Journal of Chromatography, 340 (1985) 321—359
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2464

REVIEW

CHROMATOGRAPHIC ANALYSIS OF ANTITUBERCULOSIS DRUGS IN BIOLOGICAL SAMPLES*

MACK R. HOLDINESS

Louisiana State University Medical Center, Department of Pharmacology and Experimental Therapeutics, 1901 Perdido Street, New Orleans, LA 70112 (U.S.A.)

(Received August 1st, 1984)

CONTENTS

*Parts of this manuscript have been previously presented at the Southeast—Southwest Regional American Chemical Society Meeting in New Orleans, LA, U.S.A., December 10—13, 1980.

0378-4347/85/$03.30

1. INTRODUCTION

Tuberculosis is an infectious disease that has plagued man since the earliest of times. References have been made to this condition by the ancient Chinese [1] and found in Egyptian skeletons dating from 2500 B.C. [2], yet it was not until the discovery in 1882 by Dr. Robert Koch [3] that the etiological agent was defined as *Mycobacterium tuberculosis*. Since that time many advances have been made toward the reduction of this disease; however, it is still prevalent and found in large numbers in developing countries. In 1972, 1 070 548 active cases of this disease existed in the world and 140 222 deaths resulted the same year due to complications from tuberculosis [4]. In the United States the number of new cases in 1983 was 23 532 of which there has been no substantial decline in deaths from 1979 to 1982 [5,6]. Tuberculosis was the leading cause of death among 38 communicable diseases reported in 1979 to the Centers for Disease Control in the United States and in fact the number of tuberculosis deaths exceeded the combined total for the other 37 communicable diseases. Therefore, as a cause of morbidity and mortality, tuberculosis still remains one of the more prevalent infectious diseases in the world. For an excellent review of the epidemiology and pathophysiology of tuberculosis the reader is referred to the book by Youmans [7].

Only since the 1940s has effective chemotherapy been applicable for this disease. The bacteriostatic effect of sulfonamides in guinea pigs infected with the tubercle bacilli was demonstrated with a derivative of dapsone known as promin (glucosulfone sodium). This agent was capable of arresting the progress of otherwise fatal tuberculosis in the experimental animals [8]. In 1944,

TABLE 1

LIST OF ABBREVIATIONS

ACINH	Acetylisoniazid	PAS	*p*-Aminosalicylic acid
CAM	Capreomycin	PTA	Prothionamide
CYS	Cycloserine	PZA	Pyrazinamide
EMB	Ethambutol	RIF	Rifampicin
ETA	Ethionamide	SM	Streptomycin
INH	Isoniazid	TAZ	Thiacetazone
KNM	Kanamycin	TCA	Trichloroacetic acid
PA	Pyrazinoic acid	VOM	Viomycin

streptomycin was noted to have a striking therapeutic effect in human patients [9] and the discovery of *p*-aminosalicylic acid lead to the use of two-drug therapy for adequate prevention of tuberculosis. Isoniazid was first described in 1952 by three different laboratories [10—12] (40 years following its original synthesis) and as an effective anti-tuberculosis drug has proven to be the most valuable and widely used agent in nearly all types and cases of tuberculosis [13,14]. As time progressed, a number of other drugs have been used with moderate to excellent results as chemotherapeutic agents. The compounds that will be described in this review are isoniazid, rifampicin, ethambutol, *p*-aminosalicylic acid, pyrazinamide, streptomycin, kanamycin, ethionamide, cycloserine, viomycin, capreomycin, and thiacetazone. For the abbreviations used see Table 1.

There are a number of ways to classify these drugs according to popular use, bactericidal or bacteriostatic capabilities, naturally occurring or as synthetic compounds. All of the naturally occurring antibiotics (RIF, SM, CYS, CAM, VOM, KNM) are produced by different strains of bacteria and generally isolated from crude fermentation fractions of culture medium. The synthetic compounds, listed in order of discovery of anti-tuberculosis activity, are PAS, TAZ, PZA, INH, ETA, and EMB. Another method of classification is that of use (first-line, second-line, and third-line) or decreasing potency with increasing side-effects. First-line drugs (INH and RIF) are those with proven excellence of antibacterial action (both of which are bactericidal) and minimal or controllable side-effects. In this group, INH is bactericidal to all populations (including slowly or intermittently growing organisms in solid caseous areas of neutral pH) [14]. Second-line drugs consist of those chemotherapeutic agents which are less effective for potency but used to supplement the action of first-line drugs and prevent resistance from developing. These compounds (EMB, PAS, PZA, SM) are less acceptable to patients and may produce undesirable or dangerous side-effects in some cases. Third-line drugs (CYS, ETA, CAM, VOM, KNM, TAZ) generally have inferior potency, are prone to produce more side-reactions and are used in special circumstances such as intolerance or resistance to several first- or second-line agents. It should be immediately pointed out to the reader that this last method of classification by popular use is a changing system with different sources listing different drugs among the three categories. As an example, Youmans [7] listed INH, RIF, EMB and SM as first-line and classified all other agents as second-line drugs whereas recently it has been suggested to advance PZA from second-line to first-line drug due to its modified use in short-course daily and intermittent chemotherapy with INH and RIF for pulmonary tuberculosis [15]. Others simply classify the drugs as either commonly used (INH, RIF, SM, PZA, EMB) or less commonly used agents (CAM, KNM, PAS, ETA, CYS) [14]. There are a number of instances in which second-line drugs are very useful such as in short-course chemotherapy and resistance, and in technically developing countries. Also, the third-line agents do have a role in anti-tuberculosis treatment regardless of inferior potency and ill effects. These compounds would not be chosen for initial treatment unless some contra-indication prevented the use of first- and second-line drugs such as resistance, allergy, and intolerance or in case of non-availability of more appropriate treatment as occurs in developing countries. As an example, TAZ is not used in the

TABLE 2

METABOLITES AND PHARMACOKINETICS OF ANTITUBERCULOSIS DRUGS [50]

Drug	Metabolites	Oral dose	T_{max} (h)	C_{max} (μg/ml)	$t_{½}$
INH	Acetylisoniazid	300 mg	1–2	0.8 (slow acetylator)	45–110 min
	Pyruvic hydrazone				
	α-Ketoglutaric acid			0.2 (rapid acetylator)	2–4.5 h
	Isonicotinic acid				
	Isonicotinoyl glycine				
	Monoacetylhydrazine				
	1,2-Diacetylhydrazone				
	Hydrazine				
RIF	25-Desacetylrifampicin	600 mg	2–4	4–32	2.3–5.1 h
	3-Formylrifamycin SV				
	3-Formyl-25-desacetylrifamycin				
	N-Desmethylrifampicin				
EMB	2,2′-(Ethylenediimino)dibutanoic acid	800 mg	2–4	2–5	3.5–5.2 h
	2,2′-(Ethylenediimino)dibutanal				
PAS	*m*-Aminophenol	4 g	1–2	75	26–64 min
	Acetyl *p*-aminosalicylic acid				
	p-Aminosalicyluric acid				
	Acetyl *p*-aminosalicyluric acid				
	2,4-Dihydroxybenzoic acid				
PZA	Pyrazinoic acid	1 g	2	45	9–9.8 *h*
	5-Hydroxypyrazinoic acid				
	5-Hydroxypyrazinamide				
SM	Unknown	1 g*	1–2	25–50	2–3 h
KNM-A	Kanamycin A**	1 g*	1	20–35	2–4 h
	Kanamycin B**				
	Kanamycin C**				
ETA	S-Oxocarbamoyl dihydropyridine	250 mg	2	2	2.1 h
	Carbamoyl dihydropyridine				
	Thiocarbamoyl dihydropyridine				
	Ethionamide sulfoxide				
CYS	Unknown	750 mg	3–4	20	21–25 min
CAM	Capreomycin IA**	1 g*	1–2	20–47	–
	Capreomycin IB**				
	Capreomycin IIA**				
	Capreomycin IIB**				
VOM	Unknown	1 g*	2	25–50	–
TAZ	Unknown	150 mg	4–5	1.6–3.2	8–12.9 h

*Intramuscular injection route.

**Isomeric components produced from fermentation broth.

United States and for the most part has been neglected in American literature [7]; however, this drug has found extended use in Ethiopia, India, and Pakistan [16] and East Africa [13,17].

Therapeutic drug monitoring and pharmacokinetic studies represent a major reason for development of clinically useful assay methodologies. As an example, these procedures are applied for determination of clearance calculations in hemodialysis [18], absorption, excretion, metabolism, and disposition [19—22] of INH and EMB in diseased and normal patients. Also, the methods have been adapted for analysis of INH in pediatric [23] and RIF in elderly populations [24,25]; others have observed changes in the fundamental pharmacokinetic properties of these agents when given in combinations with other anti-tuberculosis drugs [26] and in patients with liver disease and renal insufficiency [27]. Via therapeutic drug monitoring techniques the optimal dosage, frequency and duration of KNM therapy has been studied for the treatment of pediatric meningitis and necrotizing enterocolitis [28,29]. The above listed studies are just a few of the applications for monitoring the compounds.

Chromatographic methods have become increasingly important for quantitation and selectivity of analysis. In this review the different types of chromatographic procedures for analyzing these drugs in biological samples will be described along with their applications, and the chromatographic procedures will be compared with the non-chromatographic ones. Also discussed in this paper will be the problems that arise in clinical studies of these compounds such as sample handling and storage, and metabolism and pharmacokinetics which ultimately effect the blood levels of these components.

2. ANALYTICAL CONSIDERATIONS

2.1. Pharmacokinetics and metabolites

The quantitation of these components in biological fluids is greatly influenced by factors such as metabolism, protein binding and interactions with other agents. Drug interactions may alter the level of free compound in tissue fluids by increasing or decreasing protein binding and by induction or inhibition of enzymatic metabolism. INH is susceptible to metabolism at different rates by an enzyme acquired through inherited genetic mechanisms which ultimately effects the therapeutic blood concentrations. Table 2 contains a summary of the anti-tuberculosis drugs, known metabolites, maximal plasma concentrations (C_{max}), time required to achieve this level (T_{max}), plasma half-life ($t_{½}$) along with the concentration and route of administration.

An example of these mechanisms and interactions are demonstrated with INH of which its proposed metabolic pathway is given in Fig. 1. The major urinary excretion products are INH, acetylisoniazid (ACINH), pyruvic acid hydrazone, hydrazine, α-ketoglutaric acid hydrazone, isonicotinic acid, isonicotinoyl glycine, monoacetylhydrazine and 1,2-diacetylhydrazine [21]. Pronounced inter-individual variation in INH plasma concentrations and clearance rates following dosing are associated with hereditary differences in the acetylator status. This variation in acetylation is due to different genotypes, the differences of which are primarily due to a genetically controlled poly-

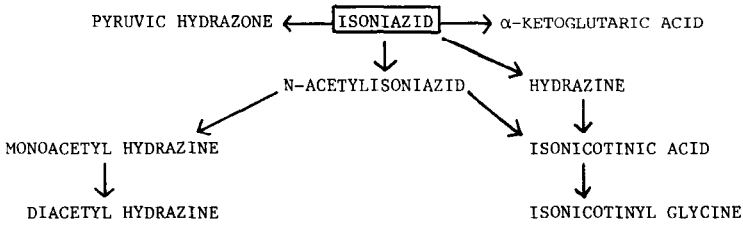


Fig. 1. Pathways for the metabolism of isoniazid in man [21, 50].

morphic N-acetyltransferase (E.C. 2.3.1.5) in the liver and small intestine. There is a bimodal distribution of activity of the enzyme which divides populations into rapid and slow acetylators [30—33]. This drug achieves C_{max} (0.8 μg/ml, slow acetylator or 0.2 μg/ml, rapid acetylator) within 1—2 h following oral dosing [34] and, depending upon the acetylator status, may be inactivated and eliminated rapidly as not to produce sustained effective therapeutic levels. INH is not appreciably bound to plasma proteins, yet increased concentrations of the free drug (which prolongs the serum $t_{1/2}$) are produced when it is administered with PAS which acts to reduce the degree of enzymatic acetylation [21,35,36]. Coadministration of insulin with INH is also known to increase the free concentrations of this component in the liver and lungs [37], whereas the $t_{1/2}$ value is observed to decrease when given to patients who have received ethanol or sodium salicylate, due to their effect of increased activity upon the acetylator enzyme [38].

RIF half-life is increased in hepatic disfunction and is decreased in patients who are slow acetylators of INH [39]. This drug is known to induce the metabolism of warfarin, tolbutamide, oral contraceptives, methadone, digitoxin and other drugs [39]. The major metabolites of EMB are a dibutyric acid derivative and an aldehyde intermediate as shown in Fig. 2. Both of these metabolic products are devoid of antimicrobial activity [22] and approximately 8—15% of a given dose appears in the urine as the dibutyric acid derivative. Red blood cells serve as a depot and contain 1.1—1.6 times the concentration of this compound than is normally present in plasma which serves to slowly allow this component to flow back into the plasma [19,20,40].

The metabolites and pharmacokinetic data of PZA [41,42], PAS [43,44], ETA [44,45] and TAZ [46,47] are also listed in Table 2. Of these compounds, over 80% of PAS is excreted in urine with approximately 5% being in the acetylated form. Coadministration with probenecid decreases renal elimination, therefore increasing $t_{1/2}$ and prolonging toxic effects [44]. Also, less than 1% of ETA is found in urine due to its rapid conversion to metabolites. This drug is known to increase free concentrations of INH due to an inhibitory action upon the acetylating enzyme. In states of malnutrition, such as kwashiorkor, the

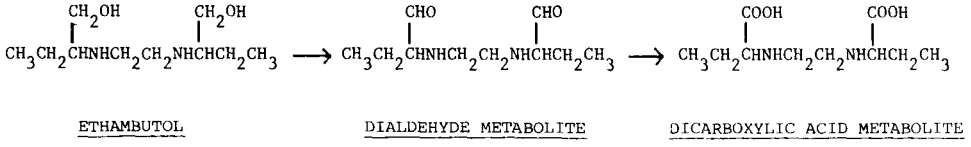


Fig. 2. Metabolites derived from the biotransformation of ethambutol. These metabolites are devoid of antimicrobial activity [50].

protein binding of PAS decreases from 15% to essentially unbound and decreases of 6% are noted for ETA; thus an increase in C_{max}, T_{max} and possible prolongation of $t_{½}$ may be observed. TAZ is the only one of the synthetic compounds which is mainly used in developing countries and little is known of its metabolism. Scanty data are available which suggest that the thiosemicarbazide ($NH_2CSNHNH_2$) is the pharmacologically active moiety whereas the free aldehyde ($CH_3CONHC_6H_4CHO$) moiety, which by itself is inactive, seems to enhance the activity of the former in combination [46,47].

SM and KNM are aminoglycosides that also have been used for tuberculosis therapy [48]. Approximately one third of SM is protein-bound and both drugs are eliminated by the kidneys. However, in kwashiorkor there is a 15% increase in the free level of SM. CYS [48], VOM [49], and CAM [48] are antibiotic derivatives that are mainly excreted by the kidney.

Therefore, it is evident that the clinician must be aware of the pharmacokinetic interactions of these anti-tuberculosis medications. Drug interactions may lead to unwanted increases or decreases in the C_{max}, T_{max} and $t_{½}$ values of these agents such that toxic effects may become evident or therapeutic levels may not be obtained and lead to inappropriate treatment for tuberculosis. In the case of organ (renal and liver) disfunction, toxic effects may be noted due to impairment of the main route of elimination of these agents and lead to build-up of toxic metabolites as may well be observed in the case of malnutrition. Also, the effects of these agents upon commonly administered drugs (e.g., digitoxin, warfarin and oral contraceptives) may lead to their subtherapeutic effects and hence treatment failure for other underlying pathological conditions.

For a recent detailed review of the clinical pharmacokinetics of these drugs the reader is referred to the paper of Holdiness [50].

2.2. Sample handling and storage

Sample handling and storage are major concerns for investigators when one considers that improper treatment may lead to irreversible protein binding, degradation or conversion to labile metabolites and thus a true estimate of the blood levels may not be obtained. A number of studies have been performed concerning this matter for the compounds in question. Controversy still exists over the stability of INH in frozen samples; earlier workers have published conflicting data concerning serum stability of INH at −20°C [51—55]. Concerning fluorometric detection methods using salicylaldehyde as the complexing agent, if serum is deproteinized within 6 h of sampling by 5% trichloroacetic acid (TCA), the levels of INH usually remain constant over a period of three weeks at −20°C [56,57]; however, in non-deproteinized serum, INH is not detectable after 12 h due to irreversible protein binding. Also, INH is not detectable in plasma or whole blood by this fluorometric procedure since it is believed that whole blood or plasma possibly contains substances which quench the fluorescence of the INH—salicylaldehyde complex. Assay results indicate that serum samples which contain drugs such as aspirin, diphenylhydantoin, tolbutamide and phenobarbital do not interfere with the quantitation of INH. The above fluorometric method [57] was modified for use in pediatric studies [25]. By

increasing the concentration of TCA to 10%, ACINH in addition to INH can be monitored in 200-μl samples of both blood and heparinized plasma. Optimization of the intensity of the INH—salicylaldehyde complex is achieved by incubation at 50°C for 10 min and interference is not detectable in plasma samples which also contain RIF, EMB, PAS, prednisone, diphenylhydantoin, quinidine, imipramine or procainamide. It was once thought that INH could be generated from its labile hydrazone due to sample storage [31]; however, this has not turned out to be the case but rather the degradation of ACINH to isonicotinic acid [25]. This instability of ACINH can be prevented if the specimen is deproteinized within 24 h after collection. High-performance liquid chromatographic (HPLC) methods for INH and ACINH extraction utilize sodium hydroxide and ammonium sulfate for protein precipitation [58]. Specimens extracted on the day of sampling can be stored at 4°C before chromatographic analysis without significant loss for two weeks. Recoveries are found to be 101 ± 4% and 98 ± 5% for INH and ACINH, respectively. However, for INH samples not extracted within 24 h or stored at 4°C the recovery drops to 75 ± 18%, yet the recovery of ACINH remains unchanged. It is demonstrated that INH, ACINH, acetylhydrazine, and diacetylhydrazine in urine samples can be stored up to 24 h after collection at room temperature or subsequently stored at −15°C for three months without deterioration of the samples [59].

Ascorbic acid is required as an antioxidant for the extraction and analysis of RIF and its metabolites in plasma, saliva and urine when monitored by HPLC [60]. If plasma and urine are immediately frozen at −20°C, no decrease in RIF content is observable after three months of storage. EMB is a relatively stable molecule and its content in biological samples is unlikely to be altered by oxidation or reaction with exogenous agents. Girgis et al. [61] developed a colorimetric procedure in which a color complex is formed between EMB and bromthymol blue; compounds such as CYS, INH and PAS do not interfere with the assay. When analyzed by gas chromatography (GC) with electron-capture detection (ECD) it is demonstrated that EMB samples can be extracted and stored in a refrigerator for 24 h (non-derivatized) without sample loss [62]. Lee and Benet [63] demonstrated that plasma samples can be frozen for at least one week without sample deterioration while others [64] have observed that plasma specimens can be frozen at −4°C for up to five months without observable loss.

Collection and storage of PZA, pyrazinoic acid (PA), ETA and prothionamide (PTA) present few problems. These samples can be collected in serum, plasma or urine and stored frozen at −20°C for an indefinite period of time. Degradation of PZA into PA is not observable in plasma samples for up to eight months when refrigerated to −20°C [26] and preservatives are not required for urine or serum [65]. ETA and PTA in plasma may be stored over liquid nitrogen whereas urine specimens can be stored at −20°C for at least two months [66]. For analysis of PAS, one of the major contaminants is the breakdown product, *m*-aminophenol. When analyzed spectrophotometrically this decomposition product can interfere with assay results; however, by passing the sample through a weak cation-exchange column, it is possible to obtain only PAS for accurate quantitation [67]. When PAS is used as an internal standard

for monitoring 5-aminosalicylic acid in human plasma it is noted that a decrease in sample content is not observable after seven days at either room temperature or 4°C [68]. TAZ can present a problem for quantitation due to the formation of partial acid hydrolysis products of thiosemicarbazone and others. This may be overcome by extraction with ethyl acetate for HPLC analysis or incubation of a sample with 2 *M* hydrochloric acid in order to totally hydrolyze TAZ to *p*-aminobenzaldehyde which is further derivatized for spectrophotometric analysis. Monitoring of TAZ samples in aqueous solutions and urine when stored at −20°C in the dark for periods up to one year have demonstrated that serum specimens exhibit a decrease in mean TAZ concentration of approximately 2% per week [69]. However, once the samples are extracted into chloroform—amyl alcohol or chloroform—2-propanol it is found to be stable almost indefinitely at −20°C.

Little information is available concerning storage and stability studies of SM, KNM, CAM, VOM and CYS. Information is published concerning tritium-labeled dihydrostreptomycin in which the sample is stored frozen in water [70]; however, the duration of time before noted decomposition occurred or storage temperature is not specified. Isomeric kanamycins A, B and C are produced simultaneously and isolated as a complex from fermentation broths. Only KNM-A is of therapeutic value whereas the others are considered undesirable contamination. When CYS is determined by the colorimetric method of Jones [71] in blood, plasma, cerebrospinal fluid (CSF), urine and fermentation media, degradation products of amino acids, antibiotics, SM, neomycin and a number of other compounds to not interfere with the selectivity of this assay.

3. NON-CHROMATOGRAPHIC ASSAY PROCEDURES

This section is devoted to descriptions of analysis of each of these compounds in order of existing microbiological, spectrophotometric, fluorometric and radiochemical procedures. Microbiological techniques generally involve inhibition of a strain of bacteria (not necessarily *M. tuberculosis*) previously sensitized to the drug under study. For serial dilution methods the sensitized strain is grown within a medium and a quantity of plasma, serum or urine containing the compound is added to this sample and incubated for a specified period of time. Standards are simultaneously compared which have known quantities of this agent and the minimum inhibitory growth concentration of the unknown sample can be derived. Agar diffusion techniques work on a similar principle except the samples are either deposited in wells within the agar or on paper discs and the diameter of the zone with inhibited growth determined and compared with similarly treated standards for quantitation. High sensitivity may be obtained, yet problems associated with this type of assay are concurrent inhibition of the bacterial strain not only by the drug under investigation but also by any metabolites that may possess anti-tuberculosis activity and lengthy incubation periods (up to ten to fourteen days) may be required before analysis. Numerous spectrophotometric, fluorometric and radiochemical assays have been developed for quantitation of these components in various biological media. In general, the detection limits range from 100 to 0.01 μg/ml depending upon the drug monitored, biological specimen or derivatization

agent used. Also, these procedures may involve lengthy extraction or sample preparation before the compound in question can be quantitated. Methods such as these, however, have been applied for routine monitoring and pharmacokinetic analysis. Discussion will be made of the non-chromatographic procedures including their advantages and disadvantages for routine analysis.

3.1. Isoniazid

A vertical agar diffusion assay for INH developed by Lloyd and Mitchison [72] was applicable to serum and urine with a detection limit of < 0.1 μg and required approximately ten days for completion. However, it was observed that INH may completely deteriorate in serum at 37°C during the long incubation time which at best makes the results questionable [73].

Spectrophotometric analysis of INH and ACINH using a number of complexing agents have been reported for enhancing spectral absorption properties with detection limits in the low μg/ml range for media such as serum, urine, plasma, blood and body tissues such as liver, kidney and muscle. Eidus and Harnanansingh [73] have extracted INH from 3 ml of serum and plasma with *n*-butanol—chloroform (3:7) following reaction with *trans*-cinnamaldehyde to form a hydrazone derivative absorbing maximally at 340 nm with a detection limit of 0.5 μg/ml. This method was five times more sensitive than previous procedures utilizing vanillin or piperonal as coupling reagents; however, no mention was made of possible interference from other drugs or serum components. Urinary glucose can interfere with the estimation of ACINH, yet extraction with *n*-butanol—chloroform (3:7) allows for a clean-up step in which the recovery of ACINH is quantitative both in the presence or absence of glucose. Interference due to hydrazones and isonicotinyl glycine is substantially reduced by this extraction step which would normally lead to an overestimation of the concentrations of these components [74]. Spectrofluorometric procedures have been used for quantitation of INH and ACINH in serum and plasma with detection limits of 0.2 μg/ml. Miceli et al. [57] utilized salicylaldehyde as the fluorescent agent and determined that components such as aspirin, diphenylhydantoin, tolbutamide and phenobarbital do not interfere with the determination of INH in serum. The procedure of Miceli et al. [57] was later modified by Olsen et al. [23] to include analysis of ACINH in similar media which only required a sample volume of 200 μl. Modifications included increasing the content of TCA from 5% to 10%, using ascorbic acid instead of 2-mercaptoethanol (which avoided the use of a hood) and optimization of the incubation procedure for maximum fluorescence.

3.2. Rifampicin

Iwainsky et al. [75] utilized a serial dilution method for serum RIF with *M. tuberculosis* H37Rv as the test organism which required eight days for completion. INH was administered after the last collection of blood samples in order to avoid inadvertent simultaneous detection of its antimycobacterial activity. Acocella et al. [76] utilized *Sarcina lutea* ATCC 9341 as the test microorganism. This latter organism was 40% less sensitive to the deacetylated

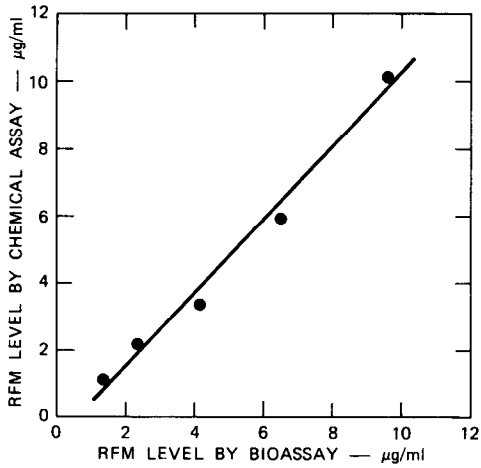


Fig. 3. Correlation between rifampicin (RFM) levels in patients measured by chemical (HPLC) and bioassay methods ($r = 0.994$). (Reproduced with permission from ref. 77.)

form of RIF and greater specificity of the assay was assured by the prior thin-layer chromatographic (TLC) separation of the drugs and metabolites followed by incubation of the separated products on agar plates. A comparison of measurement for RIF was made between a microbiological agar diffusion technique with *Staphylococcus aureus* and HPLC by Peters et al. [77]. The correlation between RIF concentrations in patients determined by the two methods is presented in Fig. 3. A linear correlation ($r = 0.944$) was obtained suggesting that the two methods yield essentially equivalent results. Plasma samples from rats were also tested by these two methods and again a linear correlation ($r = 0.918$) was obtained. In another study [78] comparing serum RIF concentrations via microbiological (*Bacillus bervis* as test organism) versus a TLC method found a mean regression coefficient of 1.02 with a standard error of the mean of 0.03 indicating excellent agreement between the procedures.

Iwainsky et al. [75] have utilized spectrophotometry for determination of RIF and its biotransformation products of 25-desacetyl-RIF and dimethyl-RIF in urine following oral administration. This drug and its metabolites were extracted with cyclohexane—chloroform (5:1) and determined quantitatively at 465 nm with a detection limit of 1 μg/ml. The metabolites and RIF were simultaneously monitored by this procedure and the excretion in urine was found to be proportional to the increase in dosage.

3.3. Ethambutol

M. smegmatis ATCC 607 [79] and 607B [80] have been used for the quantitation of EMB in serum, urine and CSF with an agar diffusion technique. Parallel assays with both organisms revealed the variant *M. smegmatis* 607B to be less sensitive than the 607 culture with reduction of zone diameters, due to the use of 607B, of 32.6%, 30.0% and 38.0% with levels of EMB at 6.4, 3.2 and 1.6 μg/ml, respectively. Also, Sauton's agar medium gave consistently larger zones of inhibition as compared to the zone sizes of Middlebrook and Cohn's 7H10 agar and Cohn's 7H11 agar medium which were consistently equal. Thus

the 607B variant gave poorer results and the commonly used media (7H10 and 7H11) invariably resulted in reduced sensitivity.

EMB has been quantitated in serum and urine [81] and CSF [40] spectrophotometrically using bromthymol blue as a complexing agent. The samples (3 ml), in general, were extracted into chloroform before reaction with the reagent and the detection limit was 0.5 μg/ml. Later Gundert-Remy et al. [40] dried the final solutions of the EMB—bromthymol blue complex, separated and quantitated them by TLC which confirmed the assumption that EMB alone was determined by the chemical assay.

^{14}C-Radiolabeled EMB has been studied for absorption, excretion and metabolic fate in man [22]. At 6 h following i.v. injection, two metabolites were isolated by counter-current distribution: a dialdehyde and dicarboxylic acid derivative. Within a 12—24 h period, 4% of the administered dose was in the form of these two metabolites and the limit of detection was <1 μg/ml. Another study [82] comparing the drug concentrations of EMB in the lungs of monkeys via radiochemical and microbiological analysis found that the microbiological assay values were 75% of those of the radiochemical values which suggested that most of the radiolabeled EMB equivalents were either parent or bioactive metabolites.

3.4. p-Aminosalicylic acid

Mattila et al. [83] have used sodium nitroprusside for determination of PAS in serum with a detection limit of <15 μg/ml whereas Held and Fried [27] have utilized Ehrlich's reagent for estimation of PAS by spectrophotometric and fluorometric procedures. In the latter method the urinary elimination of PAS was studied following 20 mg/kg i.v. injections of the drug and it was found by comparison of these methods that the $t_{1/2}$ determined fluorometrically was 93% longer than that determined spectrophotometrically. The longer $t_{1/2}$ observed by fluorometry along with the TLC studies indicate the additional estimation of metabolites via the extraction method [27].

3.5. Pyrazinamide

The absorption, metabolism and excretion of PZA and its metabolite PA have been quantitated in serum and urine by Ellard [84]. This spectrophotometric assay was based upon differential solvent extraction followed by reaction with alkaline nitroprusside which yielded detection limits of 1 μg/ml in serum and 5 μg/ml in urine. At least 1—3 ml of sample were required for analysis and interference from each other or other anti-tuberculosis drugs (INH, PAS, SM, ETA, CYS, TAZ) were not noted in this assay.

3.6. Streptomycin

A paper microdisc method utilizing *Staphylococcus albus* KS 462 as the test organism was developed by Jalling et al. [85] for serum and plasma which required overnight incubation. Only 10 μl of sample were required for analysis and SM could be quantitated in the presence of ampicillin and cloxacillin by

the addition of penicillinase which inhibited the activity of the latter two antibiotics. The limit of detection was not specified in this procedure nor were interferences from other anti-tuberculosis drugs determined.

Thiobarbituric acid reagent has been used for the quantitation of SM by spectrophotometry [86]. This method makes use of the development of the chromogens at 37°C and was able to quantitate the drug in human and bovine sera in the 20—100 μg/ml range. This assay was designed to eliminate interferences observed from various sugars and glycoproteins present in serum; interferences from other anti-tuberculosis medications were not specified.

^{3}H-Radiolabeled SM was incubated with *Streptomyces griseus* for the study of the oxidation of this compound in vivo [87]; following incubation the compound was separated from cellular extracts by descending paper chromatography and analyzed by scintillation counting of which approximately 1 μg of sample could be detected by this method. Buchanan and Van der Walt [88] have also studied the binding of radiolabeled SM to normal and kwashiorkor serum and found that there was a 15% increase in the free drug in the latter serum. Albumin was determined by electrophoresis to be the predominant binding protein. Limits of detection were not specified.

3.7. Kanamycin

Maitra et al. [89] have reviewed microbiological assay methods for determination of KNM in serum and plasma. This compound was quantitated in the presence of penicillins and cephalosporins that were inactivated by the enzymes β-lactamase I and II. The procedure could be performed in 3—4 h with a mean error of ± 11% and a detection limit of approximately 2 μg/ml. Interferences from other anti-tuberculosis drugs were not specified.

3.8. Ethionamide

Colston et al. [90] have evaluated the activity of ETA and PTA using a mouse footpad model. Both of these compounds were found to have minimum inhibitory concentrations (MIC) against *Mycobacterium leprae* of 0.05 μg/ml and to be bactericidal against this organism at dietary concentrations of 0.1%. It required approximately sixty days following inoculation for assessment of bactericidal activity.

^{14}C-Radiolabeled ETA has also been analyzed following oral administration of the drug in dietary preparations of mice [90]. The compound was analyzed by scintillation counting from 1-ml serum samples with a detection limit of 0.05 μg/ml. The extracting solvent (diethyl ether) was chosen to minimize any potential contribution from sulfoxide metabolites of this drug to the assay.

3.9. Cycloserine

CYS concentrations in serum and urine have been determined by agar diffusion with *Bordetella bronchiseptica* ATCC 4617 as the test organism [83]. Incubation required 16 h and the sensitivity was < 8 μg/ml. This microbiological technique was compared to that of a spectrophotometric assay in which CYS was derivatized with sodium nitroprusside and determined at 625 nm. Both

procedures gave similar results with C_{max} at 2 h of 14—36 μg/ml by bioassay and 19—33 μg/ml by chemical assay. The $t_{1/2}$ in serum ranged from 3 to 15 h by bioassay and 4 to 30 h by the chemical assay. The advantage of the chemical method is the shorter time period required to perform it. Assay interferences were not determined for either procedure.

Jones [71] directly treated samples of plasma, CSF, urine and fermentation media with nitropentacyanoferrate and quantitated the derivative at 625 nm. This procedure was able to determine 2—3 μg of CYS with an accuracy of ± 2% and a precision of ± 1%. When standards were compared with a bioassay using *Micrococcus pyrogenes* ATCC 6538, the two procedures gave good agreement for crystalline CYS. The color of the solutions was found to be stable at least 4 h at room temperature and with the exception of urine where a large number of antibiotics and similar compounds (e.g. serine, serineamide, serine hydroxamic acid) did not produce any noticeable interference in the assay results in blood, plasma, CSF or fermentation mediums.

3.10. Capreomycin

M. tuberculosis H37Rv was used as the test organism for determination of CAM in urine and serum. Little information was given concerning selectivity of the assay procedure except that the agent could inhibit the bacterial strain for 6 h following a 1-g intramuscular injection and that no significant accumulation was noted in the absence of functional renal impairment [91]. Interferences from other anti-tuberculosis compounds or serum components were not specified.

3.11. Viomycin

Gyselen et al. [92] have quantitated VOM along with other anti-tuberculosis drugs in Lowenstein—Jensen medium. *M. tuberculosis* H37Rv was added to resistant samples to determine sensitivity and the samples were incubated for three weeks; the MIC of VOM was 10 μg/ml. Contributions from interferences of other anti-tuberculosis medications were not indicated.

3.12. Thiacetazone

A microbiological application of TAZ was performed by Leat and Marks [93] in which incubation was required at 4°C for three weeks before analysis could be performed; the detection limit was < 2 μg/ml. Few specifics were given about this assay procedure including interference studies.

Ellard et al. [69] have described spectrophotometric and fluorometric methods for analysis of TAZ in serum and urine with detection limits of 0.3—0.6 μg/ml. By the first method, TAZ was hydrolyzed to *p*-aminobenzaldehyde which was diazotized and coupled with N-(1-N-naphthyl)ethylenediamine in acetone—water (1:1) to give the Bratton—Marshall chromophore which was determined at 550 nm. Also, TAZ was determined at 330 nm following extraction with chloroform—amyl alcohol (4:1). For the latter method the compound was oxidized to *p*-acetylaminobenzoic acid and derivatized with

hydroxylamine hydrochloride. Analysis of variance for 216 serum samples for the two methods revealed an overall error of ± 0.27 μg/ml. The correlation coefficients (r) of the linear regression between fluorometric and spectrophotometric analysis were 0.974 and 0.980, respectively.

Colston et al. [94] fed [^{35}S]TAZ to mice for 24 h and analyzed the serum for its concentrations; limits of detection were < 0.2 μg/ml and estimated MICs were between 10 and 50 μg/ml for *M. leprae* infected mice. Interferences from radiolabeled metabolites were neither studied nor specified.

4. CHROMATOGRAPHIC METHODS

In this section chromatographic methods will be described for each of these compounds in order of existing planar chromatography (paper chromatography and TLC), classical column chromatography, HPLC, gas chromatography (GC) and GC—mass spectrometry (MS) with an emphasis on the latter three. Chromatographic applications are finding wide utility in many laboratories; these methods offer similar sensitivity as non-chromatographic methods and generally better selectivity for determining the concentrations of these drugs and metabolites in various biological media.

4.1. Isoniazid

Paper chromatography has been applied for the analysis of urine from patients who received tritium-labeled INH and ACINH by Mitchell et al. [95]. Descending Whatman No. 3 paper chromatography was used for separation of these urinary products with a solvent system of 2-propanol—*n*-butanol—water (13:5:3) for 2 h and the spots were visualized with Ehrlich's reagent or *o*-phenylenediamine before quantitation with scintillation counting. Recovery was 99 ± 2%, yet limits of detection were not specified. Isonicotinic acid, isonicotinoyl glycine, α-ketoglutarate isoniazid hydrazone and pyruvate isoniazid hydrazone did not coelute or interfere with the analysis. It was noted that ACINH was metabolized identically in either rapid or slow acetylators and that approximately 45% of this metabolite was hydrolyzed to isonicotinic acid and free acetylhydrazine.

Quantitation of INH and ACINH by *HPLC* in biological samples has been explored by numerous investigators [58,96—100]. Saxena et al. [96] were able to analyze INH and ACINH in spiked plasma and urine samples with UV detection at 266 nm. The mobile phase consisted of methanol—water (3:2) with dioctyl sodium sulfosuccinate (ion-pairing reagent) and separations were completed in less than 10 min on a < 10-μm μBondapak C_{18} column. Using 1-benzoyl-2-isonicotinoylhydrazine as internal standard, the minimum detectable quantities were 200 and 50 ng of INH and ACINH, respectively. Moulin et al. [58] and Holdiness [97] have measured INH and ACINH in tuberculosis patients following oral administration of this drug. Both procedures utilized μBondapak C_{18} columns and 1-benzoyl-2-isonicotinoylhydrazine as internal standard, yet the latter method required an ion-pairing agent of dioctyl sodium sulfosuccinate for effective separation. Plasma samples were extracted into *n*-butanol—chloroform (3:7) and then reextracted in 0.25 *M* sulfuric acid.

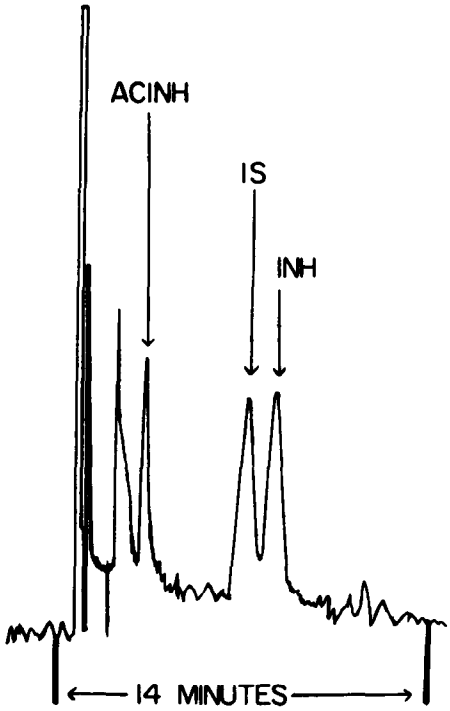


Fig. 4. HPLC profile of ACINH, 1-benzoyl-2-isonicotinoylhydrazine, internal standard (IS) and INH extracted from human plasma 4 h following oral dosing with INH. The mobile phase consisted of 0.001 *M* dioctylsulfosuccinate in distilled water—ethanol (11:9) adjusted to pH 2.50 at a flow-rate of 4.0 ml/min. Separation was performed on a Waters Assoc. reversed-phase 10-μm μBondapak radial compression column [97]. Concentrations: INH 1.50 μg/ml; ACINH 2.09 μg/ml. Retention times: ACINH 205 sec; IS 535 sec; INH 640 sec.

A chromatogram of INH, ACINH and internal standard isolated from human plasma 4 h following oral dosing is shown in Fig. 4 [97]. Positive identification of the respective peaks was achieved by addition of ACINH and INH standards (500 ng) to previously extracted samples and observing the increased peak area at the corresponding retention time. Concentrations (μg/ml) of INH and ACINH after an oral dose of 300 mg INH are given in Table 3. The parent drug concentration reached a maximum within 1—2 h after administration and the metabolite concentration was maximal within 3—4 h in this patient. INH was still detectable 12 h after dosing and using a 2-ml sample the detection limit was 100 ng/ml [97]. Moulin et al. [58] identified INH and ACINH (in the presence of EMB, RIF and their metabolites) by MS analysis following collection of fractions as they eluted from the column. Hutchings et al. [99] used iproniazid as internal standard and effected separation of the drug and its metabolite (detection limit 20 ng/ml each) on a 5-μm Spherisorb nitrile column whereas Guillaumont et al. [98] used a 10-μm μBondapak column (without internal standard) for quantitation in human serum extracts, polymorphonucleocytes and alveolar macrophages. In the latter study, differential solvent extraction was used to obtain RIF and 25-desacetyl-RIF from INH and ACINH in the same 1 ml of serum or cell suspension and *n*-heptanesulfonic acid was required for effective separation. Detection limits were listed as 95 and 85 ng/ml of

TABLE 3

CONCENTRATIONS OF ISONIAZID (INH) AND ACETYLISONIAZID (ACINH) IN HUMAN PLASMA [97]

Concentrations are expressed as μg drug per ml plasma (average of three determinations) ± standard deviation.

Time* (h)	INH	ACINH
0	–	–
1	1.71 ± 0.51	0.30 ± 0.12
2	2.92 ± 0.72	1.41 ± 0.40
4	1.50 ± 0.50	2.09 ± 0.68
6	0.97 ± 0.43	0.61 ± 0.34
9	0.52 ± 0.33	–
12	0.25 ± 0.11	–

*Time after an oral dose of 300 mg INH.

serum and 19 and 17 ng per 10^6 cells for INH and ACINH, respectively. The latest procedure of Lacroix et al. [100] utilized 50 μl of plasma without a solvent extraction step and INH was condensed with cinnamic aldehyde to form isonicotinoyl hydrazone after TCA deproteinization. This product was separated on a 6-μm Zorbax cyano column (without internal standard) and when compared with a fluorometric procedure [100] had a correlation coefficient of 0.992.

Common anti-tuberculosis drugs such as RIF, PZA, SM and EMB did not interfere with assay results. All of the above listed HPLC methods have utilized UV detection.

Methods for *GC and MS analysis* of INH and associated metabolites have been available since the mid-1960s. Calo et al. [101] reported GC conditions for INH determination but unfortunately the method was not adaptable to biological samples with relatively low concentrations. INH and ACINH have been assayed in rat urine by the method of Frater-Schroder and Zbinden [102]. Eicosan was the internal standard and the compounds were derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS). Separation was effected with a 4% OV-17 column at 210°C and the samples were quantitated by flame ionization detection (FID) and GC—MS; the detection limit was approximately 50 μg/ml INH. Others have utilized nitrogen—phosphorus detectors for quantitation of INH, ACINH, hydrazine, acetylhydrazine and diacetylhydrazine in spiked human urine [103]. *p*-Chlorobenzaldehyde was used to directly derivatize INH, hydrazine and acetylhydrazine whereas ACINH and diacetylhydrazine were similarly determined following hydrolysis to INH and acetylhydrazine, respectively. Internal standards were synthesized by reacting *p*-bromobenzaldehyde with the above mentioned compounds and detection limits for the components were < 8 μg/ml. Although this latter technique was more sensitive, its disadvantages included a tedious extraction and derivatization procedure and 25 ml of urine were required per extraction. This would probably make it an unacceptable method for routine quantitation of plasma samples especially for pharmacokinetic studies. GC—MS methods have become popular since the 1970s [59,104—107].

Timbrell et al. [59] identified urinary monoacetylhydrazine as a potential hepatotoxin of INH metabolism (utilizing the above described *p*-chlorobenzaldehyde derivatization procedure) whereas Noda and co-workers [105,107] have monitored INH, ACINH, hydrazine, acetylhydrazine and diacetylhydrazine in human urine and rat hepatocytes utilizing deuterated internal standard (except in the case of hydrazine which was quantitated using [^{15}N]hydrazine as internal standard). This procedure also involved a tedious extraction and derivatization procedure with benzaldehyde plus an additional step of further derivatization with N,O-bis(trimethylsilyl)acetamide (BSA) to form the corresponding trimethylsilylates prior to GC injection. Lauterburg et al. [106] observed INH, ACINH, acetylhydrazine and diacetylhydrazine via selected-ion monitoring (SIM) techniques in human plasma following an oral dose of 300 mg. The trimethylsilyl derivatives (BSTFA) of diacetylhydrazine and ACINH and benzaldehyde hydrazones of acetylhydrazine and INH were separated on a 1% OV-17 column (programmed from 90°C to 270°C). Deuterated analogues served as internal standards and the total-ion current with single-ion tracings of a standard mixture can be observed in Fig. 5. The ions monitored were diacetylhydrazine/d_6-diacetylhydrazine (*m/z* 245/251), acetylhydrazine/d_3-acetylhydrazine (*m/z* 219/222), acetylisoniazid/d_3-acetylisoniazid (*m/z* 308/311) and isoniazid/d_6-isoniazid (*m/z* 282/288). This procedure was well suited to determine the hepatotoxic hydrazino metabolites of INH in human plasma following oral administration of therapeutic doses of the drug and the assay was sensitive enough to quantitate acetylhydrazine and diacetylhydrazine in plasma concentrations in the range 10—20 ng/ml.

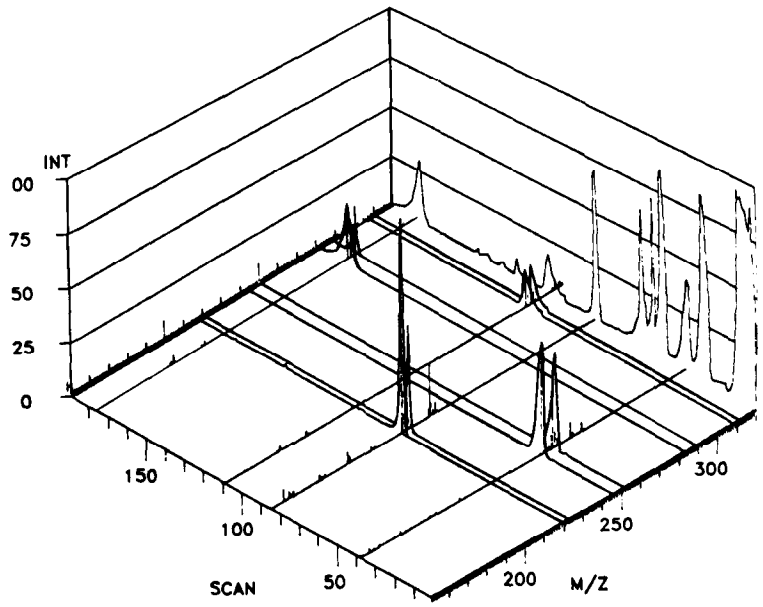


Fig. 5. Total-ion current and single-ion tracings of a standard mixture of diacetylhydrazine (*m/z* 245), acetylhydrazine (*m/z* 219), acetylisoniazid (*m/z* 308) and isoniazid (*m/z* 282) and their deuterated internal standards d_6-diacetylhydrazine (*m/z* 251), d_3-acetylhydrazine (*m/z* 222), d_3-acetylisoniazid (*m/z* 311) and d_6-isoniazid (*m/z* 288). Concentration of standards not listed. (Reproduced with permission from ref. 106.)

Although GC—MS techniques provide for more selectivity of analysis, the rather involved extraction and derivatization procedures along with the expense of instrumentation will probably minimize its use for routine therapeutic drug monitoring as compared to the less expensive and technically less complex methods of HPLC.

4.2. Rifampicin

A number of *TLC* methods have been developed for quantitation of these compounds and their metabolites [76,108,109]. Kolos and Eidus [108] separated RIF and 25-desacetyl-RIF from urine samples on 100-μm layer sheets of silica gel with a solvent system of chloroform—ethanol—0.1 *M* hydrochloric acid (84.0:15.9:0.1). The compounds were well resolved and as little as 2 μg of each component were detectable spectrophotometrically. Wilson et al. [109] utilized a chloroform—methanol—water (16:4:5) solvent system with commercially available precoated silica gel 60F plates for resolution of RIF from its possible degradative and synthetic by-products formed during drug manufacturing. RIF was resolved from rifamycin O, rifamycin S, rifampin quinone, 25-desacetyl-RIF quinone, 25-desacetyl-RIF, 3-formylrifamycin SV, rifamycin B, rifampin N-oxide and 1-amino-4-methylpiperazine present in commercial formulations. Concentrations up to 300 μg could be chromatographed without spot distortion or streaking and the minimum amount of RIF detectable was 0.30 μg when the plate was sprayed with ninhydrin and observed under short-wavelength UV light. The length of time required for development of the plates was not listed for any of the above procedures as were possible interferences from other components in biological samples.

Effective *HPLC* separations of RIF and metabolites in biological media have been reviewed [110] and various column packings were used such as LiChrosorb silica [60,77], reversed-phase C_{18} [98,111,112] and C_8 [113] and Micropak NH_2 [114]. Ratti et al. [113] developed a procedure that allowed for the separation of RIF, 25-desacetyl-RIF, 3-formylrifamycin SV, 3-formyl-25-desacetylrifamycin and N-desmethylrifampicin. Good baseline separation could be obtained for most of the compounds except for RIF and N-desmethylrifamycin; however, only RIF and 25-desacetyl-RIF appeared in sufficient quantities in plasma to be assayed. Butyl-*p*-hydroxybenzoate was the internal standard and the sensitivity of the method for both compounds was 200 ng/ml. Guillaumont et al. [98] developed a method in which RIF, 25-desacetyl-RIF, INH and ACINH could be extracted from the same human serum sample. RIF and 25-desacetyl-RIF were extracted in acetone, and INH and ACINH extracted into *n*-butanol—chloroform (3:7). Following differential solvent extraction the two sets of samples were separated on a reversed-phase C_{18} column using different mobile phases; internal standards were not used for either method. Detection limits were found to be 17 and 10 ng/ml and 3.4 and 2 ng per 10^6 cells for RIF and 25-desacetyl-RIF, respectively. Lecaillon et al. [60] described a simple and sensitive HPLC procedure for simultaneous and specific determination of RIF, 3-formylrifamycin SV (FR), 3-formyl-25-desacetylrifamycin SV (FDR) and 25-desacetyl-RIF (DR) on a 5-μm LiChrosorb silica 60 column. Following oral dosing, 1 ml of plasma, urine or saliva was extracted with an isooctane—dichloro-

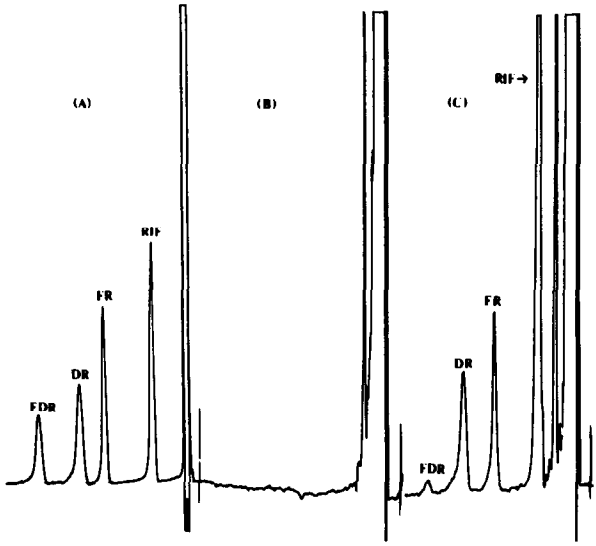


Fig. 6. (A) HPLC profile of a synthetic mixture of RIF and three of its metabolites. Peaks: RIF = Rifampicin; FR = 3-formylrifamycin SV; DR = 25-desacetylrifampicin; FDR = 3-formyl-25-desacetylrifamycin SV. (B) Chromatogram of blank plasma extract. (C) Chromatogram of a plasma extract of a subject given an oral dose of rifampicin. RIF, found: 1.45 μg/ml; FR, found: 0.41 μg/ml; DR, found: 0.45 μg/ml; FDR, found: 0.05 μg/ml. Retention times: RIF 2.5 min, FR 5.5 min, DR 7.5 min, FDR 9.3 min. (Reproduced with permission from ref. 60.)

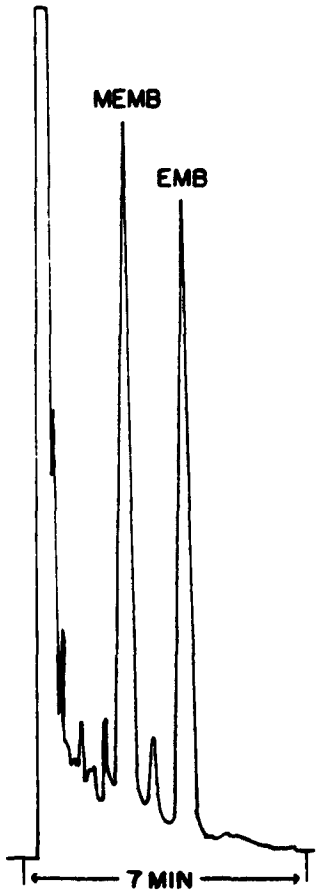
methane solution (3:2) and injected onto the chromatographic column. The mobile phase consisted of dichloromethane—isooctane—ethanol—water—acetic acid (36.6:45:16.8:65:0.02) at a flow-rate of 3 ml/min. Fig. 6 demonstrates the resolution of RIF from the three metabolites and components from the biological matrix. None of these interfered with the analysis. A total of 10 min was required for analysis and the procedure allows an absolute sensitivity of RIF down to 5 ng; calibration was performed daily with external standards and precision of the measurement was ± 5%.

4.3. Ethambutol

As far as this author can ascertain, HPLC methods have not been developed for quantitation of EMB in biological fluids.

GC and *GC—MS* methods for EMB analysis have been developed in various biological media [62,63,101,115—122]. Calo et al. [101] and Richard et al. [116] formed trimethylsilyl derivatives of EMB and used GC with flame ionization detection (FID) for measurement; however, the sensitivity of the method did not allow for quantitation of drug concentrations expected in biological fluids nor were the limits of detection, extraction procedure or internal standard specified. Strind and Salvesen [115] developed a GC—FID procedure for quantitation of the drug in urine using diphenydramine as internal standard; however, the detection limit was only 50 μg/ml, the drug, however, could only be detected in urine following a 2-g dose (a 2.5-fold increase over the normal dose). Lee and Benet [63,117] developed a sensitive

GC—ECD method for quantitation in biological fluids using trifluoroacetic anhydride (TFAA) as the derivatizing agent and 2,2'-(ethylenediimino)-di-1-propanol (MEMB) as the internal standard. The limit of detection of this procedure was 10 ng and it has been applied for the determination of disposition kinetics and hemodialysis clearance in dogs and human patients [18—20,118]. The authors suggest that with the sensitivity of this detector only 1—2 μl of plasma would be required to quantitate EMB in the therapeutic range of 3—5 μg/ml; this claim, however, has not been substantiated in vitro. Blair et al. [119] applied GC—MS techniques for quantitation of the agent in biological media using methane chemical ionization (CI) with trimethylsilylimidazole derivatization. The investigators first attempted to analyse the component using methane CI via direct probe insertion yet under CI conditions the problem of contaminants from plasma carry over in the extraction step enhanced the $[M + 1]^+$ ion of the deuterated internal standard (d_4-EMB). However, following derivatization and prior GC separation on either a 3% OV-17 or 3% OV-225 column, the characteristic $[M + 1]^+$ ion of EMB (*m/z* 349) and internal standard (*m/z* 353) could be quantitated without matrix interference. Detection limits were not specified. Ohya et al. [120] quantitated EMB in dog plasma using d_4-EMB as internal standard with TFAA as the derivatizing agent. SIM was carried out at 30 eV and *m/z* 294 and 296 corresponding to the fragment ions $[M/2]^+$ of EMB and internal standard were observed, respectively. Separation was effected at 150°C on a 2% OV-17 column and analyses were completed within 4 min. The method was utilized successfully for studying the bioavailability and pharmacokinetics of the drug in dogs. In this study it was observed that the stability of the trimethylsilyl derivative was inferior to that of the TFAA derivative and gradually decomposed over a three-day period yielding no observable peaks. Holdiness and co-workers [121,122] developed a GC—MS technique for assay of this drug in human plasma following oral administration and compared it with the GC—ECD method of Lee and Benet [63]. For GC—ECD determination MEMB was used as the internal standard and for GC—MS a deuterated analogue (d_4-EMB) was synthesized for quantitation. TFAA was the derivatizing agent for both methods; a 3% OV-17 column (160°C) was used for resolution of the components by GC—MS and a 3% SE-30 column (170°C) for GC—ECD. Following oral administration of 800 mg EMB, plasma samples were obtained, the appropriate internal standard was added and extracted with chloroform. Plasma for GC—ECD analysis contained 3 μg MEMB whereas 3 μg of deuterated EMB was added to plasma samples for GC—MS quantitation. SIM was used for GC—MS at an acceleration voltage of 70 eV and the ions recorded were *m/z* 307 (EMB), *m/z* 280 (MEMB) and *m/z* 310 (d_4-EMB). Fig. 7 presents a typical GC—ECD profile of EMB extracted from plasma with MEMB as internal standard; the retention times are 2.73 and 4.31 min for MEMB and EMB, respectively. For GC—MS analysis of plasma, an SIM for trace EMB and deuterated internal standard can be seen in Fig. 8 with a retention time of 2.5 min for each compound. The GC—ECD samples with MEMB as internal standard could be quantitated via the GC—MS technique. The concentrations of EMB (from a tuberculosis patient collected over a 24-h period) following an oral dose of 800 mg of the drug are listed in Table 4. The concentrations (μg/ml) in plasma of EMB were determined by GC—MS with



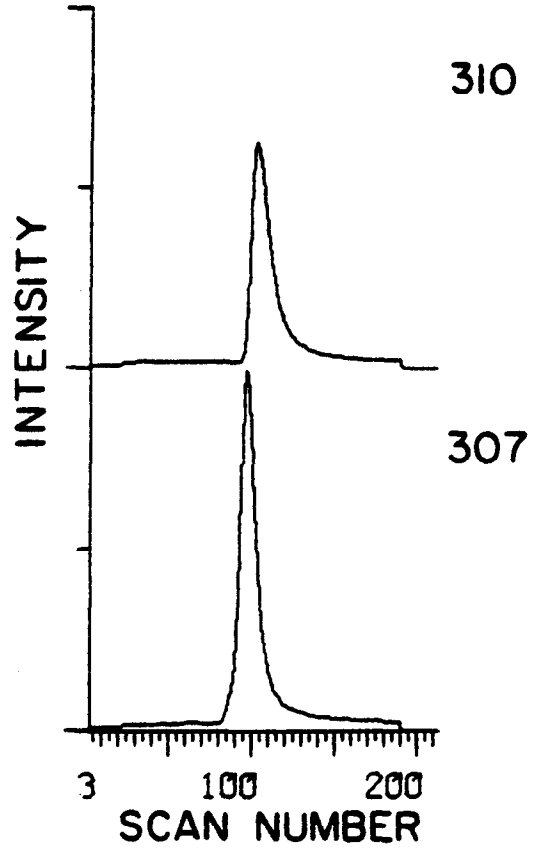


Fig. 7. GC—ECD profile of ethambutol (EMB) and MEMB as internal standard from plasma (3.71 μg/ml of ethambutol).

Fig. 8. Single-ion chromatogram from plasma of ethambutol (EMB) (*m/z* 307) and d_4-ethambutol (*m/z* 310) as internal standard (3.36 μg/ml ethambutol).

TABLE 4

CONCENTRATIONS OF ETHAMBUTOL IN HUMAN PLASMA DETERMINED BY GC—MS AND GC—ECD PROCEDURES [122]

Time* (h)	GC—MS**		GC—ECD
	[$^2H_4$] EMB	MEMB	MEMB
0	0.30 ± 0.10	0.33 ± 0.07	0.31 ± 0.11
1	2.66 ± 0.27	2.58 ± 0.20	2.78 ± 0.29
2	3.36 ± 0.39	3.66 ± 0.44	3.71 ± 0.40
4	1.68 ± 0.30	1.71 ± 0.29	1.81 ± 0.35
6	1.15 ± 0.31	1.17 ± 0.38	1.19 ± 0.28
12	0.80 ± 0.20	0.80 ± 0.19	0.86 ± 0.17
24	0.48 ± 0.09	0.42 ± 0.07	0.52 ± 0.12

*Time after an oral dose of 800 mg of EMB and 300 mg INH. Concentrations expressed as μg EMB per ml plasma (average of three determinations) ± standard deviation.

**Either [$^2H_4$] EMB or MEMB was used as the internal standard.

either d_4-EMB- or MEMB-containing samples. These data are compared with the values obtained via GC—ECD using MEMB as internal standard, and showed excellent agreement obtained by either method regardless of the internal standard used for quantitation. Limits of detection with a 2:1 signal-to-noise ratio are 36 ng/ml (GC—MS) and 11 ng/ml (GC—ECD) and interferences were not observed following concurrent administration of INH or RIF.

4.4. p-Aminosalicylic acid

PAS has been used as an internal standard by three investigators [68,123, 124] for quantitation of 5-aminosalicylate and metabolites in plasma and urine samples using *HPLC*. In these three cases either reversed-phase C_{18} or LiChrosorb silica 60 columns were used for resolution of PAS from 5-amino-

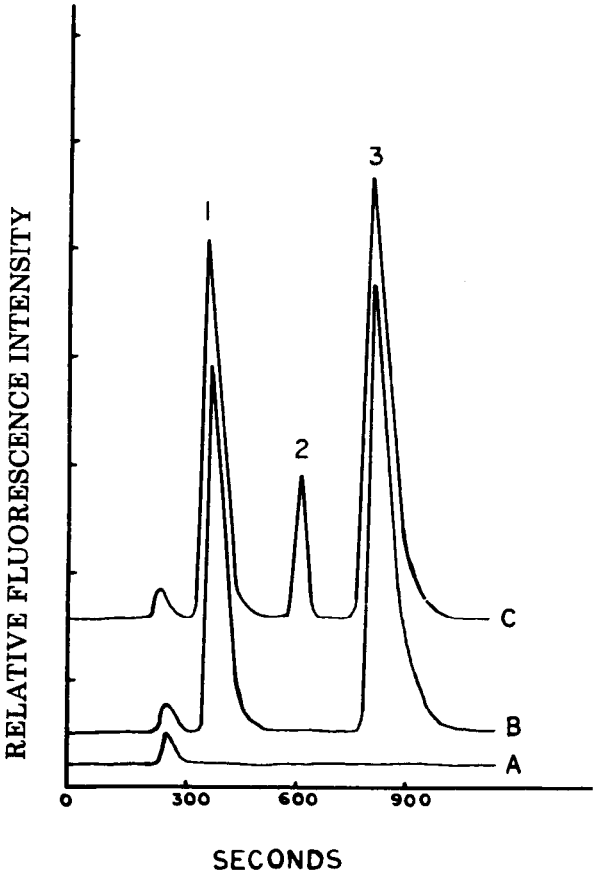


Fig. 9. Typical chromatogram of PAS (1) and anthranilic acid, internal standard (3) in spiked human plasma sample (B). For comparison, chromatograms of blank plasma (A) of a plasma sample (C) also containing N-acetyl metabolite of PAS (2) are shown. Conditions: column, LiChrosorb C_{18} (2.50 mm × 3.2 mm I.D.), eluent, absolute methanol—distilled water (1 : 4) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate adjusted to pH 5.5 with concentrated phosphoric acid; flow-rate 1 ml/min; excitation and emission wavelengths 270 and 385 nm, respectively. Concentrations not listed. (Reproduced with permission from ref. 125.)

salicylate and its metabolite, acetyl-5-aminosalicylate, with detection limits of 20 ng/ml plasma. Also, ion-pairing reagents were required for separation such as N,N,N-trimethylcetylammonium bromide [124] or N,N,N-trimethylhexadecylammonium bromide [123]. Honigberg et al. [125] have developed a method for monitoring PAS in plasma samples with fluorometric detection using a <10-μm LiChrosorb C_{18} column with a mobile phase of methanol—water (1:4) containing 0.005 *M* tetrabutylammonium hydroxide (ion-pairing agent) and 0.01 *M* disodium phosphate (pH 5.5) for effective separation. Anthranilic acid was the internal standard and plasma samples spiked with PAS were extracted for analysis. Fig. 9 presents a typical chromatogram for the resolution of PAS and internal standard from spiked plasma. Under these conditions, the N-acetyl metabolites of PAS, INH and ascorbic acid were found not to interfere with the assay and the minimum detectable amount of PAS via this procedure with a 2:1 signal-to-noise ratio was 500 ng. Spiked plasma samples containing INH and ascorbic acid did not interfere with assay results. The applicability of HPLC in the assay of plasma concentrations of this component using fluorometry and ion pairing with tetrabutylammonium ion is clearly demonstrated with an accuracy of 1—5% which is within the range of accuracy for the earlier mentioned papers [123,124].

4.5. Pyrazinamide

PZA and its metabolites have been monitored by *paper chromatography* following i.v. administration to dogs [42]. Plasma and urine samples were collected and separated on Whatman No. 1 paper in 0.05 *M* ammonium formate buffer, pH 3.4, for 1 h. The separated spots were viewed by UV light, cut and analyzed for content by polarography. This procedure had a sensitivity of < 0.1 μg/ml PA and 5-hydroxypyrazinoic acid.

PZA has also been quantitated by *TLC* methods in bacterial media suspensions [126]. This method involved the incubation of PZA in *M. tuberculosis* broths and quantitation of MIC. The procedure provided a means of determination of drug concentrations by silica gel plates with the solvent system isopropanol—ammonium hydroxide (7:3) following development for 7 h. The plates were examined by UV light and PA was also detectable by this method; limits of sensitivity were not specified.

An *anion-exchange classical column technique* was developed which permits determination of PZA and its major metabolites of PA and 5-hydroxypyrazinoic acid [127]. Patients were administered 3 g of PZA orally and the urinary products separated first on a Dowex 50 column from which a specific fraction was further separated on a Bio-Rad Cl^- column. Eluted materials were identified by UV absorption following coelution with authentic samples added to urine and via TLC of spiked samples. A chromatogram of the eluted materials from urine after chromatographic separation on the Bio-Rad Cl^- column is shown in Fig. 10. The dark line represents a xanthinuric patient and the dashed line is that of a control patient. The four peaks were identified as unmetabolized PZA (I), 5-hydroxypyrazinoic acid (III) and PA (IV), respectively. Peak II was not identified. This study presented evidence that PA was the likely agent causing xanthine retention and that the mechanisms of renal transport of

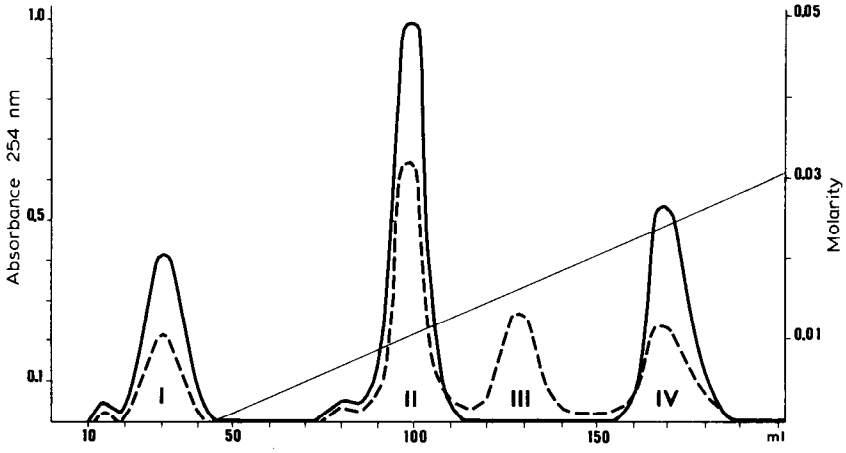


Fig. 10. Chromatogram of the eluded materials from the urine after separation in BioRad Cl^-. The peaks were identified as unmetabolized PZA (I), 5-hydroxypyrazinoic acid (III), and PA (IV); Peak II was not identified. The urine samples were collected 3 h after PZA ingestion: (——) xanthinuric patient (3 g); (--------) control patient (2 g). Concentrations not listed. (Reproduced with permission from ref. 127.)

xanthine and hypoxanthine were different. Neither of the above listed procedures examined the interferences due to other commonly administered antituberculosis medications.

An *HPLC* assay for metabolites of PZA in human plasma has been developed by Ratti et al. [26]. Using a LiChrosorb NH_2 column, PA and 5 hydroxypyrazinoic acid were well resolved in biological samples with retention times of 8.44 and 10.74 min, respectively. This assay required only 300 μl of plasma for extraction and analysis, and was studied in the concentration range 1—10 μg/ml. PZA, RIF, DR, INH, ACINH and SM did not interfere with the assay.

Pitre et al. [128] and Roboz et al. [65] quantitated PZA, PA, 5-hydroxypyrazinoic acid and 5-hydroxypyrazinamide in human serum and urine and rat liver via *GC—MS*. By the method of Roboz et al. [65] serum was deproteinized, evaporated and the crude residues were silylated with BSTFA and TMCS. Selected ions of PZA (*m/z* 196), PA (*m/z* 197), 5-OH-PA (*m/z* 285), nicotinamide (internal standard No. 1, *m/z* 195), and nicotinic acid (internal standard No. 2, *m/z* 196) were monitored as seen in Figs. 11 and 12 for serum and urine, respectively. CI was used with isobutane as both the chromatographic carrier and reagent gas. Detection limits for PZA and PA were 10 ng/ml and for 5-OH-PA 20 ng/ml using 200 μl serum or 20 μl urine. The reproducibility (mean ± S.D.) in serum at 500 ng/ml was 0.71 ± 0.102, 0.82 ± 0.052, and 0.33 ± 0.030 for PA, PZA and 5-OH-PA, respectively.

4.6. Streptomycin

SM has been analyzed in biological fluids by several investigators [129,130]. Heding [129] separated SM mixtures in 1 h by *TLC* on silica gel plates using distilled water. SM was well resolved from drug formulations which contained streptidine, dihydrostreptomycin, dihydro-N-dimethylstreptomycin, hydroxystreptomycin, streptomycyclamin, dihydrodesoxystreptomycin and methyl-

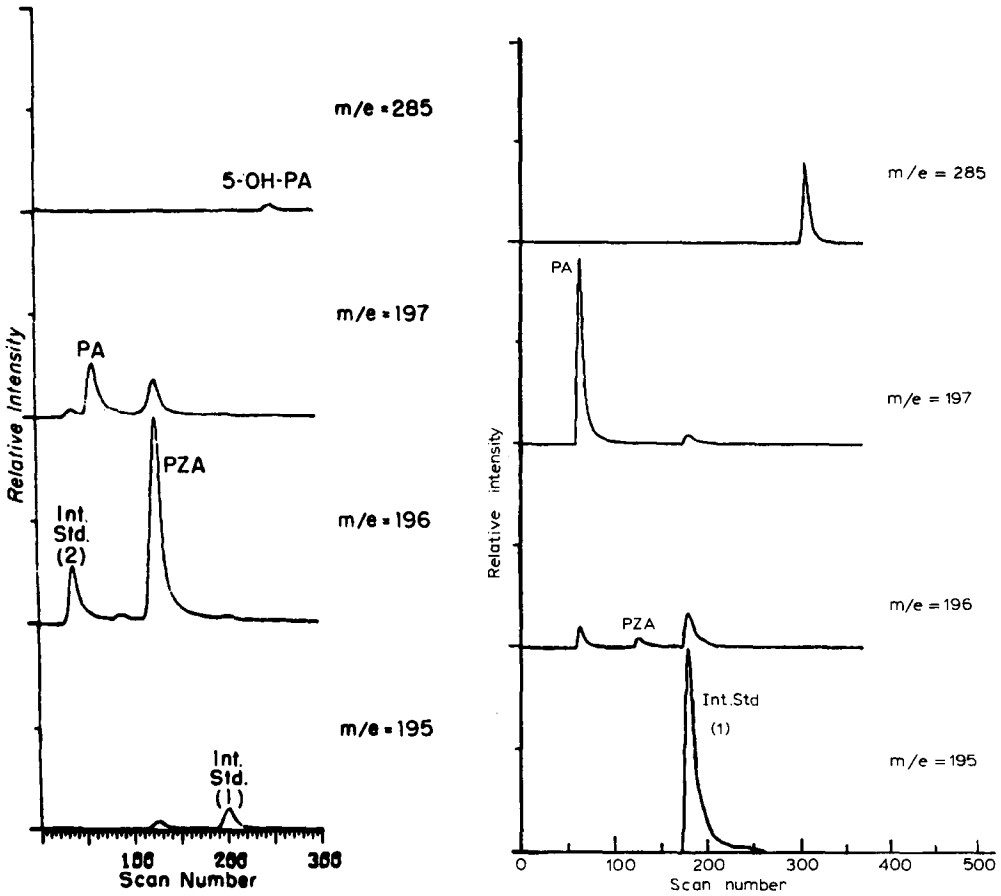


Fig. 11. Single-ion chromatogram of PZA and its metabolites in the serum of a patient receiving PZA. Nicotinic acid (2) and nicotinamide (1) are internal standards. Concentrations not listed. (Reproduced with permission from ref. 65.)

Fig. 12. Single-ion tracing of PZA and its metabolites in the urine of a patient receiving PZA. Nicotinamide (1) is the internal standard. Concentrations not listed. (Reproduced with permission from ref. 65.)

streptomycin. The components were visualized with α-naphthol-diacetyl reagent and < 20 μg of samples were observable. SM and dihydrostreptomycin's interactions were studied on *DNA-cellulose columns* [131]. Dihydrostreptomycin and methyl green were initially retained on this column and later eluted with increasing sodium chloride gradient whereas SM was not eluted from this column; the nature of the binding of dihydrostreptomycin was ascribed to electrostatic forces. Interference studies were not performed in any of the above listed studies.

4.7. Kanamycin

Benjamin et al. [132] have quantitated KNM (as a mixture) in urine and serum samples using 2-chloro-4-nitrobenzo-2-oxa-1,3-diazole as the fluorescent reagent. Samples less than 10 μg/ml were measurable and good correlation was

obtained between this *TLC* procedure and a microbiological method from serum. Glycine, tobramycin and gentamicin did not interfere with assay results and the time required to process one sample was approximately 60 min.

Kanamycins have been separated by *classical column chromatography* by Rothrock et al. [133] who separated KNM-A, -B and -C on a 1.2-m strong anion-exchange resin column. The procedure was later improved by Inouye and Ogawa [134] with a strong anion-exchange column of 39 cm length requiring 3.5 h for complete separation of the components. Detection limits were not specified, yet the KNM isomers were well resolved from interferences such as neamine, neomycin B and C, and paromomycin I and II. Bryant et al. [135] analyzed KNM in urine, plasma, kidney and cochlear perilymph of rats by ion-exchange chromatography; following protein separation the samples were passed through a CM-Sephadex C-25 resin and KNM eluted with a buffer of 0.2 *M* sodium sulfate—0.01 *M* sodium hydroxide. The eluate was derivatized with fluorescamine and quantitated fluorometrically which method was capable of detecting 80—100 ng/ml KNM (KNM-A, -B and -C were not separated but rather detected as a combined mixture). Although fluorescamine

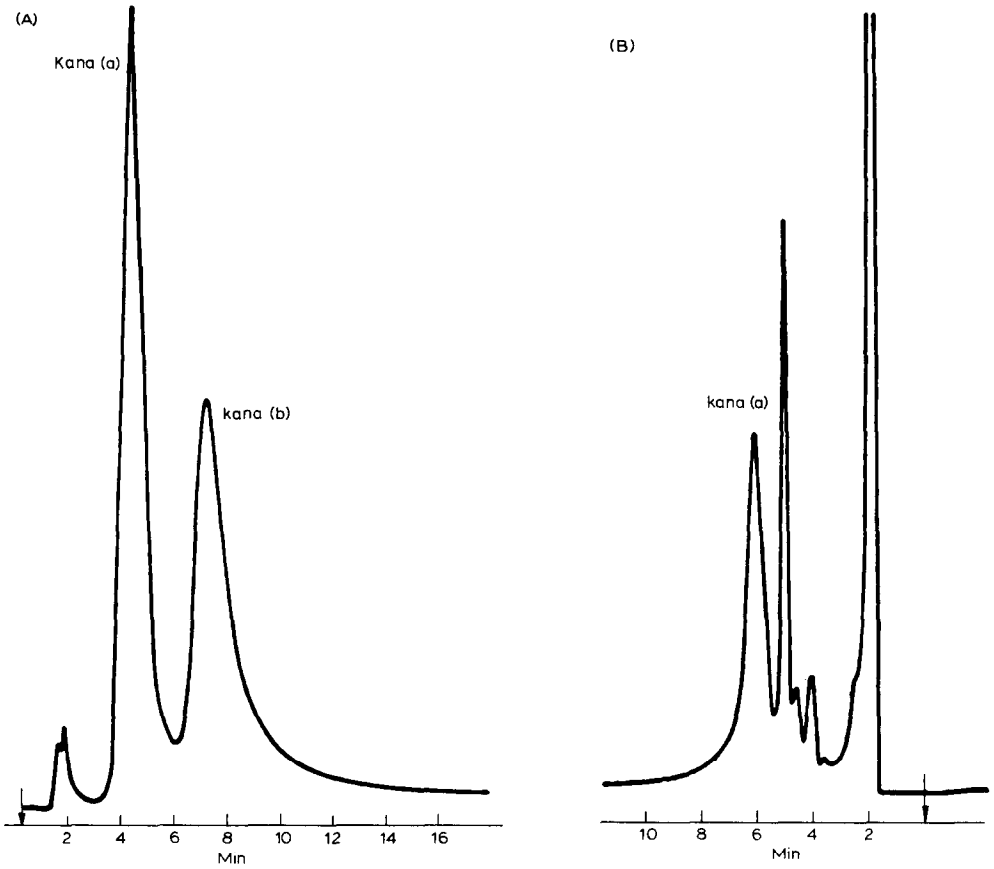


Fig. 13. (A) Typical chromatogram showing separation of kanamycins (KANA) A and B at high levels. (B) Chromatogram of kanamycin process samples. Column, HS Pellionex SCX. Detection, fluorescence with *o*-phthalaldehyde. Mobile phase, 0.01 *M* K^+EDTA (pH 9.5). Concentrations not listed. (Reproduced with permission from ref. 137.)

is not selective and reacts with primary amines, the protein precipitation and chromatographic separation isolates KNM sufficiently from other primary amines such as amino acids in the fluids and tissues studied to provide selectivity for this assay.

HPLC methods have also been utilized for quantitation of KNM from human sera [136] and fermentation broths [137]. By the former method, cefaperazone was quantitated in the presence of penicillins and aminoglycosides (including KNM) which could potentially be coadministered with this cephalosporin. Separation was effected via a 10-μm μBondapak C_{18} column with UV detection. Since the assay was specifically designed for cefaperazone, few specifics were given concerning the quantitation of KNM; regardless, the potential exists for using the assay to the advantage of analyzing KNM in human serum and urine.

Mays et al. [137] have developed a fast and selective HPLC technique for determination of KNM-A and KNM-B from processed fermentation samples. Resolution was accomplished on a HS Pellionex strong cation-exchange column with a mobile phase of 0.01 *M* EDTA at pH 9.0—9.5. Post-column derivatization was achieved with *o*-phthalaldehyde and the components monitored via fluorescence detection. Fig. 13A presents a typical chromatogram of the resolution of KNM-A from KNM-B whereas Fig. 13B demonstrates a chromatographic separation of KNM process samples. Chromatographic analysis required approximately 15 min per sample and the detection limit of KNM-A was < 20 ng. Reproducibility of the chromatographic system was $\pm 1\%$ based upon repetitive injections of standards. Processed samples were assayed for KNM by the HPLC procedure and by a biological turbidimetric method. Positive correlation obtained from 24 samples over several weeks revealed the HPLC assay to be approximately 6% lower than the bioassay. Whether this difference was due to small amounts of KNM-B present, possible interferences in the bioassay or some other factor was not determined. The samples analyzed, however, did not demonstrate measurable amounts of KNM-B.

As far as this author can ascertain, usable *GC* or *GC—MS* methods have not been developed for determination of this component in biological samples.

4.8. Ethionamide

HPLC determination of ETA and its sulfoxide metabolite from urine and plasma has been performed using PTA as internal standard [66,138]. Following extraction of the component with organic solvents, the samples were resolved on a 10-μm μPorasil silica column with a mobile phase of diethyl ether—methanol (24:1) and monitored via UV detection at 295 nm. A typical chromatogram of the plasma extract obtained 2 h following ingestion of 500 mg ETA is shown in Fig. 14. The detector response was linear over a 500-fold range and the practical limit of detection was 10 ng/ml. Compounds such as isonicotinamide, sulfoxide metabolites of ETA and PTA, clofazimine, EMB, INH, ACINH, isonicotinic acid, PAS, RIF, SM and TAZ did not affect the assay; however, dapsone (which coeluted with ETA) and PZA (which coeluted with the internal standard) were found to interfere with the assay results and, therefore, should not be concurrently administered when estimating ETA or

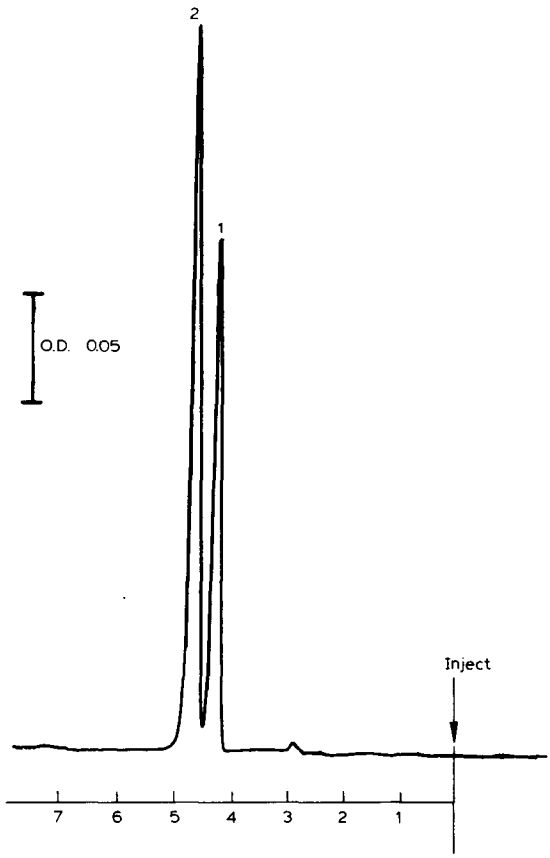


Fig. 14. Chromatogram of an extract of plasma from a volunteer 2 h after ingestion of 500 mg ethionamide. Peaks: 1 = prothionamide (internal standard); 2 = ethionamide. Concentrations not listed. (Reproduced with permission from ref. 66.)

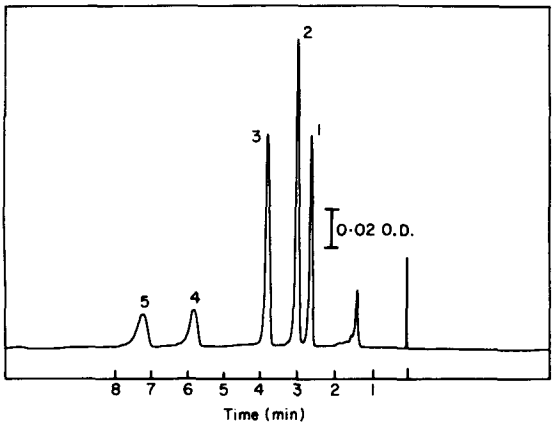


Fig. 15. Chromatogram of an extract of plasma from a volunteer 4 h after ingestion of a combined dose of 250 mg ethionamide plus 250 mg prothionamide. Peaks: 1 = prothionamide; 2 = ethionamide; 3 = 2-methylthioisonicotinamide (internal standard); 4 = prothionamide sulfoxide; 5 = ethionamide sulfoxide. Concentrations not listed. (Reproduced with permission from ref. 138.)

PTA by this method. Jenner et al. [138] later modified the above procedure to include analysis of ETA, PTA and the sulfoxide metabolites of each of these compounds using 2-methylthioisonicotinamide as internal standard. When both thioamides and their metabolites were being determined, chloroform was used for extraction instead of diethyl ether (as in the former method which extracts only the unchanged drugs). Separation was effected with a 5-μm Hypersil spherical column with a mobile phase consisting of chloroform—propan-2-ol—water (229:20:1) using UV detection. A typical chromatogram of this extract from plasma is seen in Fig. 15 requiring only 8 min for complete analysis. The obvious advantage of the latter technique is that of simultaneously quantitating four components as opposed to two in approximately the same length of time. This assay was applied for pharmacokinetic analysis of these drugs in man.

A *GC* method has also been utilized for estimating ETA or PTA in serum samples [90]; again, one drug can be used as an internal standard for the other. The components were extracted into diethyl ether and derivatization was not required for thermal stability or detection. Separation was effected on a 3% OV-225 column with FID and a linear relationship of test drug to internal standard was obtained in the range 0.2—5 μg/ml. The sulfoxide metabolites were not detectable and interferences from other anti-tuberculosis drugs were not studied.

4.9. Cycloserine

Few useful procedures have been developed for quantitation of CYS in biological media. A *paper chromatographic* technique was used to quantitate the drug in human urine following collection after oral dosing [139]. It required 4 h to develop the samples utilizing a solvent system of *n*-butanol—acetic acid—water (4:1:5) and the final product was developed with sodium nitroprusside forming a blue complex. Little quantitative data are given concerning detection limits or interferences; however, the technique was applied to determine the quantity of CYS excreted and the percentage change to unknown metabolized products.

Sondack et al. [140] utilized *GC*—FID and *GC—MS* for identification and quantitation of this drug. The samples were derivatized with BSTFA and TMCS, and hexamethylbenzene was the internal standard. The stationary phase was 3.8% UC-W98 held isothermally at 115°C and the retention times of CYS and internal standard were 2.8 and 4.1 min, respectively. A typical chromatogram is presented in Fig. 16 in which A is a derivatization by-product, B is N,N′-bis(trimethylsilyl)-CYS and C is the internal standard. Detection limits were not specified; however, the method was precise to within ± 0.5%. This method has yet to be applied for quantitation of the levels of this drug that exist in a biological system.

4.10. Capreomycin

Black et al. [91] have utilized a *paper chromatographic* technique for quantitation of the unaltered drug in the urine of ten patients following i.m. injection. However, few specifics were given concerning the solvent system,

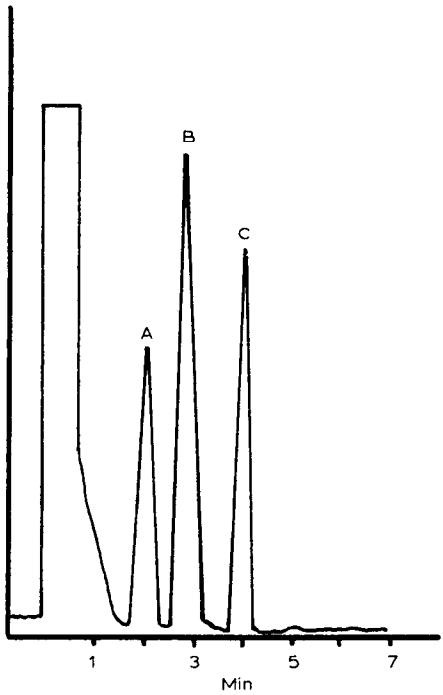


Fig. 16. Typical gas chromatogram of trimethylsilyl-derivatized cycloserine. Peaks: A = derivatized by-products; B = N,N'-bis(trimethylsilyl)cycloserine; C = hexamethyl benzene (internal standard). Concentrations not listed. (Reproduced with permission from ref. 140.)

detection limit or method of detection. An additional study was performed using paper chromatography to resolve the four components of CAM (IA, IB, IIA, IIB) from fermentation broths; yet again few specifics were given concerning the methodology [141].

4.11. Viomycin

VOM has been analyzed by an enzyme-coupled immunoassay by Kitagawa et al. [142]. This compound was converted to N-β-monoacetylviomycin and isolated by Sephadex column chromatography. The derivative was reacted with bovine serum albumin and separated via disc electrophoresis to give the bovine serum albumin N-β-acetylviomycin conjugate with an estimated molecular weight of 74 000. Competitive bindings of VOM and conjugate to rabbit antiserum of VOM were performed. Utilizing the antiserum and the chromatographically purified VOM enzyme conjugate, the detection limit was listed as 4 ng. It is unlikely investigators will see much about this compound in the future concerning anti-tuberculosis therapy since the drug has recently been discontinued from the market.

4.12. Thiacetazone

Thus far only a single *HPLC* method [143] has been developed for quantitation of TAZ in human samples following oral dosing. The method has been

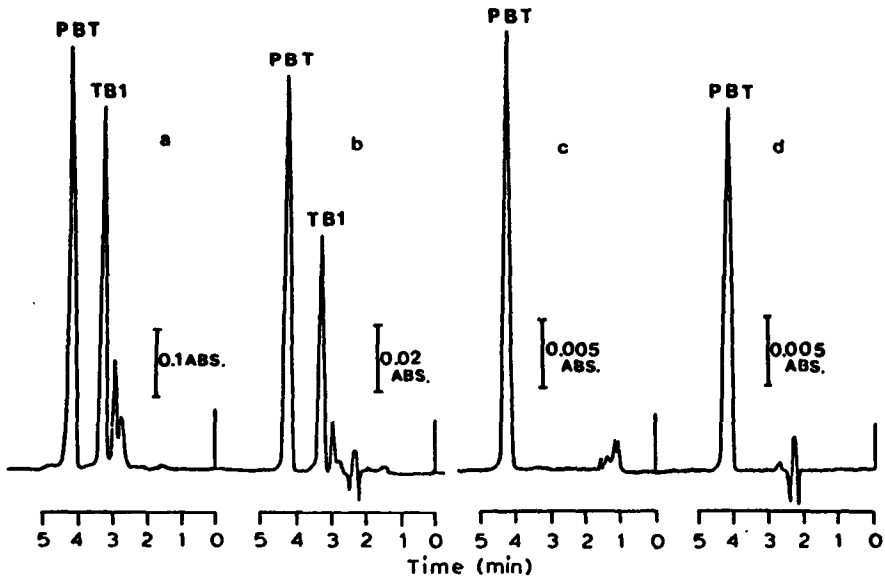


Fig. 17. (a) Chromatogram of an extract of a urine sample from a volunteer obtained 71—73 h after ingestion of the final (sixth) dose of 150 mg thiacetazone (TB1); 6 μg PBT were added as internal standard. (b) Chromatogram of an extract of a plasma sample obtained 72 h after ingestion of 150 mg thiacetazone; 1.5 μg PBT were added as internal standard. (c) Chromatogram of an extract of a pretreatment urine sample; 0.3 μg PBT has been added to the sample. (d) Chromatogram of an extract of a pretreatment plasma sample; 0.3 μg PBT has been added as internal standard. (Reproduced with permission from ref. 143.)

applied for measuring this compound after a 150-mg dose in urine, plasma and feces. TAZ can be extracted from urine with ethyl acetate and the organic layer dried and the residue taken up in the mobile phase. In the case of plasma and feces samples, in order to minimize the extraction of lipophilic components that may cause rapid deterioration of the analytical column, the dried residues were extracted in mobile phase and 2% ethanol in hexane and the centrifuged aqueous layer was injected onto the column. The analysis of TAZ in urine and plasma extracts using 4-propionylaminobenzaldehyde thiosemicarbazone (PBT) as internal standard [143,144] as outlined in Fig. 17a—d. Resolution was achieved on a 10-μm μBondapak C_{18} column with acetonitrile—water (3:7) as mobile phase. The detection limit was 3 ng/ml and solutions of 100 μg/ml EMB, INH, ACINH, isonicotinic acid, PAS, PZA, RIF, SM, ETA, PTA, clofazimine and dapsone did not interfere with the method.

5. MISCELLANEOUS DRUGS AND ASSAYS

Over the years a number of drugs have been investigated in tuberculosis treatment programs with or without success. Amikacin has been used in combination with other agents to suppress cross resistance and a number of HPLC assays have been developed for quantitation with either UV or fluorometric detection [145—147]. Wong et al. [145] analyzed this drug in human serum on either silica or reversed-phase columns; 1-fluoro-2,4-dinitrobenzene was used for pre-column derivatization and the detection limit by UV monitoring at 360 nm was 2 μg/ml. Maitra et al. [146] derivatized the drug with *o*-phthal-

aldehyde and resolved the compound from serum components on a silica column. The detection limit was 1.0 μg/ml and a comparison of this method with that of a bioassay for five patients revealed variation in the two procedures of < 10%.

PTA, the propyl analogue of ETA, has been used for tuberculosis chemotherapy although the drug has been found to be more appropriate for treatment of leprosy. Rossi and Rübsamen [148] utilized TLC for separation of PTA and its sulfoxide metabolite in physiological fluids using UV absorbance photometry for detection. Plasma or urine samples (following oral administration of drug) were extracted into ethyl acetate and applied to silica gel 60F plates and separated with a solvent system of acetic acid—acetone—methanol—benzene (1:1:4:14) in 45 min. A developing reagent was not required and detection limits were 0.75 μg for both components. Recovery from both plasma and urine was approximately 98% and the inter-assay reproducibility was within 4.5%. Interferences were not noted from plasma or urine, yet contributions from other anti-tuberculosis drugs were not specified.

A number of HPLC procedures have been developed for PTA [66,138] of which chromatograms have been presented in Figs. 14 and 15 and previously discussed in section 4.8. However, Peters et al. [149] have recently described an HPLC assay for PTA and its sulfoxide metabolite in rat and armadillo plasma. The samples were extracted into chloroform; an internal standard was not used. Separation was effected with a 5-μm LiChrosorb SI-60 column with a mobile phase of chloroform—methanol—water (187:14:1) and monitored at 340 nm. Recoveries were 97% and 88% for PTA and its metabolite, respectively, with a detection limit of 10 ng/ml for each component. Other drugs such as 2-propylisonicotinamide, ETA, dapsone and monoacetyldapsone did not interfere with the assay.

6. CONCLUSIONS

Quantitative methods for anti-tuberculosis drugs have been described in detail along with features that affect the concentration of these agents in various biological matrices. Two important aspects which will be considered for proper quantitation of these components along with selected examples are (1) separation and interferences from the biological matrix and (2) reliability of chromatographic techniques as compared with the classical methods for measuring these components in various biological tissues. Drug interactions and their effect upon the blood levels of these agents have been previously discussed elsewhere [50].

In consideration of the drug to be selected for analysis, the investigator should also ponder the potential problems of the biological matrix from which the components will be extracted. As an example, in the case of analysis of serum containing INH via a microbiological assay, INH may completely deteriorate during the incubation period thus casting doubt upon the final results [73]. It is well known that controversy exists over the stability of this drug in non-deproteinized frozen samples and various investigators have suggested methods for extraction before irreversible protein binding or decomposition to other metabolites occur. Pre-extraction may well lead to more reliable data con-

cerning tissue levels of the agent. Therefore, stability studies should be performed in order to determine the length of time and types of media in which samples can be stored without degradation. Extraction systems should be devised to clean up the sample to avoid interferences. It was noted that the $t_{1/2}$ of PAS was 93% longer by a fluorometric over that of a spectrophotometric method of which TLC demonstrated this increase to be due to the additional extraction of metabolites via the organic solvent. Thus, selective sample extraction should lead to better reproducibility of the method in addition to enrichment of the components for analysis. Another example consists of components in tissues which interfere with analysis of the compound in question via masking or quenching the response. Urinary glucose was demonstrated to interfere with estimation of ACINH, yet selective extraction with *n*-butanol—chloroform (3:7) cleaned up the samples to such an extent that equivalent recoveries of the metabolite were obtainable in the presence or absence of glucose [74]. Interferences with various sugars and glycoproteins have given false values of SM in serum, yet the addition of *m*-periodate-sulfuric acid precipitated proteins and other endogenous serum components which lead to inaccurate estimation of the drug in this specimen [86]. Selective chloroform solvent extraction of radiolabeled ETA and PTA has lead to the exclusion of the quantitation of radiolabeled sulfoxide metabolites [90]. By the same token, diethyl ether extracted both of these drugs and metabolites and HPLC allowed for simultaneous separation and detection of all four components in one chromatographic run [138]. In another study it was found that dapsone coeluted with ETA as did PZA with the internal standard via HPLC; therefore, simultaneous administration of these drugs would have to be avoided or taken into consideration for accurate quantitation. Thus, the biological matrix possesses problems for proper analytical assessment of drug content which requires careful consideration for extraction procedures and sample stability.

Methods are generally devised or selected for use according to the investigator's expertise and possible prior experience with non-chromatographic or chromatographic techniques. Therefore, of the chromatographic procedures available major consideration must be given to the reliability, selectivity and reproducibility among the various instrumental techniques and their comparison in different tissues. As an example three different HPLC procedures by

TABLE 5

CONCENTRATIONS OF INH AND ACINH FOLLOWING 300 mg OF ORAL INH (ALL VALUES IN μg/ml)

Time* (h)	Serum (n = 1) [58]		Plasma (n = 1) [97]		Plasma (n = 4) [100]	
	INH	ACINH	INH	ACINH	INH	ACINH
2.0	7.4	1.4	2.92	1.41	–	–
3.0	–	–	–	–	4.23	2.66
4.0	–	–	1.50	2.09	–	–
4.5	4.0	2.0	–	–	–	–
6.0	–	–	0.97	0.61	2.01	2.39

*Time after an oral dose of 300 mg INH.

three different research laboratories [58,97,100] are compared for the analysis of serum and plasma INH and ACINH content as seen in Table 5. Although not studied under optimum conditions by analyzing the same extract in the same laboratory using each different set of HPLC conditions, the results are consistent in which similar values for INH and ACINH are found by the three methods, thus demonstrating inter-laboratory reproducibility of data obtained by different methods to some degree. As previously presented in Fig. 3, a linear correlation of $r = 0.944$ was found in plasma RIF levels between an HPLC method and that of a microbiological assay [77]. Comparison of RIF levels from rat plasma by the same two techniques revealed a correlation of $r = 0.918$ demonstrating that the two methods again yield essentially identical results within experimental error [77]. In another study, a TLC method [78] was contrasted with a bioassay for serum RIF content yielding a mean regression coefficient of 1.02 ± 0.03 (S.E.) indicating excellent agreement between the non-chromatographic and chromatographic techniques.

Different chromatographic assays have been compared for analysis of EMB in plasma samples of which the results are presented in Table 4. It was found that regardless of the technique chosen or internal standard used (GC—ECD or GC—MS) that similar results were obtainable [82].

The last example consist of the comparison of an HPLC method for KNM-A with that of a bioassay for the same [76]. The HPLC method was approximately 6% lower than the bioassay which could possibly be accounted for by the presence of KNM-B although this was never confirmed [76].

Thus, chromatographic methods offer similar reproducibility with non-chromatographic methods, similar sensitivities and generally better specificity for quantitation for the compounds in question in biological fluids.

7. ACKNOWLEDGEMENTS

The author wishes to express his appreciation to the editors and authors of the following articles who provided permission and photographs for the figures reproduced in this manuscript: (1) Proceedings of the Western Pharmacological Society, Drs. J.H. Peters, G.R. Gordon, J.F. Murray, W. Ichikawa, R.H. Gelber, T.M. Welch and C.R. Goucher (ref. 77); (2) Biomedicine, Drs. C. Aucher, C. Pasquier, P. Pehuet and F. Delbarre (ref. 127); (3) Journal of Chromatography, Drs. J.B. Lecaillon, N. Febvre, J.P. Metayer and C. Souppart (ref. 60); P.J. Jenner and G.A. Ellard (ref. 66); I.L. Honigberg, J.T. Steward, T.C. Clarke and D.Y. Davis (ref. 125); P.J. Jenner (ref. 143); B.H. Lauterburg, C.V. Smith and J.R. Mitchell (ref. 106); J. Roboz, R. Suzuki and T.F. Yu (ref. 65); D.L. Mays, R.J. Van Apeldoorn and R.G. Lauback (ref. 137); (4) Journal of Pharmaceutical Sciences, Drs. D.L. Sondack, F.E. Gainer and H.J. Wesselman (ref. 140); and (5) Journal of Antimicrobial Chemotherapy, Drs. P.J. Jenner, G.A. Ellard, P.J.K. Gruer and V.R. Aber (ref. 138).

8. SUMMARY

Numerous chromatographic and non-chromatographic methods of analysis for anti-tuberculosis drugs and metabolites in biological tissues have been dis-

cussed in this review. Depending upon the analytical methodology selected, limits of detection range from microgram to picogram levels. A number of examples have been given of the correlation between different types of assay procedures. The metabolism and pharmacokinetics have been described along with some of the commonly associated problems of sample collection and storage.

REFERENCES

1 R. Dubos and J. Dubos, The White Plague. Tuberculosis, Man and Society, Little, Brown and Company, Boston, MA, 1952.
2 D. Morse, D.R. Brothwell and J.P. Ucko, Amer. Rev. Tuberc., 90 (1964) 524.
3 R. Koch, Berl. Klin. Wochenschr., 19 (1882) 221.
4 L.B. Reichman, Chest, 76 (1979) 737.
5 Tuberculosis - United States 1983, Morb. Mort. Week. Report, 33 (1984) 77.
6 K.E. Powell, D. Brown and L.S. Farer, J. Amer. Med. Assoc., 249 (1983) 1455.
7 G.P. Youmans, Tuberculosis, Saunders, Philadelphia, PA, 1979.
8 V.C. Barr, Chemotherapy of Tuberculosis, Butterworth, London, 1964.
9 W.H. Feldman and H.C. Hinshaw, Proc. Mayo Clin., 19 (1944) 593.
10 J. Bernstein, W.A. Lott, B.A. Steinberg and H.L. Yale, Amer. Rev. Tuberc., 65 (1952) 357.
11 H.H. Fox, Science, 116 (1952) 129.
12 H.A. Offe, W. Siefkin and G. Domagk, Z. Naturforsch., 7b (1952) 462.
13 S.M. Aquinas, Med. Prog., 71 (1982) 79.
14 W.C. Bailey, R.K. Albert, P.T. Davidson, L.S. Farer, J. Glassroth, E. Kendig, Jr., R.G. Loudon and L.S. Inselman, Amer. Rev. Respir. Dis., 127 (1983) 790.
15 P.E. Hermans, Proc. Mayo Clin., 58 (1983) 3.
16 W. Fox, A.J. Stark, R. Tall, J.L. Bhatia, J.H.C. Clarke, T.O. Donea, K.V. Krishnaswani and N. Oussedik, Tubercle, 55 (1974) 29.
17 D.J. Girling, Drugs, 23 (1982) 56.
18 C.S. Lee, T.C. Marbury and L.Z. Benet, J. Pharmacokin. Biopharm., 8 (1980) 69.
19 C.S. Lee, D.C. Brater, J.G. Gambertoglio and L.S. Benet, J. Pharmacokin. Biopharm., 8 (1980) 335.
20 C.S. Lee, J.G. Gambertoglio, D.C. Barter and L.Z. Benet, Clin. Pharmacol. Ther., 22 (1978) 615.
21 W.W. Weber and D.H. Hein, Clin. Pharmacokin., 4 (1979) 401.
22 E.A. Peets, W.M. Sweeney, V.A. Place and D.A. Buyske, Amer. Rev. Respir. Dis., 90 (1964) 51.
23 W.A. Olsen, P.G. Dayton, Z.H. Israili and A.W. Pruitt, Clin. Chem., 23 (1977) 745.
24 C. Advenier, C. Gobert, G. Houin, D. Bedet, S. Richelet and J.P. Tillement, Ther. Drug Monitor., 5 (1983) 61.
25 G. Houin, A. Beucler, S. Richelet, R. Brioride, C. Lafaix and J.P. Tillement, Ther. Drug Monitor., 5 (1983) 67.
26 B. Ratti, A. Toselli, F. Beretta and A. Bernareggi, Farmaco Ed. Sci., 37 (1982) 226.
27 H. Held and F. Fried, Chemotherapy, 23 (1977) 405.
28 P.F. Wright, A.B. Kaiser, C.M. Brown, K.T. McKee, H. Trujillo and Z.A. McGee, J. Infect. Dis., 143 (1980) 141.
29 E.A. Egan, G. Mantilla, R.M. Nelson and D.V. Eitzman, J. Pediatr., 89 (1976) 467.
30 G.A. Ellard, Clin. Pharmacol. Ther., 19 (1976) 610.
31 H.E. Boxenbaum and S. Riegelman, J. Pharmacokin. Biopharm., 4 (1976) 287.
32 G.A. Ellard and P.T. Gammon, J. Pharmacokin. Biopharm., 4 (1976) 83.
33 W.W. Weber, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 43 (1984) 2332.
34 J.M. Robson and F.M. Sullivan, Pharmacol. Rev., 15 (1963) 169.
35 J.C. Bell, D.K. Riemenschnider and N.S. Mitchell, Amer. Rev. Tuberc., 76 (1957) 152.
36 A. Hanngren, O. Borga and F. Sjoqvist, Scand. J. Respir. Dis., 51 (1970) 61.

37 A. Dansyz and K. Wisniewski, Mater. Med. Polon., 2 (1970) 35.
38 P.D. Hansten, Drug Interact. Newslett., 3 (1983) 7.
39 W. Zilly, D.D. Breimer and E. Richter, Clin. Pharmacokin., 31 (1976) 95.
40 U. Gundert-Remy, M. Klett and E. Weber, Eur. J. Clin. Pharmacol., 6 (1973) 133.
41 T.F. Yu, J. Perel, L. Berger, J. Roboz, Z.H. Israili and P.G. Dayton, Amer. J. Med., 63 (1977) 723.
42 I.M. Weiner and J.P. Tinker, J. Pharmacol. Exp. Ther., 180 (1972) 411.
43 E.L. Way, P.K. Smith, D.L. Howie and R. Weiss, J. Pharmacol. Exp. Ther., 93 (1948) 368.
44 G.L. Mandell and M.A. Sande, in L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, p. 1200.
45 A. Breder, P. Brumel and L. Mazeau, Ann. Pharmacol. Fr., 24 (1966) 493.
46 P.K. Sen, R. Chatterjee, J.R. Saha and H.S. Roy, Ind. J. Med. Res., 62 (1974) 557.
47 Y. Asahi, Chem. Pharm. Bull. Tokyo, 11 (1963) 1241.
48 M.A. Sande and G.L. Mandell, in L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, p. 1162.
49 P.B. Storey and R.L. McLean, Antibiot. Med. Clin. Ther., 4 (1957) 223.
50 M.R. Holdiness, Clin. Pharmacokin., 9 (1984) 511.
51 N.F. Poole and A.E. Meyer, Proc. Soc. Exp. Biol. Med., 104 (1960) 560.
52 J.W. Jeane, Amer. Rev. Respir. Dis., 83 (1961) 906.
53 J.W. Jeane, E. Lapinski, J. Dexter and W.H. Hall, Amer. Rev. Respir. Dis., 86 (1962) 567.
54 W.C. Morse, J.M. Lacerte, N.J. Pickering and L.R. Kuhn, Amer. Rev. Respir. Dis., 86 (1962) 564.
55 J.H. Peters and R.C. Good, Amer. Rev. Respir. Dis., 86 (1962) 573.
56 T.P. O'Barr, D.J. Keith and E.B. Blair, Amer. Rev. Respir. Dis., 107 (1973) 472.
57 J.N. Miceli, W.A. Olsen and W.W. Weber, Biochem. Med., 12 (1975) 348.
58 M.A. Moulin, F. Albessard, J. Lacotte and R. Camsonne, J. Chromatogr., 226 (1981) 250.
59 J.A. Timbrell, J.M. Wright and T.A. Baillis, Clin. Pharmacol. Ther., 22 (1977) 602.
60 J.B. Lecaillon, N. Febvre, J.P. Metayer and C. Souppart, J. Chromatogr., 145 (1978) 319.
61 E.H. Girgis, Z. Gad and M. Maran, J. Pharm. Sci., 63 (1974) 1764.
62 J.N. Miceli, D.M. Ryan and A.K. Done, Chromatogr. Newslett., 7 (1979) 9.
63 C.S. Lee and L.Z. Benet, J. Chromatogr., 128 (1976) 188.
64 M.R. Holdiness, unpublished results.
65 J. Roboz, R. Suzuki and T.-F. Yü, J. Chromatogr., 147 (1978) 337.
66 P.J. Jenner and G.A. Ellard, J. Chromatogr., 225 (1981) 245.
67 C.R. Pasqualucci, A. Vigevani, P. Radaelli and G.G. Gallo, J. Pharm. Sci., 59 (1970) 683.
68 C. Fischer, K. Maier and U. Klotz, J. Chromatogr., 225 (1981) 498.
69 G.A. Ellard, J.M. Dickerson, P.T. Gammon and D.A. Mitchison, Tubercle, 55 (1974) 41.
70 J.H. Hash, Meth. Enzymol., 43 (1975) 466.
71 L.R. Jones, Anal. Chem., 28 (1956) 39.
72 J. Lloyd and D.A. Mitchison, J. Clin. Pathol., 17 (1964) 662.
73 L. Eidus and A.M.T. Harnanansingh, Clin. Chem., 17 (1971) 492.
74 G.R. Sarma, C. Immanuel, S. Kailasam, M. Kannapiran, N.G.K. Nair and S. Radhakrishna, Ind. J. Med. Red., 62 (1974) 945.
75 H. Iwainsky, K. Winsel, E. Werner and H. Eule, Scand. J. Respir. Dis., 55 (1974) 229.
76 G. Acocella, R. Mattiusi and G. Segre, Pharmacol. Res. Commun., 10 (1978) 271.
77 J.H. Peters, G.R. Gordon, J.F. Murray, W. Ichikawa, R.H. Gelber, T.M. Welch and C.R. Goucher, Proc. West. Pharmacol. Soc., 20 (1977) 221.
78 R.P. Mouton, H. Mattie, K. Swart, J. Kreukniet and J. deWael, J. Antimicrob. Chemother., 5 (1979) 447.
79 V.A. Place and J.P. Thomas, Amer. Rev. Respir. Dis., 88 (1963) 901.
80 P.R.J. Gangadharman and E.R. Candler, J. Antimicrob. Chemother., 3 (1977) 57.

81 I. Strauss and F. Erhardt, Chemotherapy, 15 (1970) 148.
82 R.H. Liss, R.J. Letourneau and J.P. Schepis, Amer. Rev. Respir. Dis., 123 (1981) 529.
83 M.J. Mattila, E. Nieminen and H. Tiitinen, Scand, J. Respir. Dis., 50 (1969) 291.
84 G.A. Ellard, Tubercle, 50 (1969) 144.
85 B. Jalling, A.S. Malmborg, A. Lindman and L.O. Boreus, Eur. J. Clin. Pharmacol., 4 (1972) 50.
86 E. Duda, L. Marton and G. Kiss, Biochem. Med., 15 (1976) 330.
87 S. Maier and H. Grisebach, Biochem. Biophys. Acta, 586 (1979) 231.
88 N. Buchanan and L.A. van der Walt, S. Afr. Med. J., 52 (1977) 522.
89 S.K. Maitra, T.T. Yoshikawa, L.B. Guze and M.C. Schotz, Clin. Chem., 25 (1979) 1361.
90 M.J. Colston, G.A. Ellard and P.T. Gammon, Lepr. Rev., 49 (1978) 115.
91 H.R. Black, R.S. Griffith and J.F. Brickler, Antimicrob. Agents Chemother., 18 (1975) 70.
92 A. Gyselen, L. Verbist, J. Prignot and J. Cosmans, Tubercle, 46 (1965) 243.
93 J.L. Leat and J. Marks, Tubercle, 51 (1970) 68.
94 M.J. Colston, G.R.F. Hilson, G.A. Ellard, P.T. Gammon and R.J.W. Rees, Lepr. Rev., 49 (1978) 101.
95 J.R. Mitchell, U.P. Thorgursson, M. Black, J.A. Timbrell, W.R. Snodgrass, W.Z. Plotter, D.J. Jollow and H.R. Keiser, Clin. Pharmacol. Ther., 18 (1975) 70.
96 S.J. Saxena, J.T. Stewart, I.L. Honigberg, J.G. Washington and G.R. Keene, J. Pharm. Sci., 66 (1977) 813.
97 M.R. Holdiness, J. Liquid Chromatogr., 5 (1982) 707.
98 M. Guillaumont, M. Leclercq, Y. Forbert, B. Guise and R. Harf, J. Chromatogr., 232 (1982) 369.
99 A. Hutchings, R.D. Monie, B. Spragg and P.A. Routledge, J. Chromatogr., 277 (1983) 385.
100 C. Lacroix, G. Laine, J.P. Goulle and J. Nouveau, J. Chromatogr., 307 (1984) 137.
101 A. Calò, C. Cardini and V. Quercia, J. Chromatogr., 37 (1968) 194.
102 M. Frater-Schroder and G. Zbinden, Biochem. Med., 14 (1975) 274.
103 J.A. Timbrell, J.M. Wright and C.M. Smith, J. Chromatogr., 138 (1977) 165.
104 R.M. DeSagher, A.P. DeLeenheer, A.E. Claeys and A.A. Cruyl, Biomed. Mass Spectrom., 2 (1975) 82.
105 A. Noda, T. Goromaru, K. Matsuyama, K. Sogabe, K.Y. Hsu and S. Iguchi, J. Pharm. Dyn., 1 (1978) 132.
106 B.H. Lauterburg, C.V. Smith and J.R. Mitchell, J. Chromatogr., 224 (1981) 431.
107 A. Noda, K.-Y. Hsu, Y. Aso, K. Matsuyama, S. Iguchi and N. Hirata, J. Chromatogr., 230 (1982) 345.
108 O.T. Kolos and L.L. Eidus, J. Chromatogr., 68 (1972) 295.
109 W.L. Wilson, K.C. Graham and M.J. Lebelle, J. Chromatogr., 144 (1977) 270.
110 A. Aszalos, J. Liquid Chromatogr., 7S (1984) 69.
111 J.A. Schmit, R.A. Henry, R.C. Williams and J.F. Dieckman, J. Chromatogr. Sci., 9 (1971) 645.
112 M. Gidoh, S. Tsutsumi and S. Takitani, J. Chromatogr., 223 (1981) 379.
113 R. Ratti, R.R. Parenti, A. Toselli and L.F. Zerilli, J. Chromatogr., 225 (1981) 526.
114 V. Vlasáková, J. Benès and K. Živný, J. Chromatogr., 151 (1978) 199.
115 V. Strind and B. Salvesen, Med. Nor. Farm. Selsk., 36 (1974) 44.
116 B.M. Richard, J.E. Manno and B.R. Manno, J. Chromatogr., 89 (1974) 80.
117 C.S. Lee and L.Z. Benet, J. Pharm. Sci., 67 (1978) 470.
118 C.S. Lee and A. Varughese, J. Pharm. Sci., 73 (1984) 787.
119 A.D. Blair, A.W. Forrey, T.G. Christopher, B. Maxwell and R.E. Cutler, Methodol. Dev. Biochem., 5 (1976) 231.
120 K. Ohya, S. Shintani and M. Sano, J. Chromatogr., 221 (1980) 293.
121 M.R. Holdiness, Z.H. Israili and J.B. Justice, Southeast. Southwest. Amer. Chem. Soc. Meet., 28 (1980) 100.
122 M.R. Holdiness, Z.H. Israili and J.B. Justice, J. Chromatogr., 224 (1981) 415.
123 S.H. Hansen, J. Chromatogr., 226 (1981) 504.

124 C. Fischer and U. Klotz, J. Chromatogr., 162 (1979) 237.
125 I.L. Honigberg, J.T. Stewart, T.C. Clark and D.Y. Davis, J. Chromatogr., 181 (1980) 266.
126 J.K. McClatchy, A.Y. Tsang and M.S. Cernich, Antimicrob. Agents Chemother., 20 (1981) 556.
127 C. Auscher, C. Pasquier, P. Pehuet and F. Delbarre, Biomedicine, 28 (1978) 129.
128 D. Pitre, R.M. Facino, M. Carini and A. Carlo, Pharmacol. Res. Commun., 13 (1981) 315.
129 H. Heding, Acta Chem. Scand., 24 (1970) 3086.
130 S. Maier, U. Matern and H. Grisebach, FEBS Lett., 49 (1975) 317.
131 A. Inagaki and M. Kageyama, J. Biochem., 68 (1970) 187.
132 D.M. Benjamin, J.J. McCormack and D.W. Gump, Anal. Chem., 45 (1973) 1531.
133 J.W. Rothrock, R.T. Goegelman and F.J. Wolf, Antibiot. Ann., 3 (1959) 121.
134 S. Inouye and H. Ogawa, J. Chromatogr., 13 (1964) 536.
135 G. Bryant, L. Glade, C. Norris, M. Tachibana and P. Guth, Res. Commun. Chem. Path. Pharmacol., 22 (1978) 155.
136 J. Dokladalova, G.T. Querica and J.P. Stankewich, J. Chromatogr., 276 (1983) 129.
137 D.L. Mays, R.J. van Apeldoorn and R.G. Lauback, J. Chromatogr., 120 (1976) 93.
138 P.J. Jenner, G.A. Ellard, P.J.K. Gruer and V.R. Aber, J. Antimicrob. Chemother., 13 (1984) 267.
139 P. Georgescu, E. Savuleanu and I. Daniello, Scand. J. Respir. Dis., 71S (1970) 63.
140 D.L. Sondack, F.E. Gainer and H.J. Wesselman, J. Pharm. Sci., 62 (1973) 1345.
141 E.B. Herr, M.O. Redstone, Ann. N.Y. Acad. Sci., 135 (1966) 940.
142 T. Kitagawa, T. Fujitake and H. Taniyama, J. Antibiot., 29 (1976) 1343.
143 P.J. Jenner, J. Chromatogr., 276 (1983) 463.
144 P.J. Jenner, G.A. Ellard and O.B. Swai, Lepr. Rev., 55 (1984) 121.
145 L.T. Wong, A.R. Beaubien and A.P. Pabuts, J. Chromatogr., 231 (1982) 145.
146 S.K. Maitra, T.T. Yoshikawa, C.M. Steyn, L.B. Guze and M.C. Schotz, Antimicrob. Agents Chemother., 14 (1978) 880.
147 J.P. Anhalt and S.D. Brow, Clin. Chem., 24 (1978) 1940.
148 M. Rossi and K. Rübsamen, J. Chromatogr., 132 (1977) 562.
149 J.H. Peters, G.R. Gordon and J.F. Murray, Jr., Int. J. Lepr., 51 (1983) 54.