Journal of Chromatography, 194 (1980) 387–398
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,768

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE MYCOTOXIN PENICILLIC ACID AND ITS APPLICATION TO BIOLOGICAL FLUIDS

PING KWONG CHAN*, MOHAMED Y. SIRAJ and A. WALLACE HAYES

Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS 39216 (U.S.A.)

(First received January 17th, 1980; revised manuscript received February 20th, 1980)

SUMMARY

Penicillic acid (PA) is a carcinogenic food contaminant produced by several food-borne fungi. PA was resolved as a sharp peak by reversed-phase high-performance liquid chromatography on a small-particle (10 μm) column in 3–4 min by an elution system composed of acetonitrile (AN), water and glacial acetic acid (AC) with detection by ultraviolet (UV) absorbance at 254 nm. Peak height and peak area were related linearly to the amount of PA injected over a range of 5–500 ng. Reproducibility of retention time, peak height and peak area was demonstrated. The lower detection limit was 5 ng in two elution systems [AN–H_2O–AC (45:55:2), flow-rate (F) 1 ml/min; AN–H_2O–AC (40:60:2), F = 1.2 ml/min] and 10 ng in a third system [AN–H_2O–AC (25:75:2), F = 1.6 ml/min]. Based on the sensitivity and separation of PA from interfering peaks, the following systems were optimal: plasma and bile, AN–H_2O–AC (40:60:2), F = 1.2 ml/min; urine, AN–H_2O–AC (25:75:2), F = 1.6 ml/min. Good recovery (89–98%) over a range of 1–50 μg/ml of PA was obtained from PA-spiked plasma samples treated first with 25% HPO_3 followed by extraction with chloroform. A single peak detected either by UV absorbance or by radioactivity was obtained when plasma samples spiked with [^{14}C]PA were extracted. Good recovery (92–105%) of PA also was obtained from spiked urine and bile samples.

INTRODUCTION

Penicillic acid (PA), a secondary fungal metabolite of several *Penicillia*, was discovered by Alsberg and Black[1] in 1913. PA (Fig. 1) has a variety of activities and toxicities, including antibacterial[2], antiviral[3], antitumor[3] and antidiuretic[4] properties and a digitalis-like action on cardiac muscle, and dilating action on coronary and pulmonary vessels[4]. PA also is cytotoxic[5], hepatotoxic[6–8] and carcinogenic in mice and rats[9–11]. The potential human health hazard of PA was suggested when it was isolated from agricultural products including blue-eye diseased corn, poultry feed, commercial corn, dried beans and tobacco products[12–16].

Procedures for the estimation of PA in natural products and in semi-synthetic

0021-9673/80/0000–0000/$02.25

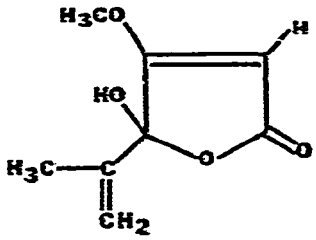

Fig. 1. Structure of penicillic acid, $C_8O_4H_{10}$. Molecular weight, 170.16.

growth media, include colorimetric methods[17], bioassays[18], thin-layer chromatography (TLC)[12,19–22], gas–liquid chromatography (GLC)[13–16,23–25] and GLC–mass spectrometry (MS)[15]. However, a simple, convenient, specific and sensitive method for extraction and estimation of PA in biological fluids has not been reported. This paper describes procedures for the extraction of PA from rat plasma, urine and bile and a high-performance liquid chromatographic (HPLC) technique to quantitate PA in these biological fluids.

EXPERIMENTAL

PA, purchased from Makor Chemical (Jerusalem, Israel), was recrystallized from benzene and hexane. Its purity (>99%) was established by ultraviolet (UV) absorption, melting point, TLC and HPLC. The radiopurity (>99%) and chemical purity (>99%) of ^{14}C-labeled penicillic acid, a gift from Dr. A. Ciegler (USDA, New Orleans, LA, U.S.A.), was confirmed by radiochromatography and HPLC, respectively. Standard solutions of PA were prepared by accurately weighing a specific amount of PA and dissolving it in glass-distilled water to prepare standards containing 1 mg/ml. Lower concentrations of PA were prepared by diluting the standard stock solution with the appropriate elution solvent system.

Equipment

Reversed-phase chromatography was performed using a Waters Assoc. HPLC system, including a U6K septumless injector, a Model 440 UV absorbance detector, a Model 660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe dual-pen recorder equipped with an electronic integrator (Houston Instrument, Austin, TX, U.S.A.). Separations were achieved with a μBondapak C_{18} (particle size 10 μm) column (30 cm × 39 mm I.D., Waters Assoc.) at an elution rate of 1.2–1.6 ml/min at a normal pressure of 750–1000 p.s.i.

A pre-column (Whatman, Clifton, NJ, U.S.A.) connected just before the μBondapak column increased the retention time of PA only 1–2 sec. PA was detected at 254 nm, with the UV absorbance detector at sensitivities of 0.005–0.5 absorbance units full scale (a.u.f.s.). PA standards and sample solutions in a volume of 2–10 μl were injected with a microsyringe (Hamilton, Reno, NV, U.S.A.).

Elution solvent

The elution system(s) consisted of distilled-in-glass acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), glass-distilled water and glacial acetic acid

(Fisher Scientific, Fairlawn, NJ, U.S.A.). The elution system(s) was prepared by stirring under vacuum the appropriate ratio of acetonitrile (AN), water and glacial acetic acid (AC). The pH of the elution system(s) was 3.6.

RESULTS AND DISCUSSION

Optimization of the elution system

Solvent programming was used to establish optimum solvent ratios. The elution system was optimized further by separation of the PA peak from interfering peaks in the biological samples. The capacity ratios (k') and t' values of PA in various HPLC systems are listed in Table I. Except in the two systems with methanol, PA was resolved in 3–5 min. The t' value is an approximate reflection of peak tailing. A t' value of 1 represents a symmetrical peak. k' was calculated by dividing the difference between the retention time of the PA peak and the non-retained peak by the non-retained retention time. As indicated in Table I, tailing in all systems was acceptable. Although systems containing methanol gave higher k' values, the peaks were broadened if the solvent system contained less than 40% methanol. Column pressure also was high and sensitivity low in these systems. On the contrary, PA was resolved as a sharp peak in 3–4 min in systems containing acetonitrile. Sensitivity in these systems, however, decreased as polarity of the solvent system increased.

TABLE I

PARAMETERS OF PENICILLIC ACID IN VARIOUS SOLVENT SYSTEMS WITH A μBONDAPAK C_{18} COLUMN

AN = Acetonitrile; AC = acetic acid. t' = (Total peak width − tail width)/total peak widt h; $k' = (V_1 - V_0)/V_0$ where V_0 = void volume and V_1 = volume required for peak elution; t_R = retention time.

AN–H_2O–AC Ratio (v/v)	*Flow-rate (ml/min)*	*t_R (sec)*	*t'*	*k'*	*Sensitivity*	
					*mm/ng**	*mm^2/ng***
45:55:2	1.0	245	0.58	0.49	1.09	0.28
45:55:2	1.2	215	0.54	0.44	1.08	0.29
45:55:2	1.4	190	0.61	0.41	1.09	0.22
40:60:2	1.2	218	0.67	0.66	0.98	0.23
40:60:2	1.4	190	0.5	0.53	0.95	0.23
40:60:0	1.2	220	0.5	2.4	0.84	0.23
35:65:2	1.2	240	0.63	0.71	0.92	0.29
30:70:2	1.4	230	0.66	0.92	0.84	0.25
25:75:2	1.6	235	0.66	2.75	0.69	0.21

* Peak height.
** Peak area.

Analysis of chloroform extracted PA-spiked plasma and bile samples revealed that although elution system I (AN–H_2O–AC, 45:55:2) gave good sensitivity, separation of PA from interfering peaks was unacceptable. System II (AN–H_2O–AC, 40:60:2) gave adequate separation of PA from interfering peaks with good sensitivity. Chloroform extracted PA-spiked urine samples revealed that only solvent system III

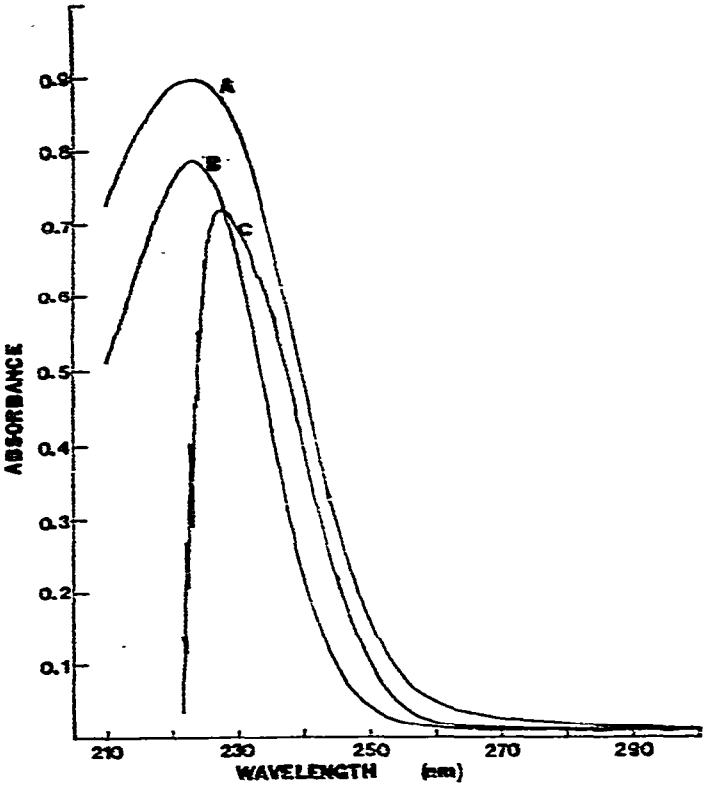

Fig. 2. UV absorbance spectra of penicillic acid (10 μg/ml) in water (A), acetonitrile (B) and in elution solvent system II, acetonitrile–water–glacial acetic acid (40:60:2) (C).

containing AN–H_2O–AC (25:75:2) gave satisfactory separation of PA from interfering peaks even though sensitivity was somewhat low.

Detection

The UV absorption spectra of PA in water, AN and in elution solvent system III are illustrated in Fig. 2. Maximum absorption was at 227 nm. Although the molar coefficient of absorptivity (ε_{254}) at 254 nm, in both elution solvent systems II and III,

TABLE II

ABSORBANCE CHARACTERISTICS OF PENICILLIC ACID IN WATER, ACETONITRILE AND TWO HPLC ELUTION SOLVENT SYSTEMS

Systems: II = acetonitrile–water–glacial acetic acid (40:60:2); III = acetonitrile–water–glacial acetic acid (25:75:2).

Characteristic	*Water*	*AN*	*Elution solvent system*	
			II	*III*
$\lambda_{max.}$ (nm)	224	224	227	227
$\varepsilon_{max.}$ (M^{-1} cm^{-1})	12,180	10,550	9665	9937
ε_{254} (M^{-1} cm^{-1})	1225	204.2	340.3	476.5

was less than 5% of maximum absorption (Table II), 254 nm was used because it was the closest wavelength available. Thus, the lower limit of detection of PA by an HPLC system equipped with a variable-wavelength detector should be increased if similar sensitivities are available. Although many biological substances absorb UV light at 254 nm, the extraction procedures described below coupled with optimum elution solvent systems provided good separation of PA from interfering peaks.

Retention time

Retention times were highly reproducible with the HPLC solvent systems and column conditions employed (Table III). Mean retention times for PA in system II, flow-rate (F) = 1.2 ml/min, and in system III, F = 1.6 ml/min, over a period of several days were 218.3 and 234.5 sec, respectively, with coefficients of variation [(σ/mean)·100] of 0.92% and 1.22%, respectively.

TABLE III

REPRODUCIBILITY OF RETENTION TIME FOR PENICILLIC ACID BY HPLC

Systems: I = acetonitrile–water–glacial acetic acid (45:55:2), flow-rate 1.0 ml/min; II = acetonitrile–water–glacial acetic acid (40:60:2), flow-rate 1.2 ml/min; III = acetonitrile–water–glacial acetic acid (25:75:2), flow-rate 1.6 ml/min.

Statistic	*Elution solvent systems*		
	I	*II*	*III*
Injections, N*	44	67	51
Retention time (sec)			
Range	242–248	213–220	230–240
Mean	244	218.3	234.5
Mean retention time (min)	4.06	3.64	3.91
Standard deviation (sec)	1.69	2.02	2.87
Coefficient of variation (%)**	0.69	0.92	1.22

* Successive injections of penicillic acid, 10 ng to 10 μg over 4 days.

** (Standard deviation/mean) × 100.

Linearity

Peak height and peak area (Figs. 3 and 4) were related linearly to the amount of PA injected over a range of 5–500 ng of PA in systems I, II and III. For each system, the correlation coefficients for both peak height and peak area exceeded 0.999.

Precision and sensitivity

Precision was evaluated by injecting 5-μl aliquots of standard solution containing 100 ng of PA in each of the three elution solvent systems (Table IV). Reproducibility of both peak height and peak area measurements was good, with coefficients of variation of 1.6–4.1% for peak height and 2.1–3.5% for peak area, representing the combined errors of dilution, HPLC resolution, injection and detection.

Mean peak height sensitivities were 1.09, 0.99 and 0.69 mm/ng for systems I, II and III, respectively, while peak area sensitivities were 0.28, 0.23 and 0.2 mm^2/ng, respectively (Table IV). The chromatograms shown in Fig. 3 indicate that 10–20 ng of PA can be detected in systems II and III. Detection limits, defined as the quantity

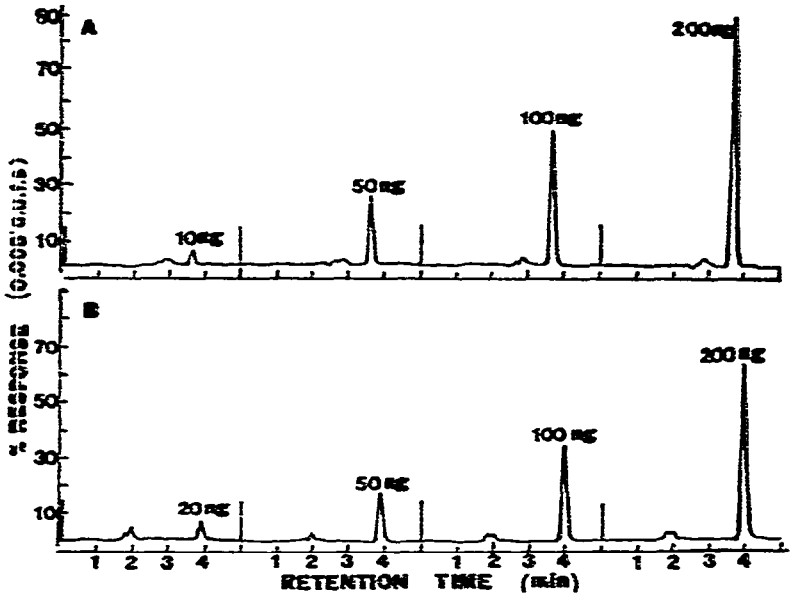

Fig. 3. HPLC resolution of penicillic acid on μBondapak C_{18} column. A, elution solvent system II. acetonitrile (AN)–water–glacial acetic acid (AC) (40:60:2), flow-rate 1.2 ml/min; B, elution solvent system III, AN–H_2O–AC (25:75:2), flow-rate 1.6 ml/min. Detection at 254 nm.

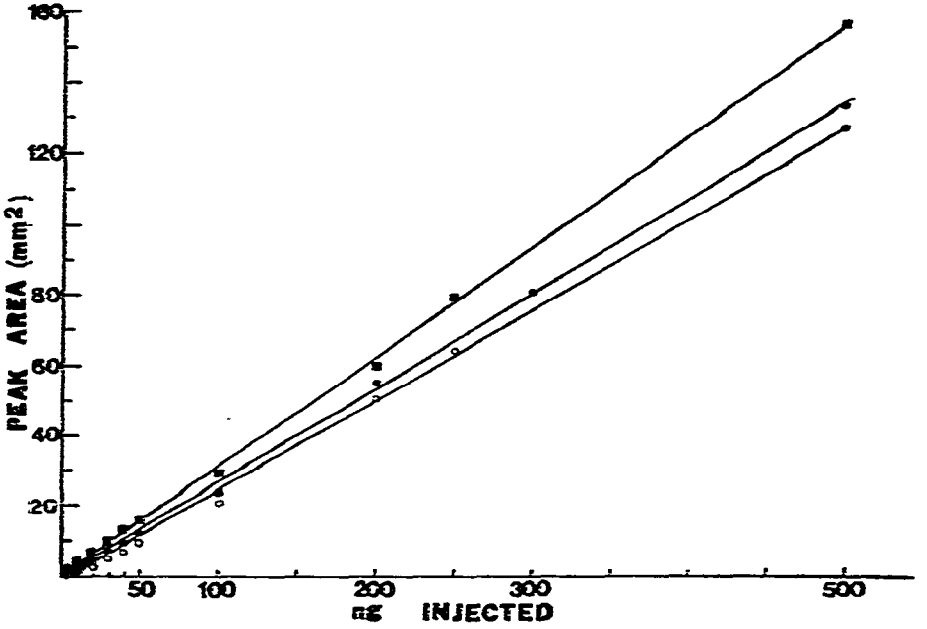

Fig. 4. Linearity, peak area *vs.* quantity of penicillic acid (5–500 ng) at 254 nm in: ■, AN–H_2O–AC, (45:55:2), flow-rate (*F*) 1.0 ml/min; ●, AN–H_2O–AC (40:60:2), $F = 1.2$ ml/min; ○, AN–H_2O–AC (25:75:2), $F = 1.6$ ml/min.

TABLE IV

PEAK HEIGHT AND PEAK AREA REPRODUCIBILITY OF PENICILLIC ACID SEPARATION BY HPLC

Systems I–III as in Table III.

Statistic	*Elution solvent systems (AN–H_2O–AC)*		
	I	*II*	*III*
Injections, *N**	10	10	5
Peak height (mm)			
Range	103.5–110	96–110	67–70
Mean	108.8	98.4	68.8
Standard deviation (mm)	2.8	4.1	1.1
Coefficient of variation (%)**	2.5	4.1	1.6
Sensitivity (mm/ng)***	1.09	0.99	0.69
Peak area (mm^2)			
Range	27.2–28.8	21.8–24.4	19–20
Mean	28.03	22.9	19.8
Standard deviation (mm^2)	0.6	0.8	0.6
Coefficient of variation (%)**	2.1	3.5	3.0
Sensitivity (mm^2/ng)***	0.28	0.23	0.2

* Successive injections of 100 ng of penicillic acid in 5 μl of an elution solution system.
** (Standard deviation/mean) × 100.
*** Calculated to maximum sensitivity, 0.005 a.u.f.s.

of PA which gave a peak height 2.5 times above background, were 5 ng in systems I and II and 10 ng in system III (data not presented).

Biological applications

Extraction of PA from plasma. Ethyl acetate and chloroform were used to extract PA from plasma samples. Chloroform-extracted samples gave cleaner chromatograms and better separation of PA from interfering peaks in the plasma in system II but not in system I. Several protein precipitation procedures, including acidification and heating, were tested. Strong acids such as HCl (3 *N* or 4 *N*) and metaphosphoric acid (HPO_3, 25%) gave quantitative recovery of PA from plasma; however, with HCl, excessive foaming occurred. HPO_3 gave better protein precipitation; indeed, HPO_3 has been used to percipitate protein in the determination of tissue glutathione levels[26]. To determine the amount of HPO_3 (25%) needed for optimal extraction of PA from plasma, 10, 15, 50, 100, 200 and 300 μl of HPO_3 were added to 100 μl of plasma spiked with PA and then the samples were extracted twice with 1.5 ml of chloroform each time. Fifty and 100 μl of HPO_3 gave the best recovery and the cleanest chromatograms. The following extraction procedure, therefore, was used to extract PA from plasma.

Spiked rat plasma (100 μl), containing 1, 2, 5, 10, 25 or 50 μg/ml of PA in a test-tube (15 × 12 mm), was treated with 100 μl of HPO_3 (25%), mixed and then diluted with 0.3 ml of glass-distilled water. The mixture was extracted twice with 1.5 ml of chloroform by mixing for 2 min each time. After centrifugation at 600 *g* for 10 min, the lower chloroform layers were pooled in a tube (15 × 10 mm) and evaporated to dryness under a stream of N_2. The residue was taken up in 50 or 100 μl of acetonitrile and a 5–10-μl aliquot was analyzed by HPLC. No increase in system

back pressure was observed despite many days of repeated injections, indicating sample preparation was adequate. Typical HPLC tracings of chloroform-extracted plasma and chloroform-extracted PA-spiked plasma are illustrated in Fig. 5. Recovery of PA was 89–98% (Table V).

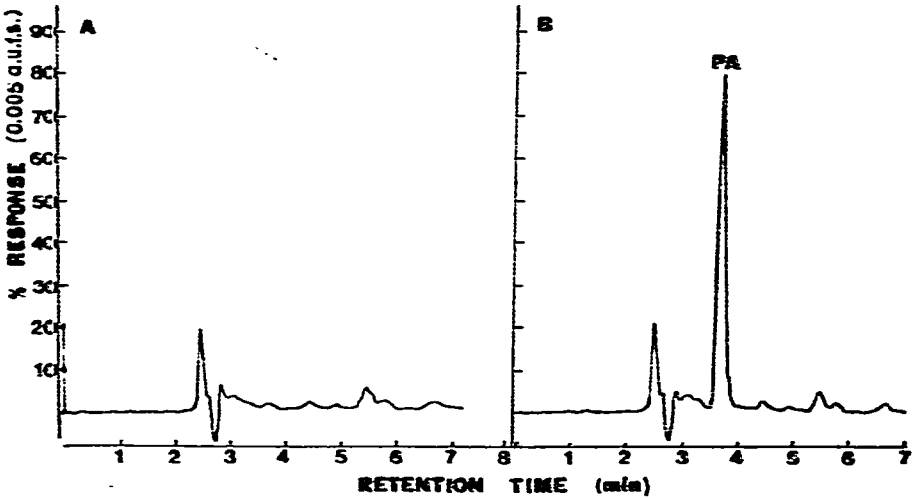

Fig. 5. Chromatogram tracing of: A, chloroform-extracted plasma; B, extract of plasma spiked to 50 μg/ml penicillic acid. Elution solvent system II: AN–H_2O–AC 40:60:2, F = 1.2 ml/min. Detection at 254 nm.

TABLE V

RECOVERY OF PENICILLIC ACID FROM PLASMA, BILE AND URINE

Penicillic acid (μg/ml)	*Recovery (%), Mean ± S.E.**		
	Plasma	*Bile*	*Urine*
1	88.9 ± 5.0	ND	ND
2	97.2 ± 9.1	91.8 ± 3.3	99.9 ± 6.2
5	91.7 ± 2.0	92.6 ± 3.0	102.6 ± 13.6
10	91.7 ± 4.3	ND	105.5 ± 6.4
25	95.2 ± 4.5	103.1 ± 3.7	104.2 ± 2.0
50	97.4 ± 3.2	96.7 ± 5.2	101.1 ± 0.8

* n = 3. ND = Not done.

To determine the radioactivity purity of [^{14}C]PA, 10 μg of [^{14}C]PA in 10 μl of elution solvent system II were injected and the column then eluted with the same solvent system. Fractions, collected at 10-sec intervals, were added to 10 ml of PCS (Amersham, Arlington Heights, IL, U.S.A.) for liquid scintillation spectrometry. Lag time between UV detection and collection of the sample was estimated by injecting 10 μg of non-labeled PA and collecting fractions at 5-sec intervals. Aliquots of 10 μl were re-injected for PA analysis. The lag time, calculated by substracting the PA retention time from the retention time corresponding to the eluate fraction with the

highest PA concentration, was 50 sec. After correction for lag time, all radioactivity was eluted as a single peak corresponding to the retention time of PA as detected by UV absorbance, indicating that [^{14}C]PA was at least 99% pure (Fig. 6, A and B). [^{14}C]PA (100 μg) was added to 100 μl of plasma, extracted and analyzed by HPLC as described above. Chromatograms similar to those obtained for the standard [^{14}C]PA (Fig. 6, C and D) were obtained, indicating that all added radioactivity was extracted from the plasma as the parent compound.

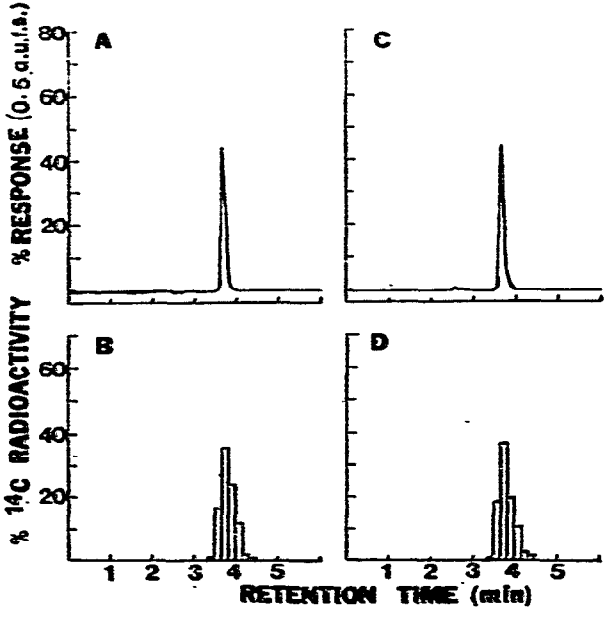

Fig. 6. A, Chromatogram tracing of 10-μl aliquot of 10 μg of ^{14}C-labeled penicillic acid in elution solvent system II. See text for column and conditions. B, Radioactivity collected at 10-sec intervals after injection of 10 μg penicillic acid. Retention time was corrected for the lag time between UV detection and collection. C, Chromatogram tracing of extract of plasma spiked with 100 μg [^{14}C]PA. See text for column and conditions. D, Radioactivity collected at 10-sec intervals after injection of a 10-μl aliquot of N_2-evaporated extract of plasma spiked with [^{14}C]PA and extracted as in C.

Extraction of PA from bile. Recovery of PA from spiked bile samples was dependent on pH with quantitative recovery only at pH 4.4 or below (adjusted with 0.2 *M* acetate buffer) (Fig. 7). PA was added to 50 μl of bile, collected from a cannulated common bile duct of male rats under pentobarbital anesthesia. The bile then was adjusted to pH 4.4 and extracted twice with 1.5 ml of chloroform. The chloroform layers were pooled and evaporated to dryness under N_2. The residue was taken up in acetonitrile for HPLC analysis using elution system II. A typical chromatogram of a chloroform-extracted bile sample containing 50 μg/ml of PA is illustrated in Fig. 8. Recovery of PA was 92–103% over a range of 2–50 μg/ml (Table V).

Extraction of PA from urine. Not only was extraction of PA from urine dependent on pH (Fig. 9), peaks in the urine which interfered with PA detection also were pH dependent. Large amounts of interfering substances were extracted below pH 3. As the pH of urine (adjusted either with 0.2 *M* acetate buffer or 0.2 *M* phosphate

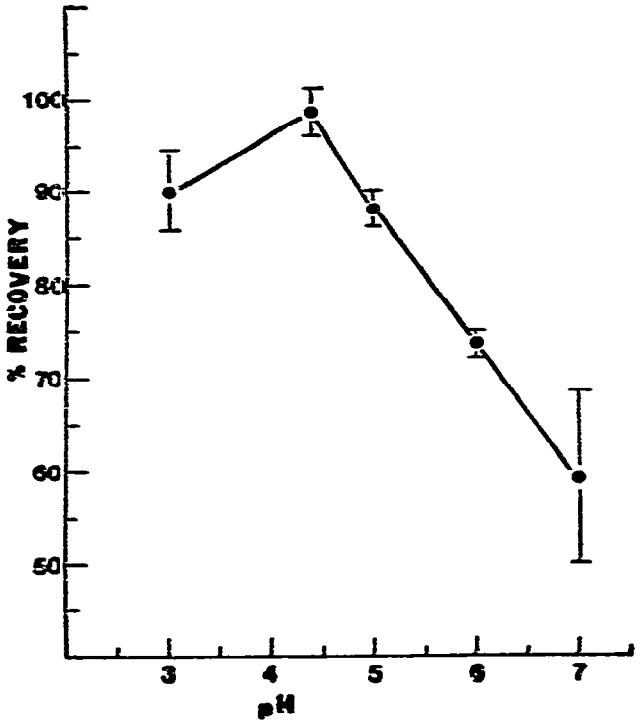

Fig. 7. Effect of pH on the recovery of penicillic acid from bile (50 μg/ml). Each point represents the mean ± S.E. of three individual extractions.

buffer) increased, acceptable HPLC chromatograms were obtained (Fig. 10). Maximal recovery of PA from urine samples containing 50 μg/ml of PA was at pH 5.0 (Fig. 9). Small volumes (100 μl) of rat urine, to which PA was added, were adjusted to pH 5.0

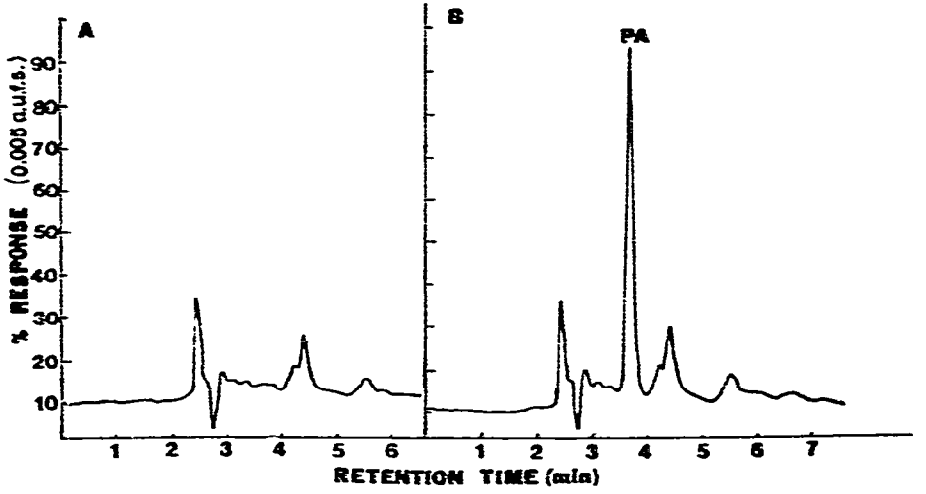

Fig. 8. Chromatogram tracings of: A, bile extract; B, extract of bile spiked to 50 μg/ml penicillic acid Elution solvent system: AN–H_2O–AC (40:60:2), F = 1.2 ml/min. Detection at 254 nm.

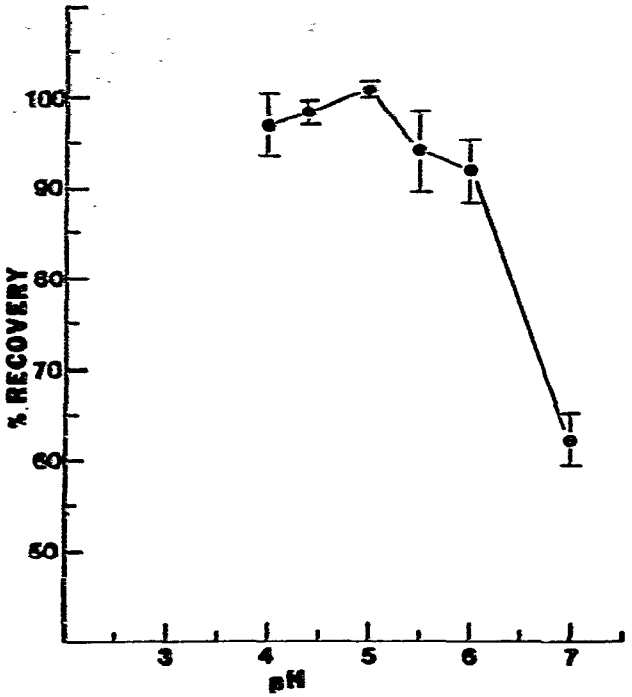

Fig. 9. Effect of pH on the recovery of penicillic acid from urine (50 μg/ml). Each point represents the mean $\pm$ S.E. of three individual extractions.

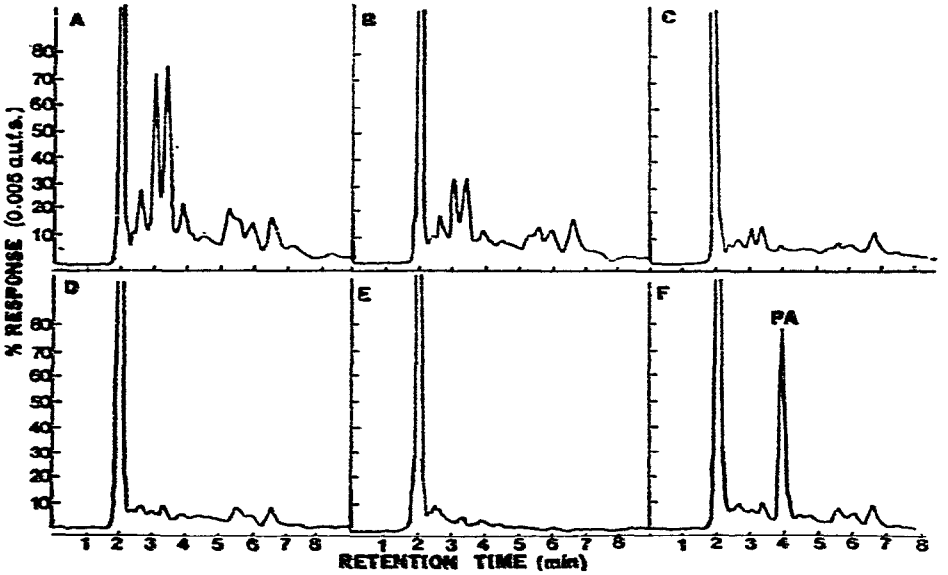

Fig. 10. Chromatogram tracings of extract of urine or of penicillic acid-spiked urine (F). The urine was adjusted to different pHs with either 0.2 *M* acetate buffer or 0.2 *M* phosphate buffer. A, Urine, pH 3; B, urine, pH 4.0; C, urine, pH 4.4; D, urine, pH 5; E, urine, pH 7; F, spiked urine (50 μg/ml), pH 5. Elution solvent system: AN–H_2O–AC (25:75:2), F = 1.6 ml/min. Detection at 254 nm.

and then extracted three times with 1.5 ml of chloroform. The chloroform layers were pooled and evaporated to dryness under N_2. Residues were taken up in 50–100 μl of acetonitrile and 5–10-μl aliquots were analyzed. Elution system III was the best system for the analysis of PA in urine because most of the interfering peaks in urine eluted before PA in this system. The recovery of PA from urine was approximately 100% over a range of 2–50 μg/ml (Table V).

Various analytical methods have been developed for detection of PA. Although TLC methods are specific, such methods are not particularly sensitive; colorimetric methods and bioassays are neither specific nor sensitive. The only sensitive methods currently available for PA are GLC and GLC–MS procedures which are difficult and often laborious. The HPLC method described here is simple, rapid, specific and has equal or greater sensitivity than some previously reported GLC methods. The extraction procedures for PA from bile, blood and urine are simple one-step extraction, thus minimize the loss of toxin during extraction. This HPLC method should be well-suited for field monitoring of PA in biological fluids from animals and humans and for pharmacokinetic studies.

ACKNOWLEDGEMENTS

This research was supported by U.S.P.H.S. Grants ES 01351 and ES 02191. P.K.C. was supported by Training Grant ES 07045.

REFERENCES

1 C. L. Alsberg and O. F. Black, *Dept. Agr. Bur. Plant, Ind. Bull.*, 270 (1913) 7.
2 A. E. Oxford, *Biochem. J.*, 36 (1942) 438–444.
3 S. Suzuki, T. Kimura, F. Saito and K. Ando, *Agr. Biol. Chem.*, 35 (1971) 287–290.
4 M. F. Murnaghan, *J. Pharmacol. Exp. Ther.*, 88 (1946) 119–132.
5 M. Umeda, *Jap. J. Exp. Med.*, 41 (1971) 195–201.
6 H. Kobayashi, H. Tsumoda and T. Tatsumo, *Chem. Pharm. Bull.*, 19 (1971) 839–842.
7 A. Ciegler, H. J. Mintzlaff, D. Weisledes and L. Leistner, *Appl. Microbiol.*, 24 (1972) 114–119.
8 P. K. Chan, C. S. Reddy and A. W. Hayes, *Toxicol. Appl. Pharmacol.*, 52 (1980) 1–9.
9 F. Dickens and H. E. H. Jones, *Brit. J. Cancer*, 15 (1961) 85–95.
10 F. Dickens and H. E. H. Jones, *Brit. J. Cancer*, 17 (1963) 100–108.
11 F. Dickens and H. E. H. Jones, *Brit., J. Cancer*, 19 (1965) 392–403.
12 C. P. Kurtzman and A. Ciegler, *Appl. Microbiol.*, 20 (1970).
13 R. W. Pero, D. Harvan, R. G. Owens and J. P. Snow, *J. Chromatogr.*, 65 (1972) 501–506.
14 C. W. Bacon, J. G. Sweeney, J. D. Robbins and C. Burdick, *Appl. Microbiol.*, 26 (1973) 155–160.
15 C. W. Thorpe and R. L. Johnson, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 861–865.
16 J. P. Snow, G. B. Lucas, R. W. Harven and R. G. Owens, *Appl. Microbiol.*, 24 (1972) 34–36.
17 R. Bentley and J. G. Keil, *J. Biol. Chem.*, 237 (1962) 86.
18 V. Betina, *J. Chromatogr.*, 15 (1964) 379–392.
19 S. Neelakantan, T. Balasubramanian, R. Balasarawathi, G. I. Jasmine and R. Swaminathan, *J. Food Sci. Technol.*, 15 (1978) 125–126.
20 A. Ciegler and C. P. Kurtzman, *J. Chromatogr.*, 51 (1970) 511–516.
21 P. M. Scott and E. Somers, *J. Agr. Food Chem.*, 16 (1968) 483.
22 P. M. Scott, J. W. Lawrence and W. Van Walbleck, *Appl. Microbiol.*, 20 (1970) 839.
23 T. Suzuki, M. Takeda and H. Tenabe, *Shokuhin Eiseigaku Zasshi*, 12 (1971) 495.
24 R. W. Pero and D. Harvan, *J. Chromatogr.*, 80 (1973) 255–258.
25 Y. Fujimoto, T. Suzuki and Y. Hoshino, *J. Chromatogr.*, 105 (1975) 99–106.
26 V. H. Cohn and J. Lyte, *Anal. Biochem.*, 14 (1966) 434–440.