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Quantification of pyrazinamide and its metabolites in plasma by ionic-pair high-performance liquid chromatography. Implications for the separation mechanism

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Abstract

Pyrazinamide, the amide of pyrazinoic acid, is one of the basic therapeutic agents currently used in combination for chemotherapy of tuberculosis. A reversed-phase high-performance liquid chromatography method based on ionic pair chromatography, was developed after solid-phase extraction of the analytes from plasma with prior addition of internal standard. The main metabolites, pyrazinoic acid, 5-hydroxypyrazinoic acid and 5-hydroxypyrazinamide, were included as well as uric acid and other purine derivatives to allow detailed study of the pharmacokinetics of the drug, especially in patients with impaired kidney function. Some interesting features of the chromatographic system giving some insight in the retention mechanism and of the solid-phase extraction are discussed in detail. © 1998 Elsevier Science B.V.

Keywords: Pyrazinamide; Pyrazinoic acid; 5-Hydroxypyrazinoic acid; 5-Hydroxypyrazinamide

1. Introduction

Pyrazinamide (PZA), the amide of pyrazinoic acid (PC), is widely used in combination with other drugs for chemotherapy of tuberculosis. A complete block of the secretion of uric acid and other oxypurines in renal tubules has been observed as a side effect [1–3]. In order to avoid hyperuricaemia, xanthine oxidase inhibitors, such as allopurinol, are given in combination with pyrazinamide. Thus, allopurinol and its metabolite oxypurinol are detectable in plasma samples. Detailed pharmacokinetic and metabolic studies, especially in patients with impaired kidney function, are of interest to optimize dosage and to minimize side effects. Methods available for determination of pyrazinamide range from simple photometric measurement [4,5], open column-chromatography [6], gas chromatography–mass spectrometry [7] and high-performance liquid chromatography [8–16]. All have major drawbacks, such as expensive equipment, long and laborious analysis procedures or lack of quantification of the metabolites. Furthermore, a method has not yet been established to produce kinetic data for uric acid and other purine derivatives. Therefore we developed a high-performance liquid chromatographic method for quantification of pyrazinamide and its main metabolites, pyrazinoic acid, 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid, in plasma after solid-phase extraction of the analytes. It differentiated

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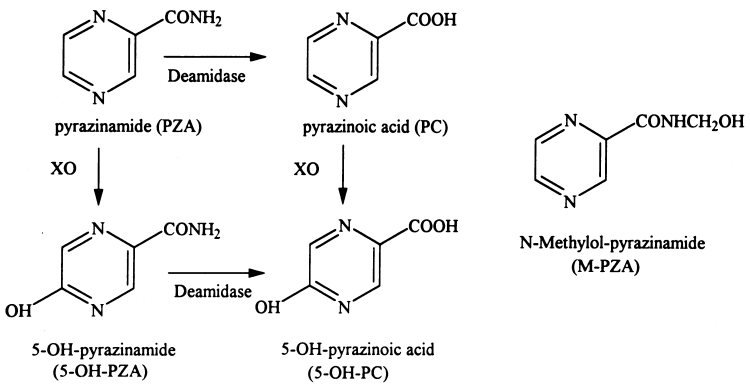

Fig. 1. Chemical structures and metabolic relations of the analytes (XO, xanthine oxidase). Structure of the internal Standard M-PZA.

allopurinol and oxypurinol as well as uric acid in one run. Internal standardization was used to enhance precision of the analysis. The chemical structures of the analytes and of the internal standard are given in Fig. 1.

2. Experimental

2.1. Chemicals

PZA, PC and tetrapentylammonium bromide were obtained from Aldrich (Steinheim, Germany). 5-OH-PZA and 5-OH-PC were synthesized chemically (see below). All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) in the highest available purity or in HPLC-grade. ODS-Silica (3 μm) was from Shandon (Frankfurt/Main, Germany). Phenyl-solid-phase extraction columns (3 ml) were from Varian (Frankfurt/Main, Germany). Water was used in aqua ad injectabilia-quality delivered in glass containers from Pfrimmer (Erlangen, Germany).

2.2. Sample preparation and storage

Blood was collected in heparinized tubes and centrifuged (3000 *g*, 10 min) immediately after drawing. Plasma samples were stored at −20°C until analysis.

2.3. HPLC system

Separation was achieved on a coupled column consisting of two stainless steel columns (15 cm×4.6 mm, Bischoff Analysentechnik, Leonberg, Germany) filled with ODS-Silica (3 μm, Shandon) and attached to each other by a low-dead volume coupling (Bischoff). The columns were packed according to the 'balanced density slurry' method [17]. The column was preconditioned with a 1 m*M* solution of tetrapentyl-ammonium bromide in water at a flow-rate of 0.8 ml/min for 3 days, followed by 100% methanol for 6 h at the same flow-rate. Mobile phase was delivered by a Gynkothek 600 HPLC pump (Gynkothek, Munich, Germany) at a flow-rate of 0.8 ml/min. Samples were applied to the column by means of an automatic sampler (Kontron HPLC autosampler 465, Kontron, Munich, Germany) with a sample volume of 50 μl. Detection at 268 nm was achieved by Kratos Spectroflow 773 photometer with an 8-μl cell (Kratos, Ramsey, NJ, USA). Chromatograms were recorded by a Shimadzu CR6a integrator (Shimadzu, Kyoto, Japan).

The mobile phase was a mixture of 0.02 *M* potassium dihydrogenphosphate buffer (pH 6), methanol (4%, v/v) and 6 μ*M* tetrapentylammonium bromide, added as 1 m*M* solution in water. Dissolved gases were removed by 10 min sonication in an ultrasonic bath (Bandelin, Berlin, Germany). Each week, the column was eluted for 6 h with pure methanol, thus restoring the initial properties of the column.

2.4. Synthesis of 5-hydroxypyrazinoic acid and 5-hydroxypyrazinamide

2-Aminoquinoxaline was prepared from 2-phenylendiamine via *N*-cyanomethyl-2-phenylendiamine [18] and acetylated by refluxing with acetic anhydride to 2-acetylaminoquinoxaline [19]. This was oxidized by potassium permanganate to 2-acetylaminopyrazin-5,6-dicarboxylic acid, followed by deacetylation and decarboxylation to 2-aminopyrazin-5-carboxylic acid. Deamination was achieved by diazotization and hydrolysis to 5-hydroxypyrazinoic acid. Methylation was achieved by treatment with excess diazomethane in diethyl ether. The corresponding amide was prepared by heating the carboxylic acid methylester in a saturated solution of ammonia in methanol in an autoclave for 8 h at 180°C [20]. Both end products were characterized by ^{1}H and ^{13}C NMR and checked for purity by HPLC (purity greater than 99%, data not shown).

2.5. Synthesis of N-methylol-pyrazinamide (M-PZA, internal standard)

Five g pyrazinamide (40 mmol) were suspended in 50 ml water [21] and dissolved by dropwise addition of 40% sodium hydroxide at room temperature. Twenty ml of a 40% formaldehyde solution were added, by which the reaction product began immediately to separate. After standing overnight, the solids were removed by filtration and washed with water until the pH was neutral. M-PZA was checked for purity by HPLC and ^{1}H and ^{13}C NMR (purity >97%, residual unreacted PZA, cf. Fig. 3a). Its purity was sufficient enough for use as an internal standard without further processing.

2.6. Solid-phase extraction

Phenyl solid-phase extraction columns were stored for 1 week immersed in 100% 2-propanol to achieve thorough and homogeneous wetting. Conditioning of the columns was done with 3×3 ml of methanol in a Baker vacuum manifold (10 columns, Baker Chemicals, Deventer, The Netherlands), followed by 3×3 ml of potassium chloride solution (0.1 *M*, adjusted with hydrochloric acid to pH 1.8). Two ml of potassium chloride solution (0.1 *M*, adjusted with hydrochloric acid to pH 1.2) were added to 0.5 ml plasma and 10 μg of M-PZA in 100 μl methanol as internal standard, and the mixture was applied to the preconditioned extraction columns. After having passed the columns they were washed with 2×0.5 ml of potassium chloride solution (0.1 *M*, adjusted with hydrochloric acid to pH 1.8), followed by 400 μl of Tris–buffer (0.1 *M*, pH 9.5). For recovery studies, blank plasma was spiked with known amounts of the analytes, and processed as described above.

Application of vacuum (200 mbar) for 10 min removed traces of humidity prior to elution with subsequent 4×400 μl methanol. The eluates were collected in 1.5-ml Eppendorf tubes, followed by addition of 30 μl phosphate buffer (1 *M*, pH 5.5). They were then vortexed and dried under nitrogen at room temperature. The residue was dissolved in 1 ml of mobile phase, vortexed and centrifuged for 10 min at 3000 *g* prior to HPLC analysis.

3. Results

3.1. HPLC

The mobile phase contained the ionic pair reagent tetrapentylammonium bromide for interaction with the carboxy groups of PC and 5-OH-PC, thus allowing baseline separation of the analytes within 16 min (Fig. 2). Solvent front appeared at 2.95 min, retention times were determined as 3.5 min for 5-OH-PC, 4.31 min for PC, 6.92 min for uric acid, 7.76 min for 5-OH-PZA, 9.32 min for oxypurinol, 10.64 min for allopurinol 12.55 min for PZA and 15.91 min for M-PZA, respectively. The standard curves of peak areas against varying concentrations of the analytes in water were strictly linear for the concentration ranges used:

PC	$y=20.1x-2.19$	$r=0.9999$, 1–40 μg/ml
PZA	$y=23.7x-33.42$	$r=0.9991$, 1–80 μg/ml
M-PZA	$y=19.9x-22.9$	$r=0.9958$, 1–50 μg/ml
5-OH-PC	$y=6.48x+0.37$	$r=0.9997$, 1–50 μg/ml
5-OH-PZA	$y=7.08x-0.625$	$r=0.9989$, 1–50 μg/ml

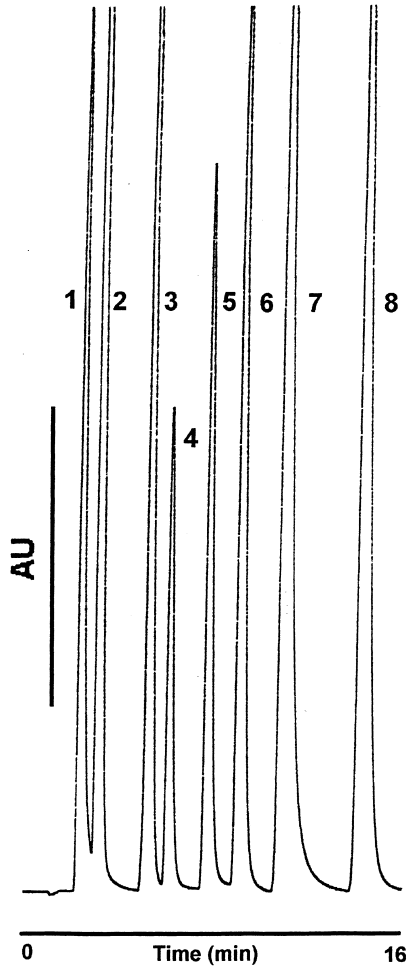

Fig. 2. Chromatogram of the analytes in an aqueous solution (10 μg/ml of each compound). **1**, 5-OH-PC; **2**, PC; **3**, uric acid; **4**, 5-OH-PZA; **5**, oxypurinol; **6**, allopurinol; **7**, PZA; **8**, M-PZA.

All determinations were done in 12-fold replication. Detection limits for all analytes were at 0.3 μg/ml.

Other tuberculostatics were tested for possible interference in the chromatography including ethambutol, isonicotinic acid hydrazide (INH), streptomycin and rifampicin. The latter two compounds did not elute from the column with the used mobile phase, ethambutol was not detectable due to lack of UV absorption and INH eluted between PC and uric acid.

3.2. Solid-phase extraction

Recoveries of the analytes from plasma were determined by spiking a blank plasma with appropriate concentrations of the analytes, followed by the described extraction procedure and HPLC analysis. Recovered concentrations were calculated with respect to the standard curves above for the analytes in water.

The resulting standard curves for spiked plasma samples showed linear characteristics for all analytes:

PC	$y=0.890x+0.0662$	$r=0.9999$, 1–40 μg/ml, $n=5$
PZA	$y=0.814x+1.619$	$r=0.9988$, 1–80 μg/ml, $n=5$
M-PZA	$y=1.05x+0.161$	$r=0.99$, 5–25 μg/ml, $n=5$
5-OH-PC	$y=0.0297x+0.0788$	$r=0.99$, 1–50 μg/ml, $n=5$
5-OH-PZA	$y=0.363x+0.397$	$r=0.999$, 1–50 μg/ml, $n=5$

The slope of the different equations indicates the average recoveries of the analytes; 100% recovery is equality of added and found concentration, thus leading to a recovery function with slope 1. The individual recoveries were found to be 89% for PC, 81% for PZA, 105% for M-PZA, 36% for 5-OH-PZA and 3% for 5-OH-PC. The absolute recovery data are given in Table 1.

Recovery of INH, the only tuberculostatic drug with possible interference, showed a neglectable recovery (below 0.5%) under the analytical conditions.

3.3. Internal standardization

Internal standardization was chosen in order to enhance the analysis of patients' samples.

A series of compounds with similar chemistry to

Table 1
Absolute recoveries of PZA and related compounds from spiked plasma after solid-phase extraction and HPLC-analysis ($n=5$, mean±S.E.M.)

Substance	Concentration (μg/ml)	
	Added	Found
PZA	1	2.49±1.59
	10	9.55±1.81
	20	17.48±1.08
	40	33.93±3.14
	60	52.63±3.24
	80	65.31±5.44
PC	1	1.07±1.05
	5	4.55±1.13
	10	8.76±1.59
	20	17.92±2.59
	40	35.74±3.43
5-OH-PZA	1	0.44±0.13
	5	2.19±0.35
	10	4.17±0.62
	20	8.02±1.23
	50	18.38±3.28
5-OH-PC	1	0
	5	0.23±0.03
	10	0.45±0.02
	20	0.75±0.06
	50	1.52±0.34
M-PZA	5	6.14±1.57
	10	10.57±1.68
	15	15.36±1.72
	20	19.60±2.45
	25	27.87±3.26

PZA was tested for use as internal standard, including pyrazinoic dicarboxylic acid, nicotinic acid, isonicotinic acid, picolinic acid and the corresponding amides, as well as alkylated pyrazines and 5-fluorouracil. All compounds as well as oxy- and allopurinol proved to be unsuitable for use as internal standard due to lack of sufficient recovery under the analytical conditions (cf. INH). The solid-phase extraction procedure seems to be highly specific for the monosubstituted pyrazine moiety. Only M-PZA showed sufficient recovery from spiked plasma.

The synthesis product was contaminated with a small amount of unreacted PZA (<1%) as demonstrated in Fig. 3a. This content was considered to be neglectable for our analytical purposes, but has led to a somewhat higher recovery of PZA in the lowest concentration (Table 1). Furthermore it was questionable if this compound would stand the analytical procedure due to pH-dependent decomposition. Therefore we investigated the influence of the buffer added after solid-phase extraction. Indeed, at pH values >7 a cleavage of the compound was found, but at lower pH values it was stable over the whole analytical process (Fig. 4). The standard curve of M-PZA showed linear characteristics for solutions in water as well as for spiked plasma. Therefore this substance was suitable for internal standardization. A constant amount of M-PZA (10 μg/ml) was added to each spiked plasma sample and, after solid-phase extraction and HPLC analysis, the area quotients of the analytes and M-PZA were taken for quantification. In spiked plasma, these relations were strictly linear for all analytes (data not shown).

3.4. Application to patients' samples

A patient undergoing treatment with a combination of five tuberculostatics, including a dose of PZA and allopurinol, was investigated with this method. A HPLC run of processed plasma samples is given in Fig. 3, including plasma before therapy. No interference due to endogenous compounds or to the tuberculostatic drugs occurred. The resulting pharmacokinetics of PZA and the main metabolites are shown in Fig. 5.

4. Discussion

The new HPLC method allows separation and quantification of PZA and its main metabolites in one run. Interference of uric acid, as well as of allopurinol, oxypurinol and INH and other tuberculostatics, was excluded. Internal standardization was achieved by addition of constant amounts of M-PZA to plasma samples. The HPLC method is sensitive and reproducible. Its applicability to clinical samples is demonstrated. Some interesting and

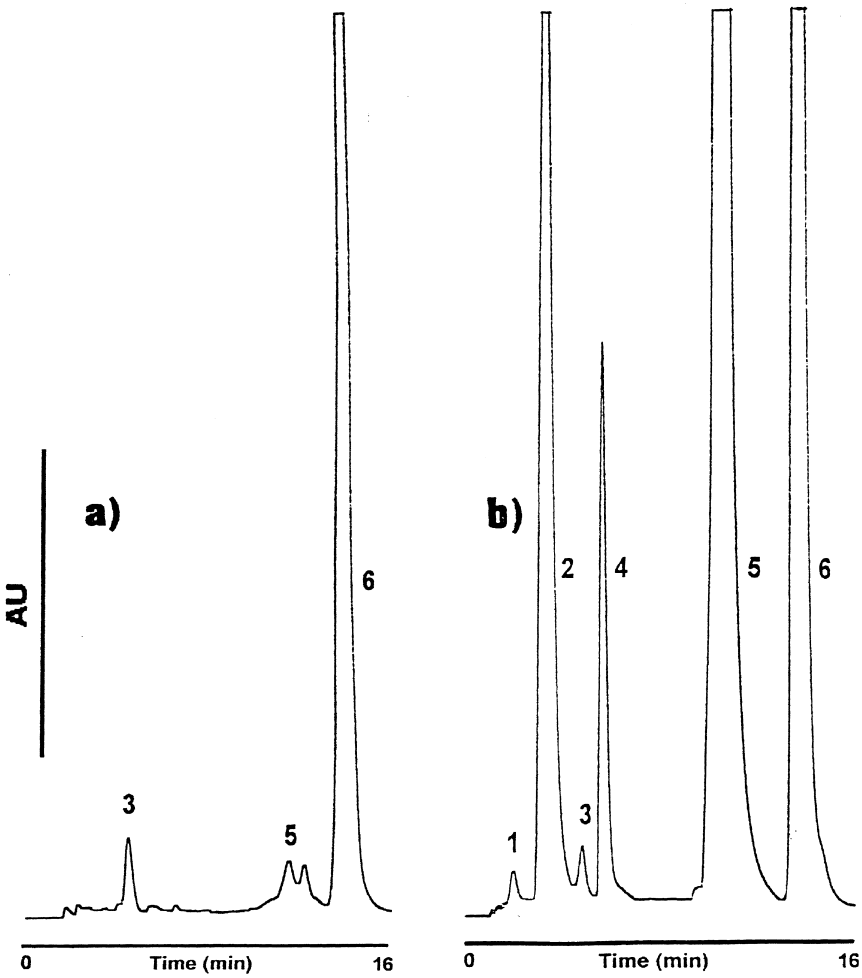

Fig. 3. Chromatograms of plasma samples from a patient (a) prior to therapy and (b) following a single oral dose of 2 g pyrazinamide in combination with ethambutol, isonicotinic acid hydrazide, streptomycin, rifampicin and allopurinol. Internal standard M-PZA (10 μg/ml) was added to both samples. **1**, 5-OH-PC; **2**, PC; **3**, uric acid; **4**, 5-OH-PZA; **5**, PZA; **6**, M-PZA. In (a) a small, neglecticable amount of **5** was found due to contamination of M-PZA.

unusual methodological features should be discussed in detail.

4.1. HPLC

The HPLC separation of PZA and the main metabolites makes use of the ionic pair reagent tetrapentylammonium bromide for interaction with the carboxylic functions. Without ionic pair reagent, PC showed strong peak tailing nearly independent of the pH of the mobile phase (Fig. 6a). For suppression of this property, several tetraalkylammonium salts including the propyl-, butyl-, pentyl- and hexyl-derivatives were tested, indicating that the pentyl-derivative has the best effect.

All compounds have similar effects with respect to resolution and tailing suppression, but differ in their kinetic behaviour towards the column (see below). Initial attempts to establish stable separation of the analytes with the default concentration of 1 m*M* of ionic pair reagent in the mobile phase failed due to an instability of retention times, especially for the carboxylic acids depending of the working time of the column. Since the tetrapentylammonium cation is

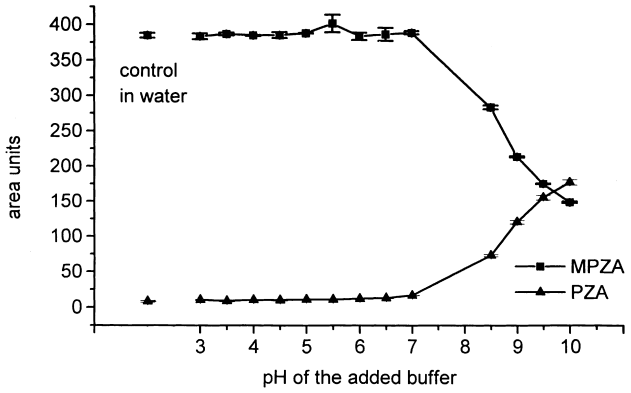

Fig. 4. Stability of the internal standard M-PZA after solid-phase extraction and HPLC analysis from spiked plasma. Influence of the added buffer pH after solid-phase extraction.

very lipophilic, we hypothesize that a continuous absorption of ionic pair reagent to the C_{18} stationary phase occurs, thus inducing a continuous shift of the column's properties. This assumption was supported by the fact that elution with methanol for 6 h restored the initial properties of the column. Since some ionic pair reagent might have been absorbed irreversibly to the column, the methanol-eluted column has slightly different properties compared to a freshly packed column (less peak tailing for the carboxylic acids). Therefore, each freshly packed column was saturated with ionic pair reagent by recycling a mobile phase with a high concentration of ionic pair reagent followed by elution with methanol.

In order to minimize the absorptive processes, and to stabilize the retention times of the analytes, we investigated the influence of the concentration of the ionic pair reagent in the mobile phase used for quantitative analysis. Concentrations far below 1 m*M* were capable of suppressing peak tailing, but without major change in retention time of the analytes. These findings are summarized in Fig. 6, showing the chromatograms of a mixture of PC, PZA and M-PZA under different states of the column and with respect to different ion pair reagent concentrations. The strong effects of very low concentrations were demonstrated, as well as different column states. A freshly packed column (Fig. 6a) showed strong peak tailing of PC, which could be suppressed by con-

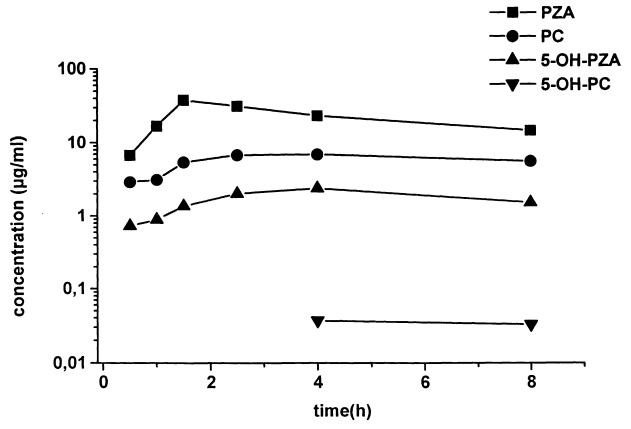

Fig. 5. Time course of pyrazinamide and its main metabolites in plasma following a single oral dose of 2 g in combination with ethambutol, isonicotinic acid hydrazide, streptomycin, rifampicin and allopurinol.

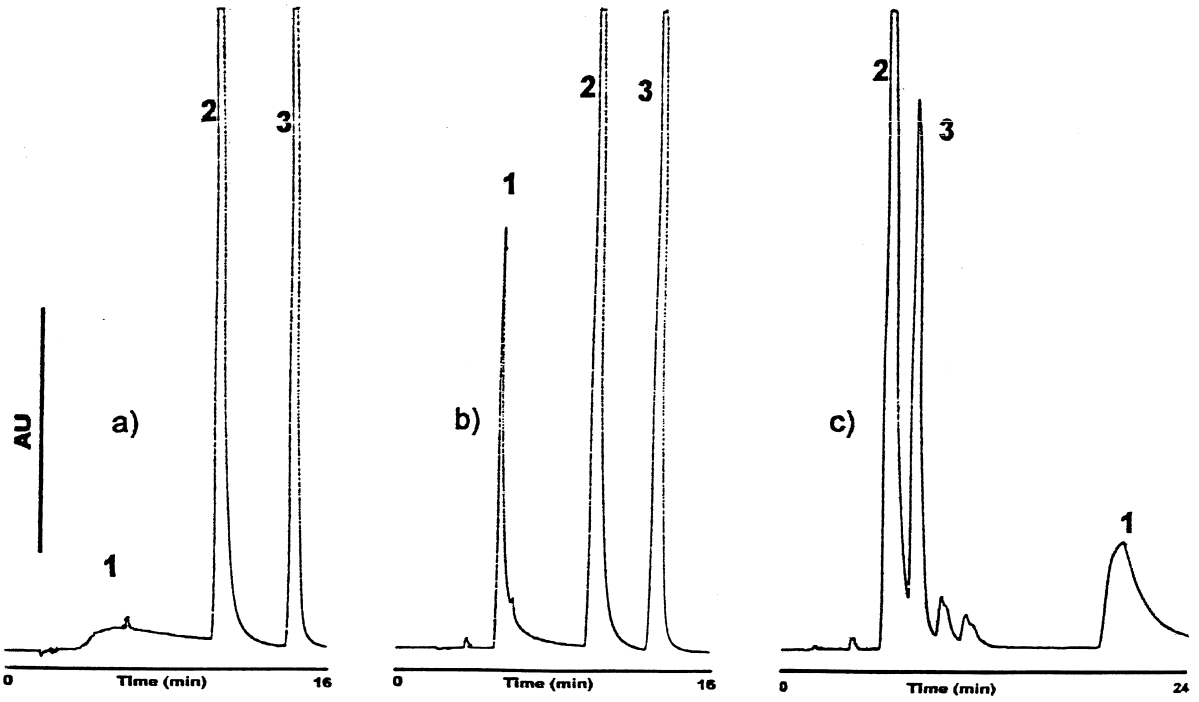

Fig. 6. Influence of the ionic pair reagent concentration on the chromatographic resolution. (a) Freshly packed column, mobile phase without ionic pair reagent (tetrapentylammonium bromide); (b) used column after regeneration, mobile phase with 0.01 m*M* ionic pair reagent; (c) used column after regeneration, mobile phase with 0.15 m*M* ionic pair reagent. All other chromatographic conditions were maintained; sample was a mixture of 25 μg MPZA (**3**), 30 μg PZA (**2**) and 10 μg PC (**1**) per ml water.

centrations as low as 6 μ*M* tetrapentylammonium bromide (Fig. 2). The effects of higher concentrations are demonstrated in Fig. 6b,c. The higher ionic pair reagent concentrations have led to a shift of the elution time of PC even behind M-PZA. The same result was seen if an intermediate concentration was used for a longer time period (2–3 weeks). This state was reversible by flushing the column with pure methanol. Interestingly, retention times of PZA and M-PZA were distinctly shortened when eluted with the higher ionic pair concentration, possibly due to loss of binding sites masked by the absorbed ionic pair reagent.

These results led us to the above-mentioned hypothesis of a continuous absorption of ionic pair reagent to the column. Furthermore, we think that a concentration-dependent equilibrium in this absorptive process is reached after some time. The velocity of this process could be minimized by using very low concentration of ionic pair reagent combined with a low content of methanol in the mobile phase disturbing the absorption process, thus achieving stable chromatographic conditions. The other investigated tetraalkylammonium salts showed major differences in the velocity of this putative absorption process, but not in chromatographic specificity. The longer the alkyl chain, the quicker a stable absorption equilibrium was achieved, and vice versa, the easier the desorption with pure methanol was achieved. Therefore, the pentyl-derivative was used. Nevertheless, the analytical columns were regenerated each week with methanol. In Table 2 the stability of our chromatographic system is demonstrated with respect to intra-day and inter-day variability. The latter includes several cycles of column regeneration not affecting the resulting data.

4.2. Solid-phase extraction

Phenyl-conjugated solid-phase extraction columns proved to be best suited for extraction of the analytes

Table 2
Intra- and inter-day-variability of the chromatographic system expressed by the area units of the individual peaks

Substance	Concentration (μg/ml)	Area units (mean±S.D., C.V.%)	
		Intra-day variability	Inter-day variability
5-OH-pyrazinoic acid	1	6.62±1.24, 10.8	6.12±0.48, 0.9
	5	33.65±0.55, 1.6	33.12±1.05, 0.6
	10	61.19±2.66, 4.3	64.37±2.10, 3.3
	20	130.72±1.53, 1.1	127.46±4.91, 3.8
	50	330.93±3.83, 1.1	317.91±7.73, 2.4
5-OH-pyrazinamide	1	6.49±0.21, 3.3	6.44±0.27, 4.3
	5	33.39±1.97, 5.8	32.43±2.75, 8.4
	10	66.70±5.53, 8.3	64.54±1.46, 2.2
	20	144.39±11.63, 8.0	151.29±1.58, 1.0
	50	351.02±20.67, 5.8	341.42±29.68, 5.0
Pyrazinoic acid	1	19.31±1.22, 6.3	18.31±1.67, 9.1
	10	200.63±11.14, 5.5	201.31±5.77, 2.8
	20	404.12±24.37, 6.0	404.81±12.19, 3.0
	40	851.91±46.11, 5.4	793.17±12.47, 1.5
Pyrazinamide	1	21.25±0.27, 1.2	20.18±0.67, 3.3
	10	208.43±2.78, 1.3	200.26±3.70, 1.8
	20	420.02±3.45, 0.8	399.16±7.05, 2.8
	40	816.48±6.69, 0.8	801.10±13.52, 1.6
N-Methylol-pyrazinamide	1	19.55±0.89, 4.6	18.11±0.56, 3.1
	10	180.30±11.95, 6.6	176.02±4.83, 2.7
	20	372.86±33.67, 9.0	354.63±7.52, 2.1
	40	789.55±28.60, 3.6	708.34±13.66, 1.9

S.D., standard deviation; C.V.%, coefficient of variation; $n=6$ for each concentration.

from plasma as compared to other modified silica columns, including C_2-, C_8- and cyclohexyl-conjugated columns as well as ion-exchange columns. None of these showed promising results which would enforce further investigation. A sufficient and homogeneous wetting of the silica is noteworthy for reasonable extraction efficiency. In retrospect, the phenyl-conjugated column filling was rather inhomogeneously wetted and partly dry and powdery after customary conditioning with methanol, similar to a badly packed chromatography column. However, immersion of the columns in other solvents like 2-propanol for at least 1 week (not methanol) resulted in visible homogeneous penetration of the silica gel, and thereafter in reproducible extraction efficiency. 2-Propanol has the advantage of being more lipophilic than methanol, but fully miscible with water. The addition of phosphate buffer, pH 5.5, to the extracted samples enabled minimization of the cleavage of the internal standard M-PZA to formaldehyde and PZA.

4.3. Recoveries and applicability

Yamamoto et al. [14] reported a HPLC method with precipitation of plasma components and subsequent analysis of the supernatant. They obtained 100% recovery for the 5-OH metabolites. In our hands, the method was not applicable due to major interferences of endogenous compounds and due to strong peak tailing of the carboxylic acids. We therefore developed this method based on solid-phase extraction. Unfortunately, recoveries of the 5-OH metabolites were rather low. Trials to achieve higher extraction efficiencies failed, probably due to the highly hydrophilic character of these compounds. Investigation of the extraction procedure revealed the main loss of substance occurring in the first step of

Table 3
Comparison of reported plasma peak concentrations [21] with our data (mean±S.E.M.)

Substance	Peak concentrations (μg/ml) and t_{max} (h)	
	[21] (n=9)	Own data (n=1)
PZA	38.7±5.9, 1.0	38.1, 1.5
PC	4.5±0.9, 4.9	6.95, 4.0
5-OH-PZA	1.8±0.3, 4.0	2.38, 4.0
5-OH-PC	0.58±0.2, 4.2	0.04, 4.0

extraction (application of the sample), supporting this explanation. In consequence, the most lipophilic substance, M-PZA, showed the best recoveries. As both metabolites showed linear and reproducible recovery functions, estimative quantification is possible in spite of the low recoveries. Furthermore, Lacroix et al. [21] reported concentrations of the metabolites in a patient undergoing treatment with 2 g PZA (27 mg/kg). Our patient received the same dose (30 mg/kg). The reported data are compared with our findings in Table 3, demonstrating good coincidence. The low concentration of 5-OH-PC may be seen in the light of the simultaneously given xanthine oxidase inhibitor allopurinol, which abolished rather specifically the production of this metabolite [14]. This strongly confirmed the reliability of our method. Nevertheless, further research is needed to circumvent this pitfall of our method.

In spite of this, our proposed analytical method is a useful tool for further investigations of the pharmacokinetics and metabolic fate of PZA. The method is applicable for drug monitoring in patients undergoing therapy with PZA in combination with other tuberculostatics.

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