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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANILINE IN ARSANILIC ACID AND SODIUM ARSANILATE

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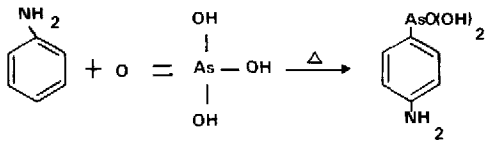
SUMMARY

A high-performance liquid chromatographic (HPLC) method for assaying aniline in arsanilic acid using an internal standard (7-hydroxycoumarin) was developed. Separation by HPLC was achieved in less than 20 min on a reversed-phase column (μBondapak C_{18}) using acetonitrile–water (10:90) as the mobile phase and ultraviolet detection at 235 nm. The method was applied to detect trace levels of aniline in arsanilic acid and sodium arsanilate used to medicate feed. The aniline content of arsanilic acid ranged from 0.1 to 0.2% (0.1–0.2 ppm equivalent in complete feed), and none was detected in sodium arsanilate. The HPLC method is rapid and accurate, and is capable of monitoring trace levels (5 ng) of aniline in arsanilic acid used to prepare medicated feed.

INTRODUCTION

Arsanilic acid and sodium arsanilate are approved for use as growth promotants and/or therapeutic agents in poultry and swine feeds. The maximum permissible level for each in animal feed is 90 g/ton (100 mg/kg), *i.e.*, 100 ppm[1].

Arsanilic acid is prepared by heating aniline and arsenic acid[2] as shown below:



The yield is seldom more than 25% and may be much smaller[3]. Therefore, potentially significant amounts of aniline may be present as the free base. However, there is also considerable oxidation of the aniline as evidenced by the deep purple colour of the reaction mixture[3]. Sodium arsanilate is prepared by treating arsanilic acid with sodium hydroxide.

Testing at the National Cancer Institute[4] has shown that aniline is a carcinogen

in animals (rats, 6000 and 12,000 ppm) and thus may be a potential risk to humans. Aniline has also been placed on the Occupational Safety and Health Administration's candidate list as a possible occupational carcinogen[5].

Aniline could accumulate in animal feeds medicated with arsanilic acid due to the use of an impure substrate or decomposition of arsanilic acid during mixing, storage, etc. Decomposition is not likely to occur during these relatively mild processes because the rupture of the arsenic–carbon bond in aromatic arsonic acids, such as arsanilic acid, does not occur even by boiling with mineral acids[6,7]. Therefore, the accumulation of aniline in medicated feeds is probably due to the use of impure arsanilic acid.

High-performance liquid chromatography (HPLC) has been used in only a few cases to separate aniline from its metabolites and other aromatic amines. Sternson and DeWitte[8] used HPLC with a mobile phase containing Ni(II) ion as a chelating agent to provide difference in reactivity to separate aniline from phenylhydroxylamine and other metabolites. Young and McNair[9] used pellicular silica gel columns to separate aniline and other aromatic monoamines and diamines.

This paper describes the HPLC separation and detection of aniline from arsanilic acid and sodium arsanilate using 7-hydroxycoumarin as an internal standard. The method involves the use of reversed-phase (C_{18} column) partition chromatography with an isocratic mobile phase of acetonitrile–water and ultraviolet (UV) detection at 235 nm.

MATERIALS AND METHODS

Apparatus

A modular HPLC system containing a M6000A solvent delivery system and U6K septumless injector (Waters Assoc., Milford, MA, U.S.A.), LC-85 spectrophotometric detector with Autocontrol (Perkin-Elmer, Norwalk, CT, U.S.A.) and 7130A strip chart recorder (Hewlett-Packard, Rockville, MD, U.S.A.) operated at 0.1 in./min chart speed was used. A 30 cm × 3.9 mm μBondapak C_{18} reversed-phase column (Waters Assoc.) operated at ambient temperature was used. The mobile phase, which was degassed by ultrasonification and under vacuum for 5 min before use, consisted of acetonitrile–water (10:90) at a flow-rate of 2.0 ml/min.

Reagents

Aniline and arsanilic acid were obtained from Matheson-Coleman & Bell (Norwood, OH, U.S.A.). 7-Hydroxycoumarin was obtained from Aldrich (Milwaukee, WI, U.S.A.). Glass-distilled organic solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and deionized water were used throughout the study. Various lots of arsanilic acid and sodium arsanilate, which are used to prepare feed pre-mixes, and the reference arsanilic acid were obtained from Abbott Labs. (North Chicago, IL, U.S.A.).

Standard solutions

A stock solution of aniline (14 mg/ml) in methanol was prepared. From this stock solution, a working solution of 0.14 mg/ml was prepared. Both solutions were stored in amber glass bottles at 4°C. The 7-hydroxycoumarin solution (2 mg/ml),

used as an internal standard, and arsanilic acid (2 mg/ml) also were prepared in methanol and were stored in clear glass bottles at 4°C.

Standard curves

Standard curve for aniline for quantitative analysis. From the working solution of aniline, 10-, 20-, 40-, 80-, 160- and 240-μl aliquots were transferred to six separate vials, 100 μl of internal standard solution (7-hydroxycoumarin) were added to each and the contents were diluted to 1.0 ml in methanol. A 5-μl volume from each of the vial was analyzed by HPLC.

Standard addition curve for aniline using arsanilic acid solution as matrix. A 15-ml volume of arsanilic acid (2 mg/ml in methanol) was prepared for use as a matrix. A 50-μl volume of aniline stock solution was diluted to 5 ml in the arsanilic acid matrix solution to serve as a working aniline solution. The internal standard (2 mg/ml) was also prepared in the matrix solution. The preparation of samples for HPLC analysis to determine linearity was carried out as described above under *Standard curve for aniline for quantitative analysis*, except that the final dilution was made in the matrix solution. The data obtained using the standard addition curve were used to calculate the recovery of added aniline.

Preparation of arsanilic acid and sodium arsanilate samples for determination of aniline

Each lot of arsanilic acid, sodium arsanilate and the reference arsanilic acid was prepared at a concentration of 2 mg/ml in methanol. A 50-μl volume of internal standard (2 mg/ml in methanol) was added to 500 μl of each sample and the solution was diluted to 1 ml in methanol. The final concentration of the samples was 1 mg/ml, and 10 μl of each sample were analyzed for aniline by HPLC.

Preparation of samples for precision analysis

Five samples of arsanilic acid, at a concentration of 2 mg/ml in methanol, were prepared. A 5-μl aliquot of each sample was analyzed by HPLC.

RESULTS

Fig. 1 shows an HPLC chromatogram of a mixture of aniline and 7-hydroxycoumarin using acetonitrile–water (10:90) as the mobile phase, a μBondapak C_{18} column and UV detection at 235 nm. Fig. 2 shows the separation from and detection of aniline in an arsanilic acid sample under the same HPLC conditions. Various combinations of acetonitrile and water as the mobile phase were examined. The above combination was chosen because it provided for adequate retention of aniline on the HPLC column and for its separation from arsanilic acid. 7-Hydroxycoumarin was selected as the internal standard because it gave a peak which was completely resolved from the aniline peak. The average retention time (min) of aniline was 11.4 $\pm$ 0.366 ($\pm$ S.D., $n = 13$) and for 7-hydroxycoumarin was 16 $\pm$ 0.347 ($\pm$ S.D., $n = 13$). When a new μBondapak C_{18} HPLC column was used, the resolution between aniline and the internal standard was significantly increased (Fig. 3).

Multi-wavelength determination of aniline

To determine the most sensitive wavelength for the detection of aniline, a

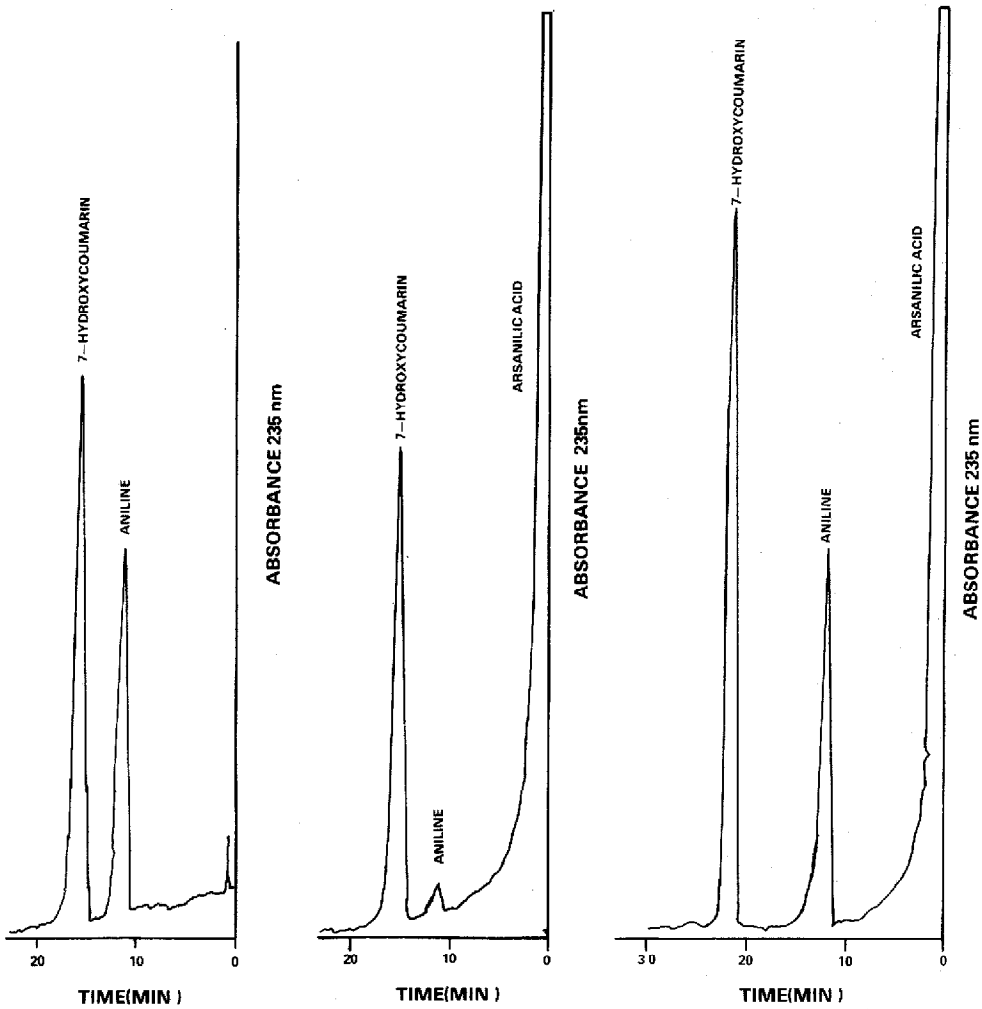


Fig. 1. HPLC separation of aniline (120 ng) and 7-hydroxycoumarin (internal standard, 1.0 μg). Conditions: column, μBondapak C_{18}; mobile phase, acetonitrile–water (10:90); detector, 235 nm; chart speed, 0.1 in./min; flow-rate, 2.0 ml/min.

Fig. 2. HPLC of arsanilic acid sample (10 μg). HPLC conditions as in Fig. 1.

Fig. 3. HPLC of reference arsanilic acid (10 μg) spiked with aniline (160 ng). HPLC conditions as in Fig. 1 except new μBondapak C_{18} column.

multi-wavelength analysis at 235 nm and 287 nm, the maxima for aniline[10], and 254 nm and 280 nm (commonly used wavelengths in HPLC), was performed. Aniline gave the most sensitive detector response at 235 nm; therefore this wavelength was used in the study.

Linearity

A linear response for aniline was established within a concentration range of 7–168 ng with a coefficient of determination, r^2, of 0.998. The response of aniline using

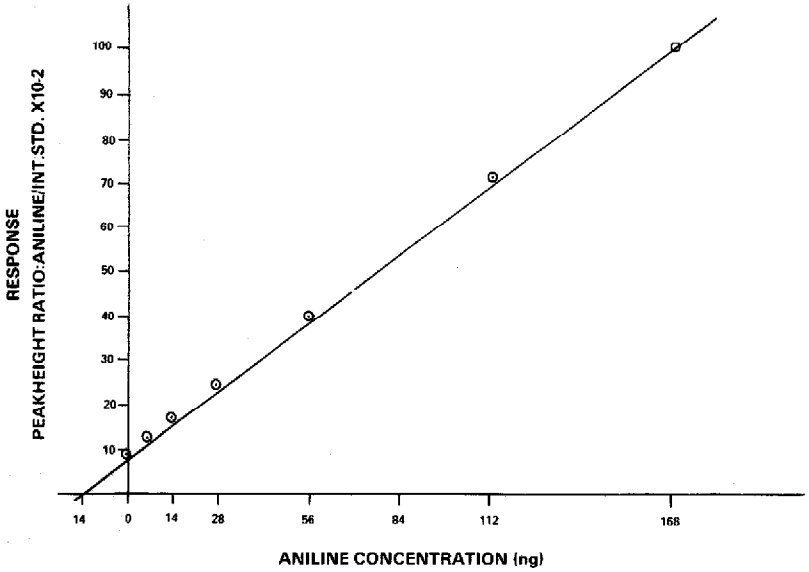


Fig. 4. Standard curve, using arsanilic acid as a matrix (standard addition calibration curve). HPLC conditions as in Fig. 1.

arsanilic acid as a matrix was linear in the same concentration range with a coefficient of determination of 0.998 (Fig. 4).

HPLC of arsanilic acid and sodium arsanilate samples

Aliquots (10 μl) of arsanilic acid (Fig. 2) and sodium arsanilate (Fig. 5) were analyzed in duplicate for aniline. A complete animal feed contains 1000 ppm arsanilic acid[1]. Based on this, the equivalent concentration of aniline in the complete feed for each sample of arsanilic acid was calculated and ranged from 0.12 to 0.18 ppm (Table I). HPLC analysis of all five lots of sodium arsanilate revealed no detectable ($<$ 5 ng) aniline (Fig. 5). A sample of the reference arsanilic acid contained no detectable

TABLE I

DETERMINATION OF ANILINE IN ARSANILIC ACID SAMPLES

*Lot No.**	*Aniline found (ng)*	*Aniline in samples (%)*
1	17.6	0.18
2**	6.1	0.12
3	13.9	0.14
4	13.7	0.14
5	12.6	0.12
Average		0.14 $\pm$ 0.03 S.D.

* 10-μg sample analyzed.
** 5-μg sample analyzed.

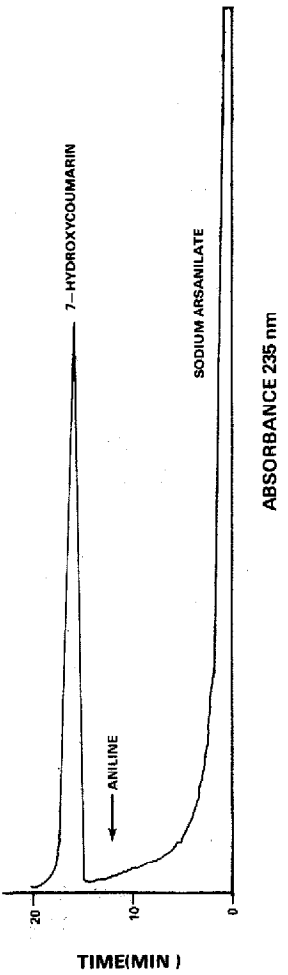


Fig. 5. HPLC of sodium arsanilate sample (10 μg). Operating conditions as in Fig. 1.

aniline; however, a sample of arsanilic acid purchased from Matheson-Coleman & Bell contained 0.12% aniline.

Specificity of assay and dual-wavelength analysis

The purity or specificity of the aniline peak was established by dual-wavelength analysis. The absorbance ratios at two wavelengths, 235 nm and 280 nm, were determined for aniline detected in the sample (arsanillic acid) and for the aniline standard. Any deviation from the absorbance ratio expected for a particular compound could indicate another compound eluting near, or simultaneously with, the compound being assayed. The absorbance ratio, A_{235}/A_{280}, for the aniline standard (0.217) was similar to that (0.219) of aniline detected in the sample, indicating that no potential contaminants were coeluted.

TABLE II

RECOVERY OF ANILINE ADDED TO ARSANILIC ACID*

Aniline added (ng)	*Total aniline found (ng)**	*Net aniline found (ng)***	*Recovery of aniline (%)*
0	13.2	0	–
7	19.7	6.5	92.8
14	26.2	13.0	92.9
28	37.5	24.3	86.8
56	61.7	48.5	86.6
112	111.7	98.5	87.9
168	160.2	147.0	87.5
Average			89.1 ± 2.9 S.D.

* Aniline added to 2 mg arsanilic acid.
** Net aniline = total aniline − aniline in arsanilic acid.

Recovery

The recovery of aniline added to arsanilic acid was calculated from the data obtained for the linearity of the aniline response in the matrix solution. The per cent recovery at each level was calculated from the standard curve for aniline for quantitative analysis. Table II shows that the recovery of aniline was 89.1 % ± 2.9 S.D. The level of aniline contaminating arsanilic acid was 13.2 μg; by the standard addition method it was found to be about 13.5 μg (Fig. 4), indicating good correlation.

Precision

The precision of the HPLC method was determined on five individual portions of an arsanilic acid sample. The mean (ng) for these determinations was 13.3 ± 1.0 S.D.

DISCUSSION

A precise and accurate quantitative HPLC method for determining aniline using 7-hydroxycoumarin as an internal standard was developed and used to detect aniline residues in samples of arsanilic acid and sodium arsanilate. The reference arsanilic acid and sodium arsanilate contained no detectable aniline. The detection limit of aniline by this method is about 5 ng. It could be improved by reaction of the primary amine group of aniline with a fluorescent reagent and using a fluorescence HPLC detector. The rupture of the arsenic–carbon bond of aromatic arsonic acids, such as arsanilic acid, does not readily occur[3,6,7]. Therefore, the detection of trace amounts of aniline in arsanilic acid samples in this study is the result of the carry-over of impurity from the synthesis of arsanilic acid. The method described is rapid and accurate, capable of monitoring trace levels of aniline in in arsanilic acid and has the potential to be applied in metabolism studies of aniline[11].

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