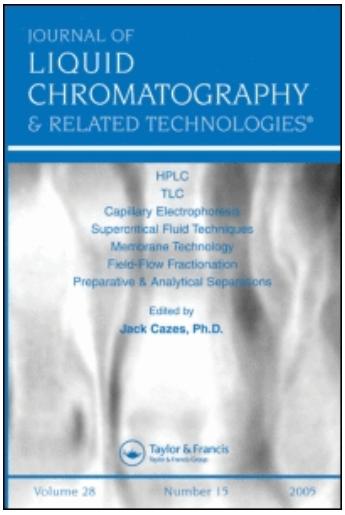

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Liquid Chromatographic Determination of 4-Amino-N-(2,6-dimethylphenyl)-benzamide in Rat Serum and Urine

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LIQUID CHROMATOGRAPHIC DETERMINATION OF 4-AMINO-N-(2,6-DIMETHYL PHENYL)-BENZAMIDE IN RAT SERUM AND URINE

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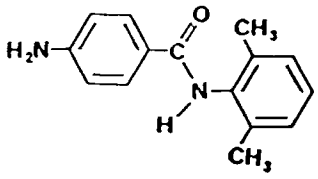
ABSTRACT

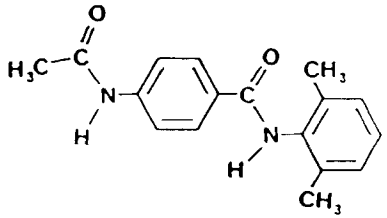
The compound 4-amino-N-(2,6-dimethylphenyl)-benzamide has shown potential as a new anticonvulsant. A method for the liquid chromatographic determination of serum and urine concentrations of the compound and its N-acetylated metabolite was developed for pharmacokinetic studies. Quantitation was achieved via UV detection at 275nm following isocratic reversed phase (C_{18}) separation using a ternary solvent system of water:acetonitrile:acetic acid (60:39:1) at a flow rate of 1.5 mL/min. The compounds were isolated from a 50 µL sample of serum using solid phase extraction with prior protein precipitation. The compounds and internal standard were eluted from the extraction column with acetonitrile. Isolation from urine was achieved similarly with the exclusion of protein precipitation. The assay procedure is useful for the determination of concentrations of parent compound from 0.68 to 204.6 µg/mL.

Introduction

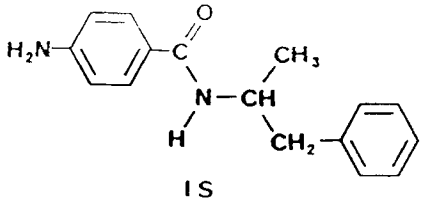
A relatively new class of anticonvulsants, the 4-aminobenzamides, are now under investigation (1-3). 4-Amino-N-(2,6-dimethylphenyl)-benzamide (DMP), a congener of d,l-4-amino-N-(α-methylbenzyl)-benzamide, shown in Figure 1, has been identified as a promising anticonvulsant agent (4). Preliminary evaluations in animal models suggests that DMP has a spectrum of anticonvulsant activity similar to that of phenytoin. Interperitoneal (ip) administration of DMP in mice produced an ED50 of 2.60 mg/kg against maximal electroshock (MES) induced seizures and provided no protection up to 20 mg/kg against subcutaneous metrazole (scMet) induced seizures. The rotorod toxicity test demonstrated a TD50 of 15.01 mg/kg after ip administration of DMP, yielding a protective index of 5.77. This is comparable to that of phenytoin (PI=6.89), and higher than that of the other prototype anticonvulsant drugs. The LD50 (24 hour lethality) for DMP is 160.83 mg/kg after ip administration, although this value is less than the other prototype anticonvulsants tested. The ratio of LD50 to HD50 (test of hypnotic dose measured by loss of righting reflex) and TD50 is higher than the ratios calculated for the other tested prototype anticonvulsants. Therefore, DMP appears to be more potent than the other anticonvulsants but possessing a wider margin between toxicity and lethality. This compound is continuing to undergo evaluation as a potential antiepileptic agent. This study reports the development of an HPLC assay for drug and acetylated metabolite



DMP



ADMP



IS

Figure I: Structures of 4-amino-N-(2,6-dimethylphenyl)-benzamide, (DMP); the acetylated metabolite, (ADMP); and the internal standard for the assay, (IS).

for quantitation in disposition studies which are essential for examining a drug's metabolic profile, its effects as to route of administration, and its persistence.

Materials and Methods

Reagents and Chemicals

All chemicals were reagent grade, pharmaceutical quality or better and were used without further purification. Nitrogen gas was obtained from Alabama Oxygen Company (Bessemer, AL). Barium hydroxide was obtained from Allied Chemical (New York, NY). Spectrophotometric grade acetic acid and zinc sulfate were purchased from J.T. Baker Chemical Company (Phillipsburg, NJ). Spectrophotometric grade acetonitrile was purchased from Fisher Scientific Company (Fair Lawn, NJ). The synthesis has been previously described for the internal standard 4-amino-N-(α-methylphenethyl)-benzamide (IS) (1), and for 4-amino-N-(2,6-dimethylphenyl)-benzamide (DMP) (2). The standard for 4-acetamido-N-(2,6-dimethylphenyl)-benzamide (ADMP) was synthesized as described below. IR spectra were recorded in chloroform solutions using matched sodium chloride cells or as fluorocarbon mulls on a Beckman 4230 spectrophotometer. All ^{1}H NMR spectra were measured in $CDCl_3$ on a Varian T-60A spectrometer with an internal standard of tetramethylsilane. Elemental analyses (C,H,N) were performed by Atlantic Microlab Inc., Atlanta, GA.

Chromatographic Procedures

The HPLC was a modular isocratic system consisting of a model 750 solvent delivery system, model 730 universal injector, a model 788 dual channel variable wavelength detector (Micromeritics, Norcross, GA), and a model 3390A integrator (Hewlett-Packard Company, Avondale, PA). The water used in the reversed phase system was double distilled. All separations were carried out at ambient temperature.

Reversed phase separation was accomplished using a 15.0 cm x 4.6 mm i.d. column packed with C_{18} chemically bonded silica (5 µm), Ultrasphere-ODS (Altex) preceded with a guard column (5 cm x 2.1 mm i.d.) dry packed with 30 to 38 m CO:PELL ODS (Whatman) and equipped with 2 µm end frits. The mobile phases consisted of mixtures of water, HPLC grade acetonitrile, and HPLC grade glacial acetic acid. The solvent system was acetonitrile-water-acetic acid (39:60:1) with a flow rate of 1.5 mL/min. and the detector operating at 275 nm.

Animals

Male Sprague-Dawley rats from Harlan Sprague Dawley (Prattville, AL) were received 1 to 2 weeks prior to use. They had free access to food and water prior to and during the experiment. The weight range of rats for this study was 201g to 292g.

Cannulation Procedure

Under ether anesthesia a 1 cm incision was made under the rat's right clavicle, the jugular vein isolated with blunt dissection and a small lateral incision made into the vein. Into this incision, with gentle rotation, approximately 3 cm of silastic (Dow Corning) tubing (0.020 in. I.D. x 0.037 in. O.D.), with end beveled, was inserted toward the heart. The tubing was then secured in place with silk surgical suture (Look, Inc.). Heparinized saline (30 μ/mL) was used during the procedure to keep the tubing free of clot formation. The incision was then closed using discontinuous suture stitches.

DMP was administered at a dose of 25 mg/kg in polyethylene glycol 400 via the cannula. The calculated dose was given over 19.9 - 22.7 minutes.

Synthesis of Acetylated Metabolite, ADMP

A solution of 2.0 g of DMP and 1.5 g of triethylamine in 30 mL of THF was added to a 250 mL 3-necked flask fitted with a reflux condensor and an addition funnel. A solution of 1.8 g of acetylchloride in 10 mL of THF was added drop wise and the resulting mixture refluxed for three hours. The mixture was cooled and diluted with 20 mL of water and the THF removed by vacuum distillation. The resulting aqueous suspension was filtered and the filtrate recrystallized from THF-ethanol mp 288-290°C. Infrared and nuclear magnetic resonance (^{1}H) spectra were consistent with the assigned structure.

Elemental analysis (C,H,N): Theoretical, C = 71.98%; H = 6.71%; N = 9.33%. Found, C = 71.90%; H = 6.84%, N = 9.23%.

Solid Phase Extraction

Added to a 50 µL serum sample in a glass test tube was 1 mL of water followed by 25 µL of IS in acetonitrile (1.2 mg/mL), 50 µL of 1.8% $Ba(OH)_2$ and 50 µL of 2.0% $ZnSO_4$ with vortexing before and after the addition of $ZnSO_4$. A reversed phase C_{18} extraction column, Sep-pak (Waters), was activated by washing with 5 mL of acetonitrile followed by 5 mL of water with the aid of a Fisher filtrator and water-aspirator vacuum. Then, without allowing the adsorbent to dry, the sample solution was charged onto the column. The column was washed with 9 mL of water to remove any water soluble substances. After the wash, DMP, ADMP, and IS were eluted from the column in 1.5 mL of acetonitrile. This solution was concentrated under a stream of nitrogen to a volume of 0.05 to 0.1 mL and 10 µL of the resulting solution was quantitated by HPLC analysis. The isolation of DMP and ADMP from urine was achieved similarly with the exclusion of protein precipitation.

Efficiency of Recovery of DMP and ADMP from Serum

The recovery efficiency of DMP and ADMP from serum according to the described solid phase extraction method above was studied by spiking 50 L serum aliquots with

known amounts of DMP and ADMP and the following concentrations (μg/mL) of each were achieved: DMP: 0.68, 0.96, 4.8, 9.6, 23.9, 47.7, 95.5, 204.6; ADMP: 0.67, 0.94, 4.7, 9.4, 23.5, 47.0, 100.8, 168.0. The stock solutions of DMP, ADMP, and IS used to prepare the samples were prepared in acetonitrile. These stock solutions were made so that the combined volume of these solutions would be less than or equal to 25 μL (i.e. the total volume of acetonitrile used in the assay procedure above), yet yield the concentration listed above. When the combined volumes were less than 25 μL, acetonitrile was added to make 25 μL. The percent recovery efficiency was calculated from the ratio of the concentrations the integrator calculated for these serum preparations to those acquired in an identical manner except for not going through the solid phase extraction procedure.

Results and Discussion

It was the goal of this project to develop a rapid and sensitive assay for DMP and its major metabolite ADMP. The assay would be used initially in pharmacokinetic studies in rats. Preliminary studies indicated that the N-acetyl derivative, ADMP, was a major metabolite resulting from DMP. Therefore, HPLC and extraction conditions for the isolation of these two compounds were developed using the internal standard IS, shown in Figure 1, since it possessed similar extraction and chromatographic properties. Table 1 shows the percent

TABLE 1

Recovery from Solid Phase Extraction (C_{18} Sep-Pak)

Compound	Description	% Recovery[1]
DMP	Parent Compound	95.2 ± 0.5
ADMP	N-Acetylated Metabolite	95.6 ± 4.1
IS	Internal Standard	93.6 ± 1.2

[1]Mean ± SD, n = 3

recovery of each compound from the C_{18} extraction column and the similarity in the efficiency of their recovery. The separation of a standard mixture is demonstrated in Figure 2.

Pharmacokinetic studies require several small volume blood samples to be collected in order to accurately describe the serum concentration-time profile of the compound of interest and its metabolites. In this study 0.2 mL blood samples were collected from each rat at various designated times via surgically placed jugular vein cannulas yielding 50 µL of serum. For isolation of DMP, ADMP, and IS from such small sample sizes, a procedure utilizing a solid phase extraction column was developed. Such procedures have been described (5) as an alternative to liquid-liquid extraction for the isolation of pharmaceuticals from a biological matrix. A reversed phase (C_{18}) extraction column, Sep-pak (Waters),

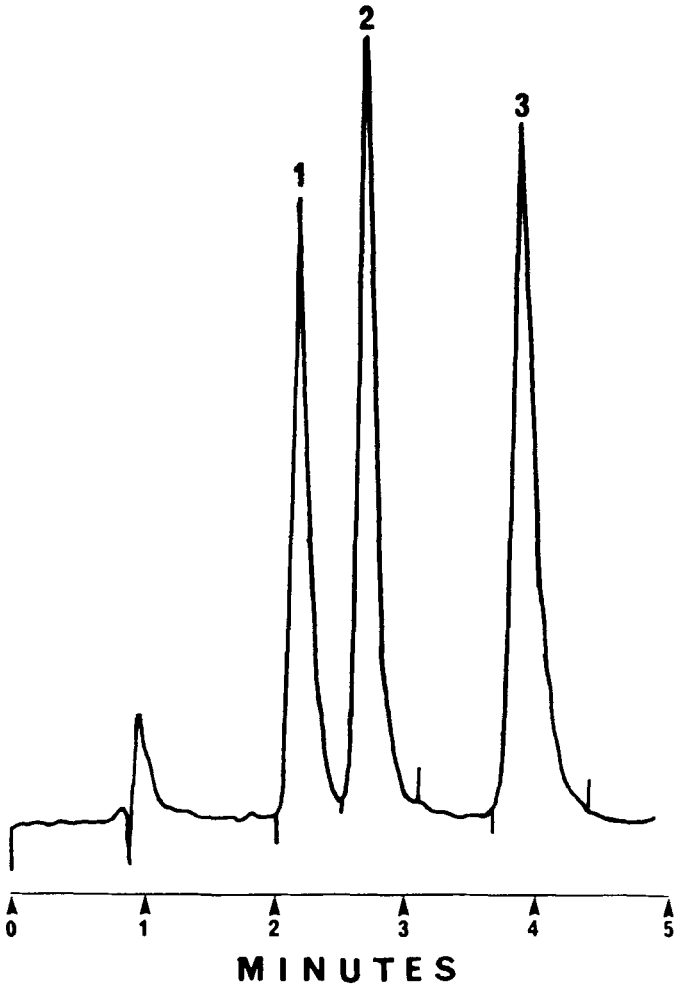


Figure II: Liquid chromatographic separation of a standard mixture of compounds DMP (peak 2), ADMP (peak 1) and IS (peak 3).

was used along with samples spiked with DMP, ADMP, and IS to develop the extraction procedure. The addition of $Ba(OH)_2$ and $ZnSO_4$ solutions caused the precipitation of serum proteins, thereby allowing total serum analysis of DMP and ADMP. The water wash removed most of the water soluble sample components. DMP, ADMP, and IS were then eluted from the extraction column with 1.5 mL of acetonitrile.

The chromatograms in Figure 3 were obtained from serum samples drawn 10 minutes, 50 minutes, 75 minutes, and 220 minutes post-intravenous dosing with DMP. This series of chromatograms reveal that significant acetylation had occurred even in the 10 minutes sample. DMP concentration is decreasing with time while ADMP increases and later begins to decline. Figure 4 shows a chromatogram resulting from the isolation of DMP and ADMP from a urine sample.

Table 2 shows predicted and observed concentrations of DMP and ADMP over a 300-fold range. The coefficient of variation (CV) varied from 0.83 to 10.00% for DMP and from 1.04 to 9.45% for ADMP. The upper limit of concentration in Table 2 is well above the highest level found in rat serum after IV dosing of DMP. Both slope and correlation show good agreement between actual and detected concentrations.

Figure 5 shows the results from a dosing study using the described assay procedure. The semi-log graph represents post-infusion serum concentrations as observed after a dose of 25 mg/kg of DMP in a polyethylene glycol

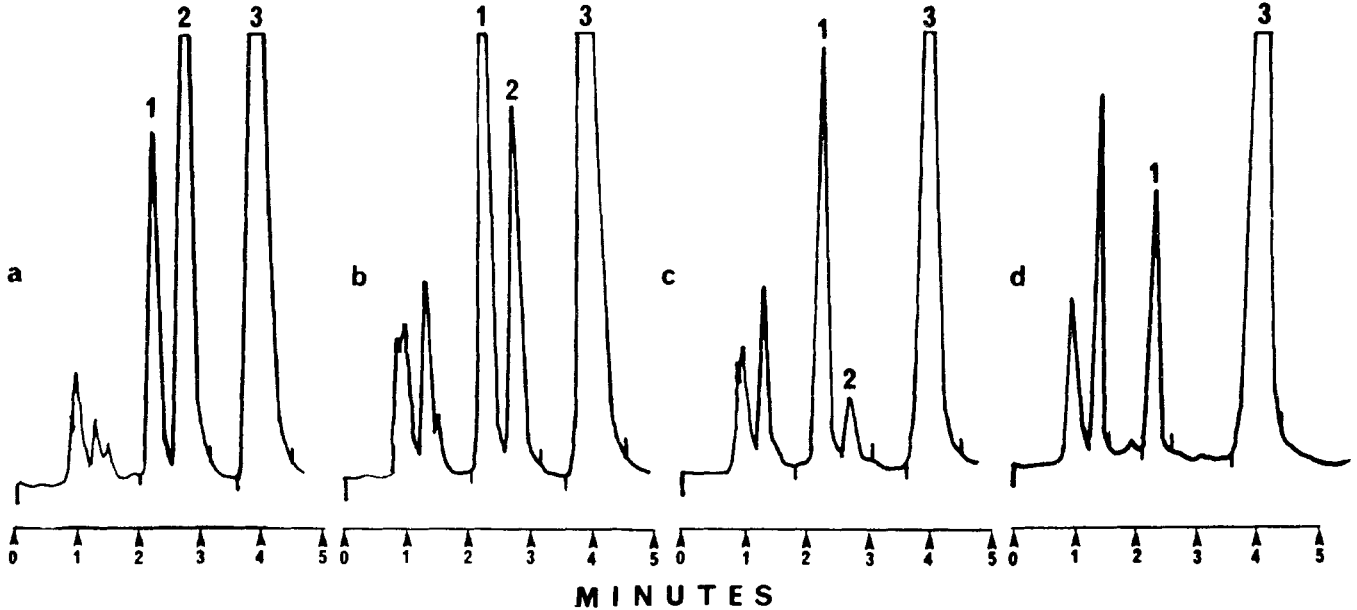


Figure III: Liquid chromatographic analysis of serum samples following intravenous dosing with DMP. a = 10 minutes, b = 50 minutes, c = 75 minutes, d = 220 minutes post-infusion. Peak 1 = ADMP, peak 2 = DMP, and peak 3 = IS.

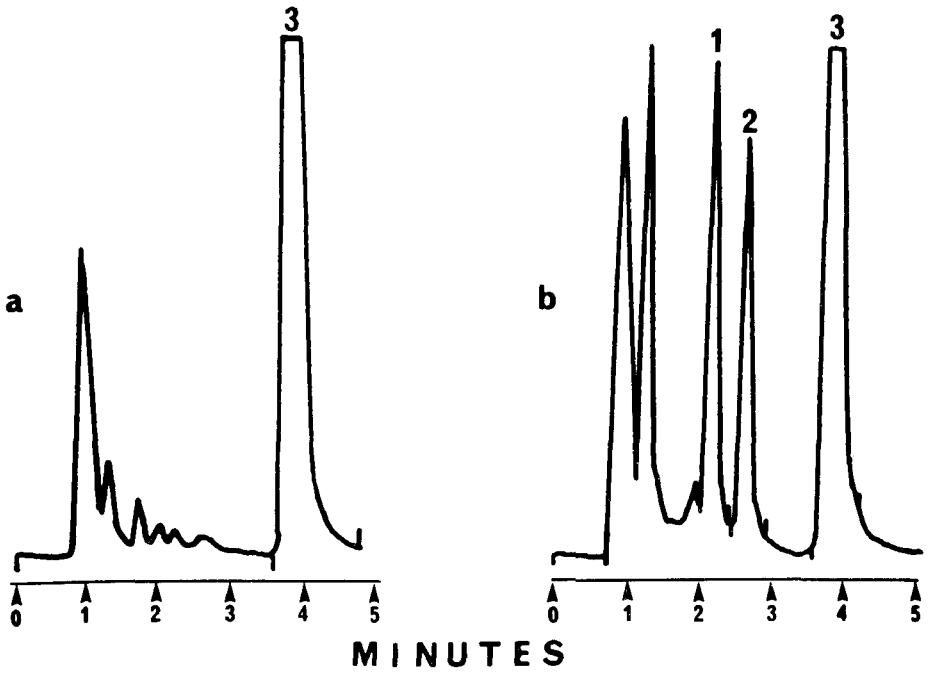


Figure IV: Chromatogram showing isolation of DMP and ADMP from urine. a = blank urine with IS, b = 1 hr urine sample after dosing. Peak 1 = ADMP, peak 2 = DMP, and peak 3 = IS.

400 solution. The metabolite levels rise quickly and exceed the level of the parent compound soon after termination of the infusion. The elimination rate of DMP is much faster than that of ADMP with a total body clearance of 1.182 L/hr/kg and a Vd of 0.434 L/kg. This difference in elimination is reflected by the marked difference in slope of the terminal phases between the two compounds. The elimination half-life of DMP was 16.1 minutes while that of ADMP was 1.7 hr.

TABLE 2

Actual and Observed Concentrations for DMP and ADMP from Serum Alliquots

DMP		ADMP	
Concentration added (μg/mL)	Detected[1] Concentration	Concentration added (μg/mL)	Detected[1] Concentration
0.68	0.66 ± 0.052	0.67	0.72 ± 0.068
0.96	0.88 ± 0.088[2]	0.94	1.04 ± 0.074
4.8	4.13 ± 0.053	4.7	4.54 ± 0.134
9.6	8.13 ± 0.067	9.4	8.55 ± 0.089
23.9	19.50 ± 0.356	23.5	20.13 ± 1.108
47.7	45.11 ± 1.388	47.0	46.79 ± 1.458
95.5	90.88 ± 2.204	100.8	97.00 ± 1.725
204.6	215.77 ± 3.034	168.0	167.36 ± 2.699
r = 0.99885		r = 0.99969	
slope = 1.049		slope = 0.992	

[1] Mean ± SD, n = 4; [2] n = 3

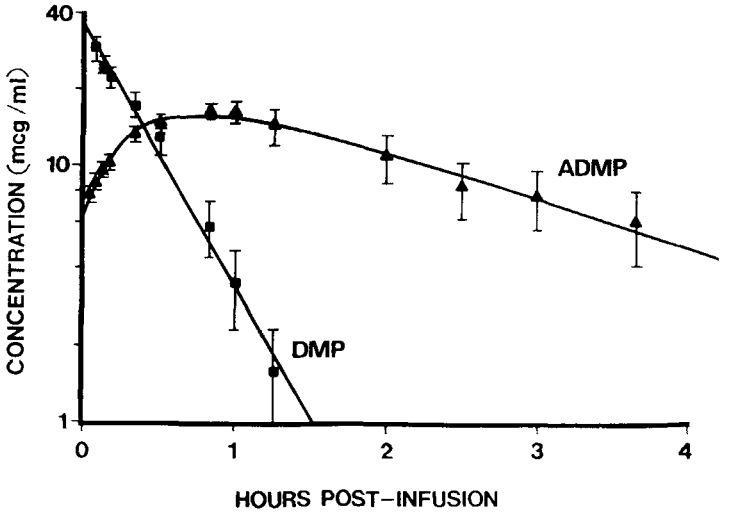


Figure V: Post infusion serum concentration profile of DMP (square) and ADMP (triangle) following intravenous infusion of 25 mg/kg of DMP.

Conclusions

A liquid chromatographic procedure has been described for the quantitative analysis of the anticonvulsant compound DMP and its N-acetylated metabolite. These compounds and the internal standard can be isolated from serum and urine in high yield using a reversed phase (C_{18}) solid extraction procedure. Chromatographic analysis was optimized using a ternary mobile phase and UV detection at 275 nm.

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