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Simultaneous Microdetermination of Rifampin, Deacetylrifampin, Isoniazid, and Acetylisoniazid in Plasma by Liquid Chromatography with Dual Electrochemical and Spectrophotometric Detection

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A rapid liquid chromatographic method for simultaneous determination of isoniazid (INH), acetylisoniazid (AINH), rifampin (RIF), and deacetylrifampin (DARIF) in microsamples of deproteinized plasma is described. The compounds and internal standard (IS) (diphenylcarbazide) were separated on a 10-μm, 8 mm × 10-cm phenyl Radial Pak cartridge in conjunction with a binary linear gradient system at a flow rate of 3 ml/min. A dual electrochemical (+800 mV) and spectrophotometric (334 nm) detection system with a computerized data station was employed to measure the above compounds in the effluent. Prior to injection, the plasma sample was diluted (2:1) with a pH 3, 0.075 *M* phosphate buffer after adding the internal standard (6.67 μg/ml of plasma) and passed through an Amicon Centrifree-MS filter at 2000*g*. Under these conditions, no interference in the analysis was observed, and the retention times of AINH, INH, IS, DARIF, and RIF were 3.95, 4.89, 15.82, 17.25, and 19.34 min, respectively. The linearity of the assay for all four compounds was excellent ($r > 0.9925$), and the between- and within-day CV was not >8% at any concentration. This method is currently being used for therapeutic monitoring and pharmacokinetic studies of INH, RIF, and their major metabolites in patients with tuberculosis.

KEY WORDS: drug monitoring; tuberculosis; microsamples; acetylator phenotype; metabolites; liquid chromatography; dual detection.

INTRODUCTION

Rifampin (RIF), 3-[[(4-methyl-1-piperazinyl)-imino]-methyl]-rifamycin SV (Fig. 1) is a bactericidal antibiotic highly potent against *Mycobacterium tuberculosis, Mycobacterium leprae,* and a host of other gram-positive and gram-negative bacteria (1). This drug is rapidly and completely absorbed from the gastrointestinal tract and is biotransformed in the liver mainly to deacetylrifampin (DARIF), a microbiologically active metabolite. Because of enzyme induction, repeated administrations of RIF lead to a pronounced acceleration of its own clearance, which often requires dosage adjustment and monitoring.

Owing to synergistic effects, RIF is often prescribed in combination with isoniazid [isonicotic acid hydrazide (INH)] (Fig. 1) for treatment of pulmonary and extrapulmonary tuberculosis (1). Indeed, this combination is commercially available as single dosage forms containing fixed doses of each of these drugs. INH is metabolized in the liver mainly by N-acetylation to form acetylisoniazid (AINH), which is biologically inactive. Because of polymorphic distribution of *N*-acetyltransferase, the enzyme responsible for this pathway, the ratio of the concentration of AINH/INH serves to classify individuals into rapid and slow acetylators. The severe adverse effects associated with INH, particularly hepatitis in older patients or in slow acetylators with renal or hepatic impairment, necessitate the monitoring of its concentration in the plasma of these patients.

Rifampin has been analyzed in biological fluids mainly by high-performance liquid chromatography (HPLC) (2–5); however, these assays, including those which deal with the analysis of both RIF and DARIF (3,5), lack an internal standard or involve laborious extraction steps. On the other hand, several techniques have been described for analysis of INH alone or with AINH in biological fluids. These comprise microbiology (6), spectrophotometry (7,8), fluorometry (8,9), gas chromatography (10), and high-performance liquid chromatography (11–15). In spite of the frequent use of these two drugs in combination, no method has been reported for the simultaneous analysis of these antituberculous agents and their major metabolites in biological fluids.

In this report, we describe an expedient and accurate method for coanalysis of rifampin, deacetylrifampin, isoniazid, and acetylisoniazid in microsamples of plasma by liquid chromatography and dual electrochemical and spectrophotometric detection. The absence of an extraction step from this assay and the efficiency of the liquid chromatography employed offer the ease and rapidity desired in an assay intended for comeasurement of multiple drugs and metabolites.

MATERIALS AND METHODS

Reagents

Analytical samples of rifampin, deacetylrifampin (both kindly provided by Ciba-Geigy, Pharmaceutical Division, Basel, Switzerland), isoniazid (Squibb Pharmaceutical, Princeton, NJ), acetylisoniazid (kindly provided by Hoffman-La Roche AG, Basel, Switzerland), and diphenylcarbazide (internal standard, (IS) (Aldrich Chemical Company, Inc., Milwaukee, WI) were used in this study. Acetonitrile, methanol, 85% phosphoric acid (all from Fisher Scientific, Fair Lawn, NJ), and anhydrous disodium hydrogen phosphate (Fluka Chemie AG, Buchs, Switzerland) were all HPLC grade. Water for HPLC was generated by passing "reverse osmosis" water through a trace organic removal cartridge and 0.45-μm membrane filter (both from Millipore Co., Milford, MA).

Apparatus

The chromatograph (Waters Associates, Milford, MA) consisted of a multisolvent delivery system (Model 600E), an au-

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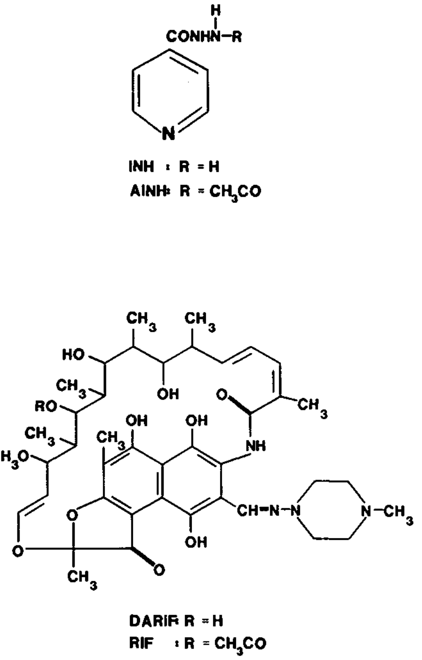

Fig. 1. The chemical structures of the four compounds analyzed.

tosampler (Wisp 712), a radial compression module (RCM) equipped with a 10-μm, 8 mm × 10-cm μBondapak phenyl cartridge preceded by a Guard Pak precolumn with a CN insert, and a variable-wavelength UV/visible detector (Model 481) set at 334 nm connected serially to an electrochemical detector (Model 656) with a potentiostatic current/voltage measuring instrument (Metrohm Ltd., Herisau, Switzerland) set at +800 mV and a 5-μA current. The oxidative mode was used with glassy carbon, Ag/AgCl, and gold electrodes as the working, reference, and auxiliary electrodes, respectively. The detector outputs were simultaneously measured and processed using a Maxima 820 interface and software with a Tandon 286 PC computer.

Procedures

Chromatography. The compounds were separated using a binary linear gradient system. Buffer A (25 m*M* disodium hydrogen phosphate solution adjusted to pH 7 with phosphoric acid) was first pumped alone for 1.7 min, then decreased from 100 to 50% over 4.3 min, with buffer B (equal volumes of buffer A and acetonitrile) constituting the remainder. This composition was maintained for 6 min, then changed over 6 min to 100% buffer B, which was kept for 5.5 min. This was changed to the initial condition (i.e., 100% buffer A) over 0.5 min and was left for reequilibration for 6 min plus a 2-min injection lag time (i.e., time needed for the autosampler to process and inject the next sample once the previous run is completed). A flow rate of 3 ml/min was employed.

Sample Preparation. To 600 μl (or a fraction thereof) of plasma, 4 μg (15 μl) of 267 μg/ml internal standard dissolved in a 10% (v/v) aqueous solution of methanol and 300 μl of a 75 m*M* aqueous solution of disodium hydrogen phosphate adjusted to pH 3.0 were added. The samples were vortex-mixed for 30 sec, then transferred to a Centrifree micropartition system (Amicon Division, W. R. Grace & Co., Beverly, MA) and centrifuged at 2000*g* for 30 min. The autosampler was programmed to inject 200 μl of the ultrafiltrate, equivalent to 133 μl of deproteinized plasma, into the chromatograph with a run time of 30 min.

Calibration Curves. To construct standard curves, we supplemented blank plasma with the following concentrations of each of RIF, DARIF, INH, and AINH: 0.05, 0.10, 0.50, 1.0, 2.0, 5.0, 10, 15, and 20 μg/ml. After addition of the appropriate amount of the internal standard (i.e., 6.67 μg/ml), the samples were treated in the same manner as described above.

Analysis of Patients' Samples

Rifampin, deacetylrifampin, isoniazid, and acetylisoniazid were analyzed in plasma samples collected 3–5 hr after the last dose from three patients who were treated orally with 600 mg of RIF and 300 mg of INH once daily for tuberculosis. Also, the applicability of the assay for studying the pharmacokinetics of these drugs was determined by measuring the concentrations of the above compounds in plasma samples collected at preselected intervals from a patient who received similar doses of RIF and INH orally. The samples were processed and analyzed as described above.

RESULTS AND DISCUSSION

Deproteinization of plasma by ultrafiltration is an expedient and efficient approach for sample preparation prior to chromatography. As demonstrated in Figs. 2 and 3, the chromatograms generated under the assay conditions used are notably clean in spite of injecting the sample into the cartridge without purification. Also, the recovery of RIF, INH, DARIF, and AINH from the plasma was close to completeness (i.e., >85%) at most concentrations and was never <75% (Table I). This parameter was determined by dividing the peak height obtained for each of the four compounds which were added to plasma by that observed for the same amount of each compound injected directly into the chromatograph. It is noteworthy that the addition of pH 3 phosphate buffer to the sample was found imperative to recover

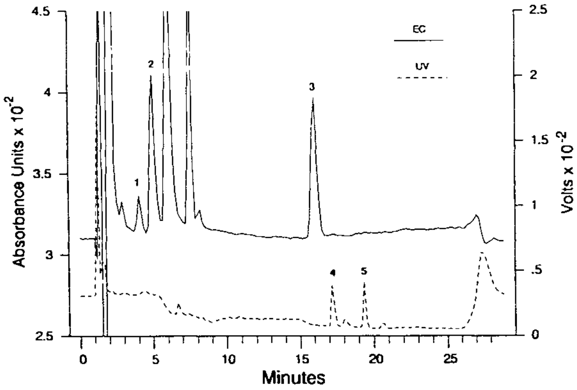

Fig. 2. Representative chromatograms with dual electrochemical and spectrophotometric detection of a blank plasma sample supplemented with 6.67 μg/ml of IS (3) and 1 μg/ml each of rifampin (5), deacetylrifampin (4), isoniazid (2), and acetylisoniazid (1).

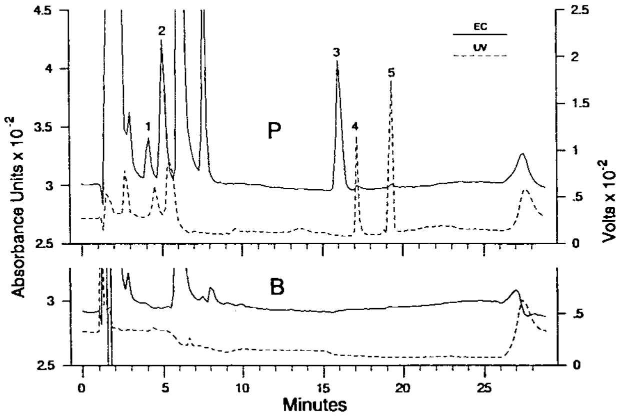

Fig. 3. Representative chromatograms with dual electrochemical and spectrophotometric detection of an intact blank plasma (B) and a patient's plasma sample (P) collected 10 hr after oral administration of 600 mg of rifampin and 300 mg of isoniazid and supplemented with 6.67 μg/ml of IS (3). The concentration determined for rifampin (5), deacetylrifampin (4), isoniazid (2), and acetylisoniazid (1) was 6.03, 2.39, 1.19, and 2.49 μg/ml, respectively.

RIF and DARIF from plasma due to the markedly elevated binding of these compounds to protein at higher pH's.

The use of a gradient buffer system under the conditions described yielded sharp and symmetrical peaks with a retention capacity factor (*k*′) for AINH, INH, IS (diphenylcarbazide), DARIF, and RIF of 3.9, 5.1, 18.8, 20.6, and 23.2, respectively. The within-day CV of the retention times for these compounds was 1.21, 0.72, 0.42, 0.27, and 0.24%, respectively. To maximize the sensitivity of the assay for all four compounds analyzed, it was essential to use, in addition to the electrochemical detector with a high sensitivity for INH and AINH, a UV detector for RIF and DARIF because of their low electrochemical response.

The specificity of the assay was investigated by determining the retention times of different drugs (antibiotic, antipyretic, antitussive, etc.) which are commonly used with RIF and INH in the treatment of tuberculosis. As shown in Table II, none of these compounds interfered, and due to the high capacity factor for all the compounds analyzed, no interference from the plasma "front" or other endogenous peaks was noted.

The assay was highly linear, with the correlation coefficient (*r*) of the peak height ratio (drug/internal standard) vs concentration standard curves constructed on 10 days for RIF, DARIF, INH, and AINH >0.9925 (Table III). The between-day precision was investigated by estimating the coefficient of variation (CV) of the concentrations measured on different days for plasma samples spiked with different amounts of these compounds. As demonstrated in Table III, the CV values obtained at these low, medium, and high concentrations were <7%, which signifies a good assay precision.

Table I. Recovery (%) of the Drugs and Metabolites from Plasma After Deproteinization by Centrifree MS Filters

Compound	Concentration (μg/ml) 1.0	5.0	20
RIF	87.3	82.9	91.2
DARIF	75.3	77.0	88.7
INH	85.7	84.9	93.0
AINH	92.0	91.8	95.7

Table II. Retention Times of Drugs Which May be Administered Concomitantly with Isoniazid and Rifampin in Treatment of Tuberculosis

Compound	Retention time (min)	Detectability
Acetylisoniazid	3.95	EC,[a] UV[b]
Isoniazid	4.89	EC, UV
Diphenylcarbazide	15.82	EC
Deacetylrifampin	17.25	EC, UV
Rifampin	19.34	EC, UV
Pyrazinamide	4.82	UV
Ethambutol		ND[c]
Clofazimine		ND
Dapson		ND
Ethionamide	10.05	EC, UV
Acetaminophen	6.83	EC, UV
Aspirin		ND
Choramphenicol	11.41	UV
5-aminosalicylic acid	2.70	EC, UV
Hydrocortizone acetate		ND
Tetracycline HCl		ND
Ampicillin		ND
Gentamicin sulfate		ND
Sulfamethoxazole	7.49	EC, UV
Mefoxin sodium	10.41	EC
Vibramycin		ND
Amoxicillin		ND
Neomycin sulfate		ND
Erythromycin		ND
Penicillin G potassium		ND
Streptomycin		ND
Cephalexin		ND
Diphenylhydramine		ND
Sulfisoxazole	7.20	EC
Acetic hydrazide	1.54	EC
Isonicotinic acid	2.13	UV
Hydrazine	1.30	EC
Gualfenesin	8.43	UV

[a] Detected electrochemically at +800 mV.
[b] Detected spectrophotometrically at 334 nm.
[c] Nondetectable during the 30-min run under the conditions described.

We examined the within-run precision and the deviation from perfect accuracy, i.e., 100 [(amount found *minus* amount added)/amount added], by supplementing blank plasma with appropriate amounts of each of the four compounds analyzed to yield the following concentrations: 0.5, 5, and 15 μg/ml. The samples were divided into five equivalent portions, and each was analyzed as a separate sample according to the above procedure. As demonstrated in Table IV, the within-run precision gave a CV below 7.2% in each case. Also, small deviations from perfect accuracy were observed (i.e., <8.8%).

The assay described is currently being used to monitor the above drugs and metabolites in patients treated for long

Table III. Linearity and Between-Day Reproducibility of the Assay Described

Compound	Linearity correlation coefficient[a]	Reproducibility Concentration (μg/ml)	Coefficient of variation (%)[b]
RIF	0.9987	0.5	5.4
	(0.9969–0.9995)	5.0	7.0
		15.0	4.4
DARIF	0.9988	0.5	5.6
	(0.9974–0.9998)	5.0	6.1
		15.0	4.2
INH	0.9982	0.5	6.0
	(0.9946–0.9998)	5.0	6.2
		15.0	4.0
AINH	0.9957	0.5	6.5
	(0.9925–0.9980)	5.0	6.2
		15.0	5.7

[a] Mean (and range) of data determined on 10 days.
[b] Determined from experiments performed on 10 days.

durations with combined doses of these antituberculous agents. The mean (SD) steady-state plasma concentrations of RIF, DARIF, INH, and AINH observed in three of these patients, who received 600 mg of RIF and 300 mg of INH, are 14.5 (4.8), 4 (1), 3.82 (1.5), and 1.7 (0.26) μg/ml, respectively. Figure 4 presents plasma concentration–time profiles for these compounds observed in a patient who received 600 mg of RIF and 300 mg of INH. While the respective maximum concentrations of INH and RIF were reached at 2 and 4 hr, respectively, after dosing, their metabolites both peaked at 6 hr with a lag time of 0.5 and 1.5 hr, respectively. The areas under the curve up to the last sample collected (i.e., 24 hr) for RIF, DARIF, INH, and AINH were 136.7, 51.3, 35.9, and 45.7 μg · hr · ml^{-1}, respectively. Thus, the accuracy and expediency (i.e., small sample size and no sample purification) of this assay make it an excellent tool, not only for simultaneous monitoring of these drugs and their major metabolites in patients, but also for pharmacokinetic investigations.

Table IV. Accuracy and Within-Run Precision of the Assay Described

Compound	Amount added (μg/ml)	Amount found (μg/ml)	Coefficient of variation (%)	Deviation from perfect accuracy (%)[a]
RIF	0.5	0.539	3.96	+7.7
	5.0	5.19	4.95	+3.8
	15.0	15.9	6.26	+5.9
DARIF	0.5	0.544	3.82	+8.8
	5.0	5.2	3.58	+4.0
	15.0	16.0	5.99	+6.5
INH	0.5	5.16	6.5	+3.2
	5.0	5.23	5.74	+4.6
	15.0	15.7	7.16	+4.9
AINH	0.5	0.526	7.19	+5.2
	5.0	4.95	7.07	−1.1
	15.0	14.36	6.92	−4.3

[a] Estimated as 100 · [(amount added minus amount found)/amount added]; number of replicate samples used = 5.

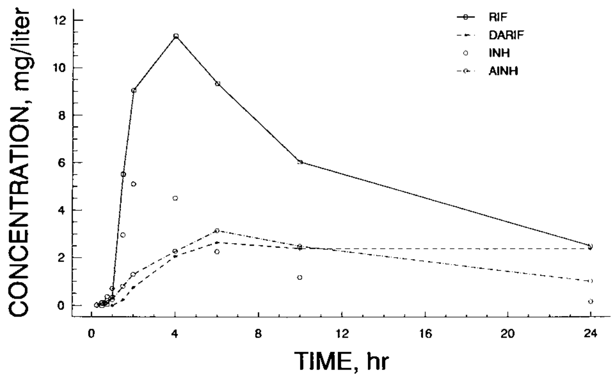

Fig. 4. Plasma concentration–time profiles for rifampin, deacetylrifampin, isoniazid, and acetylisoniazid observed in a patient who received 600 mg of RIF and 300 mg of INH.

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