Journal of Chromatography, 181 (1980) 266—271
Biomedical Applications

CHROMBIO. 450

Note

Non-extractive fluorometric measurement of *p*-aminosalicylic acid in plasma by ion-pairing techniques and high-performance liquid chromatography

IRWIN L. HONIGBERG, JAMES T. STEWART*, T. CRAIG CLARK and DON Y. DAVIS

Department of Medicinal Chemistry, School of Pharmacy, The University of Georgia, Athens, Ga. 30602 (U.S.A.)

(First received June 22nd, 1979; revised manuscript received September 26th, 1979)

The value of *p*-aminosalicyclic acid (PASA) in the treatment of tuberculosis was initially demonstrated by Lehman [1]. It has been observed that PASA in combination therapy with streptomycin and isoniazid delays the emergence of drug resistant strains of bacillus. Recently, PASA has been shown to reduce plasma cholesterol levels by 15—20% [2]. Since it is a widely available and inexpensive drug, PASA may be used as a cholesterol-lowering agent where other drugs are not effective.

A few literature methods have been reported for the analysis of PASA. These include gas chromatography [3], potentiometry [4], non-aqueous titrimetry [5], and spectrophotometry [6]. More recently, PASA has been used as an internal standard in an extractive procedure for the high-performance liquid chromatographic (HPLC) analysis of salicylazosulfapyridine metabolites in plasma [7]. Only the spectrophotometric procedure has been adapted for use in the analysis of PASA plasma levels. HPLC has been shown in these laboratories to be an effective tool for the analysis of drugs in biological fluids [8, 9]. In this paper, an HPLC separation and quantitation of PASA from plasma samples using a non-extractive approach is described. The concatenation of several recent HPLC techniques such as the use of non-extractive sample preparation, ion-pairing reversed-phase HPLC, and enhanced detector sensitivity allowed for the quantitation of therapeutic levels of PASA in as little as 100 μl of plasma.

EXPERIMENTAL

Materials

Powdered samples of *p*-aminosalicylic acid (Merck, Rahway, N.J., U.S.A.)

*To whom correspondence should be addressed.

and anthranilic acid (Chem-Service, Media, Pa., U.S.A.) were obtained for the preparation of standard solutions. PASA and anthranilic acid were recrystallized from ethanol and water, respectively, before use. An aqueous solution of tetrabutylammonium (TBA) hydroxide (40%) (Aldrich, Milwaukee, Wisc., U.S.A.) was obtained for use. All other chemicals and solvents used were the highest grade of commercially available materials.

HPLC conditions

The HPLC analyses were performed on a Waters Assoc. Model ALC 202 equipped with an M-6000 pump, a U6K injector, and a Perkin-Elmer Model 203 fluorometer adapted to contain an HPLC flow-through cell (Hellma Cells, Jamaica, N.Y., U.S.A.). The column was a LiChrosorb C_{18} column (250 × 3.2 mm I.D.) (Altex Scientific, Berkeley, Calif., U.S.A.). The column contained a packing material consisting of a C_{18} hydrocarbon bonded to a microparticulate silica gel (<10 μm) for reversed-phase chromatography. The mobile phase used was absolute methanol—distilled water (20:80) containing 0.005 *M* tetrabutylammonium (TBA) hydroxide and 0.01 *M* disodium acid phosphate. The pH of the mobile phase was adjusted to 5.5 with concentrated phosphoric acid and the flow-rate was set at 1.0 ml/min (1000 p.s.i.).

Fluorometric detector settings were: sensitivity = 10; selector = 10; excitation and emission wavelengths were set at 270 and 385 nm, respectively, and are uncorrected.

Standard solutions for calibration curve

A stock solution (1 mg/ml) of *p*-aminosalicylic acid was prepared by dissolving a weighed amount of the powder in distilled water. Quantities of 250, 50 and 10 μl of the stock solution were added to individual 5-ml volumetric flasks and blank human plasma was added to volume. In addition an internal standard stock solution (25 μg/ml) of anthranilic acid in mobile phase (see HPLC conditions) was prepared.

Plasma calibration procedure

Calibration curves were constructed by adding 100-μl quantities of each plasma stock solution into individual 15-ml centrifuge tubes to give the equivalent of 5, 1, and 0.2 mg *p*-aminosalicylic acid per 100 ml plasma. To each tube was added absolute methanol (100 μl) followed by mixing on a Vortex mixer (1 min) and centrifugation at 3000 r.p.m. (15 min). An aliquot (50 μl) of each supernatant was removed and transferred to a clean 15-ml centrifuge tube where 100 μl of the mobile phase containing the internal standard (stock solution) was added. After mixing (1 min), 50 μl of each solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

It was apparent in the approach to an HPLC analysis of PASA that the amphoteric nature of the drug would limit its extractability from a biological matrix. In order to exploit the chromatographic process for the direct injection of plasma samples into the HPLC column, a non-extractive sample preparation

prior to on-column injection was necessary. Also, a reversed-phase system was chosen so that the mobile phase would be miscible with the biological fluid and the polar components in the biological matrix would be rapidly eluted. An ion-pairing mobile phase was used to increase the capacity factor (k') for PASA so that the drug would be resolved from the background components. Because of the amphoteric nature of PASA, either a cationic or anionic ion-pair reagent could be selected. The strong acidic nature of the drug (pK_a COOH = 3.25) suggested that there would be a greater probability of success with a cationic agent and this was borne out by our results. The native fluorescence of PASA was also investigated in order to achieve detection selectivity and to increase the sensitivity of the assay. Plasma volumes as small as 100 μl could be used in the resulting procedure.

Acetonitrile was initially employed in a pretreatment step as plasma protein precipitant. It was noted that the supernate obtained after centrifugation of the denatured plasma sample was slightly milky and never became clear even upon extended centrifugation. Further, there was a possibility of drug entrapment since the precipitate formed in the denaturation process was gummy. On chromatographing the acetonitrile-treated plasma, a peak associated with the plasma sample was observed in both blank and sample at a retention time of 24 min. Efforts to remove this component by pre-extraction of the plasma sample with fat soluble organic solvents such as heptane, diethyl ether or chloroform were unsuccessful. It was decided to replace the acetonitrile with absolute methanol as protein denaturant. This was advantageous since a flocculent precipitate was obtained as well as a much clearer supernate upon centrifugation. In addition, the 24-min peak that had been observed with acetonitrile did not appear.

A study of mobile phase pH versus chromatographic characteristics of the PASA—TBA ion-pair in the mobile phase was investigated. The retention time of the ion-pair decreased with increasing pH in the pH 4—8 range. A pH of 5.5 was selected since a symmetrical peak with a reasonable retention time and adequate resolution from plasma components was obtained (see Fig. 1). With the more alkaline mobile phases, there was either lack of adequate resolution for PASA and plasma components or shoulders and multi-peaks for PASA were observed. With increasing acidity of the mobile phase, there was an increase in k' which resulted in a longer analysis time.

A plot of fluorescence intensity versus mobile phase pH (Fig. 2) showed that maximum fluorescence of the ion-pair was obtainable in the pH 5—8 range, which is consistent with the pH needed for resolution from plasma components.

A comparison of the cationic ion-pairing results to those using an anionic counterion, dioctyl sodium sulfosuccinate (DOSS), was undertaken. The mobile phase consisted of absolute methanol—distilled water (20:80) containing 0.01 *M* DOSS. The chromatographic characteristics of the PASA—DOSS ion pair were investigated at pH 2.5 and 5. Fluorescence intensity was monitored at excitation and emission wavelengths of 290 and 400 nm, respectively. A double peak with a retention time of about 600 sec was observed at pH 2.5 and a single peak at or near the solvent front was observed

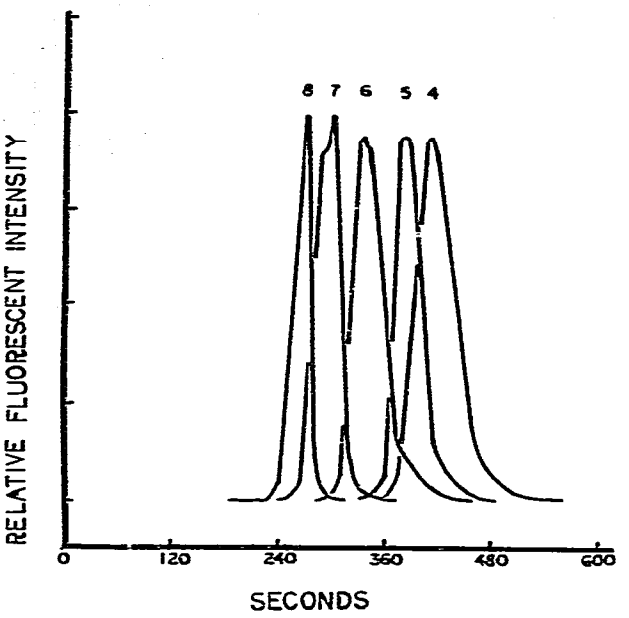

Fig. 1. Typical chromatograms of PASA in absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate using excitation and emission wavelengths of 270 and 385 nm, respectively, at various mobile phase pH values. The pH value is noted above each peak.

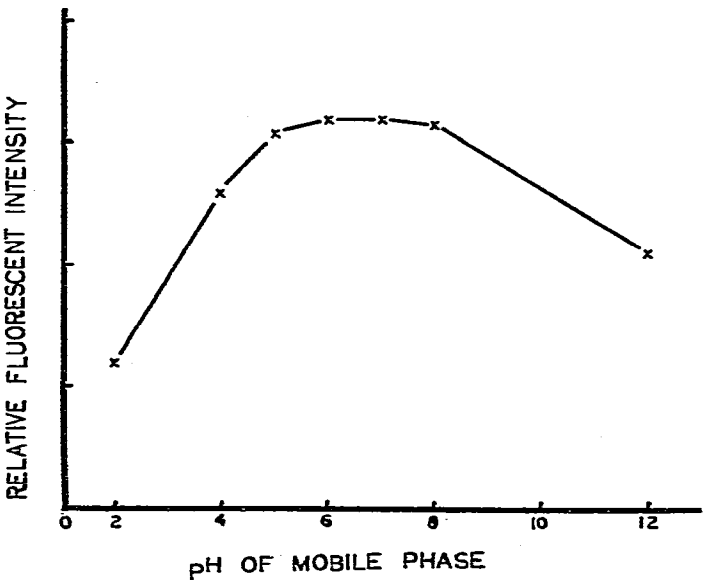

Fig. 2. pH versus PASA fluorescent intensity in absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate at excitation and emission wavelengths of 270 and 385 nm, respectively. Measurements were performed in duplicate on a Perkin-Elmer Model MPF-4 spectrophotofluorometer in the true emission mode.

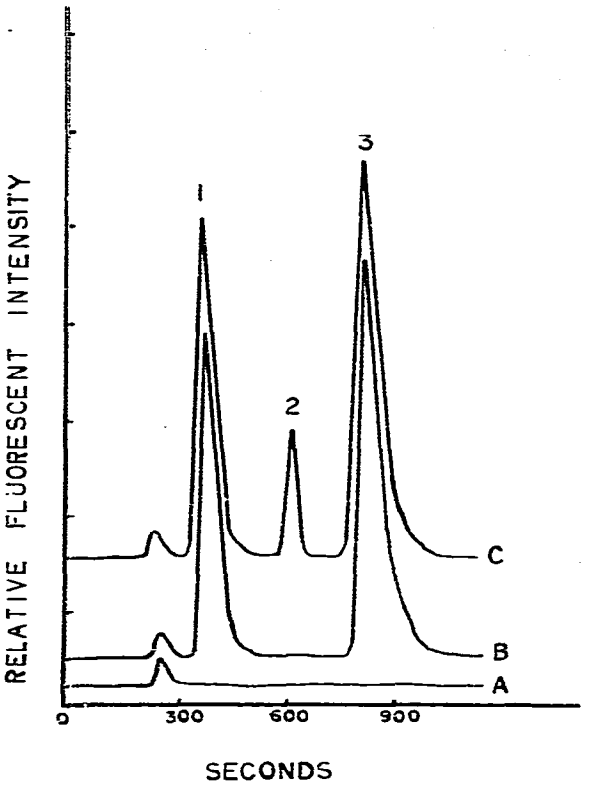

Fig. 3. Typical chromatogram of *p*-aminosalicylic acid (1) and anthranilic acid (3) (internal standard) in a spiked human plasma sample (B). For comparison, chromatograms of blank plasma (A) and of a plasma sample (C) also containing the N-acetyl metabolite of PASA (2) are shown. Conditions: Column, LiChrosorb C_{18} (250 mm × 3.2 mm I.D.), eluent, absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate adjusted to pH 5.5 with concentrated phosphoric acid; flow-rate, 1 ml/min; excitation and emission wavelength of 270 and 385 nm, respectively.

at pH 5. It was evident upon comparison that best results for the PASA assay could be obtained using ion-pairing with TBA counterion at pH 5.5.

Fig. 3 shows a typical chromatogram of the separation of PASA and anthranilic acid (internal standard) in a spiked human plasma sample using TBA cation at pH 5.5. Under the chromatographic conditions chosen, endogenous plasma constituents and the N-acetyl metabolite of PASA do not interfere with the assay. Furthermore, it has been shown in this laboratory that spiked plasma samples containing isoniazid (INH) and ascorbic acid, drugs commonly found in combination with PASA in pharmaceutical dosage forms also do not interfere.

A calibration curve for PASA in the therapeutic concentration range 0.2—5 mg per 100 ml of plasma [10] was performed. Anthranilic acid was found to be a suitable internal standard since it possessed good fluorescent intensity at the excitation and emission wavelengths of PASA. The area under the curve for each peak on the chromatograms was determined with an electronic integrator. The ratio of PASA peak area to the area of the internal standard (D/IS) was

calculated for each chromatogram. Regression analysis of these data at the various concentrations of PASA gave slope, 0.2043; intercept, —0.0066; and correlation coefficient, 0.9984 (n = 15). The standard error of estimate of y(D/IS) on x (PASA concentration) was ± 0.0216. The minimum detectable quantity of PASA that can be measured using this procedure is 500 pg (signal-to-noise ratio = 2).

Human plasma samples containing spiked quantities of PASA in the therapeutic concentration range were chromatographed concurrently with the calibration solutions and the ratios of drug peak areas to internal standard peak areas were calculated. The slope and intercept data from regression analysis for PASA calibration solutions were used to solve for drug concentration in the spiked samples: D/IS = (slope × concentration) + intercept. The data in Table I demonstrate the quantitative results obtained from these spiked plasma samples. The utility of HPLC in the assay of plasma levels of PASA using fluorometry and ion-pairing with tetrabutylammonium ion is clearly demonstrated with accuracy in the 1—5% range.

TABLE I

ANALYSIS OF *p*-AMINOSALICYLIC ACID IN SPIKED PLASMA SAMPLES

Initial concn. (mg/100 ml)	Concn. found* (mg/100 ml)	Relative standard deviation (%)	Accuracy (%)
0.500	0.5244 ± 0.0397	7.63	4.88
2.500	2.522 ± 0.0799	3.17	0.88

*Mean ± S.D. based on five replicate determinations of each sample.

REFERENCES

1 J. Lehman, Dis. Chest, 16 (1949) 684.
2 R.S. Lees and A.M. Lees, Postgraduate Medicine, 60 (1976) 99.
3 H. Kern, P. Schilling and S. Muller, Gas Chromatographic Analysis of Pharmaceuticals and Drugs, Varian Aerograph, Walnut Creek, Calif., 1968, p. 20.
4 The National Formulary, Vol. XIV, American Pharmaceutical Association, Washington, D.C. 1975, p. 40.
5 J. Hunt and M. Blake, J. Pharm. Sci., 59 (1970) 683.
6 S.H. Wan, P. Pentikainen and D.L. Azarnoff, J. Pharmacokinet. Biopharm., 2 (1974) 1.
7 C. Fischer and U. Klotz, J. Chromatogr., 162 (1979) 237.
8 S.J. Saxena, J.T. Stewart, I.L. Honigberg, J.G. Washington and G.R. Keene, J. Pharm. Sci., 66 (1977) 813.
9 I.L. Honigberg, J.T. Stewart and J.W. Coldren, J. Pharm. Sci., 68 (1979) 253.
10 E.N. Deeb and G.R. Vitagliano, Amer. Rev. Tuberc. Pulm. Dis., 72 (1955) 543.