Liquid Chromatographic Determination of Dipicolinic Acid from Bacterial Spores

A. D. WARTH

Commonwealth Scientific and Industrial Research Organization, Division of Food Research, North Ryde, New South Wales 2113, Australia

Received for publication 13 September 1979

Dipicolinic acid was determined by reverse-phase liquid chromatography. Elution was with 0.2 M potassium phosphate, pH 1.8, containing 1.5% tert-amyl alcohol or higher concentrations of lower alcohols or acetonitrile. The normal analytical range was 50 to 1,000 μM, which is equivalent to 0.1 to 1 mg of spores per ml with a relative standard error of 2 to 4% and a detection limit of <100 pmol. Dipicolinic acid was fully extracted from spores by heating at pH 1.8 for 10 min at 100°C. Sporulating cultures may be analyzed in less than 20 min without separation of cells from media. Liquid chromatography was also used to detect dipicolinic acid in more complex substrates, e.g., guinea pig feces containing *Metabacterium polyspora* spores and canned food. Dipicolinic acid could be detected in unspoiled canned salmon containing <10^6 added *Bacillus cereus* spores per g.

Dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) is a major constituent of bacterial endospores, comprising 5 to 14% of their dry weight (3, 9). Its analysis is of importance in studies of sporulation, germination, and spore structure, and its presence is considered diagnostic for bacterial endospores. Currently, DPA is determined either by spectrophotometry of the Fe2+ complex (1) or by ultraviolet spectrophotometry of the Ca2+ complex (2, 6). Spectrophotometry of the Fe2+ complex (1) is convenient but lacks sensitivity and has significant blanks. Spectrophotometry of the Ca2+ complex (2) is more sensitive but requires considerable operator time and in practice can be subject to major errors. Recently Scott and Ellar (6) improved the sensitivity by recording the differential spectrum between DPA2- and Ca DPA. A gas chromatographic method (7) was sensitive but lacked accuracy and convenience. Polargraphic determination of DPA (4) was accurate, but the equipment is not commonly available. For the determination of DPA in spores and bacterial cultures the liquid chromatographic method described here requires minimal sample preparation, is rapid and free from interference, and is more sensitive than the methods mentioned above. The improved sensitivity and versatility of liquid chromatography suggest its usefulness for the detection of low levels of DPA in more complex substrates. Some spoiled and unspoiled canned foods have been examined for the presence of DPA, and a detection limit of about 10^6 spores per g has been established for salmon. As a further example, DPA was detected in a crude preparation of *Metabacterium polyspora* spores.

MATERIALS AND METHODS

Spores. Spores were grown on sporulation medium or half-strength G medium (G/2) as described previously (8) and washed extensively with 0.01 M KCl and water. The strains used are listed in Table 3.

Reagents and apparatus. DPA was from Ralph N. Emanuel Ltd., England. 2-Methylbutan-2-ol (tert-amyl alcohol) was reagent grade, and all other solvents and chemicals were analytical reagent grade from Ajax Chemicals (Sydney, Australia). The liquid chromatograph comprised an Altex Model 110A pump, a Rheodyne model 7120 injector with a 20-μl sample loop, a water-jacketed Brownlee column (250 by 4.6 mm) containing Merck Lichrosorb RP8 10-μm, reverse-phase packing, and a Perkin-Elmer model 55LC variable-wavelength detector. Tubing and filters were of stainless steel.

DPA determination in spores. Samples containing 50 to 1,000 μM DPA (0.1 to 2 mg of spores per ml) in 0.2 M K phosphate, pH 1.8, were heated for 10 min at 100°C and either centrifuged at 4,000 × g for 5 min or filtered through a 0.2-μm membrane filter. At approximately 6-min intervals, 20-μl samples were injected onto the column. Elution was with 1.5% tert-amyl alcohol in 0.2 M K phosphate, pH 1.8, at a flow rate of 1.5 ml min⁻¹ at 25°C or room temperature. The eluant was filtered and degassed. A stock solution of pH 1.8 buffer contained 3 M H_3PO_4 and 1 M KH_2PO_4. Peak heights at 271 nm were measured at 0.02 or 0.20 absorbance units, full scale.

Efficiency of DPA extraction. Spores of *Bacillus stearothermophilus* NCA 1518 (1 mg) were heated in 4 ml of 0.2 M phosphate, pH 1.8, and centrifuged at
4000 × g. The pellet was washed with 1 ml of buffer, suspended in 0.5 ml of buffer, and autoclaved for 10 min at 120°C, readjusted to 0.50 ml and centrifuged. The percent DPA extracted was determined from analyses of the first and final supernatants.

Extraction from guinea pig feces. Fecal pellets from animals naturally colonized with *M. polyspora* were collected and homogenized with water. A preparation containing 2 × 10^6 refractile *M. polyspora* spores per ml, together with remnants of vegetable matter, was obtained by repeated fractional centrifugation. At no stage were other types of refractile spores seen. A 0.2-ml sample was extracted at pH 1.8 for 10 min at 100°C.

**DPA in canned meat loaf and pet food.** Samples of canned meat loaf and pet food were homogenized with an equal weight of water and centrifuged 15 min at 15,000 × g, and the fatty layer was discarded. The supernatant was acidified with phosphate buffer, pH 1.8, filtered, and chromatographed. The deposit was washed twice with water, acidified to pH 1.8 with H_3PO_4, heated for 10 min at 100°C, and centrifuged. Supernatant (0.5 ml) was applied × 4 to 0.8 ml of Dowex 50W-internal diameter (100 to 200 mesh) in a 50-mm plastic syringe barrel and washed with 0.5 ml of 0.2 M phosphate, pH 1.8, and 0.5 ml of water. DPA was eluted with a further 0.5 ml of water.

**Recovery of DPA from spores added to canned salmon.** Canned salmon (40 g) was diluted with 40 ml of water and homogenized. *Bacillus cereus* spores (15 μg) were added to half of the homogenate. After adjustment of the pH to 4.0 with H_3PO_4, the samples in 150-ml glass centrifuge tubes were extracted twice with ethyl acetate (40 ml). The aqueous phase was adjusted to pH 1.8 with 6 N H_2SO_4, heated at 100°C for 20 min, and extracted three times with ethyl acetate (40 ml). The dried extract was dissolved in 1 ml of citrate buffer, pH 3.5, which was then washed with ether (2 ml) and ethyl acetate (2 ml) and acidified to pH 1.8. DPA was extracted three times with ethyl acetate (2 ml), and the residue after evaporation was dissolved in 0.3 ml of water.

**Synthesis of DPA-glycine for use as an internal standard.** DPA (3 mmol) was refluxed with SOCl_2 (15 ml) for 1 h. SOCl_2 was removed by distillation and a solution of glycine (50 mmol) and NaOH (40 mmol) in 10 ml of water was added. The pH was adjusted to 2.0, and the product was recrystallized from water until DPA was <0.1% (three times).

**RESULTS**

Separation conditions. To give adequate retention of DPA on reverse-phase columns and to ensure dissociation of DPA-metal chelates, a low pH eluant was necessary. Phosphate buffer, pH 1.8, was found most suitable as the aqueous component of the eluant. Concentration was not critical, but higher pH's gave asymmetric peaks. This buffer also facilitated extraction of DPA from spores and precipitated substances which may affect the performance and lifetime of the column. Most low-molecular-weight alcohols and acetonitrile were suitable for the organic component of the eluant. Because the spores contained no substances eluting near DPA (Fig. 1a), a rapid elution was possible. With 20% methanol or 12% acetonitrile, or with lesser amounts of the longer chain length alcohols, capacity factors (k') were near 1.2, and analysis time was 5 min. The higher-molecular-weight alcohols gave slightly better reproducibility and column efficiency. Therefore, 1.5% tert-amyl alcohol in 0.2 M potassium phosphate, pH 1.8, was routinely used. Detection sensitivity was optimal at the absorbance maximum of 271 nm, but fixed wavelength detection at 254 nm resulted in only a 50% reduction.

**Quantitation.** The first one or two samples of DPA gave lower peak heights than subsequent ones. This appeared to be caused by strong binding of some DPA to the column. After the column was conditioned with DPA, reproducible peak heights of samples with lower DPA content were obtained for a number of chromatograms. At high temperature (50°C), the saturation of the column was lost quickly and recoveries were lower. For routine quantitative work, therefore, the first one or two standards should be disregarded and all samples should be preceded within approximately 30 min by a standard or by samples of similar or greater DPA concentration. Under these conditions, the relative standard error was typically 4 to 5% for >0.4 nmol of DPA. Greater precision (<2%) was obtained with the temperature controlled to 25°C and with alternate standard and sample injected at constant intervals. The standard curve (Fig. 2) was linear above 0.5 nmol. The curvature below this concentration was caused by a small asymmetry in peak shape and could be avoided by using peak area rather than

![Fig. 1. Chromatograms of extracts of spores and media on RP8. Elution was with 1.5% tert-amyl alcohol in 0.2 M potassium phosphate, pH 1.8, at 25°C. (a) *B. subtilis* B 639 spores, 0.2 mg ml⁻¹; (b) *B. cereus* sporing in G/2 medium, dilution 2x; (c) G/2 medium, dilution 2x; (d) culture supernatant after sporulation of *B. cereus*, dilution 4x.](http://aem.asm.org/).
height. A number of substances related to DPA were tested for use as internal standards, but none compensated for the previous sample effect or the small peak asymmetry. DPA-glycine (\(k' = 2.2\)) was prepared, and it and toluene-4-sulfonic acid (\(k' = 1.8\)) are suitable internal standards where quantitative injection or dilution is impractical.

No significant effects on quantitation have been found with a number of substances (Table 1). In particular, Ca\(^{2+}\) at 200 times the DPA concentration and Mn\(^{2+}\), which forms a strong complex with DPA, did not interfere.

**Extraction of DPA from spores.** DPA is released from spores by autoclaving. More conveniently, the DPA can be extracted at 100°C by using the elution buffer. Trials using the very heat-resistant species *B. stearothermophilus* (Table 2) showed that 10 min was adequate for complete extraction. For determination of the DPA content of spores, 0.1 to 1 mg of spores in 1 ml of pH 1.8 buffer was heated as described above, centrifuged or filtered, and 20 \(\mu l\) was chromatographed. DPA did not adsorb to membrane filters. Results with a number of species (Table 3) showed complete extraction, and no other peaks in the region of DPA were seen.

**Determination of DPA in sporulating cultures.** Ultraviolet absorbing compounds in complex bacteriological media were readily separated from DPA under the standard conditions. G/2 medium (Fig. 1c) and the culture supernatant after sporulation of *B. cereus* (Fig. 1d) had base lines in the DPA position of less than 0.3% of DPA peak height in the total culture (Fig. 1b). Separation of spores from the culture fluid is therefore unnecessary. At 254 nm, base-line absorbance in the media was still <1.5% of the total culture peak.

**DPA in *M. polyspora.* *M. polyspora* are commonly present in the cecum of guinea pigs and are shed in feces. Several spores, each up to 5-\(\mu\)m long, are formed in a motile cell. Although

![Fig. 2. Standard curve for the estimation of DPA. Inset shows curve for low DPA concentrations.](http://aem.asm.org/)
they are most conspicuous under phase-contrast microscopy, their successful culture in vitro has not been reported (5; J. A. Walker, M.Sc. thesis, University of Queensland, St. Lucia, Brisbane, Australia, 1973), nor has it been possible to fully purify the spores. Chromatography of the extract from a fraction of guinea pig feces which had been enriched for these spores showed a distinct peak with the same retention time as that of DPA (Fig. 3d). Added DPA cochromatographed with the peak. Repeat chromatographs with the detector set at three other wavelengths gave peak heights consistent with the spectrum of DPA. The presence of DPA in M. polyspora spores was also suggested by their response to strong acids. Observation by phase-contrast microscopy showed the spores to explode a minute or two after treatment with 2 N HCl but not after treatment with H2SO4. This phenomena is characteristic of spores containing DPA and probably results from transient osmotic pressure effects generated by the dissociation of the Ca-DPA complex in the spore (9).

Detection of DPA in spoiled foods. Three samples of canned food were examined: (i) spoiled meat loaf with a heavy bacterial growth and some spores visible by microscopy; (ii) spoiled pet food showing decomposition, gas production, pH 5.5, and a variety of cocci, bacilli, and yeasts but no spores; and (iii) unspoiled sterile salmon. Analysis of the water extract from meat loaf showed approximately 36 nmol of DPA per g free in solution (Fig. 3b), suggesting that lysis or germination of spores had occurred. After treatment of the acidified pellet at 100°C a further 200 nmol/g was extracted (Fig. 3a). The ultraviolet absorbance spectrum of the peak was very similar to that of DPA, with λmax at 271 nm and λmin at 245 nm. The DPA in the extract was concentrated and partly purified on a small ion-exchange column. No peaks corresponding to DPA were found in the pet food, either in extracts or after the ion exchange fractionation (Fig. 3c). A content of <3 μM DPA was indicated.

Recovery of DPA from spores added to canned salmon. Salmon is a product in which interfering substances such as xanthines and purines can be high. A small number of B. cereus spores (7 × 105/g) were added to half of a sample of unspoiled canned salmon. The sensitivity of the assay was increased by extraction of DPA with ethyl acetate and by concentration. At pH 2 or less, the partition coefficient was 1.2 and was unaffected by 2 mM Ca++ or Mn++, and at pH 3.5 or greater it was <10⁻⁴. Chromatography of the extracts in the standard system showed a large peak obscuring the DPA position. Aceto-nitrile (5%) in 0.4 M phosphate substantially changed the elution order and showed more than 15 peaks with a trough at the DPA position (Fig. 4b). Chromatography of the sample containing added spores showed a shoulder in this position comparable in height to that caused by the same concentration of spore extract (Fig. 4a).

Effect of samples on the position of the DPA peak. In all the extracts of food and cultures examined, DPA eluted at the same position and with a similar peak height to DPA standards. This was confirmed for each sample by the addition of DPA to a replicate. During a series of runs at constant temperature, elution times were constant, however; elution times varied slightly between different batches of eluant.

DISCUSSION

For the routine determination of DPA in spores and sporulating cultures, the standard method described above has advantages over previous methods in sensitivity, accuracy and convenience. It may be varied to optimize special requirements. At the usual concentrations of DPA found, no potentially interfering compounds eluted near DPA. Therefore, if desired, analysis time could be further reduced by the use of shorter columns, higher flow rates, or overlapping elution schedules. An additional advantage, since column efficiency is not limiting,
is greatly extended useful column life. High sensitivities may be obtained by working with small sample volumes, because only 20 μl is used for chromatography. Reverse-phase packings other than RP8 are expected to be suitable but have not been tested. Dependence of peak height on previous injections of DPA and nonlinear standard curve at low DPA concentration appear to be caused by slow adsorption and desorption to sites on the RP8. C₈- or C₂-coated silica packings could be better in this regard.

The greater sensitivity and versatility of liquid chromatography makes it useful for the detection of low concentrations of DPA. Preliminary purification and concentration of the sample and alteration of the organic phase of the eluant may be necessary for more chemically complex samples. Purification procedures using ethyl acetate extraction and ion exchange have been given.

By using the ethyl acetate extraction procedure, DPA from concentrations of 10⁶ B. cereus spores per g was detected in canned salmon. This is beyond the ready detection limit by direct microscopy but does not approach the sensitivity of viability measurements. Analysis for DPA can provide evidence for the presence of spores in particular circumstances where microscopy or culture techniques are inapplicable. Spores may not be culturable as with M. polyspora, or they may have previously germinated or lysed.

ACKNOWLEDGMENTS
I thank R. F. Adams for advice on liquid chromatography and P. Conway for the preparation of M. polyspora spores.

LITERATURE CITED