Journal of Chromatography, 147 (1978) 337–347

CHROM. 10,231

MASS FRAGMENTOGRAPHIC DETERMINATION OF PYRAZINAMIDE AND ITS METABOLITES IN SERUM AND URINE

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(First received April 12th, 1977; revised manuscript received May 23rd, 1977)

SUMMARY

A combined gas chromatographic–mass spectrometric technique is described for the simultaneous determination of pyrazinamide and its two main metabolites, pyrazinoic acid and 5-hydroxypyrazinoic acid. Serum (200 μl) is deproteinized and evaporated to dryness; urine (20 μl) is evaporated. The crude residues are silylated and selected ions are monitored in the chemical-ionization mode with isobutane as both chromatographic carrier and reagent gas. The sensitivity is 10 ng/ml for pyrazinamide and pyrazinoic acid and 20 ng/ml for the 5-hydroxy metabolite in a single analysis. Nicotinic acid and nicotinamide are internal standards. Both unchanged drug and metabolites were identified and quantified in the serum and urine of human subjects.

INTRODUCTION

The antituberculous agent pyrazinamide (pyrazine-2-carboxamide, PZA), is one of the powerful drugs available for the inhibition of urate excretion in man, consistently providing a 80–90% reduction in the renal clearance of uric acid[1]. The effect of the drug is not so striking in the dog, and in chicken there is no effect at all[2,3].

PZA is readily absorbed from the gastrointestinal tract[4]. The most important metabolite of PZA is pyrazinoic acid (pyrazine-2-carboxylic acid, PA), which has been repeatedly suggested to be the active principle leading to urate retention[1,5]. PA is formed from the parent compound by deamination effected by the hepatic microsomal enzyme pyrazinamide deaminidase (Fig. 1). Finally, PA is oxidized by xanthine oxidase to 5-hydroxypyrazinoic acid (5-hydroxypyrazine-2-carboxylic acid, 5-OH-PA). Another metabolite, pyrazinuric acid (pyrazinoylglycine), is not considered important; still another metabolite, detected but not identified, also appears insignificant[4,5].

The objective of this work was to develop a specific and sensitive technique for the simultaneous quantification of PZA, PA, and 5-OH-PA in blood and urine.

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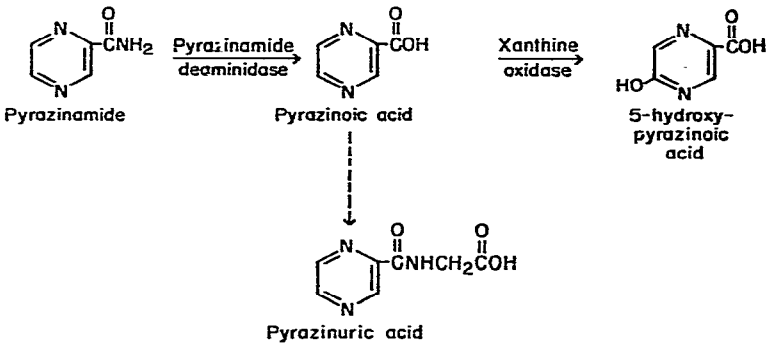

Fig. 1. Metabolism of pyrazinamide (PZA).

Previous analytical methods reported include colorimetry[6], counter-current distribution[4], and paper chromatography and electrophoresis[5]. These techniques lack specificity and are not convenient for simultaneous analyses of all components in serial samples. The inherent sensitivity and specificity of the mass spectrometric method developed permits multicomponent analysis of very small sample sizes with minimal sample preparation. Other considerations in selecting the mass spectrometric technique were the desire to utilize the same basic method for the quantification of probenecid [*p*-(di-*n*-propylsulfamyl)benzoic acid], a potent uricosuric agent, and its metabolites so that drug interactions could be studied, and also to obtain profiles of endogenous constituents which may be effected by the drugs.

In the method developed, only a minimal amount of sample preparation is required. Serum is deproteinized and the aqueous layer is evaporated to dryness; urine is evaporated directly to dryness. The crude residues are silylated and injected into a combined gas chromatograph–mass spectrometer system equipped with chemical-ionization source and operated in the selected-ion-monitoring mode. Preselected masses are monitored to identify and quantify the compounds of interest and the internal standards as they elute from the gas chromatograph. Because of the high specificity of the technique, other constituents present do not interfere, permitting the use of crude samples.

EXPERIMENTAL

Drugs and reagents

Pure samples of PZA and PA were obtained by courtesy of Merck Sharp & Dohme (Rahway, N.J., U.S.A.); 5-OH-PA was generously given by Dr. I. M. Weiner; nicotinic acid and nicotinamide were purchased from Sigma (St. Louis, Mo., U.S.A.). The silylating reagents bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), silylation-grade pyridine, and all gas chromatographic column materials and accessories were purchased from Pierce (Rockford, Ill., U.S.A.). Carrier and reagent gases were of high-purity grade from Matheson (East Rutherford, N.J., U.S.A.).

Instrumentation

Combined gas chromatography–mass spectrometry was performed using a

Finnigan Model 3300 instrument system, equipped with a chemical ionization source and a capability for selected ion monitoring (mass fragmentography). High-resolution mass spectra were obtained with a JEOL Model JSCM-1 double-focusing instrument combined with a Hewlett-Packard Model 7260A gas chromatograph.

Internal standards

Both nicotinic acid (3-pyridinecarboxylic acid) and nicotinamide (3-pyridinecarboxylic acid amide) were utilized as internal standards. The pure compounds were dissolved in water to provide a 0.5 $\mu g/\mu l$ stock solution. In all cases, adequate internal standard was added at the beginning of an analysis to yield a final concentration of the internal standard approximately in the middle of the expected concentration range of PZA.

Preparation of standards for mass spectrometry and calibration samples

The pure compounds PZA, PA, and 5-OH-PA were dissolved in a mixture of (BSTFA + 1% TMCS)–pyridine (3:1) in a PTFE-disk covered vial and kept at 70° for 2 min in a metal heating block. These samples were used to establish both high- and low-resolution mass spectra for the authentic compounds under various experimental conditions, to determine detection limits for the pure compounds, to establish sensitivities (in terms of computer-generated peak areas per unit sample quantity introduced), and also for daily routine mass range calibration.

To establish optimal analytical conditions, and subsequently to obtain calibration curves, normal pooled human serum and urine samples were spiked with known amounts of PZA, PA, and 5-OH-PA (using aqueous stock solutions) and handled in the manner described below for samples from human subjects. To compensate for irreproducible experimental errors, a full set of calibration runs with spiked serum or urine was made with every set of biological samples. The concentration range of the calibration standards covered the entire range of expected concentrations in the samples. The amount of internal standard was the same for all samples and standards analyzed, and was in the middle of the expected concentration range. A blank sample, *i.e.*, with no drug or metabolite added, was also included in the calibration runs.

Preparation of serum and urine samples

Blood was first permitted to clot at room temperature for 15–25 min, and was then centrifuged at 30 *g* for 5 min. Serum was kept frozen at —20°. Amounts of 0.2 ml of serum were needed for each determination. Urine was collected individually every 2 h and kept frozen until used; no preservative was added. Only 20 μl of urine are needed for an analysis.

Sample preparation for serum was started by adding nicotinic acid as the internal standard. Next, 0.2 ml of serum sample was added drop by drop (with a Pasteur pipette) to 1.0 ml of absolute ethanol. The precipitate was centrifuged at 755 *g* for 15 min, and the supernatant decanted. The aqueous phase was evaporated to dryness in a water-bath kept at 40° with a gentle stream of dry nitrogen. Prior to gas chromatographic–mass spectrometric analysis, the dry residue was silylated by adding 100 μl of (BSTFA + 1% TMCS)–pyridine (3:1), and heated to 70° for about 2 min in a metal heating block. Usually 4 μl of this sample were introduced into the gas chromatograph. Silylated samples should be analyzed as soon as possible.

Sample preparation for urine was even simpler. Starting with 20 μl of urine, nicotinamide standard was added and all samples were diluted with distilled water to a total final volume of 120 μl to compensate for dilution effects of the amount of internal standard needed to provide a concentration in the middle of the expected range. Next, the samples were evaporated to dryness under nitrogen stream, as in the case of serum. The resulting yellowish-whitish residue was silylated in the same manner described for serum. After heating the mixture there was no discernible solid residue.

Gas chromatography–mass spectrometry

For gas chromatographic separation a glass column, 1 m × 2 mm I.D., filled with 3% OV-17 on Chromosorb W HP, 80–100 mesh, was employed. Column temperature was programmed from 120 to 180° at a rate of 15°/min so that all compounds could be eluted in a single run. To remove other constituents present in the biological samples and also for periodic cleaning, the column was heated to 250° until no more effluent could be detected. Isobutane was used both as the gas chromatographic carrier gas and as the reagent gas in the chemical-ionization source of the mass spectrometer. There was no separator between the gas chromatograph and the mass spectrometer; the connecting tube was kept at 240°. The temperature of the inlet of the gas chromatograph was maintained at 250°.

After introducing 4 μl of sample into the gas chromatograph, most of the effluent was vented (for 0.5 min) to avoid contamination of the ion source with the solvent and reagents. The quadrupole mass spectrometer was operated in the chemical-ionization mode using isobutane (1 Torr pressure) as the reagent gas. Operational parameters were adjusted daily, using pure PZA, PA, and 5-OH-PA, to obtain maximum sensitivity. To obtain chemical-ionization spectra of all compounds for characterization and identification, the instrument was operated in the full scanning mode. For quantification, the instrument was operated in the selected-ion-monitoring mode, recording ions characteristic of the compounds being analyzed: $m/e = 196$ for PZA and nicotinic acid (same mass but different chromatographic retention times, *cf.* below), $m/e = 197$ for PA, $m/e = 285$ for 5-OH-PA, and $m/e = 195$ for nicotinamide.

High-resolution mass spectrometry

For complete identification of all compounds, initial experiments were made using high-resolution mass spectrometry. The combined gas chromatograph–mass spectrometer was operated in a routine manner, using electron-impact ionization at 70 eV, and employing photoplate detection (Ionomet ion-sensitive plates). Photoplates were analyzed with a computerized microdensitometer. All masses were determined to an accuracy of 3–5 millimass unit.

RESULTS AND DISCUSSION

Mass spectra of PZA, PA, and 5-OH-PA

First, pure samples of PZA, PA, 5-OH-PA, nicotinic acid, and nicotinamide were silylated and high-resolution mass spectra obtained using electron-impact ionization. In all cases, the molecular ions were detected, usually at low abundance. Exact mass measurements agreed with those based upon expected molecular composition

within 5 millimass units. As expected, PZA, PA, nicotinic acid, and nicotinamide each accept one silyl group to replace the active hydrogen on the side chain. The 5-hydroxy metabolite accepts two silyl groups, one on the side chain, the other on the oxygen connected to the C-5 position. Other peaks obtained in the electron-impact spectra of the compounds include the customary $(M - CH_3)^+$, $(M - COOTMSi)^+$, and other ions. Since the objective of the high-resolution analyses was to determine the molecular composition of the silylated products, detailed discussion of the electron-impact mass spectra is not warranted here.

Chemical-ionization spectra for all compounds studied are shown in Fig. 2a–e. These spectra were obtained using isobutane as the reagent gas. Essentially the same spectra are obtained with methane as the reagent gas, the isobutane spectra being of somewhat higher sensitivity than those obtained with methane. The gas chromatographic elution temperatures are somewhat lower when methane is employed. For practical purposes there is little difference between the two reagent gases and selection may be made on the basis of convenience. The chemical-ionization spectra clearly show an almost 100% abundance for the M + 1 ion; thus monitoring for quantification in biological samples presents no problem.

Gas chromatographic retention times

The approximate gas chromatographic retention times were as follows: PA: 1.4 min, PZA: 2.0 min, 5-OH-PA: 3.2 min, nicotinic acid: 1.2 min, and nicotinamide: 2.7 min. Since single ions are monitored for quantification, knowledge of precise gas chromatographic retention times is not essential. Although the same mass (m/e = 196) is monitored for both PZA and nicotinic acid, there is almost 1 min difference between the elution of the two peaks so that interferences do not present any problem. When nicotinamide is used as internal standard, there is no mass interference of any kind.

Internal standards

Nicotinic acid and nicotinamide were selected as internal standards because they are available in pure form, have both gas chromatographic and mass spectrometric properties similar to those of the compounds analyzed, and also can be extracted together with the compounds of interest from the very beginning of the analysis. Nicotinic acid is best for serum because of favorable extraction properties. Nicotinamide is favorable for urine because of its gas chromatographic properties.

Calibration curves

An individual calibration curve was obtained with every series of samples analyzed. In these curves the observed ratio of the peak area of the individual compound to that of the internal standard was plotted against the known concentration of compound used for spiking. These data points yielded a straight line within the limits of reproducibility; the slopes for PA and PZA are essentially the same, while the line for 5-OH-PA is somewhat below the line for the other two (*cf.* below). All quantifications were made using individual calibration curves.

Detection limit, sensitivity, reproducibility

Using the pure silylated compounds, the limit of detection is 100 pg for PZA

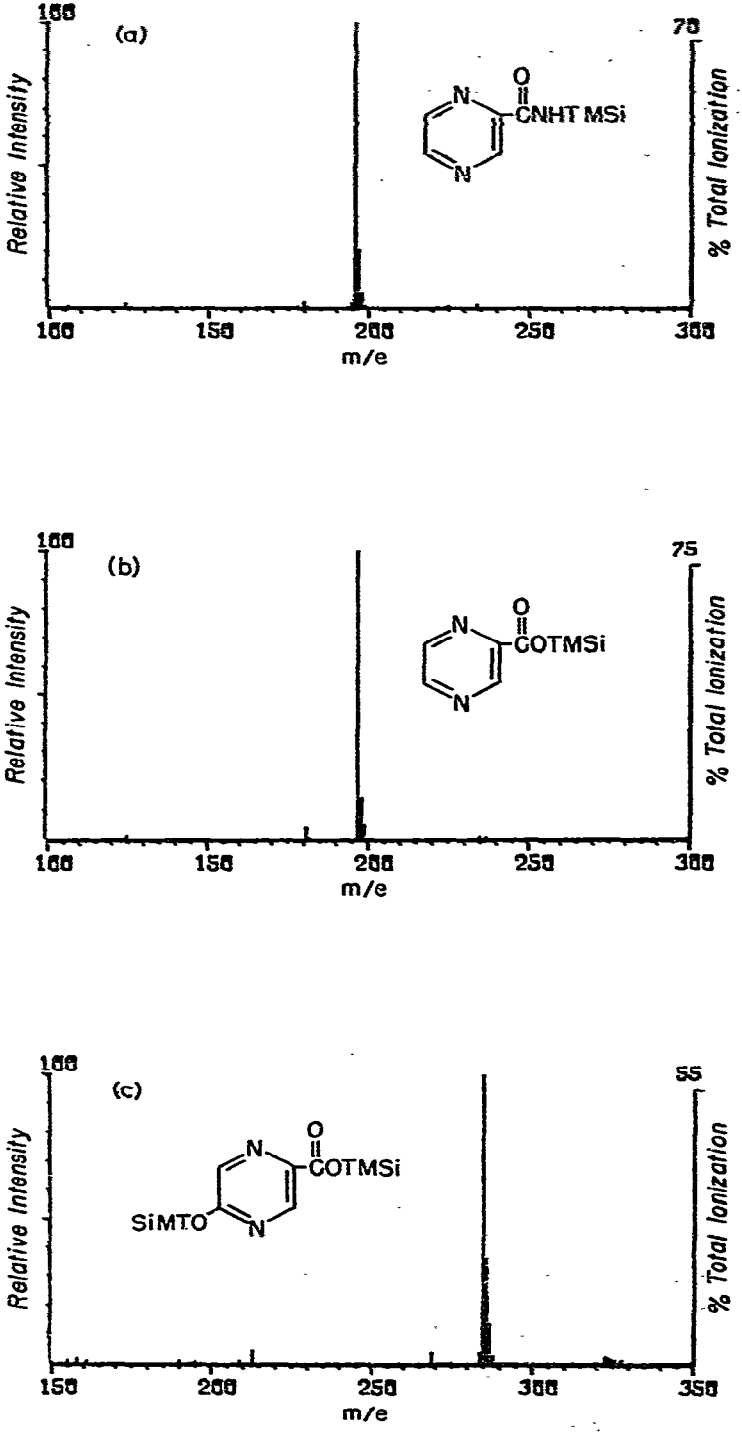
(a)
Relative Intensity
% Total Ionization
CNHTMSi
m/e
(b)
COTMSi
(c)
SiMTO
COTMSi

Fig. 2.

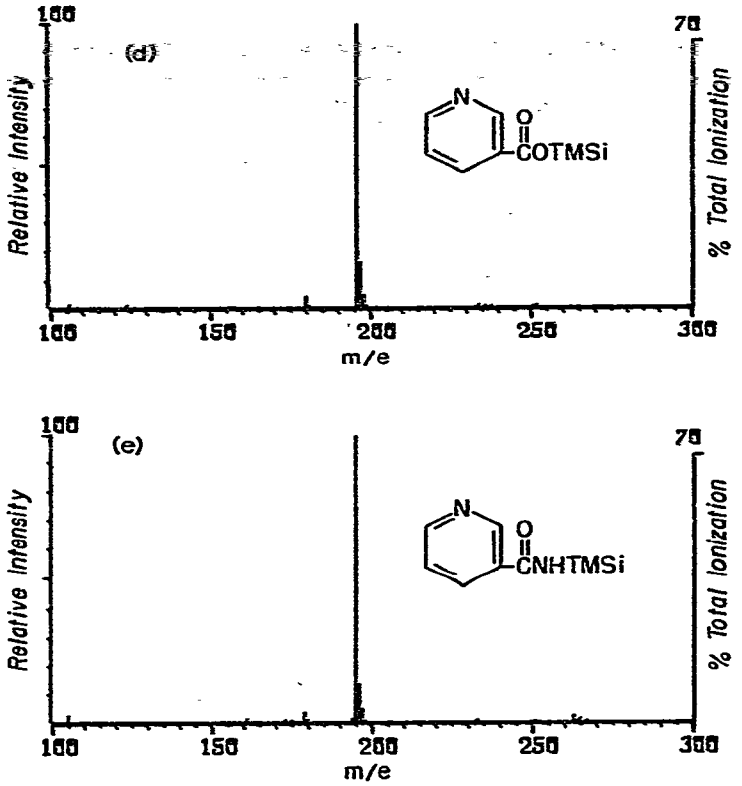

Fig. 2. Chemical-ionization (isobutane) mass spectra of the trimethylsilylated derivatives of (a) PZA, (b) PA, (c) 5-OH-PA, (d) nicotinic acid, and (e) nicotinamide.

and PA and 200 pg for 5-OH-PA; these amounts refer to the amount of material that must be introduced into the gas chromatograph in order to detect the corresponding protonated molecular ion. It is noted that the somewhat lower sensitivity for the 5-OH-PA metabolite results from the fact that somewhat compromised gas chromatographic conditions are employed to obtain all compounds in a single analysis. When optimized, the sensitivity for the 5-OH metabolite can be improved considerably; however, current sensitivity is more than adequate for all biological samples analyzed.

The sensitivity of the entire method developed is such that 10 ng/ml of PA or PZA and 20 ng/ml of 5-OH-PA can be quantified in either serum (0.2 ml starting sample) or urine (20 μl starting sample) employing the procedure described. When observing the data reported in biological samples, it is clear that this sensitivity is more than adequate. Indeed, the data on biological samples might suggest that conventional gas chromatography might be adequate. This is certainly true as far as sensitivity is concerned. However, the large number of additional constituents present, particularly in urine, presents difficult resolution problems in straight gas chromatography. The most important aspect of the technique presented here is the elimination of interference by coeluting endogenous constituents achieved by using the selected-ion-monitoring technique.

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF PA, PZA, AND 5-OH-PA IN SERUM

Data given are area ratios for compound and nicotinic acid internal standard. Each component in both concentrations was analyzed in 6 independent samples ($N = 6$).

	Sample size (μg/ml)					
	0.5			*5.0*		
	PA	*PZA*	*5-OH-PA*	*PA*	*PZA*	*5-OH-PA*
Mean	0.71	0.82	0.33	1.07	1.01	0.41
Standard deviation	0.102	0.052	0.030	0.072	0.146	0.066
Standard error	0.041	0.021	0.012	0.029	0.059	0.027

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF PA, PZA, AND 5-OH-PA IN URINE

Data given are area ratios for compound and nicotinamide internal standard. Each component in both concentrations was analyzed in 6 independent samples ($N = 6$).

	Sample size (μg/ml)					
	10			*100*		
	PA	*PZA*	*5-OH-PA*	*PA*	*PZA*	*5-OH-PA*
Mean	0.76	0.62	0.39	0.73	0.72	0.36
Standard deviation	0.119	0.024	0.040	0.036	0.015	0.030
Standard error	0.0049	0.010	0.016	0.014	0.006	0.012

Data for reproducibility are shown in Tables I and II. Observed area ratios are shown for a series of six runs for concentrations of 0.5 μg/ml and 5.0 μg/ml in serum, and 10 μg/ml and 100 μg/ml in urine. This reproducibility is routinely achievable without special precautions. No attempt was made to strive for even better reproducibility since it is believed that the present one is more than adequate for the purpose of our investigations, particularly when considering significant differences in the renal clearance of PZA. Once again, it is emphasized that the relatively high concentrations were selected because they are representative of levels expected in biological experiments. The trace analytical nature of the selected-ion-monitoring technique is utilized by using very small sample sizes and by eliminating almost all sample preparation.

Quantification

Fig. 3 shows a typical computer output of selected-ion monitoring. The compounds were monitored in a patient's serum. The 5-OH-PA metabolite appears as only a small peak due to the computer-normalized (with respect to PZA) hard-copy output. The actual area of this peak is well above that of background and is easily measured by the computer for quantification. Fig. 4 is a typical trace of selected ion monitoring in urine. Note that the 5-OH-PA peak is much larger than that found in serum.

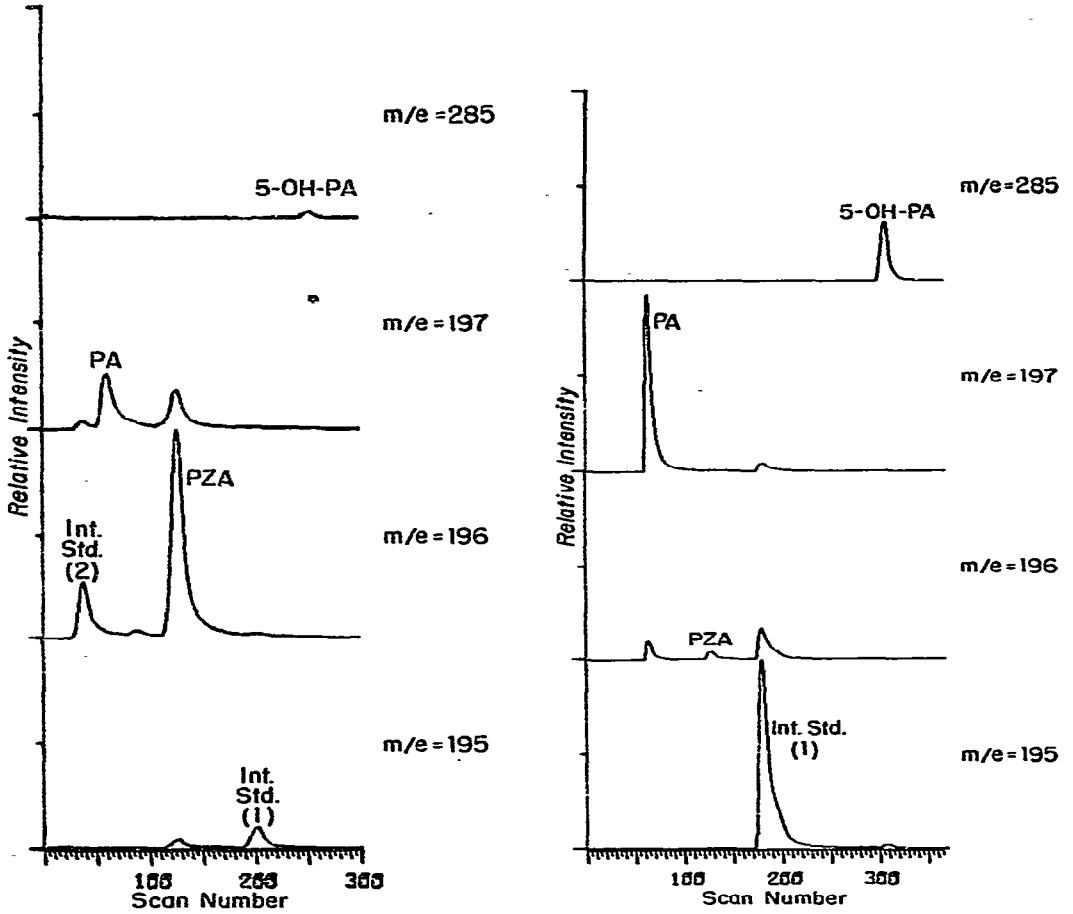

Fig. 3. Selected ion monitoring of PZA and its metabolites in the serum of a patient receiving PZA Nicotinic acid and nicotinamide are internal standards. Quantification is made by measuring peak areas. This scanning corresponds to the 4-h collection shown in Fig. 5.

Fig. 4. Selected ion monitoring of PZA and its metabolites in the urine of a patient receiving PZA. Nicotinamide is internal standard. Quantification is made by measuring peak areas. This scanning corresponds to the 2–4-h collection period shown in Fig. 6.

PZA, PA, and 5-OH-PA levels in serum and urine

Following 3.0 g of PZA in a single oral dose, serum PZA concentrations (Fig. 5) were 134 and 125 μg/ml at the end of 1 and 2 h and reached below 60 μg/ml at the end of 12 h. Serum PA concentration remained fairly constant between 20 and 30 μg/ml throughout except for the first hour. Serum 5-OH-PA ranged between 3 and 8 μg/ml. Although there has been little doubt concerning the identities of PA and the 5-OH-PA metabolites in urine in previous studies, this is the first time that the presence of these compounds in human blood have been positively confirmed by mass spectrometry. The presence of the 5-OH-PA metabolite in serum has definitely been established and speculations may be offered concerning the metabolism leading to the

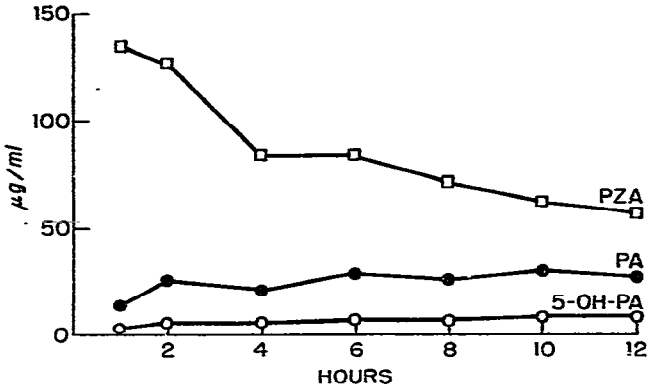

Fig. 5. Blood levels of PZA, PA, and 5-OH-PA in a patient receiving 3 g of PZA (oral).

appearance of this metabolite in serum. Where comparison is possible, the numerical values obtained appear to be of the same order of magnitude as those reported in other studies[5].

The data on urine (Fig. 6) indicate that little unchanged PZA was excreted for 12 h after 3 g of PZA was administered. PA was present in quantities several times that of PZA. PA excretion steadily increased and peaked after 6 h, then declined. The excretion of 5-OH-PA likewise increased steadily, but at a lower rate than that of PA for the first 4 h, and exceeded that of PA at 6 h and thereafter. Approximately 20% of the administered PZA was excreted as PA and 5-OH-PA by 8 h, and 30% by the end of 12 h. The unchanged PZA excretion was barely 1%. Cumulative excretion values reached 40% by 24 h. Urinary excretion patterns of PZA and its metabolites PA and 5-OH-PA obtained in the present study are quite comparable to those reported by Weiner and Tinker[5]. By giving 1.0 g of PZA intravenously to dogs of 13–16 kg, they found a plasma concentration of PZA above 100 µg/ml, with rather low

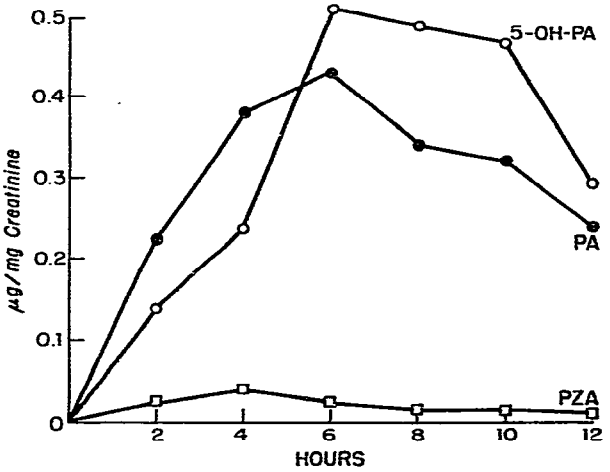

Fig. 6. Urinary excretion of PZA, PA, and 5-OH-PA in a patient receiving 3 g PZA (oral)

concentrations of PA and 5-OH-PA. As much as 30% of the administered dose was accounted for as PA and 5-OH-PA in 6 h, and 70% at the end of 24 h. Very little PZA was excreted in urine.

CONCLUSIONS

A combined gas chromatographic–mass spectrometric technique was developed for the simultaneous determination of PZA and its two main metabolites, PA and 5-OH-PA in serum and urine. The technique is specific because only selected ions are monitored; thus, only minimal sample preparation is needed. The high sensitivity (10 ng/ml for PZA and PA and 20 ng/ml for 5-OH-PA) results in small sample requirement: 200 μl serum and 20 μl urine. The use of nicotinic acid and/or nicotainamide as internal standard facilitates reproducible quantification.

The presence of both unchanged PZA and its metabolites was positively confirmed both in the serum and urine of human subjects receiving the drug. Serum levels and urinary excretion rates for both the drug and metabolites are in agreement with previous findings, based mainly on experimental animals.

The technique developed permits convenient studies of drug interactions. For example, previous studies on the influence of PZA and probenecid on urate excretion[7] are now being extended to include the investigation of the influence of probenecid on the qualitative and quantitative metabolism of PZA.

ACKNOWLEDGEMENTS

This work was supported in part by Grants AM-00162 and 5P01 CA-15936 by the National Institutes of Health, U.S.A.

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