of toxic effects or possible lack of awaited therapeutic effect of anti-tuberculosis drugs evokes the need for quantification of the drugs by a suitable technique. Different HPLC methods for determination of anti-tuberculosis drugs separately or in combinations in biological fluids are reported [1-6]. These methods are mainly devoted to the separation of single analyte and the required sensitivity is achieved through pretreatment steps based on extraction, preconcentration and derivatization. Many of this methods suffer from limitations such as lengthy and tedious procedures, high sample volumes required, large quantities of solvents involved etc. This report deals with HPLC determination of INH, PZA, PNA and RIF in human serum for routine application in therapy.

Chromatography conditions

Micro-column liquid chromatograph "MiliChrom A-02" (EcoNova, Novosibirsk, Russia) was used. The separation was performed on 2x75 mm column with ProntoSIL 120-5-C18 AQ. Gradient elution was carried out with solvent *A* 0.01M $(NH_4)_2HPO_4$ (pH 6.5), and solvent *B* acetonitrile. The program for INH, PZA, PNA determination was: 1200 μl of 5 to 80% (linear). Elution was 150 μl/min. Presample was 10 μl of 1M $(NH_4)_2HPO_4$ (pH 6.5). Detection was by UV-absorption at 254, 266, 280, 290, 310 nm. Analytical wavelength was 266 nm. The program for RIF determination was: 1100 μl of 30 to 80% (linear). Elution was 150 μl/min. Detection was by UV-absorption at 254, 320, 336, 350 nm. Analytical wavelength was 254 nm. Temperature in all cases was 35°C. Pressure was 2.5 MPa. Sample volume was 10 μl. Simultaneous multi-wavelength UV-detection at four and five wavelengths was used to improve the reliability of drugs analysis. Fig. 2 shows UV spectra of listed drugs at pH 6.5 and selected wavelengths.

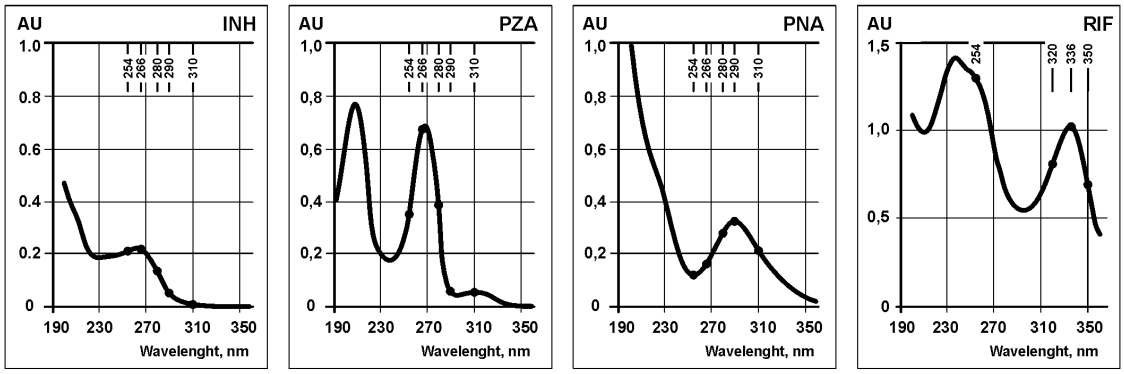

Fig. 2 Spectra of drugs

Sample preparation

INH, PZA and PNA were determined simultaneously from one portion of serum (the deproteinization with 10% trichloroacetic acid) and RIF separately from another (proteins precipitation with acetonitrile). Protein precipitation with 10% trichloroacetic acid gives an easy and rapid separation of analytes from serum samples. This procedure is convenient for many drugs escape RIF. It is known [2] that RIF spontaneously oxidizes to a quinone derivative in atmospheric oxygen above pH 8. In aqueous solutions with lower pH values, RIF hydrolyses to 3-formylrifampicin SV and amino 4-methylpiperazine. Therefore ascorbic acid (2 mg/ml of serum) was used to protect RIF from oxidative degradation and the deproteinization with acetonitrile was the method of choice for protection from hydrolysis.

The deproteinization procedure for INH, PZA and PNA includes: serum samples (200 μl, without ascorbic acid) were transferred to 1.5 ml polypropylene tube and mixed with 100 μl 10% trichloroacetic acid. The samples were briefly vortexed to obtain thorough mixing and then were centrifuged for 5 min at 2000g. The deproteinization procedure for RIF includes: serum samples (100 μl, with 2 mg/ml ascorbic acid) were transferred to 1.5 ml polypropylene tube and mixed with 200 μl acetonitrile. The samples were briefly vortexed to obtain thorough mixing and then were centrifuged for 5 min at 2000g.

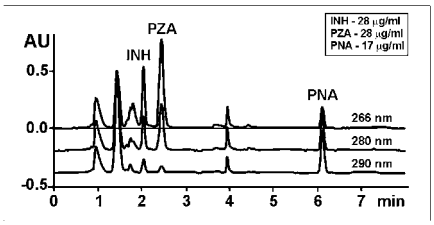

Fig.3 Chromatogram from serum spiked with INH, PZA and PNA

Calibration, precision and accuracy

Calibration curves were constructed by adding known quantities of drugs to pooled human serum and plotting peak area against the amount of drugs added. Precision of method was tested with patients sera by calculating inter-day and intra-day relative standard deviations (RSD) for different samples at each concentration level. Accuracy was estimated by the method of spiking. Detection limits were calculated as threefold signal-to-noise ratio.

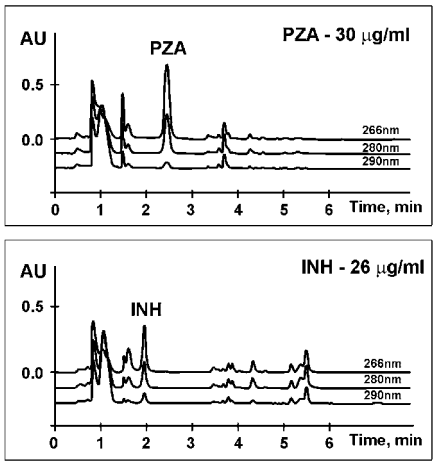

Fig.4 Chromatograms from patient serum with INH and PZA

Results and discussion

Fig. 3 shows the representative chromatogram from non-treated serum sample spiked with the large concentrations of three drugs at three wavelengths. Fig. 4 shows chromatograms from patient serum with INH and PZA. Fig. 5 illustrates the chromatograms from non-treated and treated with INH recipient serum near detection limit. Fig. 6 presents analogous chromatograms with and without RIF. Fig. 7 presents chromatogram from recipient serum with large amount of RIF.

The peaks were identified by retention time, and in addition three or four absorption ratios were calculated as ratio of peak area at one wavelength to peak area at another. Table 1 presents the values and limits of absorption ratios for listed drugs. The calculation of absorption ratios increases the reliability of drugs determination, reduces the demands for constancy of retention times, and gives a possibility to work without frequent calibration with standards.

Table 2 and 3 show precision and accuracy of assay at different concentrations. %RSD changes from 1.6

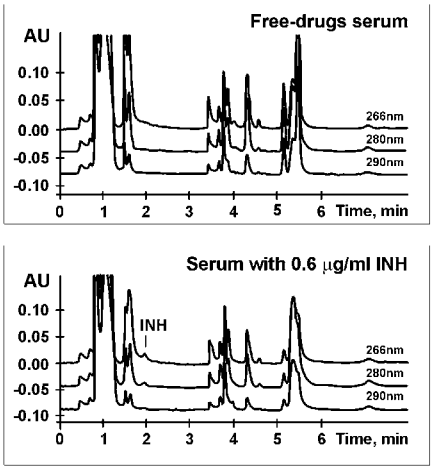

Fig.5 Chromatograms from patient serum with and without INH

to 4.6%. Recovery changes from 97 to 101%. Table 4 presents the regression statistics for the calibration curves and detection limits. Calibration curves were linear over the wide concentration range of practical interest: from 0.5 to 45 μg/ml for INH, PNA, RIF, and from 0.5 to 60 μg/ml for PZA. The limits of detection were from 0.2 to 0.5 μg/ml of serum.

Therefore, the sensitivity offered by the method is good enough to permit the determination of the amounts of interest without pretreatment (like derivatisation) or preconcentration. Good analytical parameters of the method provide the possibility of measure the pharmacokinetics of individual patients, and select for optimal dose of the most important anti-tuberculosis drugs.

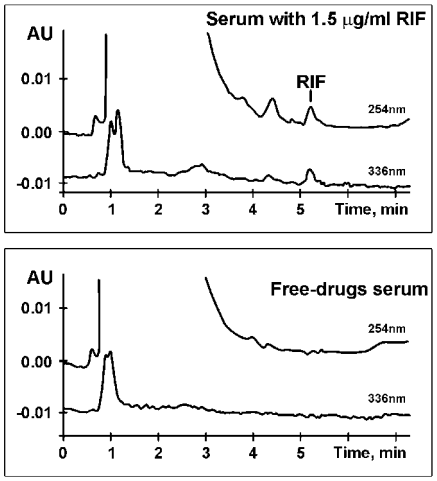

Fig.6 Chromatograms from patient serum with and without RIF

The method developed was then applied to the analysis of real patients samples. Concentration-time profiles for mean drugs values in sera of patients after standard administration are shown in Fig. 8.

Therefore, fast, simple and inexpensive method is ideally suited for selective estimation of the main anti-tuberculosis drugs (INH, PZA, RIF and PNA) in human serum in combination therapy of tuberculosis. Various features of the developed method include low volumes of serum samples required for analysis, simple and fast deproteinization procedure, simultaneous multi-wavelength detection. This makes the method very selective, rapid and economical, especially when a large number of samples are handled, and suitable for routine application in therapy.

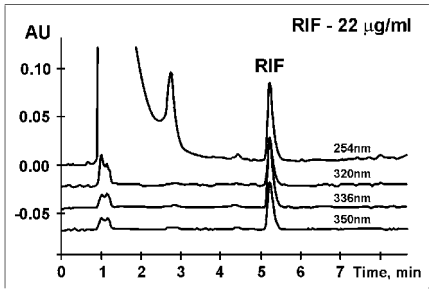

Fig.7 Chromatogram from patient serum with large amount of RIF

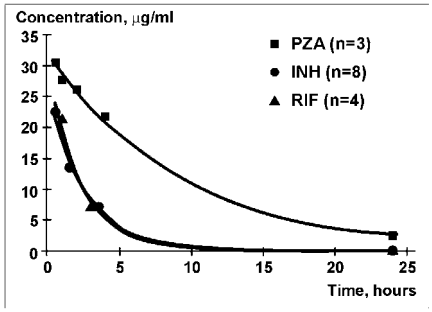

Fig.8 Concentration-time profile of mean drugs values in patients sera

Table 1

Absorption ratios

Drug name	INH	PZA	PNA	RIF
S_{254}/S_{266}	0.91 ± 0.05	0.47 ± 0.03	0.73 ± 0.02	–
S_{280}/S_{266}	0.60 ± 0.02	0.53 ± 0.01	1.76 ± 0.05	–
S_{290}/S_{266}	0.23 ± 0.01	0.07 ± 0.01	2.04 ± 0.03	–
S_{310}/S_{266}	0.06 ± 0.03	0.08 ± 0.01	1.36 ± 0.03	–
S_{320}/S_{254}	–	–	–	0.62 ± 0.03
S_{336}/S_{254}	–	–	–	0.81 ± 0.03
S_{350}/S_{254}	–	–	–	0.62 ± 0.01

S_λ is the peak area at detection wavelength λ

Table 2

Precision of assay

Drug name	Concentration range (μg/ml)	RSD (CV,%) Within day	n	RSD (CV,%) Day-to-day	n
INH	2 - 6	4.1	22	4.4	15
	6 - 15	2.5	22	3.0	19
	15 - 30	2.0	23	2.3	13
PZA	2 - 5	–	–	3.3	9
	5 - 15	2.9	10	3.0	24
	15 - 30	1.6	23	2.4	21
	30 - 40	1.8	20	–	–
PNA	2 - 5	3.6	23	–	–
	5 - 10	3.4	13	–	–
	10 - 20	2.8	23	–	–
RIF	0.5 - 5	4.3	10	4.6	10
	5 - 15	3.0	20	3.8	9
	15 - 30	3.0	20	3.0	12

Table 3

Accuracy of assay

Drug name	Concentr. added (μg/ml)	Concentr. found (μg/ml)	Recovery (%)	CV (%)	n
INH	5.2	5.1 ± 0.2	98	3.7	8
	10.2	9.9 ± 0.3	97	3.6	8
	20.8	20.9 ± 0.3	100	1.7	8
PZA	9.6	9.5 ± 0.3	99	4.2	8
	19.0	18.9 ± 0.4	99	2.6	8
	38.4	38.5 ± 0.5	100	1.6	8
PNA	3.7	3.7 ± 0.1	100	4.5	8
	7.2	7.1 ± 0.2	98	3.3	8
	14.7	14.2 ± 0.2	97	1.9	8
RIF	5.1	5.2 ± 0.1	102	3.4	10
	9.9	9.7 ± 0.2	98	2.4	10
	19.6	19.7 ± 0.5	101	3.3	10

Table 4

Regression statistics for the calibration curves y=a+bx and detection limits

Drug name	INH	PZA	PNA	RIF
Concentr. range (μg/ml)	0.5 - 45	0.5 - 60	0.5 - 45	0.5 - 45
Intercept, a	-0.012	0.002	-0.013	-0.019
S_a	0.027	0.020	0.026	0.026
Slope, b	0.1731	0.4165	0.1257	0.0878
S_b	0.0014	0.0008	0.0013	0.0007
S_0	0.061	0.046	0.057	0.059
Correlation coeffic., r	0.9998	0.9999	0.9997	0.9998
CV at mid-point (%)	2.8	1.0	3.6	2.9
Detection limits (μg/ml)	0.3	0.2	0.3	0.5

x - concentration (μg/ml), y – peak area (A.U. μl)*

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