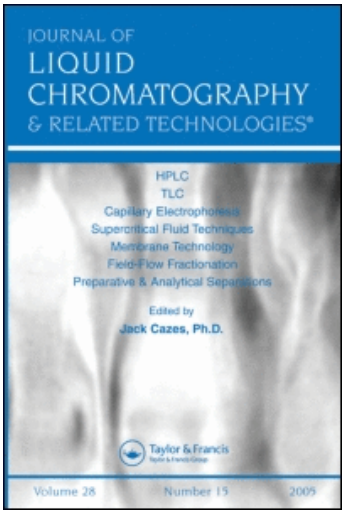

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Liquid Chromatography Analysis of Hippuric Acid

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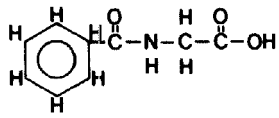
ABSTRACT

Hippuric acid analysis by TLC is demonstrated. Both normal silica and reverse phase C^{18} TLC plates are used with fluorescent quenching to visualize the hippuric acid. Urine samples may be spotted directly or extracted with $CHCl_3$ to concentrate the hippurate. Tissues may be analyzed in this same manner. Aluminum backed Merck TLC plates give best normal phase separation of tissue extracts and Whatman C^{18} give best reverse phase separation of tissue extracts. A Kontes scanner may be used to directly analyze TLC plates or the bands may be removed and analyzed by ultraviolet spectroscopy. Confirmation may be done by mass spectrometry.

INTRODUCTION

Hippuric acid (Figure one), has been cited as a metabolite of toluene and benzoic acid in humans. Agata, et al[2], showed that 70% of the toluene excreted from human volunteers was excreted as hippuric acid. Recent work[3] has shown that it is also a metabolite of ethylene glycol. The analysis for hippuric acid serves as a confirmation of a toxic exposure to these



HIPPURIC ACID

Figure 1
Chemical Formula
Hippuric Acid

compounds. Min and Schreiber[4] separated mandelic and hippuric acid by TLC.

Experimental

REAGENTS AND APPARATUS

Nanograde solvents were obtained from Mallinckrodt, (St. Louis, Mo.), or the equivalent quality was made by distillation. Thin layer plates used were (1) silica gel channeled plates from Whatman, (Clifton, N.J.), (2) aluminum backed silica gel plates, E. Merck, from Brinkmann Instruments, (Westburg, N.Y.), and (3) Reverse phase (C^{18}) TLC plates from Whatman, (Clifton, N.J.). Kontes, (Vineland, N.J.), concentrating glassware was used. Micropets and dispensers from Clay Adams Division of Becton Dickinson, (Res. riangle Park, N.C.), were used. A Varian, (Palo Alto, Calif.), Model 219 UV visible spectrometer and Finnigan, (Sunnyvale, Calif.), GC/MS Model 4000 were used. A short wave UV lamp was obtained from UV Products, San Gabriel, California. A Micromeritics, (Narcross, Georgia), HPLC with a Waters, (Milford, Mass.), C^{18} Microbondapak^{R} column was used.

METHOD OF TEST

1. Urine

Urine samples are extracted with chloroform after adjusting the pH to 2 with HCl acid (2N). The $CHCl_3$ is concentrated to 100 ul or to dryness and diluted to 100 ul. 10 ul is spotted on TLC plates. (Aluminum backed Merck plates are preferred). Normal phase developing solvent is 60/40 $CHCl_3$/MeOH (volume/volume). The visualization method is fluorescence quenching of F254 fluorescent indicator.

Reverse phase (RP) Whatman C^{18} plates may be used also. The developing solvent is 65/35/1. (Ethanol/water/acetic acid plus 0.5% Nacl).

Urine may be spotted directly in ethylene glycol poisoning where levels are 50-100 ppm in urine. Whatman preadsorbent plates are used in this work. (Samples are spiked at .2 ppm (normal urine levels).

2. Kidney

Kidneys are extracted by blending with acidic methanol (pH 2 with 2N HCl) in a Sorvall blender for two minutes. The solvent is filtered and concentrated or taken to dryness. The sample is diluted in 100 ul of chloroform or methylene chloride and spotted on a TLC plate as described in the urine analysis. Samples are spiked at 1 ppm to assure recovery.

For spectrophotometric confirmation only plates cleaned up by the oven cleaning process[5] or

aluminum backed E. Merck plates have a low enough blank to use. The hippuric acid band is removed from the TLC plates and eluted with 3 ml of Nanograde methanol made pH 2 with HCl(2N)

The methanol may be run directly on the UV-Vis spectrophotometer using quartz cells. The extracts are concentrated in inserts for the GC/MS.

3. HPLC Analysis

Ug level aliquots are injected on a Waters RP C-18 column. The mobile phase used was MeOH/HOH/Acetic Acid (65/35/1). The UV detector was set at 240 nm and 0.1 full scale. The flow rate used was 1 ml/min. and sensitivity was 1mv full scale with a chart speed of 1 cm/min.

RESULTS

Table one shows the Rf's of Hippuric Acid on normal and reverse phase plates. E. Merck silica gel plates with aluminum backs (.2mm phase) allowed for the separation of the fluorescence impurity and hippuric acid. Levels of sensitivity were 0.5ug by TLC and HPLC. The reverse phase column and TLC separated the fluorescent impurity from hippuric acid also.

The TLC plates (Whatman) with urine spotted directly on the preabsorbant area had a fluorescent band which fell on top of hippuric acid. Mass Spectral Analysis indicated it to be a substituted tyrosine molecule. Quantitation by densitometry and TLC and by HPLC was possible with very similar sensitivity.

TABLE ONE

Hippuric Acid Thin Layer Chromatography

Substrate	Development Solvent	Rf
Whatman C_{18}TLC	60/40/V/V	0.5
Reverse phase	$CHCl_3/CH_3OH$	
Merck Aluminum back Silica Gel "G"	65/35/1 V/V/V	0.3
Normal Phase	$C_2H_5OH/HOH/CH_3COOH$	

Figure 2 shows the two bands resolved by HPLC. The densitometry trace from TLC was very similar. Figure 3 shows a typical HPLC chromatogram. The Rf of hippuric acid was 0.5 with reverse phase and .30 with normal phase TLC analysis.

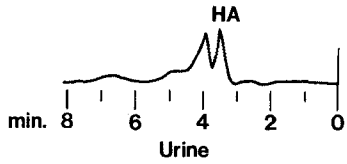


Figure 2
Separation of Urine Extract
on HPLC

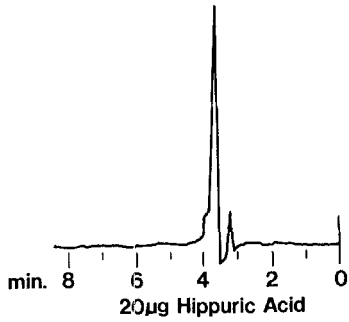


Figure 3
Hippuric Standard HPLC

SPECTRUM ID #: 1
0.02 PPM HIPPURIC ACID STD.

SCAN RATE	(NM/SEC):	2.00
SPECTRAL BAND WIDTH	(NM):	1.00
EFFECTIVE PERIOD	(SEC):	0.5

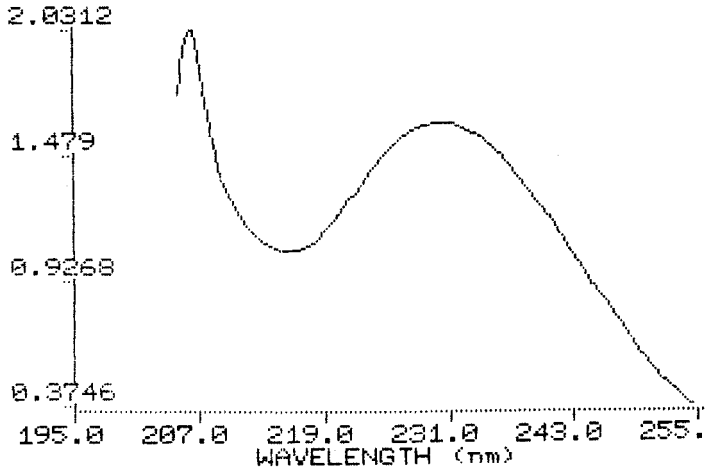


Figure 4
Ultraviolet Spectrum
of Hippuric Acid

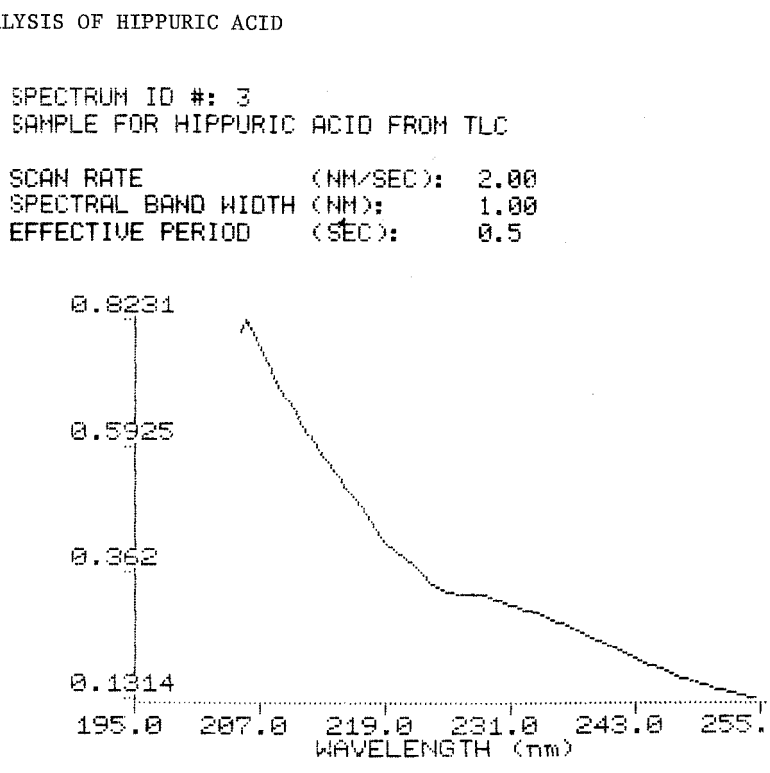


Figure 5
Ultraviolet Spectrum of Hippuric Acid
Normal Urine

Figures 4 thru 6 show the ultra violet spectra of standards and sample extracts taken from TLC plates. The mass spectrum obtained showed the fluorescent impurity to be a substituted tyrosine when referenced to the Mass Spectral Search System. Spectra were obtainable from TLC for hippuric acid at the ug level (Figure 6). Ultraviolet confirmation may be done at sub ug levels. Figure 7 shows the mass spectrum of Hippuric Acid.

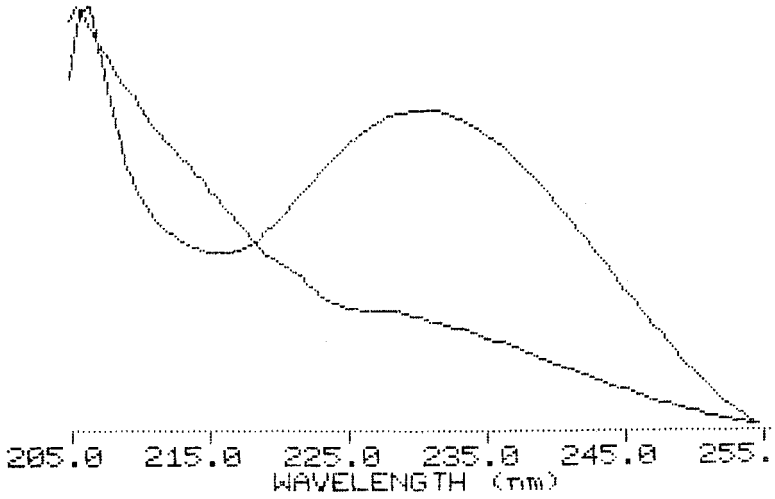


Figure 6
Standard and Urine Extract Hippuric Acid from TLC

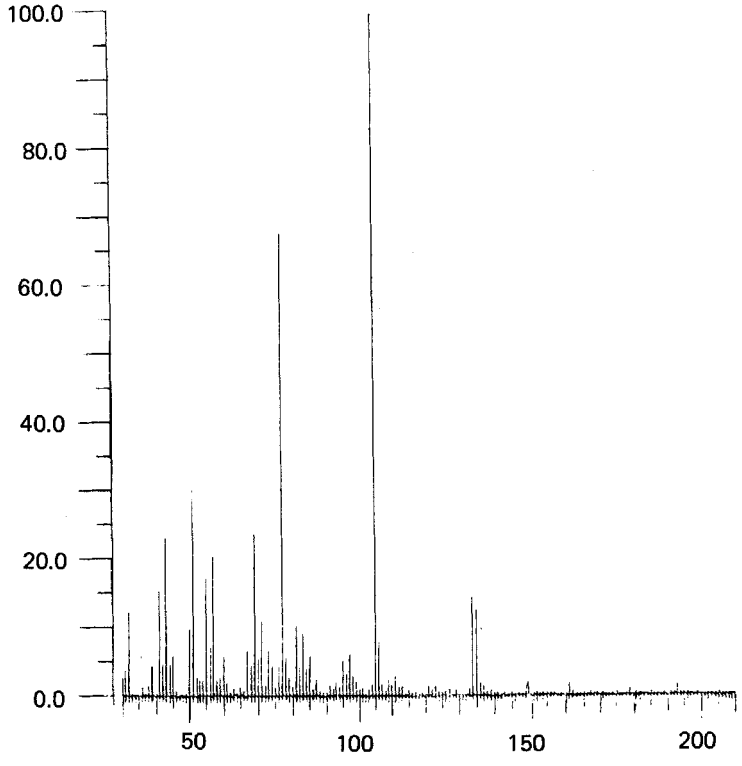


Figure 7
Mass Spectrum of Hippuric Acid

DISCUSSION/CONCLUSION

While the solvent system of Min and Schreiber worked for separation of mandelic acid and hippuric acids, it did not separate the fluorescent interference and hippuric acid. The state of the art TLC plates and HPLC allowed separation of the interference from hippuric acid.

Quantitative analysis and confirmation by UV and Mass Spectroscopy is possible from TLC bands or trapped fractions from HPLC. Sensitivity for the analysis is adequate to determine normal urine levels in humans and dogs as well as experimentally produced hippuric acid levels by ethylene glycol poisoning. The test should be applicable to other toxicological problems in which hippuric acid is produced; for example, xylene, toluene, and benzoic acid exposures.

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