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Therapeutic monitoring of antituberculosis drugs by direct in-line extraction on a high-performance liquid chromatography system

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ABSTRACT

A direct in-line pre-column extraction technique in which guanidinium and ammonium sulfate are used, followed by column switching, was employed to analyze serum, plasma and cerebrospinal fluid samples of patients treated for tuberculous meningitis. Resolution of a wide range of polar to non-polar xenobiotics was obtained on a C_8 silica column by using a linear gradient from a binary system consisting of solvent A (0.05 *M* KH_2PO_4) and solvent B (acetonitrile–isopropanol, 4:1, v/v). Apart from the antituberculosis drugs (isoniazid, pyrazinamide, ethionamide and rifampicin) the patients received up to sixteen different medicines for prevention of complications and the treatment of symptoms. Qualitative resolution of all the drugs was obtained by the chromatographic system. Quantitation of pyrazinamide and ethionamide was achieved with high precision and low inter-sample variation.

INTRODUCTION

We recently reported a novel approach to direct in-line extraction of xenobiotics from biological fluids [1]. After pre-treatment of the biological sample with a reagent consisting of 8.05 *M* guanidinium and 1.02 *M* ammonium sulfate, an aliquot is loaded onto an in-line extraction column which is pre-flushed with ammonium sulfate. The extraction column is post-flushed with ammonium sulfate whereafter the flow is diverted by column switching to pass through a C_8 analytical column. Universal high-performance liquid chromatographic (HPLC) conditions then result in separation of xenobiotics with a broad range of polarities. The guanidinium serves not only as a denaturant of protein binding sites, but also as a solvent for a wide range of xenobiotic molecules, with differing polarity, in a hydrophilic medium. The ammonium sulfate pre-flush prepares the C_{18} extraction column for hydrophobic retention of analytes and the post-flush step efficiently washes proteins and salts to waste prior to column switching.

The excellent resolution characteristics and quantitative capability of the method motivated

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its use in our routine pharmacology–toxicology laboratory for the analysis of samples containing a number of xenobiotics. It proved to be particularly useful for the analysis of anti-tuberculosis agents in children suffering from tuberculous meningitis. These patients receive many medicines simultaneously for the eradication of the microbes, the prevention of complications and the treatment of symptoms. In accordance with our major research interest it was possible, using the method, to identify and quantify isoniazid, pyrazinamide and ethionamide in 200-μl samples of either serum or plasma and cerebrospinal fluid (CSF) for the purposes of pharmacokinetic studies [2–4]. We wish to record our analytical findings in this paper.

EXPERIMENTAL

Chemicals and solvents

Isoniazid was purchased from Fluka (Buchs, Switzerland) and rifampicin as Rifadin injection from Mer National (Olifantsfontein, South Africa). Other xenobiotic standards were obtained from various pharmaceutical manufacturing companies or from Sigma (St. Louis, MO, USA). Whenever necessary chromatographic purity was achieved by recrystallization.

Acetonitrile and isopropanol were HPLC grade (LiChrosolv, E. Merck, Darmstadt, Germany). Ultrapure guanidinium (guanidine hydrochloride) was purchased from Bethesda Research Chemicals (Bethesda, MD, USA). Potassium dihydrogenphosphate and ammonium sulfate were analytical grade from Merck. HPLC-grade water was prepared from distilled, deionized water filtered through a Millipore system (Bedford, MA, USA).

Equipment and chromatographic conditions

A Hewlett Packard 1090L liquid chromatograph (Waldbron, Germany) equipped with an in-line extraction column and manual valve switching mechanism, as described by Kruger *et al.* [1], was used. A programmable filter photometer and reporting integrator (Hewlett Packard 3392) were used for detection and data recording. When necessary, similar instrumentation was used to obtain UV spectra with a diode-array detector. Separation of xenobiotics was achieved with a 10 μm pore size 250 mm × 4.6 mm I.D. Whatman Partisil C_8 column (Whatman, Maidstone, UK) maintained at 50°C. Gradient elution was achieved by using 0.05 *M* KH_2PO_4 (pH 4.5) as solvent A and acetonitrile–isopropanol (4:1, v/v) as solvent B. A flow-rate of 1.5 ml/min was used with 10% solvent B for the first minute, whereafter it was increased linearly to 70% solvent B at 16 min. The latter composition was maintained for a further 4 min until completion of the run at 20 min.

Sample preparation was as described by Kruger *et al.* [1]. A volume of 200 μl of either drug standard (dissolved in water or methanol) or biological fluid (serum, plasma or CSF) was mixed with 300 μl of pre-treatment reagent. The latter consists of 8.05 *M* guanidinium and 1.02 *M* ammonium sulfate solution in water.

Sample loading was initiated by a pre-flush of the in-line column with 500 μl of 0.5 *M* ammonium sulfate solution followed by injection of the sample and a post-flush with 500 μl of 0.5 *M* ammonium sulfate solution, Thereafter the six-port valve was switched to the analytical mode. The in-line extraction column (30 mm × 2.1 mm I.D.) was packed with 40-μm preparative-grade C_{18} bonded silica (Analytichem International, Harbor City, CA, USA).

RESULTS

Fig. 1 shows the resolution of antituberculosis drug standards dissolved in the pre-treatment reagent (upper part) and in drug-free serum (lower part). The identity of each drug, according to peak number and retention time, is indicated in Table I, as are the other drugs used in the treatment of the disease (*vide infra* Fig. 3).

It is clear from Fig. 1 that the resolution of isoniazid, pyrazinamide and ethionamide (peaks 1, 2 and 10, respectively) is adequate both in the pre-treatment reagent alone, as well as in serum. Rifampicin, however, split into two peaks in the pre-treatment reagent (peaks 15 and 17) and into

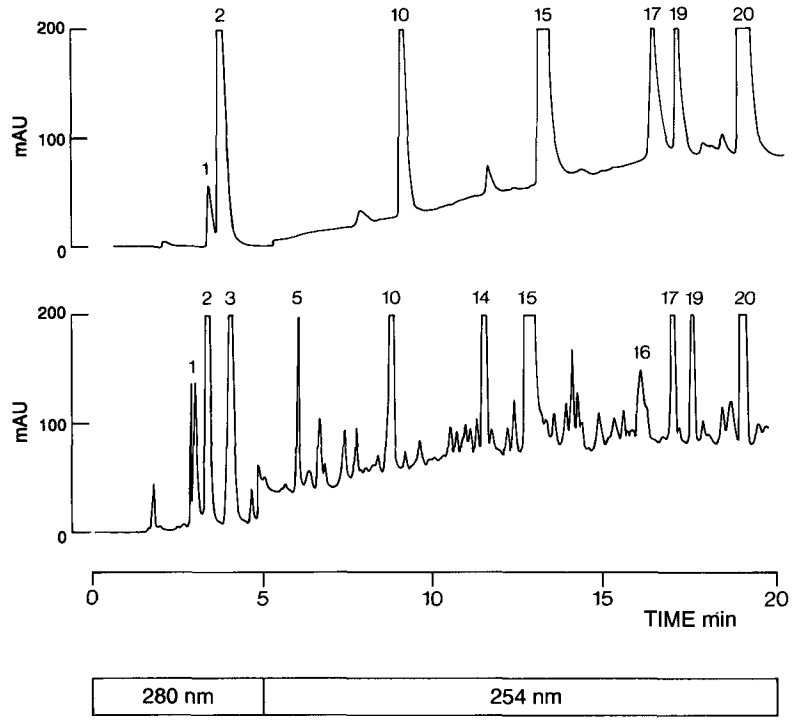

Fig. 1. Chromatograms of antituberculosis drug standards (isoniazid = 10 μg/ml, pyrazinamide = 15 μg/ml and ethionamide = 2.5 μg/ml) in pre-treatment reagent (upper part) and serum (lower part). The xenobiotics which correspond to each numbered peak are given in Table I together with their retention times. Detection was at 280 nm for the first 5 min of chromatography, whereafter it was changed to 254 nm.

TABLE I

IDENTITY OF DRUGS ACCORDING TO PEAK NUMBERS IN FIGS. 1 AND 3

Peak	Drug	Retention time (min)
1	Isoniazid	3.10
2	Pyrazinamide	3.49
3	Tryptophan	4.18
4	Chlorothiazide	5.43
5	Caffeine	6.16
6	Trimethoprim	6.75
7	Propoxyphene	6.96
8	Trimeprazine	7.53
9	Methadone	8.10
10	Ethionamide	8.93
11	Phenobarbitone	9.75
12	Chloramphenicol	10.52
13	Bromazepam	11.04
14	Substance "X"	11.57
15	Rifampicin	12.93
16	Rifampicin	16.28
17	Rifampicin	17.16
18	Diazepam	15.21
19	Prazepam	17.73
20	Heptanophenone	19.20

three peaks in serum (peaks 15, 16 and 17). This behaviour is assumed to be related to the known instability of the drug in serum [5]. Although analysis of rifampicin was not attempted because of its instability, stabilisation of the molecule [6] may make this possible.

Prazepam and heptanophenone (peaks 19 and 20, respectively) were added as internal standards. For monitoring patient samples heptanophenone was preferred (*vide infra* Fig. 3).

In chromatograms involving serum three additional peaks can be identified (lower part of Fig. 1): tryptophan, caffeine and substance "X" (peaks 3, 5 and 14, respectively). The latter is a substance commonly present in the sera of South African citizens and is thought to be of dietary origin. This is presently under investigation.

The baseline noise recorded in the serum-containing sample, shown in the lower part of Fig. 1, can be reduced considerably by switching to a lower attenuation. However, we prefer to show the most sensitive recording in order to demonstrate the presence of peak 16 of rifampicin. The

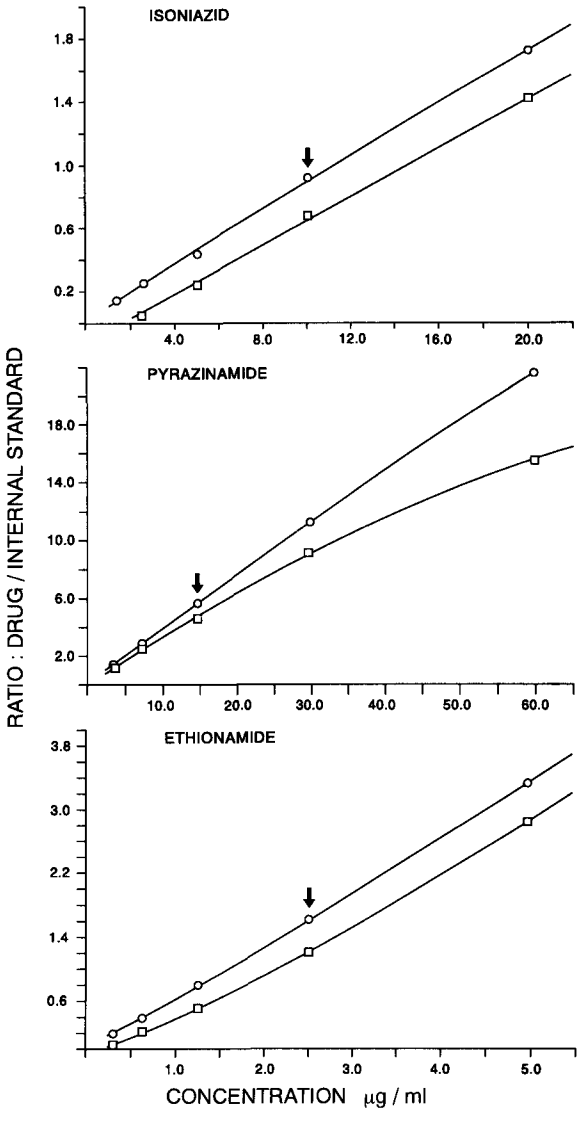

Fig. 2. Standard curves for isoniazid, pyrazinamide and ethionamide in water (○) and serum (□). The arrows indicate the concentrations used to obtain the chromatograms shown in Fig. 1.

latter has a similar UV absorption spectrum (determined by diode-array detection) to peaks 15 and 17 of rifampicin, also present in the relevant water standards.

Fig. 2 shows calibration curves for isoniazid, pyrazinamide and ethionamide of water standards and serum standards. In each of the graphs the arrows indicate the concentration of drug used to obtain the chromatogram as shown in Fig. 1. They are 10.0, 15.0 and 2.5 μg/ml for isoniazid, pyrazinamide and ethionamide, respectively, and fall approximately midway in the ranges obtained during therapy. Although the final recoveries are 10–15% lower in serum standards than in water standards for all three drugs, it is clear from Fig. 2 that quantitative relationships with r values >0.999 are obtained in concentration ranges expected to be achieved during treatment of the disease. The relatively lower extraction efficiency of pyrazinamide at a concentration of 60 μg/ml did not influence results significantly since the concentrations measured in most patient samples were found to be in the mid range [2], over which linearity prevailed. The inter-sample variation (n = 5) of values in water standards was 1.3% for isoniazid, 1.5% for pyrazinamide and 1.6% for ethionamide while the values in serum standards were 4.8, 1.9 and 2.1%, respectively.

Fig. 3 shows chromatograms of plasma and CSF taken from a patient with tuberculous meningitis. The identity and retention times of the peaks are shown in Table I. It is clear that in addition to the antituberculosis drugs (peaks 1, 2, 10, 15, 16 and 17 in the chromatogram) and tryptophan, caffeine and substance "X" (peaks 3, 5 and 14, respectively) at least ten other medicines given to the patient can be identified. The penetration of antituberculosis drugs across the blood–brain barrier is also demonstrated by their presence in the CSF. We have previously shown [1] a quantitative relationship for most of the drugs listed in Table I. Table II lists additional drugs which were identifiable in the serum of other patients with tuberculous meningitis. Comparison of analytical findings with prescription records of the patients confirmed the value of the method for identifying molecules with UV absorbance.

DISCUSSION

References in the literature to HPLC methods for the determination of antituberculosis drugs in body fluids are not rare. However, they generally deal with the extraction and determination of specific substances and, in the case of isoniazid,

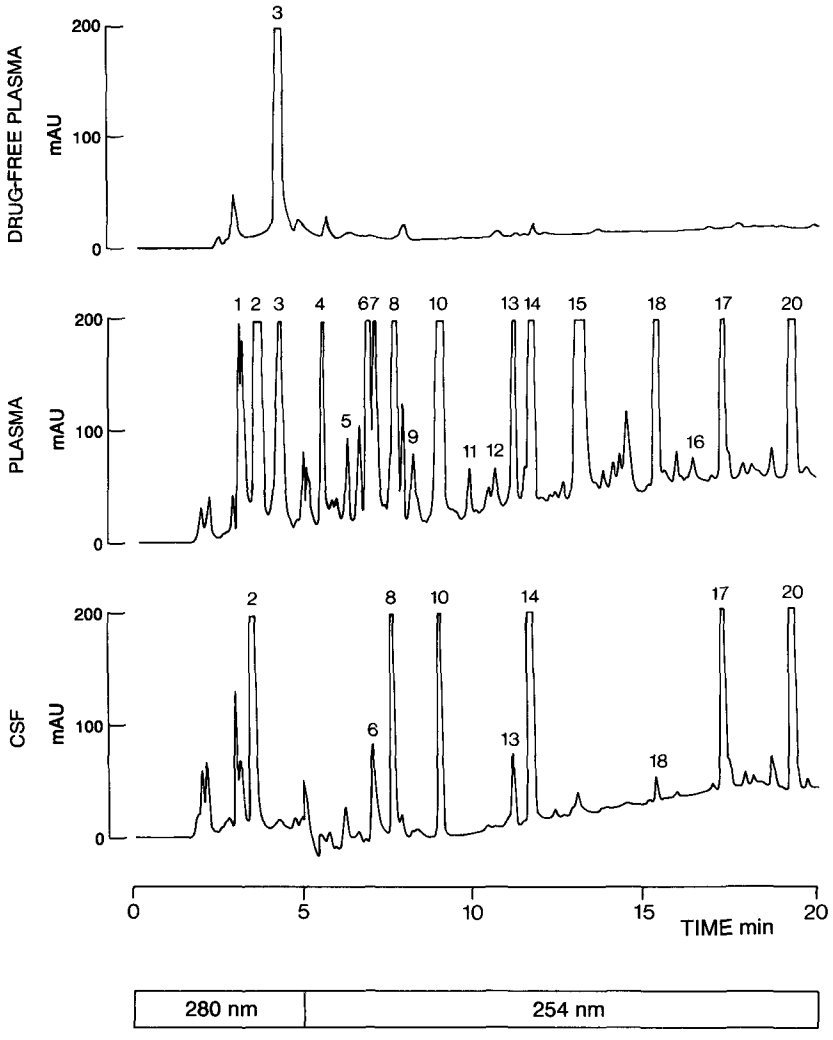

Fig. 3. Chromatograms of the plasma and cerebrospinal fluid (CSF) of a patient with tuberculous meningitis. The xenobiotics which correspond to each numbered peak are given in Table I.

TABLE II

ADDITIONAL DRUGS IDENTIFIED IN THE SERA OF PATIENTS WITH TUBERCULOUS MENINGITES BUT NOT SHOWN IN FIG. 3

Drug	Retention time (min)
Acetazolamide	4.89
Ampicillin	4.98
Furosemide	9.12
Penicillin-G	10.28
Droperidol	10.88
Carbamazepine	11.66
Phenytoin	11.90

its major metabolites. In many studies, results using only healthy volunteers or experimental animals were presented [5–9]. In contrast, the determination of antituberculosis drugs in biological fluids of patients with a disease such as tuberculous meningitis present the analyst with a complex analytical task. We have previously documented the qualitative and quantitative properties and convenience of the technique used [1]. In this report we demonstrate its application to the analysis of antituberculosis drugs in patients receiving a wide range of drug supplements. We are of the opinion that it is superior to other methods

of direct injection since a protein peak is not present during the early elution phase. For instance, in the method developed by Riva *et al.* [7] for detection of rifapentine, proteins are eluted for approximately 5 min before any xenobiotics become detectable; this may obscure early-eluting components.

Unfortunately, the method is not particularly well suited to the analysis of isoniazid as it and its metabolites elute relatively early and are not detectable. A modification of the method of La Croix *et al.* [10] was used by us for this purpose [4]. With respect to rifampicin, it is clear from the data presented here, that its instability in serum needs to be suitably addressed before the method can be used for its analysis. The accuracy and convenience of the method make it well suited to the pharmacokinetic studies of pyrazinamide and ethionamide in children with tuberculous meningitis [2,3].

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