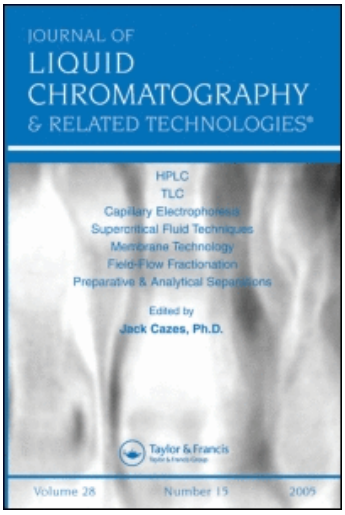

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High Performance Liquid Chromatography in Veterinary Toxicology

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INTRODUCTION

Since the late 1960's, high performance liquid chromatography (HPLC) has performed an increasingly important role as a separative analytical technique. Its range of application is far wider than that of gas chromatography which makes it attractive to those disciplines that require the capability to analyze a diversity of compound in a variety of matrices. The field of veterinary toxicology is such an area. Veterinary toxicology laboratories must have the capability to identify and quantify compounds from many different classes of toxicants. This demand requires a wide range of analytical capabilities so that inorganic, vola-

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tile organic, and complex high molecular weight toxicants can all be quickly determined. The matrices from which the analytes must be separated are frequently complex; typically feeds, biological fluids, and tissues. Often clues as to the nature of the analyte are lacking in the sample history requiring methods that can screen for many classes of compounds. This review attempts to bring together those reported applications of HPLC to veterinary toxicology.

At the 1983 Cornell University Conference for Veterinarians, Buck (1) presented data from the Animal Poison Control Center (APCC) at the University of Illinois which classified the reported poisonings to domestic animals according to their frequency of occurance in 1982. This review addresses separately those important classes of toxicants outlined by Buck for which HPLC methods applicable to veterinary toxicology have been reported.

The methods have been restricted to the analysis of feeds, tissues, biological fluids and ingesta as these represent the majority of those samples submitted for diagnosis. Methods which target particular compounds are discussed first within each section followed by the very important but all too scarce multiresidue procedures which allow for the screening of samples for a wide range of compounds.

In this regard, the use of the mass spectrometer as a detector for the HPLC has unexcelled potential. Identification of the eluting peaks can be rapidly made based on standard comparison and/or structural information derived

from the mass spectrum. The information obtained can provide unambigious confirmation of a toxicants' presence which is extremely valuable both for diagnostic and legal purposes. The marriage of HPLC and the mass spectrometer has not come as easily as that of the gas chromatograph and the mass spectrometer but great strides have been and are continuing to be made toward overcoming the difficulties with the interface. It is the authors' opinion that combined liquid chromatography mass spectrometry (LC/MS) shows great promise for the analytical toxicology laboratory. For this reason a separate section is presented devoted to a overview of the development of LC/MS interfaces in our laboratory and the application of the technique to problems of forensic interest.

APPLICATIONS

Insecticides

Organophosphates

Organophosphate insecticide poisoning represents the largest single class of compounds reported to produce toxicity in animals in 1982 in the U.S. (1). Methods of analysis include gas liquid chromatography (GLC) and thin layer chromatography (TLC). However, thermal lability is frequently a problem in GLC. Sensitivity and specificity are often compromised with TLC. HPLC methods have been reported to be used to analyze for several organophosphorous insecticides.

Azinphosmethyl (Guthion) has been determined by HPLC as a residue in crops by a method minimizing the sample

cleanup procedure. A C18 Sep-Pak clean up of the methylene chloride extract was used prior to analysis on a C18 column with acetonitrile/H20 elution and UV detection. The pesticide and its oxygen analogue were quantitated in the low ppm range. Recoveries were reported to be 75-100%. Thirty nine commonly used pesticides were shown not to co-chromatograph with the oxygen analogue but three did co-elute with the parent azinphosmethyl (2).

Multiresidue HPLC methods have been used to simultaneously separate five of the phenylphosphonothionate esters, their oxygen analogues, and degredation products from rat liver microsomal preparations (3). A RP-8 column was employed with a methanol: 5% glacial acetic acid gradient (1%-95%) with quantification in the low nanogram range using a UV detector. The insecticides analyzed were leptophos, desbromleptomphor, EPN, cyanogenphos, and EPBP.

Carbarbamates

During 1982, 15% of the reported animal poisonings by insecticides were attributable to the carbamates (Buck (1), 1983). The N-methyl carbamates are widely used as home and garden type insecticides and are thus the cause for a large number of poisonings in household pets. The carbamates are difficult to analyze by classical GC methods requiring derivatization and elevated temperatures. They can, however, be efficiently separated by HPLC. Many of the carbamates do not absorb sufficiently in the UV region which was a limiting factor in developing methods for this class of compounds until the application of post-column fluro-

metric labeling techniques (4). Carbaryl (Sevin) is one of the more common carbamate insecticides widely used as an agricultural and forest spray in addition to having home and garden applications. DeBeradinis et al. (5) have developed a fluorescence RP HPLC method to simultaneously quantitate carbaryl and its hydrolysis product, 1-napthol, from whole blood, plasma, and urine. An internal standard, napropramide, has been incorporated into the assay for quantitation in the 50-2000 ppb range.

A method for the determination of carbaryl in crops was developed by Lawrence (6). The acetone extract of the blended sample was partitioned into methylene chloride: hexane and concentrated for cleanup on a Florisil column. The fraction containing the insecticide could either be directly analyzed on a normal phase silica column with UV detection or derivatized with dansyl chloride for fluorescence detection. Ten ppb carbaryl could be detected after derivatization and 30-50 ppb could be detected without derivatization. Recoveries at 0.1 ppm average 90%.

Carbofuran is a carbamate insecticide widely used in agriculture which occasionally becomes a cause of animal intoxication. Carbofuran and its main metabolite, 3-hydroxycarbofuran, have been quantitatively determined in rape seed plants using RP HPLC. Isocratic elution of the C18 columns with methanol:water (2:7) and UV detection allowed levels at 2 ppm to be detected. The cleanup procedure was extensive involving two extractions and two carbon-silica columns (7).

Krause (8) has developed a multiresidue HPLC method for oxime and phenyl N-methylcarbamate insecticide residues. Aldicarb, bufencarb, carbaryl, carbofuran, methiocarb, methomyl, and oxamyl and four metabolites (aldicarb sulfone, aldicarb sulfoxide, 3-hydroxycarbofuran, and methiocarb sulfoxide were simultaneously determined. The method uses methanol and ultrasonic homogenizers to extract field incurred residues. Water soluble plant co-extractives and non-polar plant lipid materials are removed by liquid-liquid partitioning. Additional crop co-extractives are removed cusing a charcoal silanized Celite column. The carbamates are determined using a C8 column eluted with an acetonitrile: H20 gradient and post-column fluorometric derivatization. Recoveries were reported to be 95-100% at the 0.005 ppm level for all the residues except aldicarb sulfoxide which averaged 55%.

Rodenticides

Sodium fluoroacetate (Compound 1080) is a intensely poisonous rodenticide and predacide used in control of the rat, prairie dog, and predators. Malicious and accidental poisoning in non-target species is relatively common. Ray (9) has developed a method for detection of 1080 in vomitus, gastric contents and bait using HPLC. Chemical derivatization using O-p-nitrobenzyl-N -N -diisopropylisourea (PNBDI) was necessary for UV detection. Sample workup involved a methyl ethyl ketone extraction followed by a Sep-Pak C18 elution. Recoveries were greater than 70% at the

1 ppm level. A normal phase silica column was used eluting with 5% methyl ethyl ketone in 2,2,4-trimethyl pentane. Figure 1 demonstrates the complexity of the matrices often encountered in veterinary toxicological analysis. It was recommended that unknown samples be run spiked and unspiked to ensure that recovery from the particular biological matrix is satisfactory, since the nature of the gastric content varies considerably from animal to animal.

Another procedure for the determination of sodium fluoroacetate in poison baits was developed involving the formation of a fluorescent derivative with 4-bromomethyl-7-methoxy coumarin (10). A distillation cleanup procedure was described which overcomes extraction difficulties. A reversed phase C-8 column eluted with acetonitrile: ethyl acetate: H20 (9:2:2) was used to perform the analyses. Detection limits were described as being .2 ng per 100 ul of injection with 83-99% recovery.

Warfarin is a typical anticoagulant rodenticide with vitamin K antagonistic properties. As a class the anticoagulant rodenticides accounted for 8% of the reported poisonings to domestic animals (1). A method for the analysis of warfarin in serum or plasma was reported utilizing a 5 micron silica analytical column and a simple dichloromethane extraction. The detection limits using UV detection were reported to be 50 ppm (11).

Improved sensitivity for the determination of warfarin and its metabolites was achieved through the use of post column acid/base manipulation to enhance fluorescence. Nor-

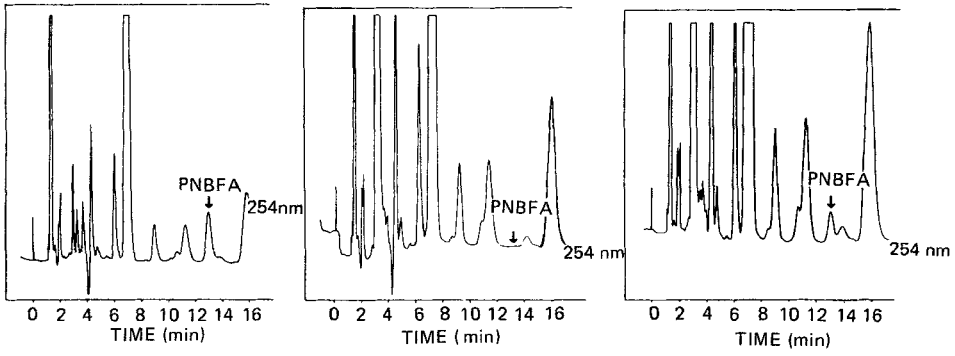


Figure 1. Typical chromatograms for HPLC analysis of 1080 as PNBFA in canine stomach contents: (left) 20 μg standard 1080: (middle) blank content: (right) content spiked with 1ppm 1080.

mal phase chromatography using a cyanobonded phase column provided detection limits in the low nanogram range in plasma and urine (12).

The anticoagulant rodenticide brodifacoum has been determined by HPLC in animal tissue and fluids (13). Clean-up involved methylene chloride extraction followed by an aluminum oxide column. Detection limits of 50 ppb were achieved using fluorescence detection and a reversed phase C18 column.

A method to determine the rodenticide chlorophancinone in tissues using a polar amino bonded phase column has been developed (14). Acetonitrile extraction and Florisil column cleanup gave recoveries greater than 95%. Acetontrile:H20 was used for the analysis with the lower limit of detection at 4 ppb.

Acenocourmarin is another anticoagulant rodenticide with vitamin K antagonistic properties. A method for quanti-

tative determination in plasma using C18 reversed phase chromatography and UV detection was developed (15). Using a single solvent extraction of the plasma a lower limit of detection of 15 ppb was reported.

Another commonly encountered anticoagulant rodenticide that has been analyzed by HPLC is phenprocoumon. Dichloromethane extraction from plasma or urine followed by C18 reversed phase chromatography and UV detection allowed for quantitation of 20 ppb in urine and 100 ppb in plasma (16).

Strychnine intoxication represented 1.1% of the reported animal poisonings in 1982 (1). Crude preparations of Strychnos nux-vomica are sometimes used in malicious poisoning cases and contain brucine as well as strychnine. Ray (17) reported HPLC conditions to detect and quantitate both alkaloids. Approximately 2 ng of strychnine and 5 ng of brucine could be detected by this method with UV absorbance and a dual channel detector. The procedure was used to confirm the presence of strychnine in ingesta, urine, and baits. A strychnine intoxication was also confirmed by a LC/MS procedure (see Glass Capillary Split Effluent DLI LC/MS).

A multiresidue procedure for the four coumarin anticoagulant rodenticides brodifacoum, difenacoum, coumatralyl, and warfarin was developed for analysis of liver and stomach contents. A relatively simple procedure involving solvent extraction and Sep-Pak cleanup was used. A two step HPLC method was devised involving a separation on a porous silica size exculsion column from which brodifacoum, dif-

enacoum, and coumatralyl co-elute. The fraction containing the co-eluents was collected for separation on a normal or reversed phase column. Warfarin resolved on the first column. The method was found satisfactory for serum, liver, brain, muscle, stomach, or rumen contents from several species. Recoveries of 80-100% were reported. Fluorescence detection allowed detection limits to approach 1 ppb for brodifacoum, difenacoum, and coumatralyl and 50-100 ppb for warfarin (18). In this procedure the use of a multidimensional HPLC procedure could possibly circumvent the fraction collection step (See Multidimesional LC/MS).

Herbicides

Herbicides represent 4.2% of the reported poisonings in 1982 (1), A figure surprisingly low considering the ubiquitious nature and potent toxicity of many of theses compounds. Chlorosulfuran is a commonly used broad spectrum weed killer. A HPLC method was developed to determine the herbicide in grain, straw, green plants, and cereals (20). The cleanup procedure involved ethyl acetate extraction and size exclusion chromatography. Recoveries of 60-100% and detection limits of .01 ppm were reported.

An HPLC procedure was reported for the determination of residues of the herbicide glyphosate and its metabolites in crops utilizing a water extraction and cation exchange cleanup (21). Post column fluorogenic labeling with O-phthalaldehyde-mercaptoethanol allowed .05 ppm to be easily detected. Recoveries average 60%. The analytical separation

utilizes an ion exchange column eluted with .09 H3P04:.01M H2S04.

Residues of difenzoquat were quantitated with an on-line enrichment method using a 7cm C10 pellicular column in place of the loop injector. The herbicide was eluted onto the C18 analytical column with the HPLC mobile phase consisting of 60:40 acetonitrile: H20 + potassium dihydrogen orthophosphate. UV detection was used to quantitate levels as low as 5 ppb (22).

The presence of the phenoxyacetic acid herbicide 2,4-D in addition to warfarin and chlorpyrifos were verified in plasma and tissue of a poisoning case by HPLC (23). The sample was directly analyzed after dilution with buffer and centrifugation. A reversed phase C18 column was used eluted with acetonitrile:methanol:phosphate buffer. Use of an internal standard and UV detection determined the concentration in the plasma of the herbicide to be 60ug/ml.

Substituted phenylurea compounds are widely used as selective herbicides in agriculture. A multiresidue method for the direct analysis of corn and other crops for eight-urea herbicides was developed by Lawerence (24). The samples were extracted with acetone, partitioned with hexane: methylene chloride, and further extracted with methylene chloride. After a Florisil column cleanup the evaporated residues were analyzed on a normal phase silica column with UV detection. Recoveries were greater than 80% in most cases. All the ureas could be detected and confirmed in the matrices studied at .05-0.1 ppm

An alternative multiresidue procedure for the determination of eight substituted phenyl urea herbicides in grains has been reported (25). A C18 analytical column is used for the analysis with a mobile phase of methanol:H20: NH3. A one step extraction procedure afforded recoveries of 85-95% at the .5 ppm level. The lower limit of detection was estimated to be 0.2 ppm using UV detection. Figure 2 shows the chromatogram of the simultaneous analysis of all eight herbicides in a grain extract.

A multiresidue method for the determination of seven herbicides in rice field waters has been developed. Simultaneous determination of Bentazon, 2,4-D, MCPA, Propanil, Molinate, and Drepamon was accomplished on a C8 column eluted isocratically with acetonitrile: 2M sodium acetate buffer (pH 4.0). Detection limits with UV monitoring of .01-.03 ppm were achieved (26).

Another multiresidue analysis of animal feed incorporating a Sweep-Co distillation apparatus for purification was developed by Eichner (27). The chromatographic separation was performed on a C8 column using a complex methanol:H20 gradient elution pattern and quantitation with a UV spectrometer at variable wavelengths. Simultaneous analysis of 36 herbicides, 2 metabolites, and 3 insecticides was reported.

Feed Problems

Adverse effects to animals resulting from a feed problem can often be traced to the feed being improperly form-

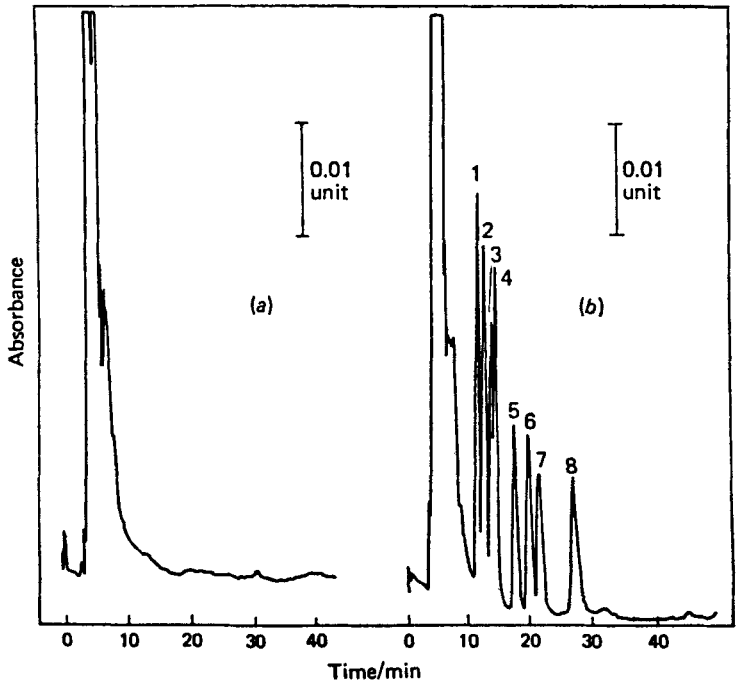


Figure 2. Typical chromatograms obtained from injections of wheat extracts: (a) unfortified, and (b) fortified with phenylurea herbicides at 2 mg/kg. 1, monuron; 2, monolinuron: 3, metabromuron; 4, chlorotoluron; 5, diuron; 6, linuron; 7, chlorbromuron; and 8, chloroxuron.

ulated or to the incorporation of toxic substances into the plant material used for feed. For instance, excessively high concentrations of vitamins, due to mistakes during feed formulation, can lead to toxic syndromes (28). Under certain environmental conditions some feed crops can overproduce toxic substances such as the plant estrogen coumestrol which could lead to reproductive problems (29).

A HPLC procedure to diagnose both hyper and hypovitaminosis A by measuring retinol levels in serum, tissue, and

milk samples was developed by Stowe (30). After hexane extraction a normal phase silica column with flourescence detection was used. The elution was run isocratically with hexane: chloroform 60:40.

Occasionally toxicity due to excessive concentration of vitamin D3 in the feed has been reported (31). Ray (32) developed a method for analysis of livestock feed which involved collecting the impure vitamin D_3 fraction eluted from a normal phase silica column, and completing the separation by injecting the fraction on a C18 column. Figure 3A shows the normal phase chromatogram marked where the fraction was collected. Figure 3b shows the completed analysis on a C18 column. This type of analysis can be achieved online by a heart cutting technique which traps the peak of interest from the first column in a loop injector and reinjects it onto a second column (see multidimensional LC/MS). Recoveries averaging 90-96% with quantitation in the low ppm range was reported.

Coumestrol is a plant estrogen found in many forage crops. High levels of estrogenic substances in feeds can have severe biological effects (29). Lookhart (33) developed an HPLC method to quickly identify and quantitate possible estrogens in feeds. Reversed phase chromatography using a C18 column, methanol:H20 eluent, and fluorescene detection allowed for quantification in the low ppb range.

Mycotoxins

The mycotoxins accounted for 2.2% of the reported cases in 1982 (1). The route of exposure of animals to

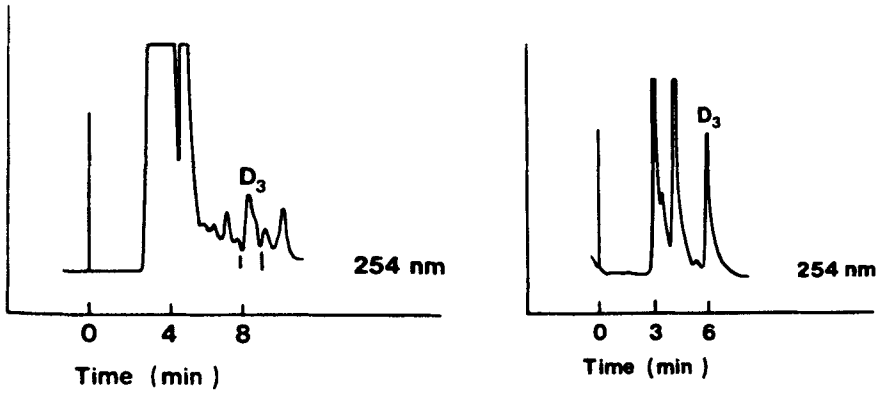


Figure 3. (A)Chromatogram of feed extract containing vitamin D_3 normal phase μPorasil column. Volume of eluate collected for reverse phase chromatography is indicated on chromatogram. (B)Chromatogram of fraction from normal phase column on a reversed phase μBondapak C_{18} column.

these compounds is predominately through the consumption of feeds that have molded or spoiled. Some of the mycotoxins pose additional concern because they leave residues in the tissues of the animals and present a potential health hazard to the consuming public.

Methods for the determination of aflatoxins by HPLC abound because of the importance to public health to monitor for the presence of this carcinogen and because the method of choice for analyzing samples containing aflatoxin is by HPLC. Aflatoxin determinations are done by both reversed and normal phase techniques; the relative merits of the two procedures are discussed by Pons (34) and Beebe (35). The two examples discussed here were chosen because of their applicability to animal feeds and products.

Gregory (36) reported a reversed phase HPLC procedure that would permit the determination of parent aflatoxin

in the various forms and conjugated metabolites in animal tissues. Several extraction steps followed by a salica acid column cleanup were required. The extracts were treated with trifluoroacetic acid (TFA) to induce fluorescence, chromatographed on a C18 column, and eluted with H20:acetoni trile: methanol. Figure 4 is an extract of beef liver showing the highly fluorescent derivatives (M_{2a}, G_{2a}, and B_{2a} and the corresponding unreacted parent aflatoxins (G_2, and B_2) after TFA treatment. The lower limit of detection of the parent aflatoxin was 1-5 pg with quantitation of the extracts in the sub ppb range.

A method for the determination of aflatoxins in animal feeds down to 2.5 ppb was reported by Cohen (37). Samples were extracted with acetonitrile:H20 and cleaned up using a Sep-Pak silica cartridge. Recoveries of 82-99% were reported. The trifluoroacetic acid induced fluorescence technique was employed for the analysis using a C18 column. Aflatoxin levels could be reported in less than 30 minutes using this procedure. Aflatoxins have been analyzed by combined liquid-chromatography/mass spectrometry. Details of the procedure are presented in this review in the Glass Capillary DLI Micro LC/MS section.

Penicillic acid is a mycotoxin which can occur in high concentrations in corn and has reported carcinogenic activity. A reversed phase HPLC method was developed by Hanna (38) for determining penicillic acid residues in chicken tissues. Optimization of chromatographic conditions was achieved using a mobile phase consisting of acetonitrile:

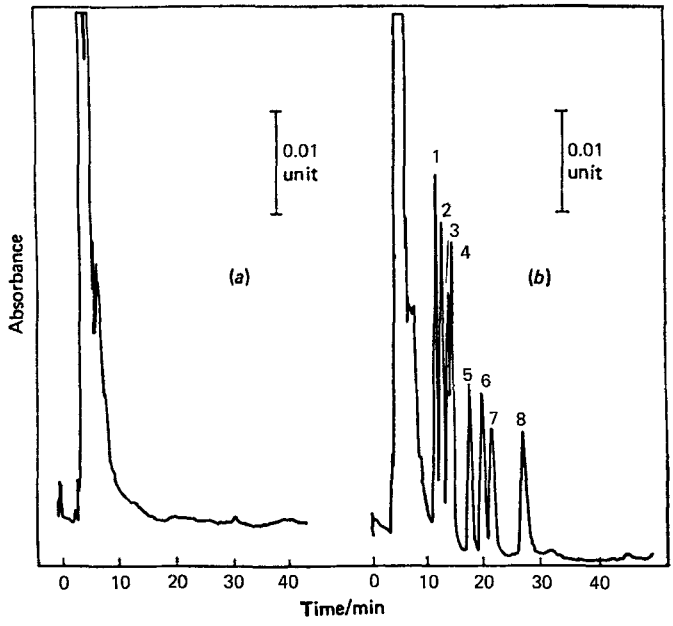


Figure 4. Typical chromatograms of beef liver extracts (TFA treated). Left = recovery sample (M_{2a}, B_{2a} G_{2a}=1ng/g; B_2, G_2 = 0.3 µg/g); Right = unspiked.

water with detection by UV absorption. Sample preparation involved acid treatment of the homogenized tissues followed by ethyl acetate extractions. Extraction efficiencies were reported to be 80-105% with quantification in the low ppb range.

Zearalenone is a mycotoxin that has recently been described as an important contaminant in corn crops. The presence of zearalenone in feedstuffs can result in reproductive disorders in many classes of livestock (39). Cohen (40) has described an HPLC method to determine zearalenone in animal feeds at levels as low as .01 ppm. An extensive

cleanup procedure is described involving solvent extractions, Sep-Pak silica cartridge purification, and Sephadex LH-20 column chromatography. Separation was achieved on a amino (NH2) bonded phase column using fluorescence detection. Recoveries at levels of .01-1.0 ppm averaged greater than 90%. Figure 5 shows a chromatogram of an animal feed sample containing .01 ppm zearalenone.

Two procedures for the analysis of zearalenone (41,42) and one for the analysis of plasma (43) have been described. In the procedure described by James (41) liver samples were extracted with methylene chloride and cleaned up on a Sephadex LH-20 column. Normal phase chromatography using a silica column and UV detection was used to achieve detection limits of 28 ppb. Turner (42) reported a reversed phase system for the analysis of zearalenone in chicken tissues. Using a C18 column and UV detection the assay could yield quantitative results in the 50-200 ppb range. A three step solvent extraction cleanup procedure was done with recoveries averaging 82-95%.

Trenholm (43) described a HPLC method for the quantitative determination of zearalenone and its metabolite zearalenol in plasma. A C8 column was used with fluorescence detection. A three step solvent extraction procedure gave recoveries averaging 76-101% on samples in the low ppb range. The limit of detection was .6 ng/ml blood plasma.

Satratoxin G and H are trichothecene fungal toxins that at times contaminate livestock feed and cause toxicity. A method for their detection and quantitative deter-

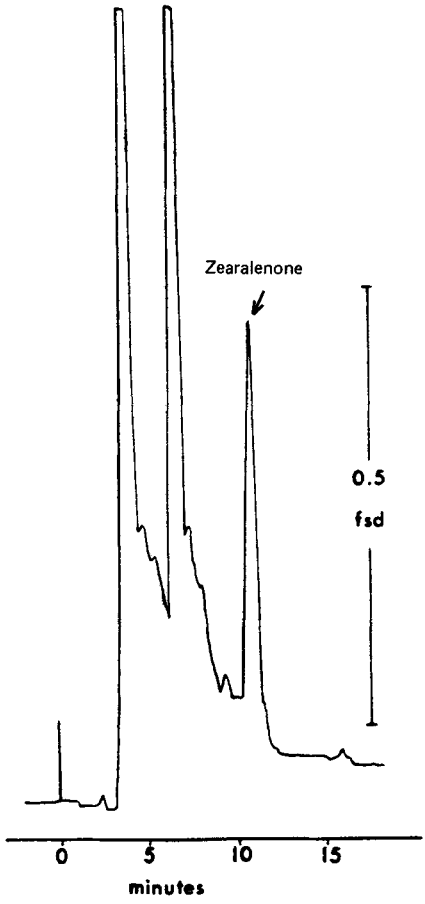


Figure 5. Chromatogram of animal feed sample with 0.01 ppm added zearalenone.

mination in grains has been described by Stack (44). The toxins were extracted with methanol:H20, cleaned up on a silica gel column and chromatrographed on a microparticulate silica column with UV detection. The recoveries averaged 65-70% with the lower limit of detection at 200 ppb.

A rapid multimycotoxin screening method for mixed feeds was reported by Howell (45). The analysis allowed for the

determination of aflatoxin B1,B2,G1,G2, zearalenone, and ochratoxin by HPLC using fluorescence detection. Detection limits of all the toxins were 1 ppb. The sample preparation is described as simple and rapid involving a chloroform:H20 extraction and Sep-Pak silica cartridge cleanup. Recoveries ranged from 70-90% for the six mycotoxins.

Fungicides

Fungicide intoxications accounted for 57 reported cases to the APCC hotline (1). A method to determine the fungicidal agents pentachlorophenol and tetrachlorophenol in animal tissues has been report (46). Ion-pair extraction followed by a silica Sep-Pak cleanup was used to prepare the samples for analysis. A porous silica column with methanol as the eluent was used for the determination. Detection limits of 1 ppb were achieved by measuring UV absorbance. Recoveries of the analytes from the tissue extracts averaged 90-100%.

Quantitative determination of captan, captifol, and folpet residues in crops could be done using a gel chromatography cleanup procedure (47). The fungicides could be simultaneously determined on a cyano bonded phase column using UV detection. Limits of determination were .02-.05 ppm with 75-120% extraction efficiencies.

A multiresidue procedure for determining residue of biphenyl, benomyl, carbendazim, 2-phenylphenol, and thiabendazole in citrus crops was developed by Farrow (48). Samples are extracted by refluxing with dilute HCL and frac-

tionated by different extraction procedures. The fungicides were determined by using both normal and reverse phase methods in the low ppm range.

Ethylene Glycol

The palatability of ethylene glycol and its ubiquitous presence as a commercial antifreeze make it a common cause of poisoning in dogs. In 1982, 55 poisonings were reported to the APCC that were cause by ethylene glycol consumption (1).

Hewlett (49) reported a HPLC method for the diagnosis of ethylene glycol intoxication based on the determination of glycolic acid, a metabolite which accumulates to a sufficient concentration with adequate half-life for reliable detection. Urine and serum are extracted with methyl ethyl ketone and the concentrated extract is derivatized with O-p-nitrobenzyl-N-N'-diisopropylisourea (PNDBI). The derivatives are chromatographed on a 5 micron silica column with 16% methyl acetate in isooctane as the eluent. UV absorbance was monitored and recoveries averaged 94-98%. Figure 6 is a chromatogram of the glycolic acid derivative standard (PNBG), spiked serum, and control serum.

Zootoxins

Toxins of animal origin such as snake venoms and insect toxins are often large complex molecules and as such are not amendable to gas chromatography. HPLC is a logical choice but few reports of its use as a diagnostic tool in

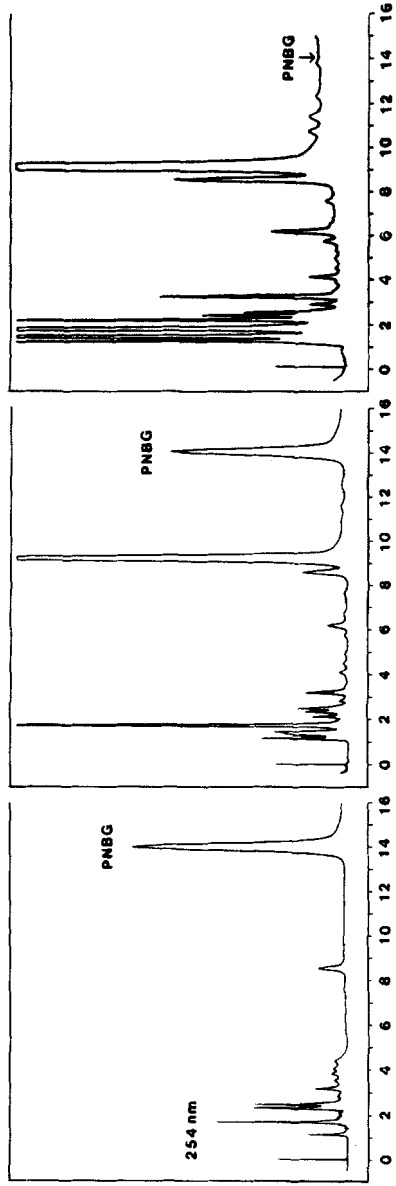


Figure 6. Typical chromatograms for HPLC analysis of glycolic acid as PNBG: (left) 1 mg standard glycolic acid; (middle) dog serum extract 3 hours after ethylene glycol administration; (right) control serum sample.

this area exist. Cantharidin is one zootoxin of veterinary importance that has been determined by HPLC. Cantharidin is the toxic principle in the three stripped blister beetle which can be trapped in alfalfa hay during the bailing process and lead to fatal poisoning of horses, cattle, and sheep. Ray (50,51) has developed a HPLC procedure for determination of cantharidin in body fluids, ingesta, and tissues of poisoned animals. An extensive extraction procedure was required to cleanup samples for analysis. A normal phase silica column was used to analyze the p-nitrobenzyloxime derivative (CAN-PNB) which was formed to enhance UV detection. Dioxane (9%) in hexane was used as the eluent. Quantitative analysis was done in the low ppm range. Figure 7 is a chromatogram of derivatized pony urine. Measurement of the peak height ratios of 254 and 280 nm offer additional evidence of peak identity.

Avicides

Avitrol (4-aminopyridine) has been used for more than a decade as a bird repellent in problem areas such as cornfields, cattle feedlots, processing plants and airports. Ray (52) has described conditions of 4-aminopyridine toxicosis in horses and established methods of extraction, identification, and quantification for this compound using TLC and HPLC. Corn bait, stomach contents, and liver were analyzed with a C18 column after methyl acetate extraction. The analysis was done isocratically with methanol:1% acetic acid and monitored by UV absorbance. Approximately .25 ng of 4-aminopyridine could be detected by this method.

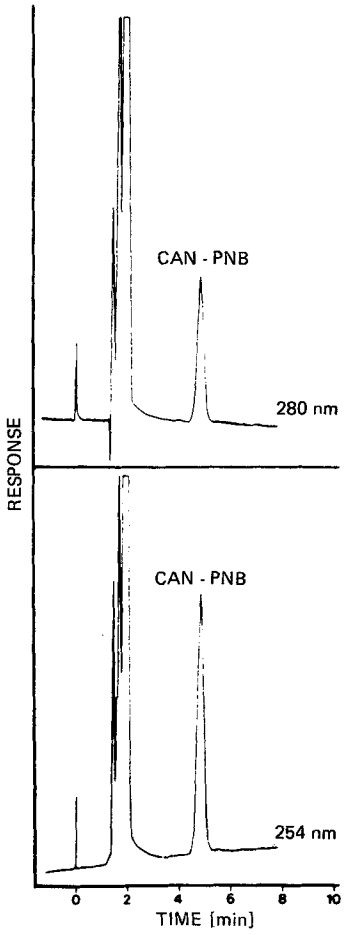


Figure 7. Chromatogram of derivatized extract of pony urine after feeding the animal with 5.3g of dried blister beetles. CAN-PNB = p-nitrobenzyloxime of cantharidin.

Medications

Occasionally undesirable or even toxic and fatal reactions to therapeutic agents occur. The combined reports of intoxication to animals from both veterinary and human medication totaled 14.5% of all the reported poisonings to the ACPP in 1982 (1). This points out that adverse drug reactions comprise a substantial number of diagnostic problems. An additional concern is the accumulation of drug residues in the tissue and products of food producing animals. Some examples where LC has been (is) used to determine drugs that pose both toxicity and residue problems are described below.

As widely used drugs, the sulfonamides occasionally present adverse therapeutic reactions. Most cases involve inadvertant overdosage resulting in renal crystallization. Some cases involve violative residues of these drugs in tissues primarily as a result of inadequate withdrawal periods from mediated feeds. Stringham (53) developed a HPLC method for sulfonamide drugs in feeds and premixes using dimethylaminobenzaldehyde (DMAB) as a post-column derivatizing agent. This derivative permits analyte detection at a wavelength where interfering substances are minimized. Thus, only a simple cleanup procedure was required involving a .15N HCL in 25% methanol extraction followed by filtration and analysis on a C18 column. Recoveries averaged 95-99% at low ppm concentrations.

A HPLC method for the determination of sulfamethazine in pork tissue by was reported by Cox (54). A cleanup proce-

dure involving a three step extraction and elution from an XAD-2 column with methanol gave recoveries of approximately 85%. The drug was reliably quantitated at 0.1 ppm on a C18 column with UV detection.

A simple reversed phase HPLC method was described for the determination of sulfanitram and Dinsed in variety of feed premixes and formulations (55). A single solvent extraction followed by filtration constituted the cleanup procedure. Acetonitrile:H20 was the mobile phase for the separation on a C18 column. Commerical feed formulations containing .02¬05ppm of the drug were analyzed by this method.

A recent trend in HPLC analysis has been toward the use of microbore columns offering advantages of solvent economy, high mass sensitivity, and high speed separation. Eckers (56) demonstrated the utility of this technique for sulfa drug analysis achieving detection limits of less than 20 pg. For further discussion of the micro HPLC technique and sulfadrug analysis see the Glass Capillary Total Effluent DLI micro LC/MS section, the Diaphragm Split Effluent DLI LC/MS section, and the LC/MS/MS section.

Adverse reactions among the antibiotics usually involve hypersensitivity or anaphalactic episodes. Oxytetracycline sometimes elicits cardiovascular responses which are manifested as acute collapse and sometimes death in both cattle and horses. An assay capable of detecting .25 ppm concentrations of oxytetracycline, chlorotetracycline, and tetracycline in plasma, urine, and tissues was develop-

ed (57). An extraction proceedure incorporating phenylbutazone as an ion-pairing reagent significantly improved extractions efficiencies over previous methods, consistently being greater than 95%. A C18 column was used for the analysis eluted with an acetonitrile:H20 mobile phase with the pH adjusted to 2.1-2.4.

Chloroamphenicol is a useful antibiotic but has been associated with a variety of adverse effects. Cats are particularly sensitive occasionally exhibiting reversable ataxia and posterior paralysis. A reversed phase method has been developed to determine chloroamphenicol and its succinate ester in biological fluid (58). A C18 column eluted with methanol:H20:acetic acid was used with UV detection. Dilution of serum samples with acetonitrile was the only sample preparation used. The limits of detection were 0.5 and 0.2µg /ml for chloroamphenicol and its succinate ester respectively with extractions efficiencies of 96-103%.

A sensitive, quantitative method has been described for the analysis of hydrochlorothiazide in equine plasma and urine using trichlorothiazide as an internal standard (59). Samples were extracted with ethyl acetate and cleaned up on convenient microcolumns made from Pasture pippettes and porous polypropylene wicks cut to serve as a frit for the microcolumn. A normal phase silica column was used for the analysis eluting with chloroform:methanol and with detection at 254nm. Figure 8 shows the practicle minimum detectable limit for hydrochlorothiazide in urine at 25

Figure 8. HPLC Chromatograms of extracts of a zero-hour equine urine (left) and a zero hour equine urine spiked with 25 ng/ml hydrochlorothiazide (right) This level of HCT in urine represents the practicle minimum detectable limit.

ng/ml. Trichlorothiazide has also been analyzed by LC/MS techniques. See Diaphragm Total Effluent DLI micro LC/MS.

Other drugs covered in this review can be found in the HPLC/MS section. Specifically, for promazine (and metabolites) and fentanyl see Glass Capillary DLI micro LC/MS; for diphenylhydantoin see Glass Capillary Split Effluent

DLI LC/MS; for corticosteroids, reserpine, indomethacin, furosemide, phenylbutazone, and oxyphenylbutazone see Diaphram Split Effluent DLI LC/MS; for butorphanol see LC/MS/MS; for antibiotics and corticosteroids see Diaphram Total Effluent DLI micro LC/MS; for corticosteroids see LC/LC/MS; and for reserpine and leucine enkephalin see thermospray LC/MS.

HIGH PERFORMANCE CHROMATOGRAPHY/MASS SPECTROMETRY

One of the more intriguing analytical methods developed over the past decade has been the combination of liquid chromatography and mass spectrometry (LC/MS). The LC/MS development has been of particular interest because it compensates for the lack of versatility in conventional liquid chromatography (LC) detectors and also complements the important role played by gas chromatography/mass spectrometry (GC/MS) in organic analysis. Although the chemist would like to utilize the same analytical benefits found in GC/MS, the combination of LC/MS is not as naturally compatible as GC/MS. The problems involved with the development of a practical LC/MS system are several orders of magnitude more difficult than those of GC/MS, and all the interfaces developed so far require some compromise in the normal operating mode of either the liquid chromatograph, the mass spectrometer, or both.

Despite the many obstacles facing the combination of the mass spectrometer and the liquid chromatograph, several

interfacing techniques have emerged. In an effort to provide effective LC/MS systems, these techniques are continually being developed and applied with varying degrees of success to difficult organic analyses. The purpose of this review is to describe briefly various examples reported thus far which afford LC/MS results from actual veterinary forensic toxicology samples. In all cases LC/MS was utilized because the analytes were not amenable to GC analysis due to thermal instability, their highly polar nature or the necessity for complicating derivatization reactions.

This review deals only with continuous, on-line LC/MS systems. There are many examples of off-line procedures that have provided useful data which may be difficult to obtain using continuous LC/MS methods (such as field desoption or high resolution mass spectrometry). Nevertheless, it is felt that the term "combined LC/MS" should be used only when the combination provides a real-time chromatographic profile of the peaks eluted from an LC column.

It would be best if the LC/MS interface did not interfere with the conventional operation of either the liquid chromatograph or the mass spectrometer. In practice all the techniques that have been proposed impose some restriction on one or another of the components. The success of any process selected depends upon the sample involved and the type of information required. The ideal operating parameters place formidable demands on both the LC system and the mass spectrometer. If one operating parameter is essential, then the partial or complete sacrifice of some other operating parameter is usually necessary.

The two most difficult obstacles to overcome for successful LC/MS are the severe flow imbalance between the LC eluant as it is vaporized upon entering the mass spectrometer and the vaporization of low-volatility or thermally labile samples. Most of the proposed LC/MS techniques have been designed to obviate or effect a compromise with respect to large differences in volume flow of gas or vapor between the two instruments. Only minimal attention has been directed toward the tolerance of the interface to buffers or ion-pair reagents, the maintenance of high system sensitivity, or the vaporization of low-volatility samples.

Direct Liquid Introduction Overview

The most straightforward way to introduce an LC effluent to a mass spectrometer is to split the effluent stream and directly inject only that amount of solvent which can be tolerated by the mass spectrometer. This method was first suggested by Tal'roze (60) in 1968. Because an electron impact (EI) source was used, the fraction of sample utilized was considerably less then 0.1%; thus the method did not attract immediate attention. McLafferty et al. (61,62,63) realized that increased sample yield could be attained if the split was directed into a chemical ionization (CI) mass spectrometer since it could accept at least 1-2 orders of magnitude more effluent. The merits of the direct liquid introduction (DLI) interface to a CI system have since been recognized and examined by several other laboratories. The direct inlet interface is currently

available as a commercial LC/MS system from Hewlett-Packard [Palo Alto, California (64)] and Nermag, [France (65)].

There are two basic interface designs that have been used for DLI LC/MS. In the earlier systems LC effluent was passed to the ion source using a capillary tube (61, 63). In some designs the tip was further restricted, while in others the flow was reduced or controlled by means of a thin coaxial wire in the capillary (66). The capillary tube technique is relatively easy and economical to fabricate but has a important disadvantage; low-volatility solutes may be deposited in the tube due to pre-evaporation of solvent. This difficultly is avoided by replacing the capillary tube with a pinhole orifice place directly adjacent to the ion source. The total effluent from the LC flows past this orifice and out of the interface. The small, acceptable amount of effluent is thus passed through the orifice into the mass spectrometer. Cooling water may be provided to prevent vaporization of the solvent and high vapor flow tnrough the aperture into the ion source.

Melera has described the Hewlett-Packard DLI interface (64, 67). The probe is inserted through a vacuum lock so that it is in direct contact with the ion source. Because of the large quantities of solvent that pass into the mass spectrometer, a cryogenic trap (cold fingers) is provided in the source region to supplement the normal CI pumping system. Samples can enter directly from the column or via an auxiliary detector placed in series. They in turn pass through a cavity which contains the orifice leading to the

mass spectrometer. The orifice consists of a small hole in a replaceable diaphragm and would normally be about 5-15 microns in diameter. The exact value is selected to be appropriate for the solvent viscosity. Approximately 0.3-3% of the effluent from a typical LC run (flow 1 mL/min) enters the CI source where the solvent acts as the CI reagent gas. The cooling water maintains the solvent in a liquid state as the effluent passes close to the heated ion source.

The split effluent DLI LC/MS interface was quite successful although not well received or extensively utilized. This was due at least in part to a "wait and see" attitude by many researchers who were not convinced that LC/MS was a viable, routine analytical technique. Since the practical detection limits by split effluent DLI LC/MS were not suitable for trace level toxicological studies, in 1976 we initiated studies to allow introduction of the total LC effluent from a micro HPLC into a CI mass spectrometer. This approach, if successful, should increase the on-column detection limits for micro LC/MS by a factor of 100 because all of the injected sample would be transferred to the mass spectrometer.

Our initial micro LC/MS experiments utilized the same glass capillary probe interface which had been used for split effluent LC/MS experiments (68). However, with the advent of the Hewlett-Packard diaphragm probe interface we developed a new micro LC/MS diaphragm probe interface patterned after that developed by Melera (67). This device has been used extensively by us for several years

(69,70,71), and is now commercially available from Hewlett-Packard (72). Micro LC/MS was until recently the only way to achieve high sensitivity (low ppb) by LC/MS. However, an entirely new approach to ionization of samples has emerged that offers a new method of LC/MS. This has been called "thermospray LC/MS" by Vestal at the University of Houston (73,74) and ion evaporation by Irabarne and Thomson of the University of Toronto (75). Thermospray LC/MS utilizes conventional LC flow rates of 1-2 mL/min, benefits from ionic modifiers such as ammonium acetate, and produces abundant molecular weight information from labile, high molecular weight compounds. Reports of detection limits equal to or better than micro LC/MS have appeared without the need to adopt micro LC techniques. Currently thermospray LC/MS is a very exciting addition to our knowledge of how to achieve sensitive, general purpose LC/MS suitable for problem solving. The following examples of drug and toxicological studies utilizing LC/MS show results obtained from the approaches to LC/MS described above.

Although many examples of LC/MS have been reported using the techniques described other than in the preceding section, we have confined our experience to the DLI method. The reasons for this choice include: (1) ease of accomplishing LC/MS with conventional CI MS hardware, low cost, ease of obtaining CI data often preferred for drug analyses, and compatibility with aqueous reversed-phase LC eluents. This paper serves to review our work with DLI LC/MS of drugs and their metabolites with both conventional LC, the newer micro LC columns, and more recently thermospray LC/MS.

Glass Capillary Split Effluent DLI LC/MS

In many of the liquid chromatography/mass spectrometer (LC/MS) systems reported to date (76), considerable effort has been expended to build or modify elaborate equipment to accomplish LC/MS. Although the appoaches already described have shown that routine LC/MS may be realized in the near future, only a few have been readily implemented on a versatile, commercially available mass spectrometer/data system without extensive modification of the mass spectrometer. We have described the combination of LC/MS/COM system on an unchanged, commercially available quadrupole mass spectrometer (69). This method has been used on five different mass spectrometers which are routine service instruments and five differenct liquid chromatographs. All instrumentation can be returned to routine service at the completion of LC/MS experiments.

Our goal with this modification of the DLI method of LC/MS was to demonstrate the method's compatibility with a standard chemical ionization quadrupole mass spectrometer. One can thus avoid the costly modifications that have been reported as associated with altering the inlet system or the ion source, or increasing the pumping speed in the ion source region. In addition, the considerable high voltage arcing problems and dangerous electrical conductance through the capillary associated with magnetic sector instruments are avoided when a quadrupole mass spectrometer is utilized. The success of our efforts suggests that any group with the appropriate laboratory equipment can inter-

face their mass spectrometer to a liquid chromatograph with a minimum of investment time or money.

Our first reports described the use of an all-glass capillary "probe interface" that was designed to fit directly into the unchanged solids probe of the Finnigan Model 3300 (Fig. 9). This system provided LC/MS results utilizing a variety of LC eluents with drug-related materials unsuitable for GC/MS analyses. The heart of this interface was a glass capillary that was inserted into the solids probe inlet of the mass spectrometer. This allowed the transfer of approximately 1% of total LC effluent directly into the ion source under chemical ionization conditions.

Typical DLI LC/MS results using this method provided the CI-LC/MS spectra shown in Fig. 10. The underivatized drugs were analyzed using 25/75 methanol/pentane as mobile phase. This reagent gas mixture generates CI mass spectra with considerably less fragmentation of these molecules than observed when acetonitrile/water reagent gas is used. As discussed earlier, this behavior appears analogous to that observed when isobutane is used as the chemical ionization reagent gas in conventional chemical ionization. The abundant $(M+1)^+$ ions displayed in Fig. 10(a-c) allow unambiguous confirmation of molecular weights of these materials.

The CI-LC/MS mass spectrum of the anticonvulsant drug diphenylhydantoin [Fig. 10(b)] readily displays its quasi-molecular ion at m/z 253 when subjected to chemical ionization by the reagent gas 25/75 methanol/pentane. As indi-

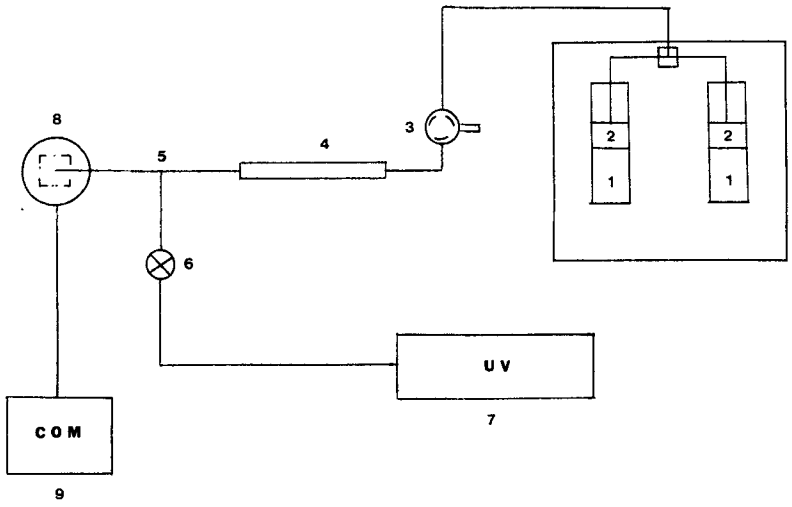


Figure 9. Schematic diagram of early DLI split effluent LC/MS system utilizing a glass capillary probe interface.

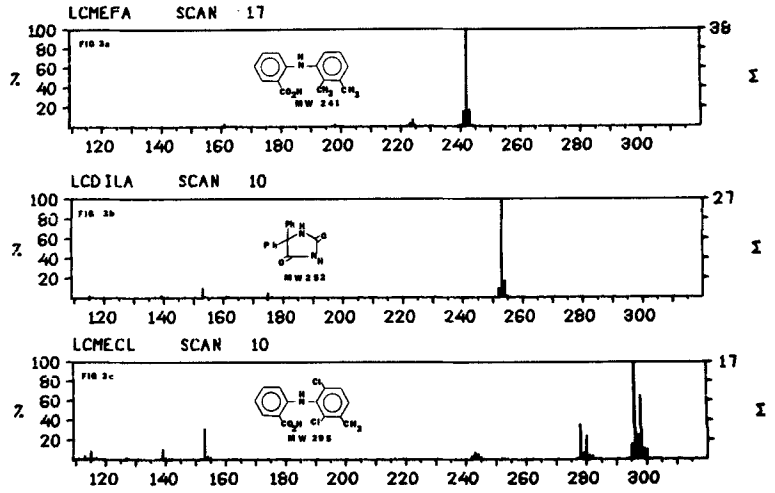


Figure 10. LC/MS chemical ionization mass spectra of (a) mefenamic acid, MW 241; (b) dilantin, MW 252; and (c) meclofenamic acid, MW 295, using 25/75 methanol/pentane as LC eluent and chemical ionization reagent gas.

cated in Fig. 10(b), the lack of abundant fragment ions suggests that the ion source temperature of 250° C does not dramatically influence fragmentation of the molecule or its protonated molecular ion. We find that the use of the methanol/pentane as eluant/reagent gas offers a useful intermediate choice which provides unambiguous molecular weight data with relatively little fragmentation.

Figure 11 shows the CI-LC/MS mass spectrum of a component found in the stomach contents of a suspect strychnine-poisoned dog. The optimum LC conditions for separating strychnine from endogenous stomach components were established off-line on a C_{18} octadecylsilyl column. An LC eluent consisting of 60/40 acetonitrile/water containing 1% acetic acid and 0.5% ammonium acetate buffer provided good separation and resolution of strychnine. When the majority of endogenous components had eluted from the LC column as determined by prior LC experiments, LC/MS COM data acquisition commenced. This experiment provided the CI-LC/MS mass spectrum of strychnine presented in Fig. 11, which is identical to that obtained from an authentic sample of strychnine (not shown here) run under the same conditions. The $(M+1)^+$ ion at m/z 335 has over 10% relative abundance and significant fragmentation ions are observed. This experiment demonstrated the feasibility of using buffers in LC/MS experiments providing the buffers or their thermal degradation products are volatile enough to be removed from the MS vacuum system.

Although the glass capillary probe LC/MS interface described earlier provides useful LC/MS CI mass spectral

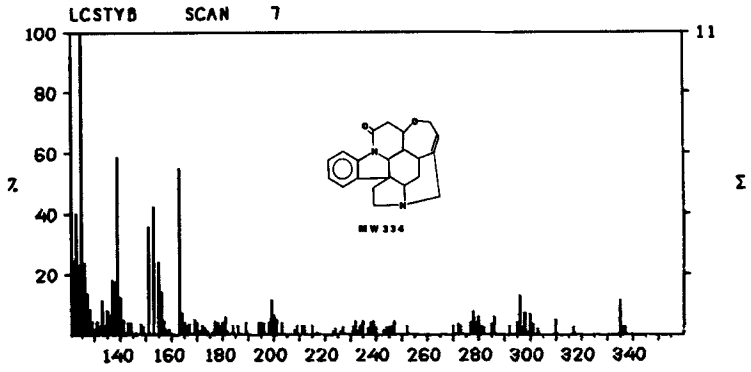


Figure 11. LC/MS mass spectrum of strychnine from the stomach contents of a poisoned dog.

results, the large split ratio of LC effluent sacrfices LC/MS sensitivity. These experiments usually required 1-5 micrograms of material injected on-column to obtain useful full scan LC/MS results. The technique of selected ion monitoring (SIM) can provide up to 100-fold increase in sensitivity by significantly increasing the integration time for selected ions in the mass spectrum.

Glass Capillary DLI Micro LC/MS

The detection limits achieved by the procedures described in the preceding section still do not match those routinely available by GC/MS, and SIM experiments lack the unique specificity afforded by a complete mass spectrum. It would be desirable to avoid the large LC effluent split necessary in these experiments which utilize conventional HPLC pumps and hardware. Conventional high-performance liquid chromatographs are usually operated at eluent flow rate

of 0.5-2 mL min^{-1}. The direct liquid introduction of total reversed-phase LC effluent from these liquid chromatographs into a mass spectrometer ion source is not practical due to the pumping limitaions of most mass spectrometers (77,78). Several recent reports have appeared which describe the use of both microbore packed columns (79) and capillary open tubular columns (80). These instruments operate at eluent flow rates from 2-40 μL min^{-1}, which are ideal for the DLI approach to LC/MS if the mass spectrometer is equipped with a cryopump (78). The limitations of this "micro" LC/MS approach include the rather small sample sizes and extended analysis time. We have demonstrated that these problems are more than offset by substantially increased sensitivity obtained from total effluent DLI micro LC/MS (81,82).

The use of micro LC/MS appeared recently (81). Takeuchi et al. described the utilization of a jet separator interface (83) to enrich the sample prior to introduction into the mass spectrometer. Later reports utilized a vacuum nebulizing interface (84,85) which significantly improved the versatility of the LC/MS system used by these workers. These experiments involve specially designed, complex hardware and do not appear to provide full-scan mass spectra at the low nanogram levels which are necessary for identification of trace level components.

Rottschaefer et al. have reported impressive CI DLI micro LC/MS results from studies of pharmaceuticals at 0.1 to 1μg levels (86). We have obtained comparable results

using the same approach. Attempts to optimize the sensitivity of the technique have provided low nanogram full scan mass spectrum detection capabilities of drugs, steroids, and toxins. Details of the equipment and applications are as follows.

A JASCO Familic-100 equipped with a 250-μL syringe pump, a UVIDEC - 100 variable wavelength spectrophotometer (UV), and a 0.3± μL quartz capillary flow cell (JASCO Inc., Easton, Maryland) was used (Fig. 12). The micro LC column was a 0.5 mm i.d. X 1.5 mm o.d. X 5 cm long polytetrafluoroethylene (PTFE) tube filled with 10 μm silica ODS SC-01 from JASCO. The exit of the UV cell was attached directly to a glass capillary interface probe (0.075 mm i.d. X 6.25 mm o.d. X 25 cm; (Wilmad Glass Company, Inc., Buena, New Jersey) described previously (77).

The JASCO micro LC and its associated detector are located as close as possible to the interface to minimize elution time to the MS (Fig. 12). A convenient removable connection is made by inserting a piece of metal tubing (0.3 mm i.d. C 0.5 mm o.d. X 2 cm) into the connecting PTFE tubing near the connector union. This connection facilitates disconnecting the UV detector when removing the glass capillary from the MS.

The use of micro HPLC for chromatographic separation may cause some skepticism from those familiar with conventional HPLC. As with all analytical techniques, micro HPLC has its limitations. Since most workers are quite familiar with the characteristic of HPLC, only the important difference between HPLC and micro HPLC are mentioned here.

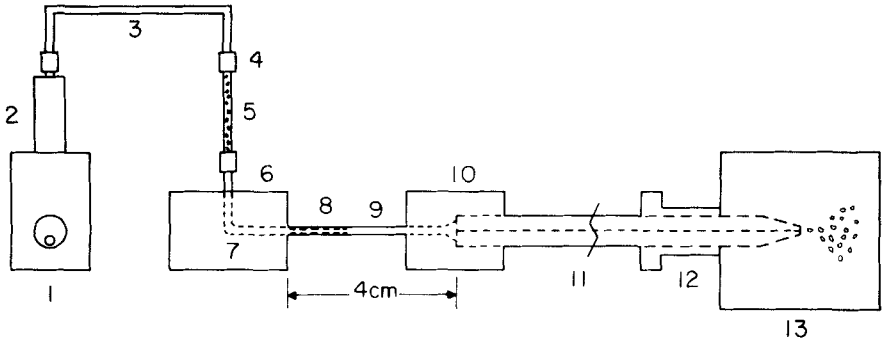


Figure 12. Schematic drawing of the JASCO micro LC/MS system: (1) pump: (2) 250- μL gas-tight syringe; (3) PTFE tubing, 0.5 mm i.d.; (4) sample inlet; (5) micro column; (6) UV variable wave-length detector; (7) micro UV cell; 0.3-μL volume; (8) stainless steel capillary; (9) PTFE tubing; (10) glass-to-teflon connector; (11) glass capillary micro LC/MS probe; (12) direct insertion probe inlet of MS; (13) CI mass spectrometer ion source. Note the close proximity of the UV exit and the micro LC/MS glass capillary probe.

In general, the detection limits of micro HPLC appear to be greater than those of conventional HPLC (87). If a variable wavelength detector is used on each instrument and all other instrumental and sample parameters are kept constant, the 0.3- μL micro UV cell (0.5 mm i.d.) detector usually yields minimum detection limits (MDL) 10 times greater than our Perkin Elmer (Norwalk, Connecticut) LC-55 detector of 6- μL cell volume and 1.35 mm i.d. The latter monitors a Waters Associates ALC-202 HPLC system. Conventional HPLC systems yield peak volumes of 0.5-2.0 mL depending upon eluent flow rate and the efficiency of the column. Peak volumes in micro LC are usually 10-30 μL and provide a more concentrated "solution" passing through the UV detector, with concommitant increased detection limits.

A limitation of micro HPLC is the availiable injection volume. As with capillary GC techniques, the micro HPLC column operates more efficiently if injection volumes are small. One-microliter injections are readily accommodated while larger injection volumes can cause reduced chromatographic performance. Of course, wide-bore HPLC columns allow larger injection volumes and therefore offset the dilution factors of the latter.

To give some idea of the chromatographic difference between micro HPLC and conventional HPLC, reference is made to the separation shown in Fig. 13. The UV traces shown in Fig. 13(a)-(c) show the effect upon the separation of three sulfa drugs caused by varying the micro LC eluent from 26/74 CH_3CN/1% acetic acid, to 24/76 CH_3CN/1% acetic acid. The rather small eluent change noted here considerably improves the separation of the last two sulfa drugs. This phenomenon is not uncommon with conventional HPLC and serves to demonstrate an important similarity between micro LC and the latter.

The CI micro LC/MS mass spectra of low nanogram amounts of the potent toxin aflatoxin B_1, the narcotic analgesic fentanyl, and sulfamethazine are shown in Fig. 14, panels (a)-(c), respectively. The LC/MS total ion current profile (TICP) and extracted ion current profiles (EICP) (71), allow comparison of component peak shapes with UV detector traces. The micro LC/MS data shown in Fig. 14 were obtained using 20/80 CH_3CN/H_2O (8 μL/min) as the micro HPLC eluent CI reagent gas. As seen from the data in Fig. 14,

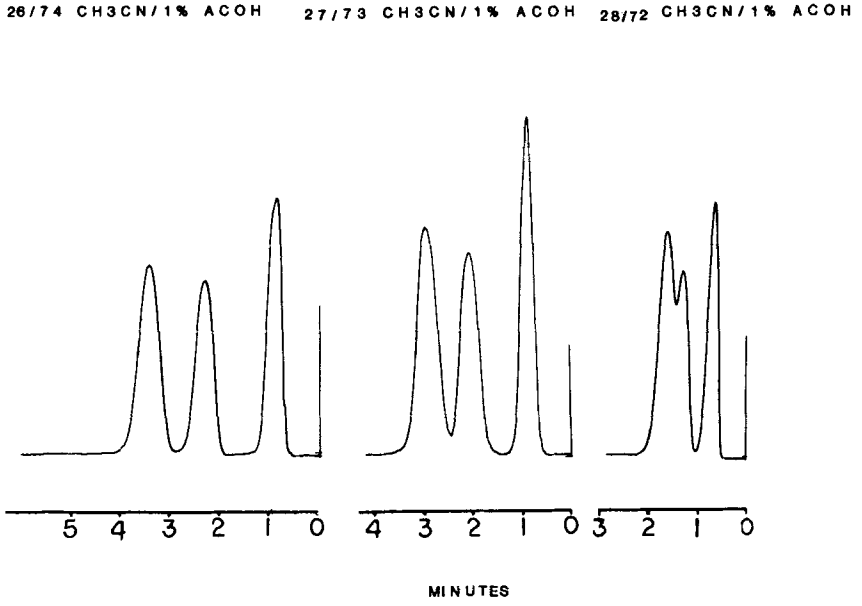


Figure 13. JASCO micro LC UV chromatograms showing the sensitvity to separation of 1% changes in eluent composition on three sulfa drugs. The eluent composition is shown above each chromatogram. The flow rate was 8 μL/min on a 0.5 mm i.d. X 10 cm SC-01 C_{18} column. The sulfa drugs were in elution order, sulfadiazine, sulfisoxazole, and sulfadimethoxine.

the LC/MS mass spectra are comprised of abundant $(M+1)^+$ ions and some fragment ions. These data can be helpful for characterizing the molecule especially when other analytical data are available.

Figure 14(a) shows the CI micro LC/MS mass spectrum for 45 ng of aflatoxin B_1 using a micro LC eluent of 20/80 CH_3CN/H_2O flowing at 8μ L/min. The minimum detectable limit (MDL) for this toxin, under the experimental conditions used, was 0.9 ng for full-scan conditions and 20 pg for

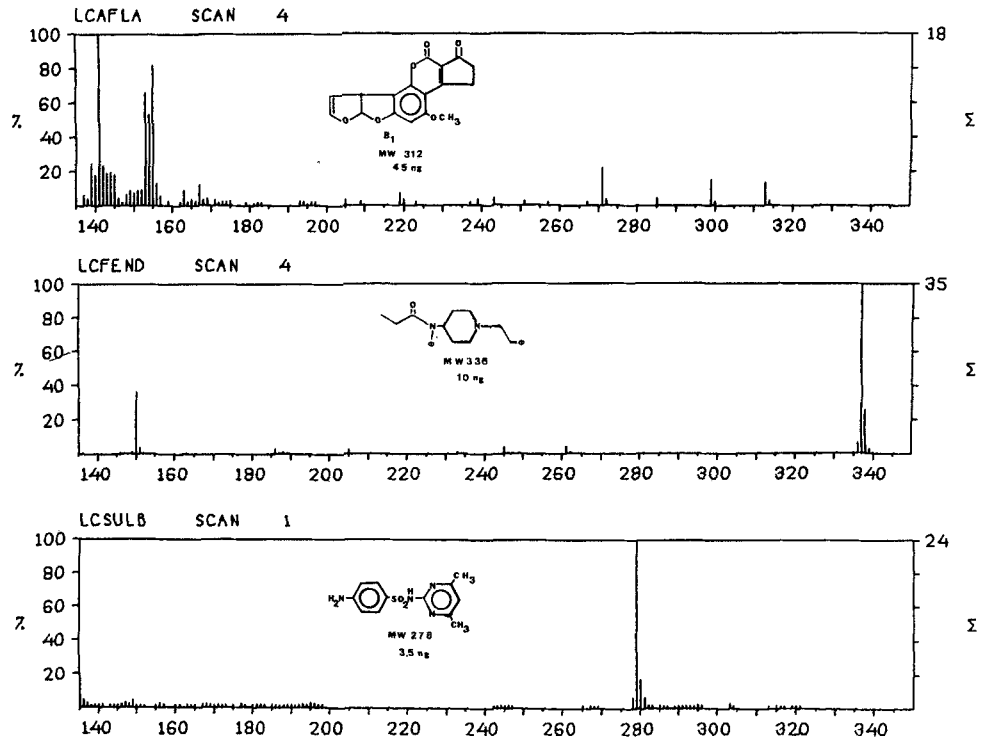


Figure 14. Micro LC/MS CI mass spectra of (a) 45 ng of aflatoxin B_1; (b) 10 ng fentanyl, and (c) 3.5 ng sulfamethazine. The micro LC eluent/CI reagent gas was 20/80 CH_3CN/H_2O for (a) and 40/60 $CH_3CN/-H_2O$ for (b) and (c).

selected ion monitoring. These detection limits are sufficient for the confirmation of toxic levels of aflatoxins found in foods and feeds.

The CI micro LC/MS mass spectrum of the narcotic analgesic fentanyl was obtained using micro LC conditions of 40/60 CH_3CN/H_2O at 8 μL/min. The abundant $(M+1)^+$ at m/z 337 readily reveals the molecular weight for this molecule. In addition, weak fragment ions were observed at m/z 261,

245,205,186 and 150. The m/z 150 may be derived from intramolecular proton transfer to the protonated N-propionyl-N-phenyl moiety followed by cleavage of the N-piperdine bond. This fragment ion combined with the abundant $(M+1)^+$ allows specific SIM detection for trace levels of fentanyl.

The CI micro LC/MS mass spectrum for 3.5 ng of sulfamethazine shown in Fig. 14(c) was obtained using 40/60 CH_3CN/H_2O at a flow rate of 8 μL/min on a 0.5 mm i.d. X 5 cm C_{18} ODS PTFE micro column. This sulfa drug is not amenable to routine GC/MS analysis without suitable derivation. Mass spectral information is usually obtained for this sulfa drug by direct insertion probe (DIP) methods for samples obtained from preparative HPLC or TLC. EI ionization often provides little or no evidence for the molecular weight of this drug due to the instability of its molecular ion, which undergoes facile cleavage of the central sulfonamide bond. Figure 14(c) shows the quality of mass spectral data obtained from the CI micro LC/MS analysis of 3.5 ng of sulfamethazine. Since HPLC is probably the method of choice for chromatographic separation of sulfonamide drugs in biological samples, micro LC/MS is a natural candidate for the separation and confirmation of these materials.

The CI/isobutane mass spectrum shown in Fig. 15(a) was obtained from authentic promazine. ALthough it is well know that parent promazine may be analyzed by GC and GC/MS without derivatization, its several oxidized metabolites preclude assay by these methods due to their thermal instability or polarity. For example, one suspected promazine

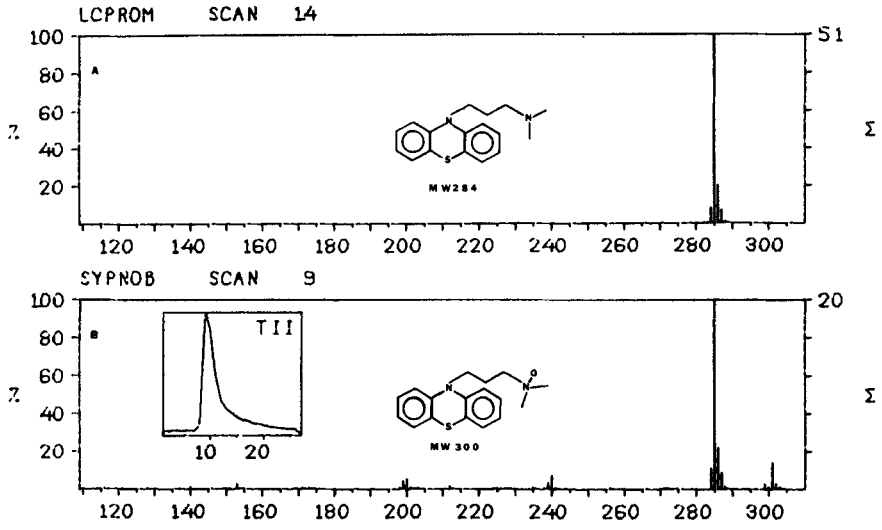


Figure 15. (a) CI/isobutane mass spectrum of promazine obtained via direct insertion probe sample introduction, and (b) micro LC/MS CI mass spectrum of the N-oxide equine urinary metabolite of administered promazine obtained using 50/50 CH_3CN/H_2O as micro LC eluent/CI reagent gas.

metabolite found in equine urine (88) appeared to be an N-oxide according to its IR and TLC behavior. NMR data supported the tertiary acyclic N-oxide structure shown in Fig. 15(b). Since DIP analysis using CI/isobutane provided the abundant $(M+1)^+$ ion for parent promazine [Fig. 15(a)], it was hoped that verification of the molecular weight of the suspected promazine N-oxide metabolite could be made in an analogous manner. However, only weak ion abundances for the molecular weight of the N-oxide metabolite could be obtained using conventional CI and EI techniques with large sample quantities. This was presumed due to the well-known expulsion of the oxygen atom from N-oxides under thermal

(89) and ionizing conditions (90). The mass spectral studies of the proposed N-oxide promazine metabolite at trace levels using conventional DIP CI methods consistently produced a mass spectrum identical to that of parent promazine [Fig. 15(a)].

The CI micro LC/MS mass spectrum of the promazine metabolite is shown in Fig. 15(b). This spectrum reveals the abundant m/z 285 ion that was observed in the DIP experiments, but there is also an m/z 301 ion of 18% relative abundance. This ion was the most definitive mass spectral support for the molecular weight of 300 for the proposed N-oxide metabolite. The m/z 240 ion may be derived form loss of the protonated tertiary N-oxide moiety, but the m/z 200 is of unknown origin.

The presence of the m/z 301 $(M+1)^+$ ion observed in the CI micro LC/MS mass spectrum, Fig. 15(b), suggests a relatively mild ionization environment under these DLI LC/MS conditions. It is believed that the desolvation of the solute molecule is not entirely complete and that the ionized solute molecules do not experience the usual thermal severity ($250^{o}C$) of the ion source chamber. This is a general experimental phenomenon and has been reported by others.

A recent report (91) demonstrated a method of analysis for sulfadimethoxine by SIM GC/MS after suitable derivatization. The authors' preliminary experiments analyzing sulfadimethoxine by micro LC/MS suggest that this method could preclude the necessity of successive derivatizations of the polar sulfonamide drugs. The mass spectrum shown in Fig.

9(a) was obtained from the micro LC/MS analysis of 3.5 ng of sulfadimethoxine using 60:40 CH_3CN/H_2O at 8 μ L/min on an SC-01 column. The abundant fragment ion at m/z 156 probably results from cleavage of the sulfonamide bond following protonation of the gaseous molecule. THe weak fragment ion at m/z 246 may result from the rather interesting expulsion of SO_2 plus hydrogen atom following initial protonation. This same loss occurs in the conventional CI mass spectrum of sulfadimethoxine (91) and other sulfa drugs.

During the course of these micro LC/MS studies of sulfadimethoxine the presence of an apparent $(M+15)^+$ ion was observed at m/z 325 using 60:40 CH_3CN/h_2O as the micro LC eluent and CI reagent gas. Similar phenomena have been observed using conventional LC/MS. Since one possible source of the apparent "methylation" is the acetonitrile molecule, the micro LC eluent was changed to 60:40 CD_3CN/D_2O. After equilibrating the SC-01 micro LC column with 60/40 CD_3CN/D_2O eluent, 10 ng of sulfadiemthoxine was injected on-column, and the micro LC/MS mass spectrum shown in Fig. 16(b) was obtained. The most abundant high-mass ion was m/z 315. If a simple "deuteration" has occurred in this experiment, analogous to the protonation which produced the $(M+1)^+$ ion at m/z 311 in Fig. 16(a), a m/z 312 ion would be expected in Fig. 9(b). The m/z 315 observed probably results from an initial rapid exchange of the three acidic hydrogens in sulfadimethoxine with deuterium atoms. This would produce sulfadimethoxine d_3, with a molecular weight of 313. When the CI conditions present in the mass

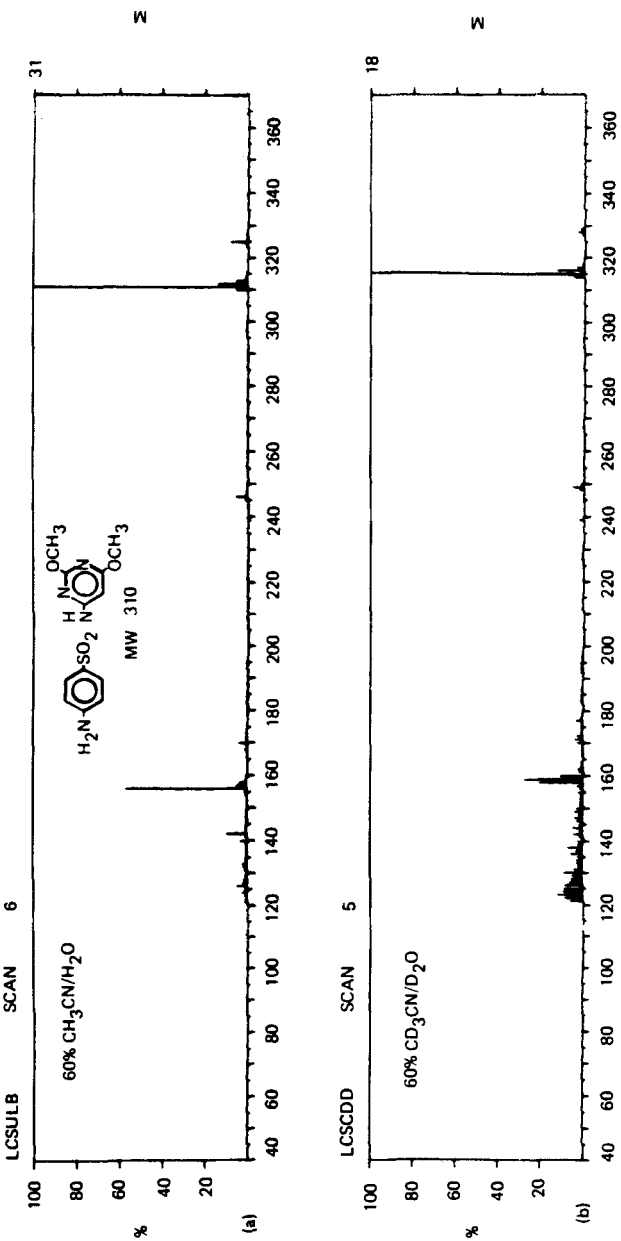


Figure 16. Micro LC/MS CI mass spectra for sulfadimethoxine using (a) 60/40 CH_3CN/H_2O as micro LC eluent reagent gas, and (b) 60/40 CD_3CN/D_2O as micro LC eluent/CI reagent gas. The SC-01 column was equilibrated with 60/40 CD_3CN/D_2O for 1 h prior to obtaining the data in (b).

spectrometer ion source transfer a deuterium ion to this molecule via an ion-molecule reaction, the resulting product ion would be the m/z 315 observed. Thus micro LC/MS conditions as described here allow the rapid determination exchangeable hydrogen atoms as has been described in previous alternative methods (92). It is interesting to note that the significant fragment ions m/z 156 and 246 observed in Fig. 15(a) are similarly moved three atomic mass units (m/z 159 and 249) and reflect the exchange of three hydrogens for three deuterium atoms.

The presence of the m/z 328 ion in Fig. 16(b) does not support the m/z 325 in Fig. 16(a) as being derived from the methylation of sulfadimethoxine by the methyl group of the acetonitrile eluent/reagent gas. An m/z 331 ion would have been expected from the micro LC/MS experiment using 60/40 CD_3CN/D_2O. This would have resulted from the addition of CD_3^+ to the deuterium-exchanged parent drug (MW 313).

The origin of the m/z 325 and 328 ions in Fig. 16(a) and (b), respectively, appears to be derived from the simple protonation of a small amount of methylated sulfadimethoxine impurity in the sample. Subsequent micro LC/MS experiments verify that parent and methylated sulfadimethoxine do not separate under the micro LC conditions used. The methylated sulfadimethoxine molecule would produce the m/z 325 ion observed in Fig. 16(a) by simple protonation. Under the micro LC/MS conditions of 60/40 CD_3CN/D_2O, a rapid initial exchange by deuterium would produce d_2 methylsulfadimethoxime (MW 326). This would yield an m/z 328 ion af-

ter accepting a deuterium ion in the subsequent ion-molecule reaction. This has been demonstrated by the micro LC/MS analysis of pure sulfadimethoxine and its pure methylated derivative prepared by treatment with ethereal diazomethane (not shown here). Thus, not only is micro LC/MS amenable to the analysis of low nanogram amounts of sulfadimethoxine but it lends itself to detecting and confirming trace levels impurities.

Diaphram Split Effluent DLI LC/MS

Although the glass capillary LC/MS probe interface described previously provided useful results, routine LC/MS operation was sometimes jeopardized by unexpected obstruction of the probe orifice and pre-evaporation of solvent exiting the probe tip. The removable metal diaphragm and cooled probe tip reported by Serum and Melera (64) offers a solution to both of these problems. A plugged diaphragm orifice can be readily opened by sonication, while water cooling of the probe tip eliminates any solvent evaporation problems. In addition, a cryogenic trap was constructed around the MS ion source to significantly increase pumping speed of the system (64).

We have analyzed a wide variety of drugs and toxic substances by LC/MS using the diaphragm DLI probe. In the split effluent mode, however, the LC/MS sensitivity is still not comparable to modern GC/MS capability. Figure 17 shows the TICP for successive injections of 5,1,0.5, and 0.2 μg of dexamethasone using 50% CH_3CN/H_2O at 1 mL/min

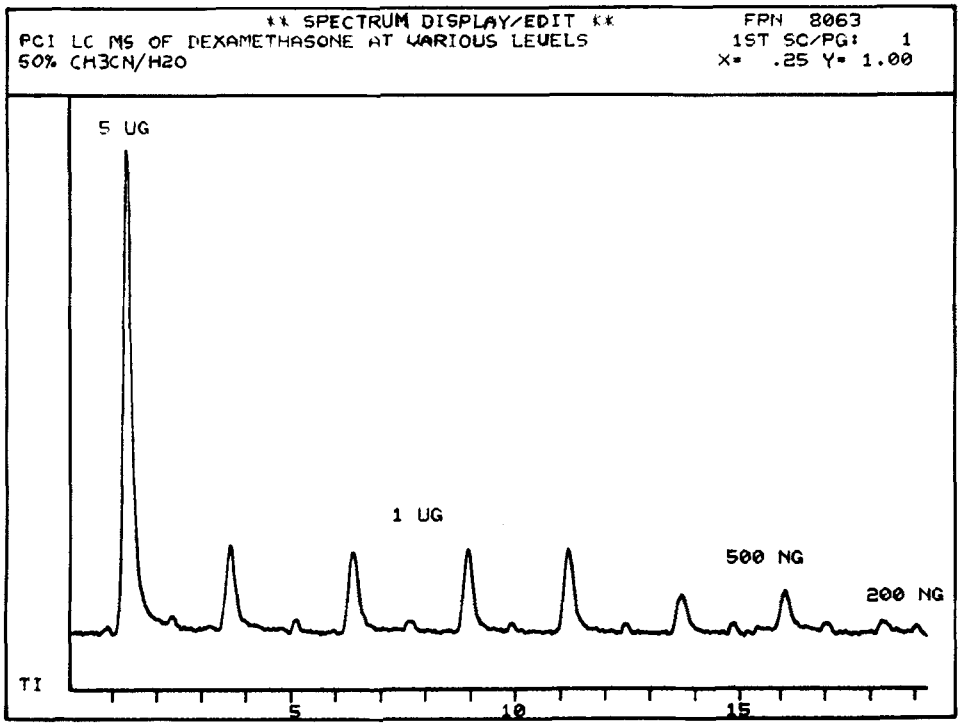


Figure 17. Diaphragm DLI LC/MS split effluent TICP for successive injections of dexamethazone at the 5-µg g, 1-µg, 500-ng, and 200-ng levels. These data were from full-scan (m/z 120-500) analysis of a 99/1 split and reveal the stability, reproducibility, and practical detection limit of this technique.

flow. Clearly the system produced a strong signal when 5 g or 1 µg of this steroid is injected onto the LC column and 1% of the total effluent enters the MS. The 0.2-µg injection of dexamethasone represents the practical detection limit of the system under these conditions of operating the MS in the full-scan mode (m/z 100-500). The reproducibility and stability of the ion current, however, is quite acceptable and should lend itself to quantitation studies.

Less volatile and/or polar molecules offer challenging problems and opportunities to LC/MS. Trichlormethiazide (TCM) and reserpine are particularly difficult to assay even by direct insertion probe MS methods. They have not as yet yielded satisfactory LC/MS results by capillary probe LC/MS, presumably due to pre-condensation in the glass capillary probe tip. The new DLI LC/MS interface easily provides useful positive ion CI and negative ion CI mass spectra of these molecules. Ion current profiles for the LC/MS analysis of a mixture of 3.0 μg each of TCM and reserpine under negative ion CI conditions are shown in Fig. 18.

When the LC was connected to the CI MS, 0.5- to 3-μg injections of reserpine yielded strong, stable TICP and EICP Chromatograms in the positive ion CI mode (PCI). One obtains an abundant $(M+1)^+$ ion at m/z 609 with the base peak at m/z 213 [Fig. 19(a)]. In the negative ion CI mode (NCI) only a weak $(M-1)^-$ m/z 607 was observed, with m/z 211 present as the base peak [Fig. 19(b)]. The sensitivity in the PCI mode for reserpine was nearly 10 times greater than in the NCI mode.

The NCI LC/MS spectrum for TCM, however, revealed considerably greater sensitivity than did the PCI results (Fig. 20). The NCI mass spectrum of TCM displayed an m/z 343 $(M-36)^-$ ion with several diagnostically useful fragment ions. In contrast, the sensitivity of a PCI mass spectrum for TCM was 100 times less.

The minimum detectable limit (MDL) obtained for TCM by NCI LC/MS was 100 ng injected onto the column. The MDL

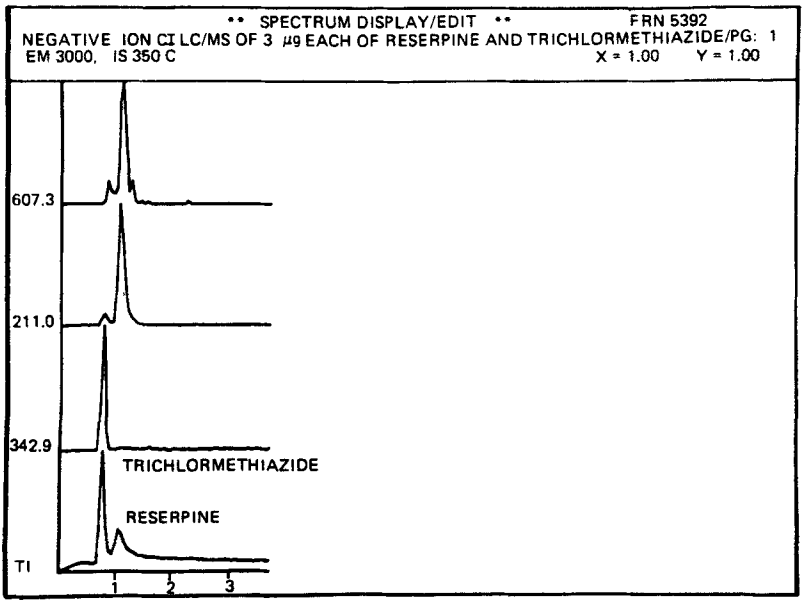


Figure 18. TICP and EICPs from the LC/MS analysis of 3 µg each of trichlormethiazide and reserpine using a conventional LC and the Hewlett-Packard DLI interface. LC eluent was 1 mL/min 95/5 CH_3CN/H_2O using a 2 mm i.d. X 5 cm C_{16} ODS column.

for parent reserpine was 500 ng in the PCI LC/MS mode. These data were full-scan mass spectra beginning at m/z 120 and could easily be improved by as much as 100 in the selected ion monitoring mode.

The LC separation and continuous LC/MS monitoring of a mixture of five parent acid drugs is shown in Fig. 21. The UV trace in Fig. 21(a) shows the separation of sulfamethazine, indomethacin, furosemide, phenylbutazone, and oxyphenbutazone using a linear gradient of 40/60-60/40 CH_3CN/1% acetic at a flow rate of 1 mL/min. Figure 14(b) shows the UV trace obtained from the LC/MS run in the NCI mode

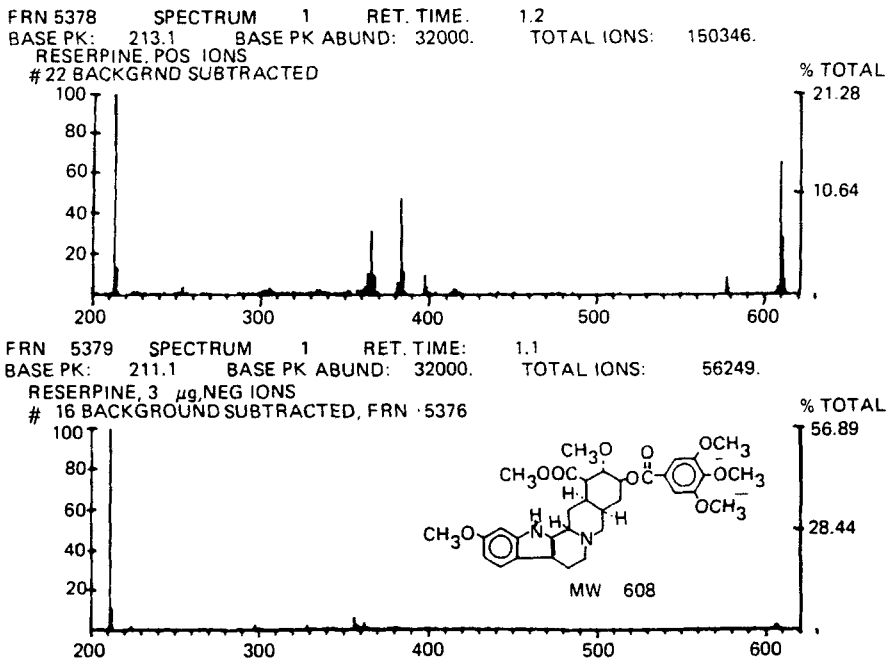


Figure 19. Positive ion CI (upper) and negative ion CI (lower) LC/MS mass spectra of 3.0 µg reserpine (MW 608).

using the diaphragm DLI interface. Although the TICP trace indicates some tailing of one component into the subsequent peak, when an abundant ion in the mass spectrum of each drug is extracted out from the TICP, the chromatographic peak shapes become quite acceptable.

The NCI mass spectra from the respective chromatographic components in Fig. 21 are shown in Fig. 22(a)-(e). These data indicate significant structural information from the fragmentation patterns and in most cases offer evidence of the molecular weight of the drug by the presence of $(M-1)^-$ ions. These data were obtained from 1- to 5-µg lev-

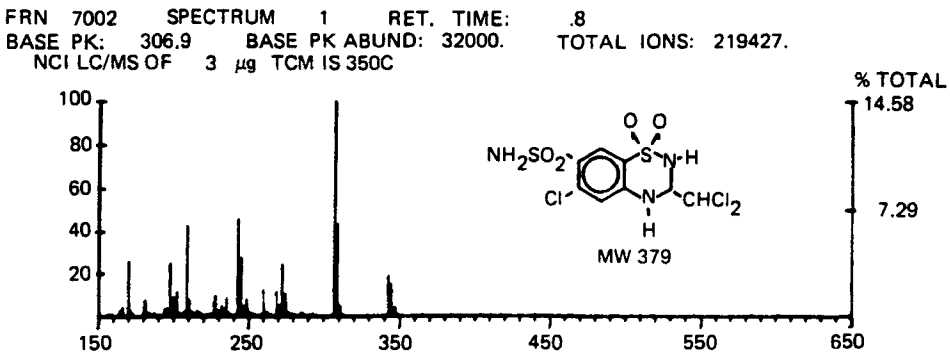


Figure 20. NCI LC/MS mass spectrum for a 3 µg trichlormethiazide using 95/5 CH_3CN/H_2O at a flow rate of 1 mL/min on a 2 mm i.d. X 5 cm C_{18} ODS column.

els of the drugs in this mixture and are representative of the quality and extent of specific information available from such experiments.

Although steroids have been analyzed by GC and GC/MS, the formation of suitable derivatives is often necessary. HPLC analysis of steroids in complex mixtures offers an alternative means of separating steroids from endogenous materials without the need to form derivatives. LC/MS lends itself nicely to the determination of steroids and their metabolites in biological samples.

Figure 23 shows the TICP and EICP data from a NCI LC/MS experiment involving the injection of several steroids including dexamethasone and its major metabolite found in the horse. It can be seen from Fig. 23 that the steroids provide acceptable ion current signals in this LC/MS experiment, which utilizes 50% CH_3CN/H_2O at a flow rate of 1 mL/min.

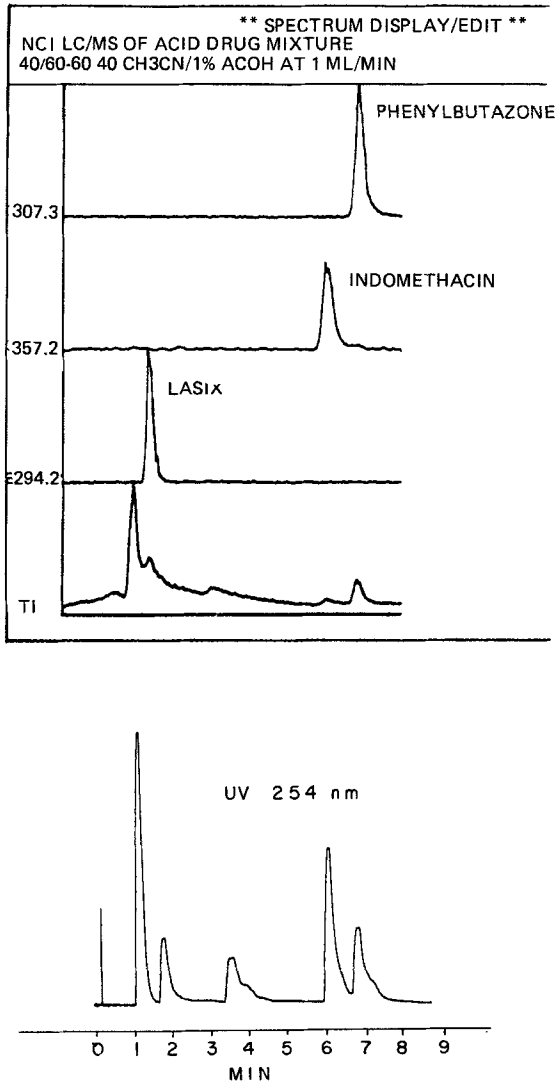


Figure 21. NCI LC/MS TICP and EICP (a) and UV chromatogram (b) for a mixture of five parent drugs, in elution order: sulfamethazine, furosemide, oxyphenbutazone, indomethacin, and phenylbutazone. The LC conditions were linear 10-min gradient from 40/60 - 60/40 CH_3CN/1% ACOH at 1 mL/min on a 2 mm i.d. X C 5 cm C_{18} ODS column.

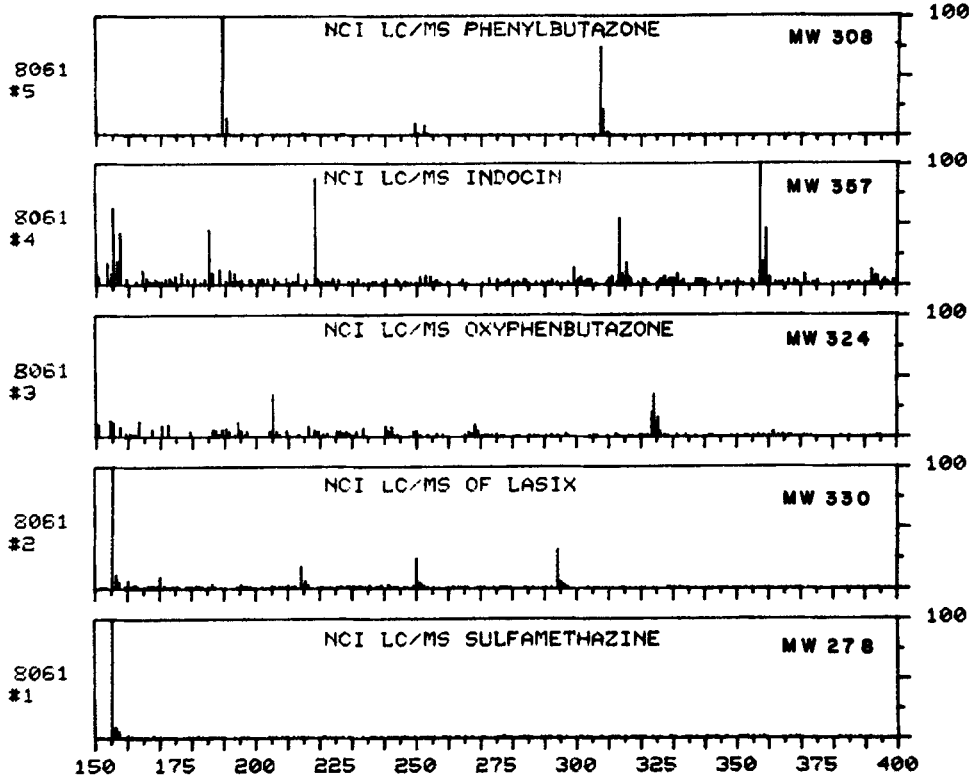


Figure 22. NCI LC/MS mass spectra of the five parent drugs shown in Fig. 21.

The m/z 364 EICP component at a retention time of 7.1 min represents the M^- ion for the dexamethasone metabolite isolated from equine urine. The molecular weight of this substance was determined by LC/MS and the structure (I; Fig. 24) was corroborated by its NMR and IR spectra. Its PCI/NCI LC/MS mass spectra are consistent with structure I and actually are diagnostic for the D-ring cyclopentanone. A comparison of NCI LC/MS mass spectra of related steroidal

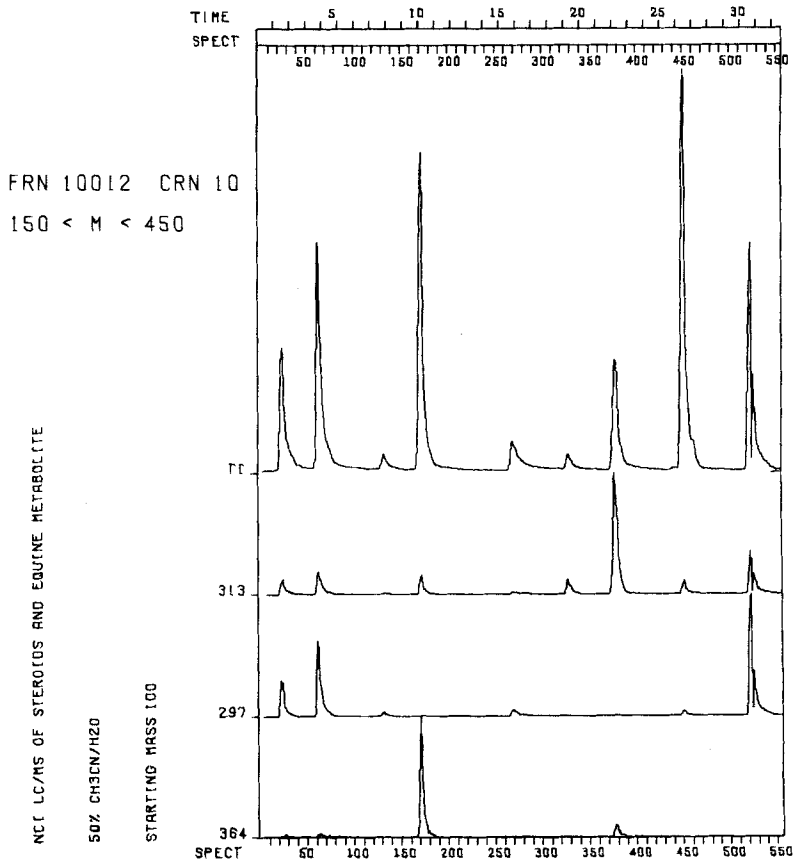
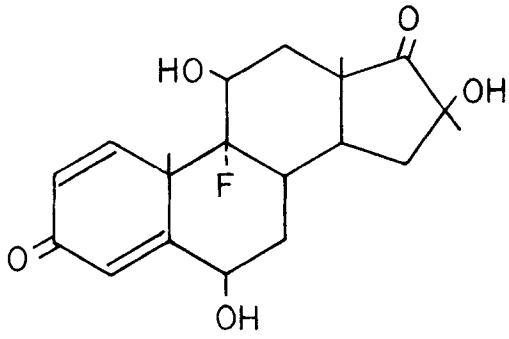


Figure 23. NCI LC/MS TICP and EICPs for several corticosteroids including dexamethasone and its major equine metabolite (peak 4). These data were obtained using 50/50 CH_3CN/H_2O at a flow rate of 1 mL/min on a 2 mm i.d. X 5 cm C_{18} ODS column.

ketones indicates a fragmentation behavior that appears specific for this structural feature.

LC/MS/MS

Although DLI LC/MS often provides the desired molecular weight information from labile compounds, it frequent-



I

Figure 24. Structure I is the major equine urinary metabolite of dexamethasone.

ly offers only limited structural information. This results because the chemical ionization conditions are very mild and hence little excess energy is transferred to the ionized molecule. Unfortunately the analyst needs some fragmentation information to characterize or corroborate the structure of the analyte.

An exciting new analytical technique is now available which appears to offer the unique combination of abundant molecular weight information plus the necessary fragmentation for structural characterization. This technique is tandem mass spectrometry or mass spectrometry/mass spectrometry (MS/MS). Typically MS/MS analysis of samples is conducted under mild ionization conditions such as chemical ionization (CI) or atmospheric pressure ionization (API) conditions. This usually provides just one abundant ion for each chemical component of a mixture which is indica-

tive of the components' molecular weights. The tandem mass spectrometer can focus the parent ion of a specific chemical component into an intermediate collision region where the parent ion collides with a neutral gas molecule. The result of this collision process is fragmentation of the parent ion into a family of "daughter ions" which are characteristic of the parent ion's structure. The daughter ions resulting from this "collision induced dissociation" (CID) are separated by the third stage mass analyser of the tandem mass spectrometer to produce a daughter ion mass spectrum for the parent ion of a particular chemical component (93).

If the parent ion described above results from on-line LC/MS introduction of sample into the mass spectrometer the daughter ion mass spectrum produced is generated by a technique referred to as LC/MS/MS. We have evaluated the potential of this technique for the identification of sulfa drugs in equine plasma and urine (94). The acid extracts of urine and plasma when sulfa drugs are isolated from the biological samples by liquid-liquid extraction are chemically very complex. Thus the "mixture analysis" capability of MS/MS should preclude exhaustive sample clean up. LC/MS/MS should provide identification of sulfadrugs from an LC chromatogram which contains unresolved interfering substances. In addition, a study was made to determine the extent of additional structural information available from LC/MS/MS over those results obtained from LC/MS. The interface used in this work was the Hewlett-Packard split ef-

fluent diaphragm probe device described above, and the MS/MS was a Sciex TAGA 6000 API instrument (95).

The LC/MS mass spectrum of sulfamethazine SM has been described previously (69). The abundant $(M+1)^+$ ion at m/z 279 readily reveals the molecular weight of this sulfa drug but lacks any real specificity because there are so few fragments ions. It is this dearth of PCI LC/MS fragmentation that prompts our interest in LC/MS/MS.
The collisionally induced dissociation (CID) mass spectrum of SM shown in Figure 25 was produced by focusing the $(M+1)^+$ ion, m/z 279, in Q_1, performing CID with nitrogen gas on this ion in Q_2, and separating the resulting daughters with Q_3. This CID or LC/MS/MS mass spectrum provides significantly more structural information than the coventional PCI LC/MS mass spectrum. In addition, the triple quadrupole allows one to observe fragment ions appearing throughout the low mass portion of the mass spectrum which is not possible in DLI LC/MS due to the presence of abundant ion molecule products resulting from the LC/MS reactant gas.

A comparison of LC/MS extracted ion current profiles (EICP) with the UV trace obtained from the LC separation of three standard sulfa drugs is shown in Figure 26. The UV trace in Figure 26A was obtained from the separation of 10μg each of sulfisoxazole (SOX), sulfadiazine (SD), and sulfadimethoxine (SDM) on a Whatman PXS 10/25 ODS column using a linear gradient of 10/90 CH_3CN/H_2O to 90/10 CH_3CN/H_2O over a 10-min interval at a flow rate of 1 mL/ min. Figure 26B

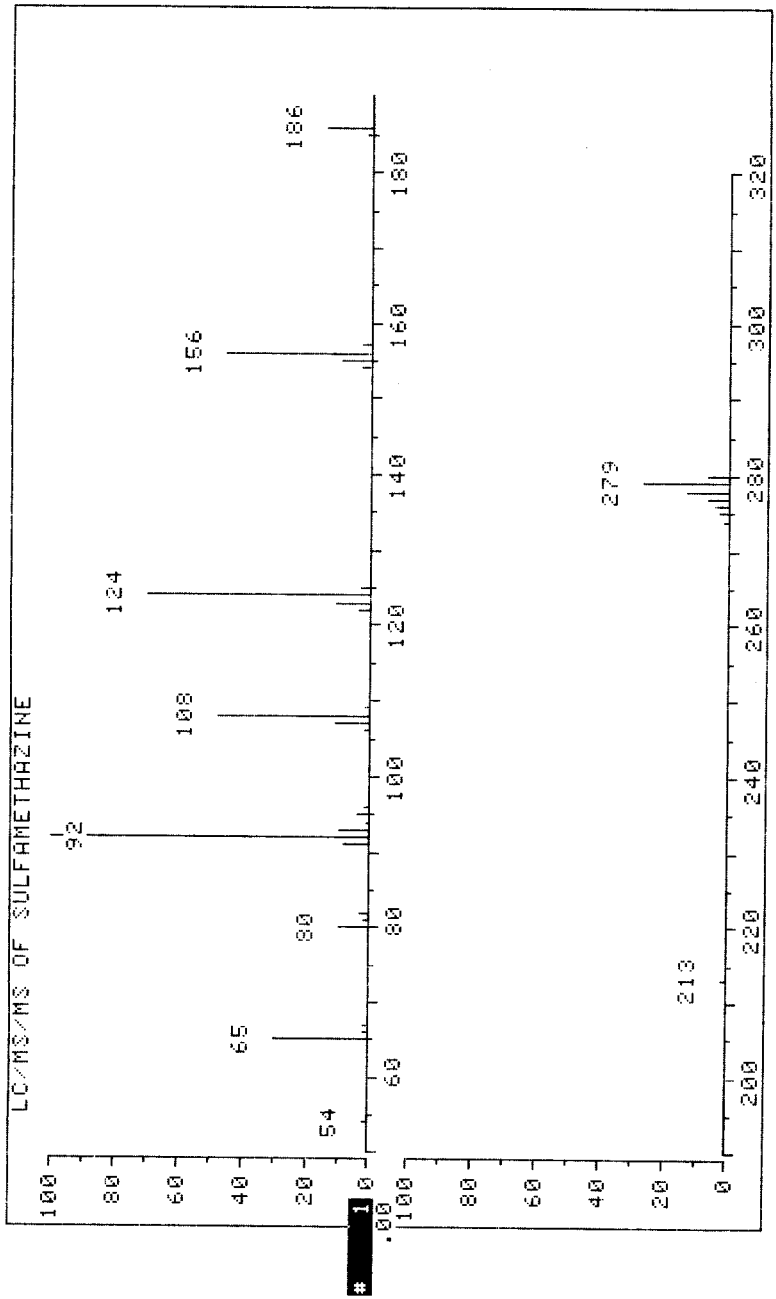


Figure 25. API LC/MS/MS mass spectrum of sulfamethazine $(M+1)^+$, m/z 279 ion. The sample was admitted via the probe using 50% CH_3CN/H_2O as CI reactant gas.

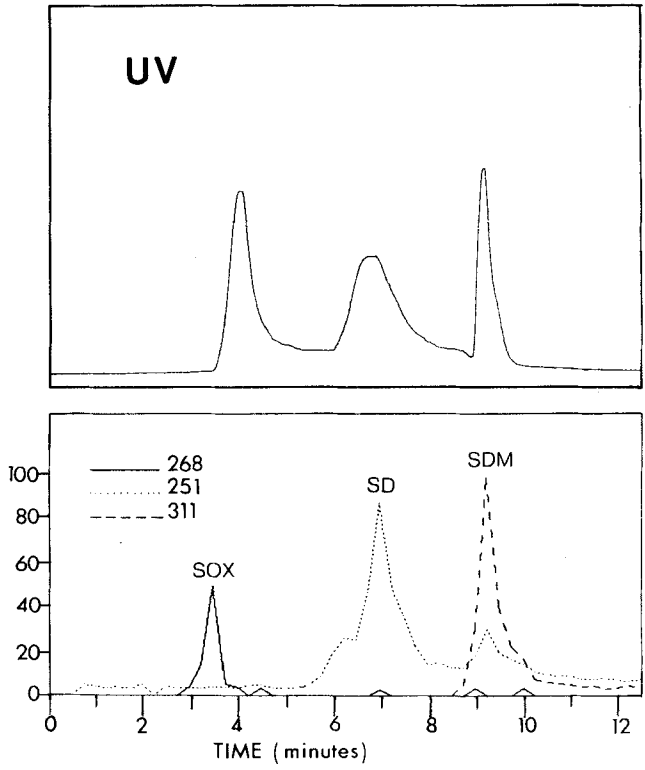


Figure 26. (a) UV liquid chromatogram at 254 nm of a standard mixture of 10μ g levels of sulfisoxazole sulfadiazine, and sulfadimethoxine using a linear gradient of 10/90 to 90/10 CH_3CN/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PCS ODS column. (b) extracted ion current profiles for selected sulfa drug ions under LC/MS conditions in the API mode under the above LC conditions.

is the EICP obtained from the DLI LC/MS analysis of the above mentioned standard mixture. This was obtained by continuously monitoring the LC effluent from the UV detector through the DLI LC/MS probe interface. The total LC effluent was split by the LC/MS interface such that approximately 10% or 100μL/min of LC efflent was admitted to the API

source. As can be seen by Figure 26B the EICPs are well resolved and show little tailing compared to the UV trace for the same mixture in Figure 26A. In addition, the ion current signal is relatively smooth and stable. It should be noted that these data are conventional LC/MS results without collision gas and with Q_1 and Q_2 operated in the RF only mode.

The LC/MS mass spectral data for the SOX,SD, and SDM are shown in Figure 27. These mass spectra reveal the simplicity often seen by such PCI LC/MS experiment. Usually one sees an abundant protonated molecular ion $(M+1)^+$ which readily reveals the molecular weight with little fragmentation resulting from the relatively mild CH_2CN/H_2O API ionizing reactant gas.

In contrast, the API LC/MS/MS mass spectra shown in Figure 28 show abundant fragment ions for each sulfa drug separated from the mixture shown in Figure 26A and a reduction of chemical noise at lower masses. The LC/MS/MS mass spectrum of sulfisoxazole shown in Figure 28A differs significantly from those of the more structurally similar SD and SDM shown in Figures 28B,C. In particular, m/z 92 is the base peak for SOX in Figure 28A with less abundant ions at m/z 113,156, and 108.

The UV trace and LC/MS/MS selected ion monitoring signals for pre-selected $(M+1)^+$ and m/z 156 ions for a standard mixture of SOX, SD, and SDM are shown in Figure 29A,B. These data were obtained from a mixture of approximately 6μ g levels of each sulfa drug injected on column with a

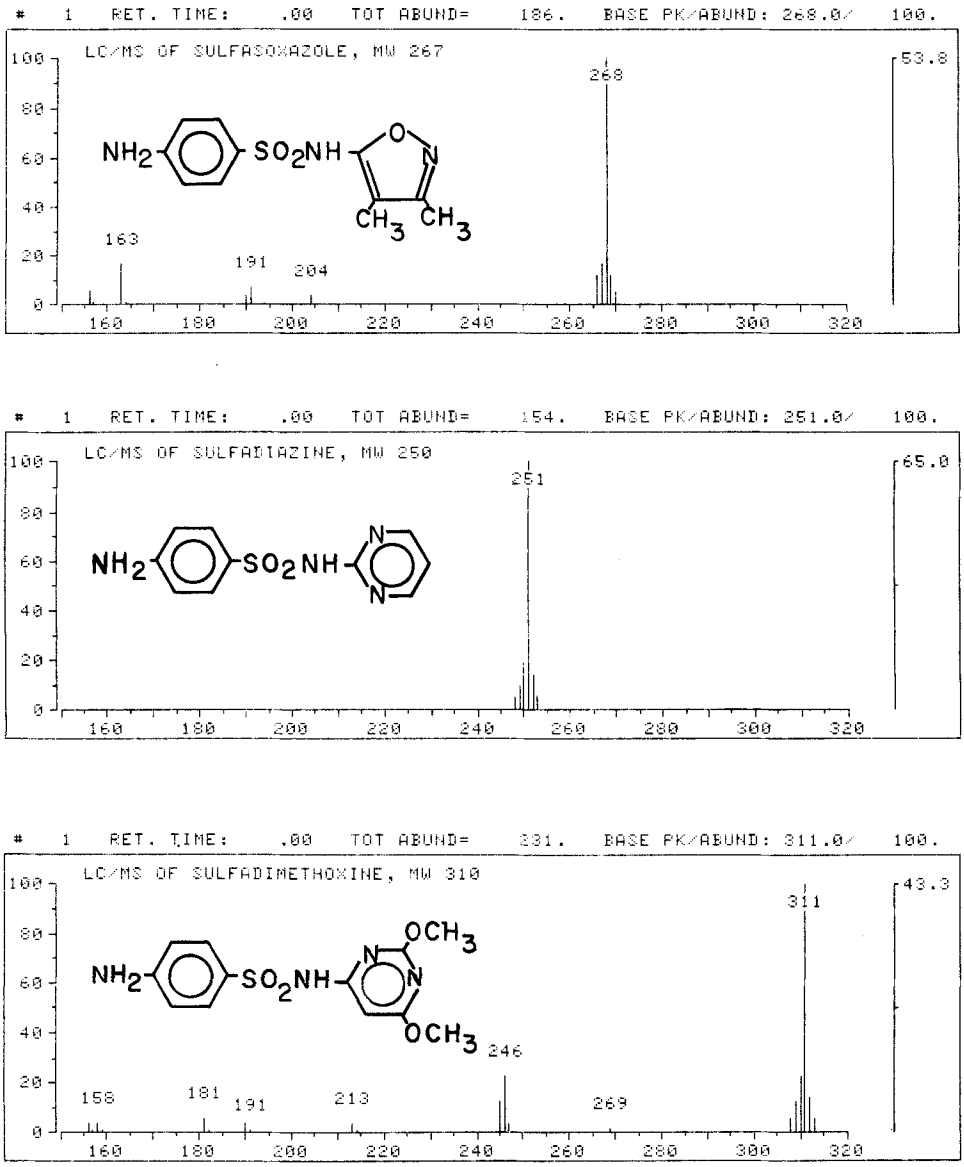
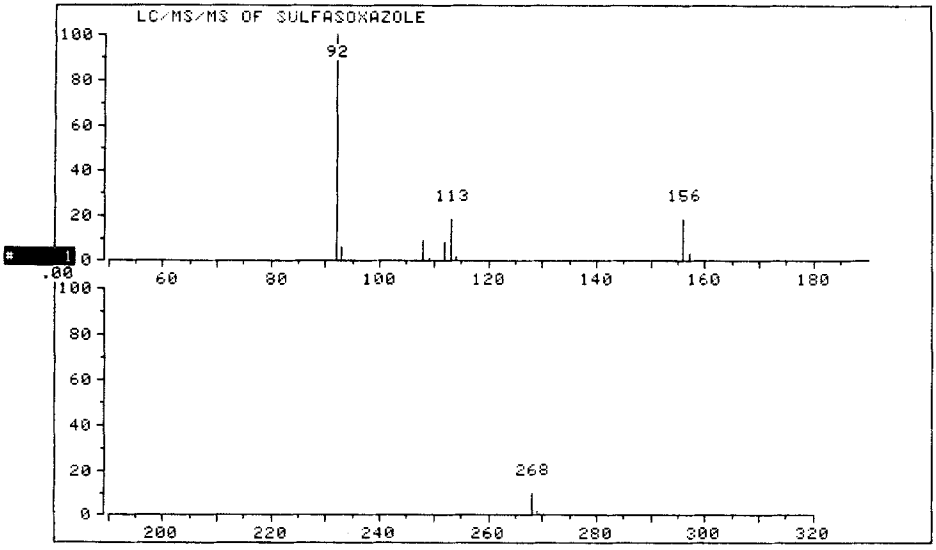
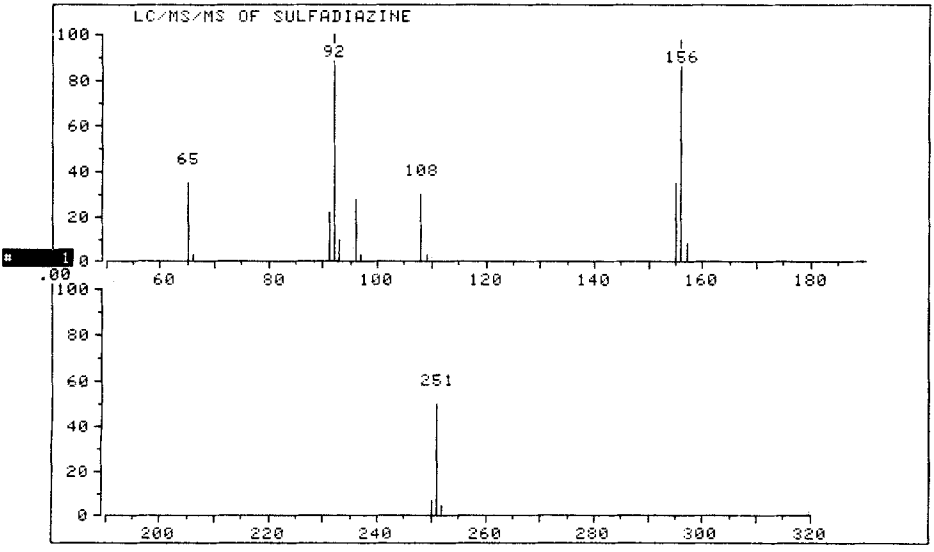


Figure 27. API LC/MS mass spectra for sulfisoxazole (a) sulfadiazine (b) and sulfadimethoxine (c) shown in Figure 26AB. The API reactant gas was aqueous acetonoitrile used as the LC eluent and the samples were admitted via the probe into the API source.





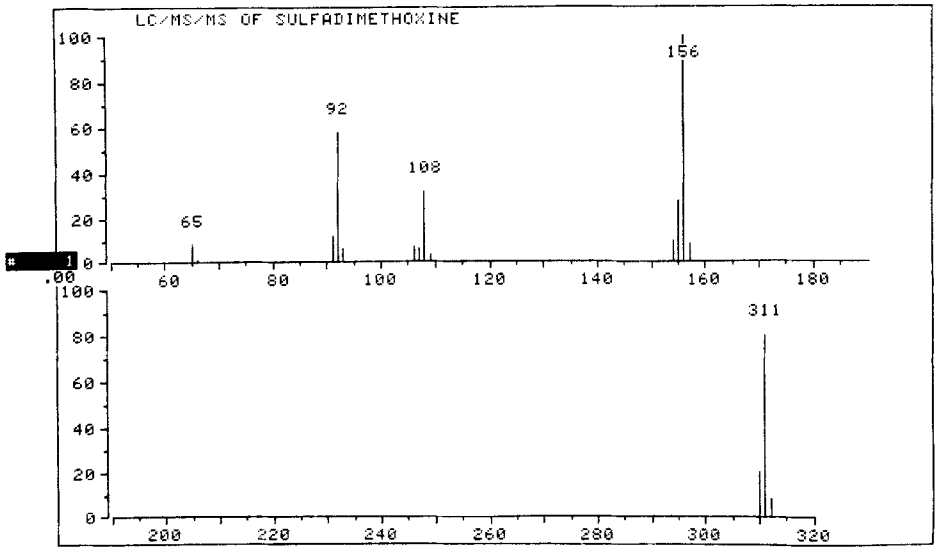


Figure 28. API LC/MS/MS mass spectra for the respective $(M+1)^+$ ions from sulfisoxazole (A), sulfadiazine (SDM) (B), and sulfadimethoxine (SM) (C) in the mixture shown in Figure 26 B.

10% split of the total LC effluent entering the API source. These data reveal the chromatographic integrity and ion current stability in the on-line LC/MS/MS mode, and the high degree of specific information offered by the presence of several fragment ions for each component.

The 254-nm UV trace shown in Figure 30A was obtained from a 10-μL injection of an organic extract of racehorse urine using the same LC conditions described for Figure 26A. The raw urine extract is very complex due to endognous materials from the horse and produced a high, broad UV absorbance signal of unresolved substances as can be seen from Figure 30A. Preliminary TLC screening methods had

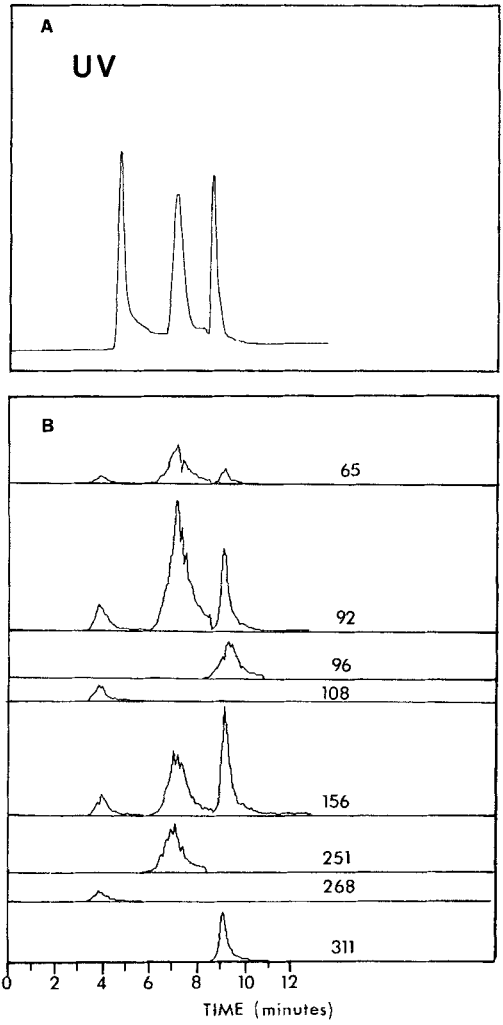


Figure 29. (A) UV liquid chromatogram at 254 nm of a standard mixture of 6 μg levels of sulfisoxazole (SOX), sulfadiazine (SD) and sulfadimethoxine (SDM) using a linear gradient of 10/90 to 90/10 CH_3CN/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PXS ODS column. (B), Extracted ion current profiles for selected sulfa drug ions under LC/MS/MS conditions in the API mode under the LC conditions described in Figure 26A.

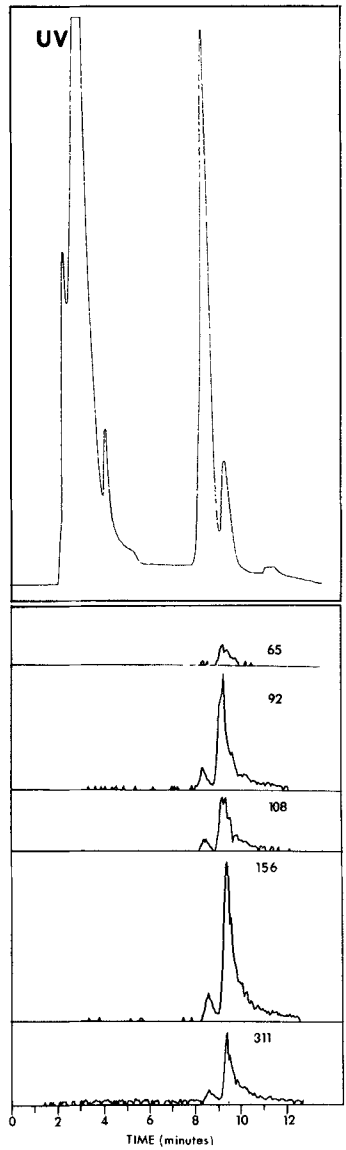


Figure 30. (A) UV liquid chromatogram at 254 nm of a hexane:dichloromethane:ether(1:1:1) extract of racehorse urine (pH 3.0) using a linear gradient of 10/90 to 90/10 CH_3CH/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PXS 2 ODS column. (B) API SIM LC/MS/MS traces for m/z 311,156 108,92 and 65 resulting from CID of the m/z 311 $(M+1)^+$ ion of sulfadimethoxine in the racehorse urine extract.

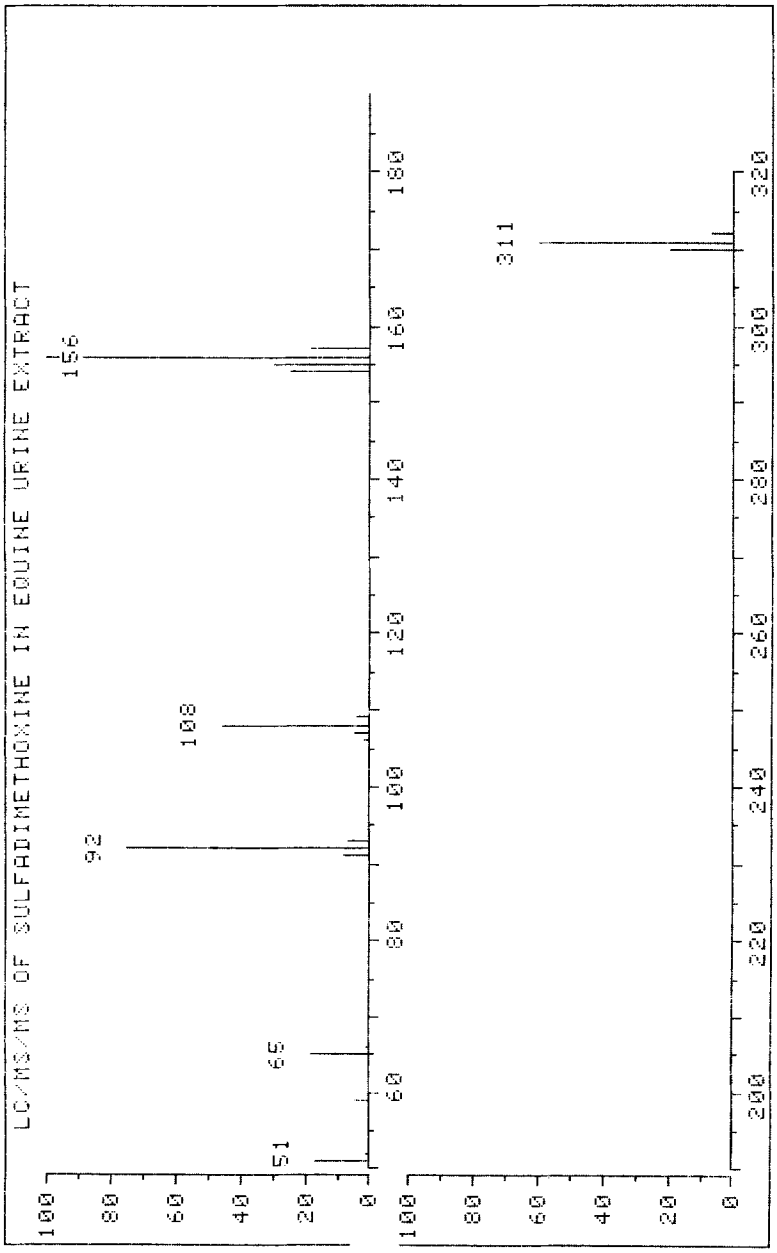


Figure 31. API LC/MS/MS mass spectrum for the 9.0 min retention time component shown in Figure 30A. This full scan LC/MS/MS mass spectrum agrees well with that of anthentic sulfadimethoxine obtained under identical conditions shown in Figure 28C.

suggested the presence of an unknown dose of sulfadimethoxine as a foreign substance in the racehorse urine. Figure 30B shows the SIM ion current traces for the LC/MS/MS analysis of the racehorse urine extract. Although the SDM dose to the horse is unknown, the levels observed were comparable to known 10 μg/mL urine levels. These data show the absence of any interfering substances in the region of the high UV absorbance with the exception of a small, resolved component eluting immediately before the SDM. A high level of an unknown component in the UV trace in this region appears to have several of the same ions as SDM, but its shorter retention time excludes this possibility. The full scan LC/MS/MS mass spectrum includes ions in the low mass portion of the spectrum that are not available from DLI LC/MS. It does not require LC resolution of SDM from co-eluting substances and is identical with that of the authentic SDM (see Figure 28C). The added benefit of both the UV and LC/MS/MS retention time compared to that of authentic SDM obtained under the same experimental conditions provides additional specificity for unequivocal identification of this sulfa drug in the racehorse urine.

Nebulizer LC/MS Interface

Recently Sciex (96) introduced a new LC/MS interface based upon conventional LC flow rates and a rapid flow of nitrogen gas which aids the nebulization of the heated total LC effluent in their API source. Their use of an API source allows facile handling of the high gas volumes produced from volatilized LC effluents.

The interface consists of a sprayer into which the entire eluent flow from the LC is directed, taking care to minimize any dead volume in the coupling. A high pressure gas (air or nitrogen at 80 psi) nebulizes the liquid into a heated spray chamber in which the small solvent droplets and entrained analyte are fully vaporized. The vapor (a mixture of solvent and analyte) flows from the spray chamber into the ion source through a heated quartz tube. A corona discharge ionizes the sample, and the ion molecule reactions (with the LC mobile phase as the CI reagent) generate MH^+ or $(M-H)^-$ ions from the compounds of interest. The ions are sampled into the MS/MS system through a gas curtain and small orifice, the gas curtain acting to prevent any sample (solvent, analyte or buffers) from entering the vacuum chamber.

The detection of drugs and metabolites in biological fluids at low levels is of interest to the biochemical, biomedical and pharmacological communities in applications ranging from kinetic metabolic studies to the detection of illegally administered drugs in racehorses. Many of the compounds of interest are involatile and thermally labile, and are present in blood and urine with much higher levels of other endogenous components. LC/MS/MS offers high specificity and positive identification even when the components cannot be completely separated on the column. Caffeine in human urine and betamethasone, hydroxybetamethasone (a metabolite) and butorphanol in equine urine, have all been successfully identified by LC/MS or LC/MS/MS. Figure 32 shows the full scan LC/MS data obtained from a urine

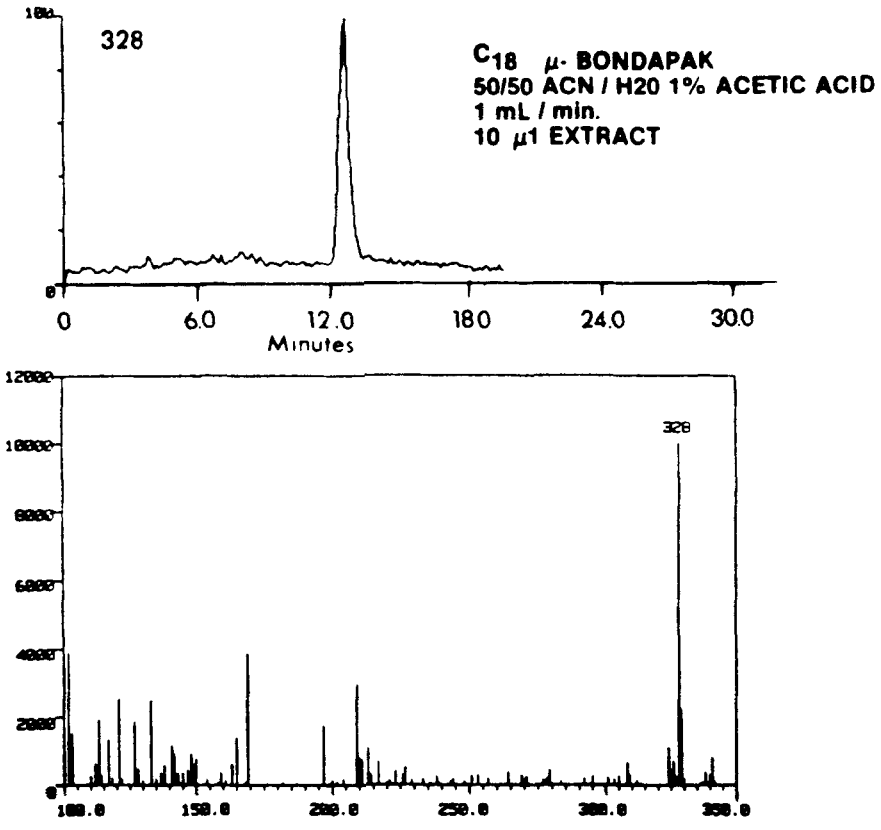


Figure 32. Full scan LC/MS of butorphanol in equine urine extract.

extract containing administered butorphanol. The full scan APCI mass spectrum is characterized by a dominant MH^+ ion at m/z 328, and this ion (along with others from co-eluting components) is readily apparent in the LC/MS spectrum for the urine extract. Unambiguous identification of butorphanol is provided by the LC/MS/MS spectrum from the extract shown in Figure 33, which matches the reference CID spectrum from the butorphanol standard. The amount of butorphanol in the

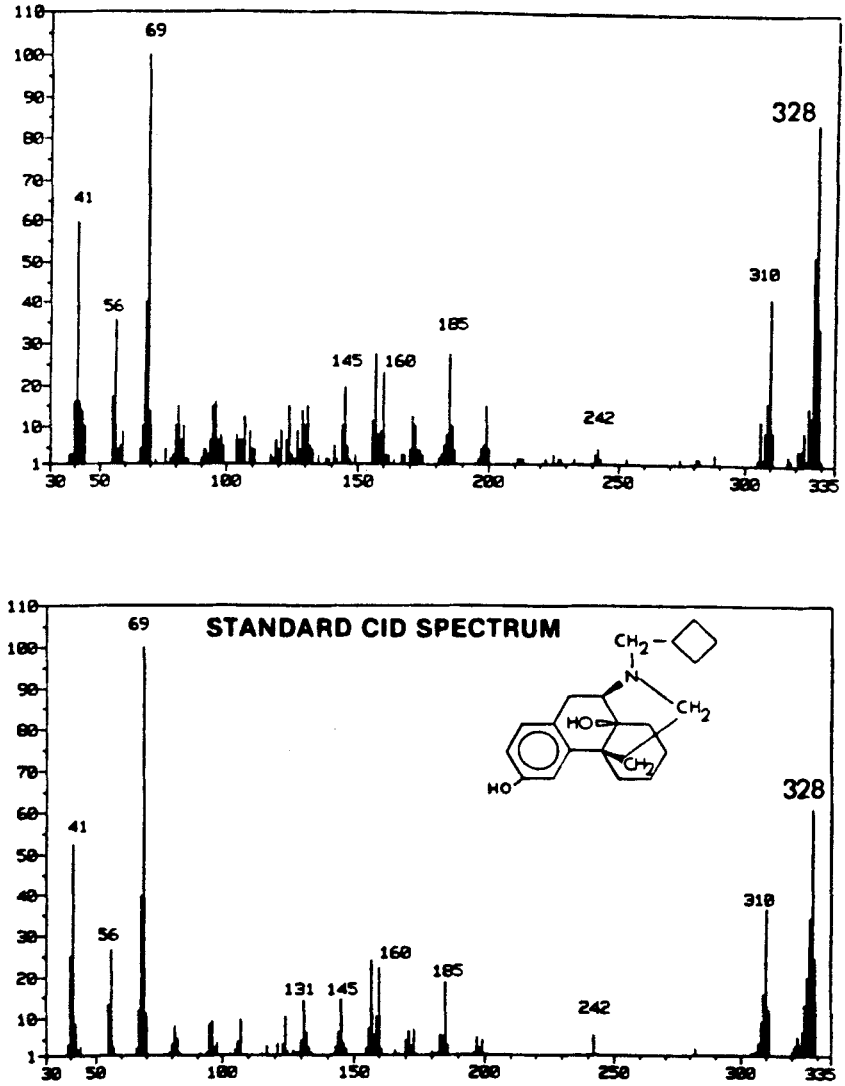


Figure 33. CID spectrum of m/z 328 from LC/MS/MS analysis of urine extract plus reference CID spectrum of butorphanol.

extract was estimated to be 2 ng per microliter, with 10 microliters injected on-column.

Diaphragm Total Effluent DLI Micro LC/MS

We have reported the construction and results from a modified DLI micro LC/MS interface patterned after the diaphragm DLI unit introduced by Hewlett-Packard Company (64). This device offers several advantages over existing commercial units, which include (1) increased routine sensitivity approaching that afforded by modern (GC/MS), (2) reduced construction and LC solvent costs, and (3) practical add-on to existing GC/MS instruments equipped with chemical ionization (CI) source. While micro LC/MS has been proposed (97) and reported (82,98), the present apparatus is the first to combine the simplicity of unchanged, commercially available LC pumps and hardware with total affluent introduction into the mass spectrometer.

Another report of metal column micro LC/MS described an adaption of the Hewlett-Packard DLI diaphragm interface to micro LC conditions (99). The simplified version of the commercially available split effluent interface is shown in Figure 34. The important salient feature of this new micro LC/MS probe is the narrowbore (0.004 in. i.d.) central throughput tube which transfers total effluent from the micro LC column to the CI mass spectrometer ion source of an unchanged commercially available quadrupole MS. The water-cooled probe tip features a removeable stainless

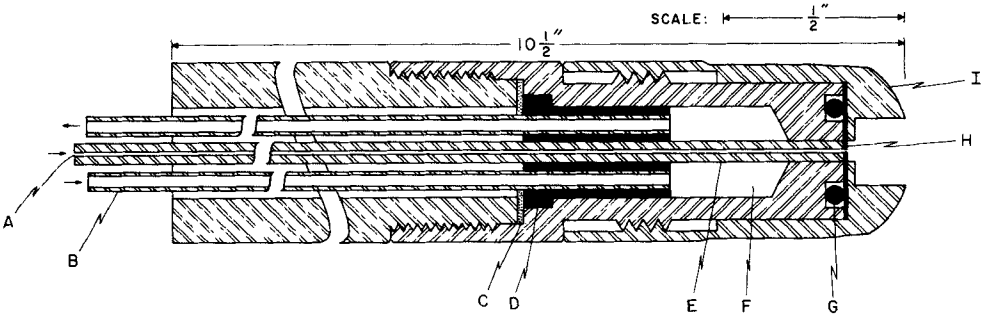


Figure 34. (A) micro LC effluent inlet line, (B) water cooling inlet tube. (C) Teflon washer for maintaining vacuum seal between probe tip/cooling chamber and probe shaft, (D) throughput tube collet, (E) 0.004 in. i.d. X 1/16 in. o.d. microbore tubing. (F) water cooling chamber, (G) Kalrez O-ring, (H) diaphragm containing 5 μ m pinhole in center, (I) removable endcap.

steel diaphram containing a precisely centered laser generated five micron pinhole. The device may be inserted into a standard one-half inch direct insertion inlet without any alteration of the MS system. This system does not suffer from the hazards of high acceleration voltage, long run times, difficulty with less volatile solutes, reported in earlier LC/MS work (100).

When a stable, short "jet" of micro LC effluent has been established through the diaphragm pinhole, the micro LC/MS probe interface may be inserted through the direct probe inlet to the cryogenically pumped CI source. The Hewlett-Packard 5985B quadrupole MS utilized in this work (70,71) operates through an eluent flow range from 10 - 60 L min^{-1} Optimum performance, however, occurs in the neighborhood of 40 l min^{-1} with any combination of aqueous meth-

anol or acetonitrile eluents. Volatile buffers such as ammonium hydroxide, trimethylamine, triethyamine, ammonium acetate, formic acid, acetic and trifluoroacetic acid offer no difficulty because they produce low molecular weight organic compounds that are readily pumped away by the MS vacuum system.

Figure 35 shows typical ion current chromatograms obtained from the DLI micro LC/MS diaphragm probe interface under negative ion chemical ionization (NCI) conditions. It should be noted that these data are acquired as full scan mass spectra (eg. m/z 80-500) and that the ion current stability and micro LC/MS sensitivity appear comparable to typical GC/MS data at these levels. The resolution of the components of dexamethasone and 6-β-hydroxyprednisolone was accomplished on a 50 cm C_{18} HRSM microbore column using a flow rate of 34μL min^{-1} 50% Ch_3CN/H_2O as micro LC/MS eluent CI reactant gas (35).

An application of micro LC/MS to actual problem solving is shown in Figure 36. The upper panel shows the micro LC UV trace from a TLC scrape of an unknown powder sample confiscated from a race track. The flow rate was 34 μL min^{-1} 50% CH_3CN/H_2O on a 50 cm C_{18} HRSM micro LC column and UV detection (15μ L flowcell, Perkin Elmer LC-55, Norwalk, CN) was 239 nm. In the lower panel of Figure 36 the corresponding micro LC/MS ion current traces for this unknown sample are shown. The major component observed at 2.8 min retention time had an NCI micro LC/MS mass spectrum identical with that of authentic dexamethasone. The minor compon-

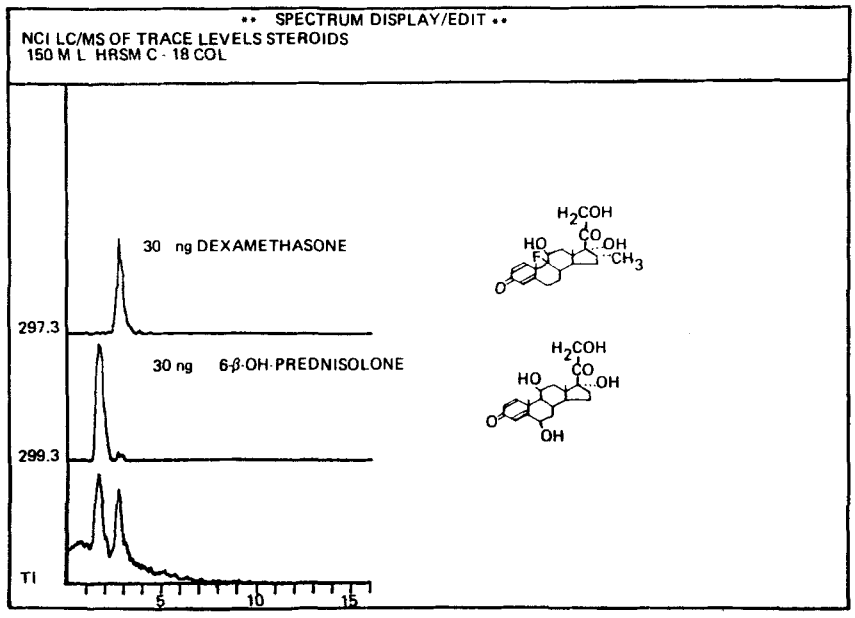


Figure 35. NCI micro LC/MS TICP and EICP for 30-ng levels of dexamethasone and 6-β-hydroxyprednisolone using 50% CH_3CN/H_2O at 34 μL/min as micro LC/MS eluent/CI reactant gas. The micro LC column was a C_{18} HRSM connected to an unchanged Waters ALC-202 pump and solvent programmer.

ent observed at 4.4 min retention time had an abundant m/z 127 ion and an apparent molecular weight of 366. Its identity is unknown and it had gone undetected by UV detector. These data demonstrate both the feasibility and versatility of micro LC/MS. The analysis times can be less than 20 minutes and sensitivity suitable for trace analysis is possible by micro LC/MS.

The DLI micro LC/MS diaphragm interface described above has been improved and additional applications reported (71). The construction of the interface was simplified

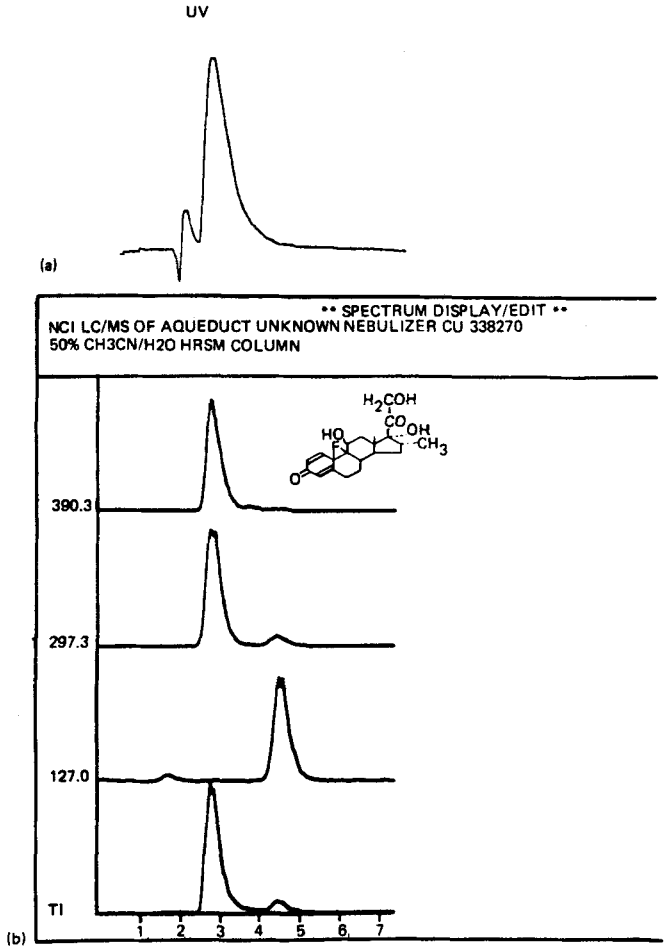


Figure 36 (A, upper) Micro LC UV trace for a TLC scrape of an unknown sample confiscated from a race track. The flow rate was 34 μ L/min 50% CH_3CN/H_2O on a C_{18} HRSM micro LC column and UV detection was 239 nm. (B, lower) NCI micro LC/MS TICP and EICP for the sample described in A. The major component observed at 2.8 min retention time was shown to be dexamethasone.

by replacing the two concentric narrowbore transfer tubes with one central throughput tube whose dimensions are 0.004 in i.d. x 1/16 in. o.d. Experimental details for accomplishing micro LC/MS were described in addition to specific in formation concerning modification of conventional Waters HPLC equipment for micro LC work. Examples of micro LC UV chromatograms for three thiazide diuretics and three corticosteroids were shown in addition to practical NCI micro LC/MS detection limits using the thermally labile, involatile compound, trichlormethiazide (TCM). The detection limit for this compound was 1.25 ng injected onto the micro LC column which provided an acceptable full scan NCI mass spectrum of this rather involatile molecule (71).

Figure 37 shows the NCI micro-LC/MS total ion current chromatograms of a TLC scrape from a zero-hour equine urine extract (lower) and an equine urine collected 2h post oral administration of TCM (upper). The micro LC/MS eluent/CI reactant gas was CH_3CN/H_2O (70/30 by vol) maintained at 40 L min^{-1} through a Chrompak 1 mm i.d. x 50 cm microbore column. Sample clean-up by preparative TLC greatly facilitates the analysis by precluding the introduction of high levels of endogenous compounds on the microbore LC column and shortens the micro LC/MS analysis time to less than 10 minutes. The determination of TCM by positive ion chemical ionization (PCI) is complicated by a 100-fold decrease in sensitivity for TCM, and interference by numerous other components that are not observed in the NCI data of Figure 37. Thus, the facility of selecting either PCI or

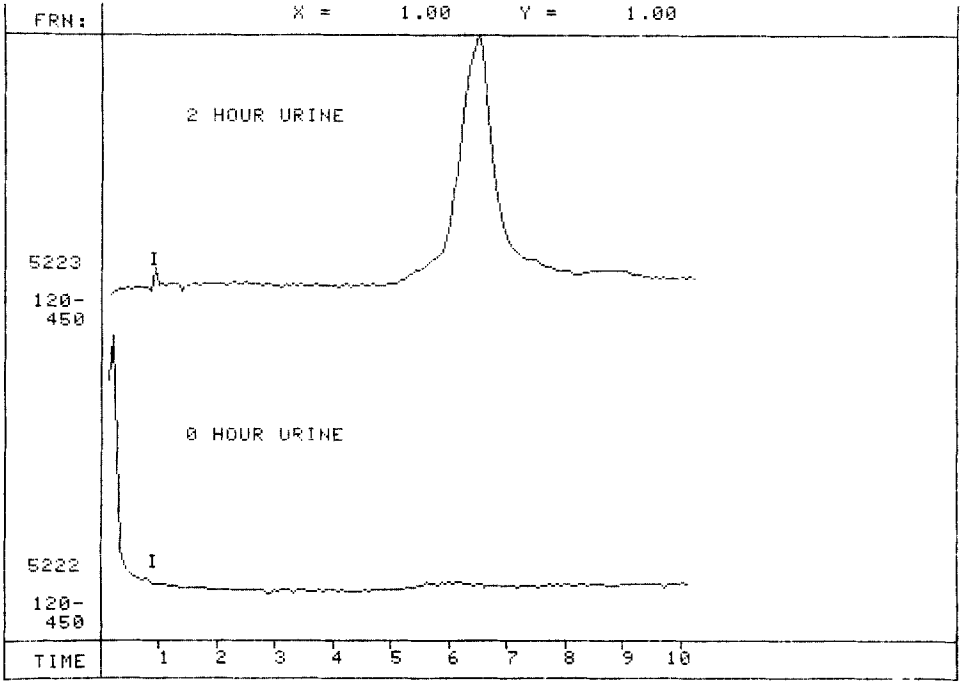


Figure 37. Negative-ion CI micro-LC/MS total ion current profiles of a TLC scrape from a zero-hour equine urine extract (lower), and an equine urine collected 2-h post oral administration of trichlormethiazide (upper).

NCI modes can improve the performance of micro LC/MS in certain instances. The above described method allows detection of TCM in racehorse urine through 24 hours post oral administration.

The application of micro LC/MS determination of betamethasone and its metabolites in equine urine, antibiotics in the crude extracts of a fermentation broth and impurities in a preparative HPLC sample obtained from a synthetic mixture of Felodipine have been reported (101). Figure 38, for example, shows both the UV chromatogram and the

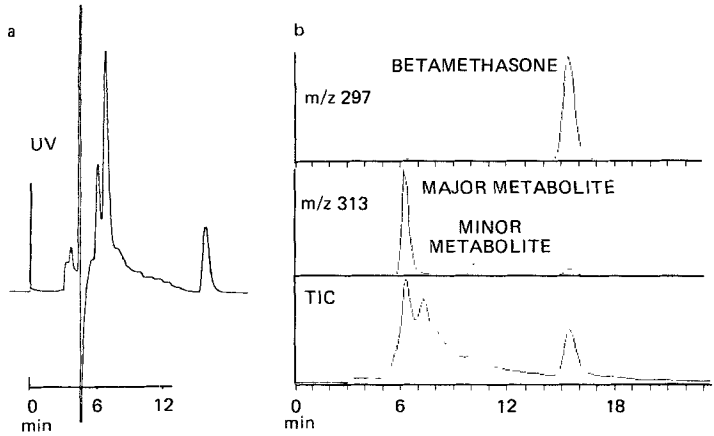


Figure 38. (a) micro LC UV chromatograms of a six hour post adminstration betamethasone equine urine extract utilizing 40 microliters per minute 70/30 methanol/water as eluent on an Alltech 1mm i.d. X 50 cm C_{18} reversed phase microbore column. (b) micro LC/MS ion current profiles of a six hour post administration betamethasone equine urine extract. Micro LC conditions as in a.

ion current chromatograms from the micro LC and micro LC/MS analysis respectively of an equine urine extract. The urine had been collected six hours after the administration of betamethasone. Both the major betamethasone metabolite and a previously undetected minor metabolite are observed in these data in addition to the parent corticosteroid, betamethasone, which is easily observed at a retention time of 15.5 min. Full scan NCI mass spectra are provided by these experiments. The micro LC conditions for these experiments were 40 L min^{-1} 60/40 CH_3OH/H_2O utilizing an Alltech 1 mm i.d. x 1/16 in
o.d. x 50 cm C_{18} reversed phase micro LC column.

Figure 39A shows the micro-LC UV chromatogram from a dichloromethane extract of crude fermentation broth. This

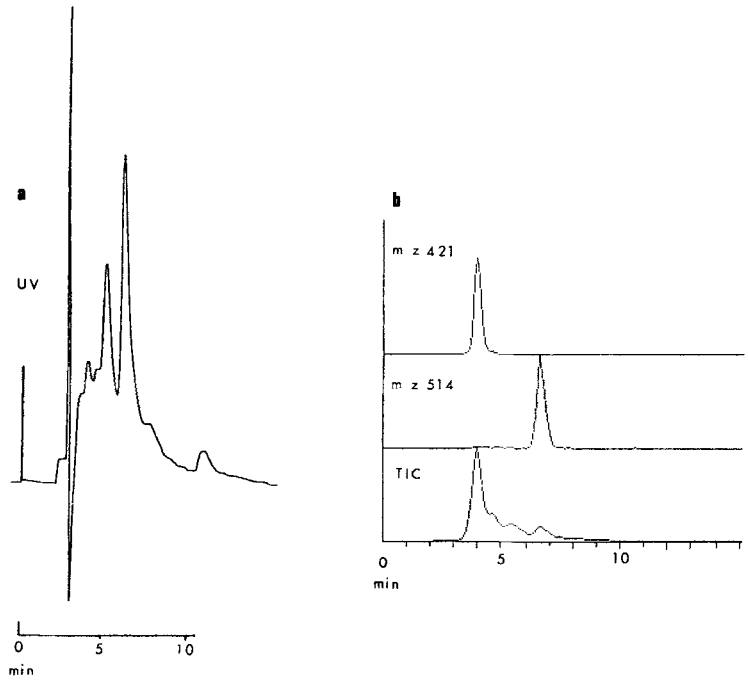


Figure 39. Micro LC UV chromatogram of a dichloromethane extract of a crude fermentation broth using aqueous methanol as eluent (b) micro LC/MS ion current profiles for antibiotics found in crude extract of fermentation broth.

chromatogram suggests the presence of many components, but only the full scan micro-LC/MS experiment shown in Figure 39B readily indicates the location for the components of interest. In particular, the $(M-1)^-$ ions for nonduscmicin and nargenicin at m/z 421 and 514 are readily "extracted" from the total ion current to locate their position in the micro-LC/MS chromatogram. Their negative ion chemical ionization (NCI) mass spectra allow them to be identified by comparison with authentic standards. A third anti-

biotic, 18-deoxynargenicin, with a molecular weight of 499 was present at such low levels that Figure 39A and 39B do not reveal its presence. However, a selected ion current (SIM) micro-LC/MS experiment readily detected this component by monitoring its $(M-1)^-$ ion at m/z 498 (not shown here).

Figure 40A shows the micro-LC UV chromatogram of a preparative HPLC residue obtained from a synthetic mixture of Felodipine. The micro-LC/MS PCI total ion current shown in Figure 40B shows a similar chromatogram. By selecting various ions from the total ion current, over twenty different components could be detected in this "preparative" HPLC sample. This is a dramatic example of the mass spectrometer's ability to detect components and provide their mass spectra when many conventional HPLC detectors can not. re-run of the sample by micro-LC duplicated the original UV chromatogram and suggested the sample had not broken down further.

Multidimensional LC/MS

Multi-dimensional chromatography in and of itself is not a novel technique. The technique has been applied to thin-layer chromatography (102) as well as gas chromatography with both packed (103) and capillary columns (104). Investigators have previously reported the technique's application to liquid chromatography (105).

Although multi-dimensional liquid chromatography is not new its coupling to mass spectrometry is a novel tech-

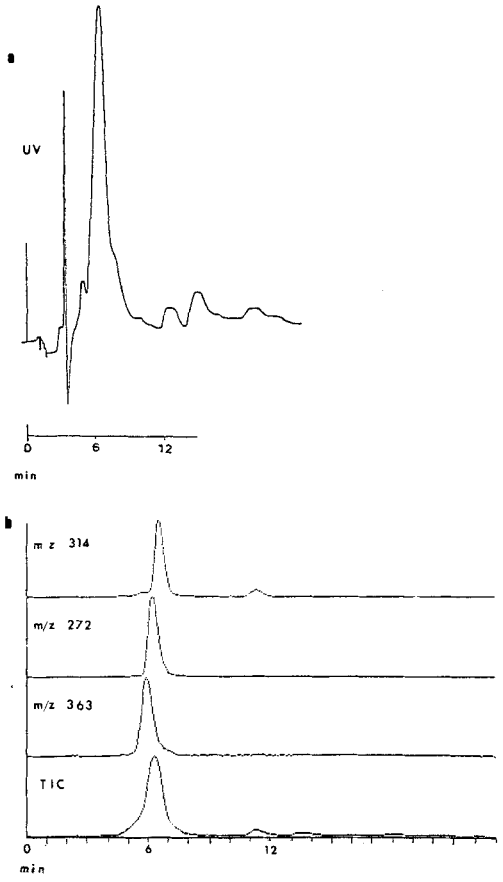


Figure 40. (a) micro LC UV chromatogram of preparative HPLC product from a synthetic mixture of Felodipine. (b) micro LC/MS ion current profiles indicating several different products detected in the preparative HPLC product.

nique which affords added versitility to conventional liquid chromatography/mass spectrometry. Bi-dimensional liquid chromatography/mass spectrometry (LC/LC/MS) is a powerful tool for determining components of complex matrices. It combines the resolving power of two LC systems with the sensitive and specific detecting abilities of the mass spec-

trometer. In this technique, fractions from one chromatographic column are selectively transferred ("heart cutting") to another for further separation, then delivered on-line to the mass spectrometer for final determination. This approach not only increases resolution but can be utilized with crude (eg. biological) extracts to avoid the degradation of expensive commerical columns. In our work, the crude biological extract is injected on an inexpensive JASCO micro LC column and with partial resolution and heart cutting minimal crude extract is then transferred to the more expensive Whatman ODS-3 microbore column.

Figure 41 shows a schematic representation of the apparatus used for micro LC/LC/MS in this work. The first micro LC was a JASCO FAMILIC 100N equipped with a 500 microliter syringe reservoir, a 0.5 mm x 21 cm silica packed PTFE microbore column connected to a variable wavelength UV detector (JASCO, UVIDEC I). The micro cell of the UV detector was a cylindrical quartz cavity with an internal volume of 0.3 microliters. The wavelength was set at 239 mm and the detector outlet was intimately connected to a Rheodyne 7413 micro loop injector valve equipped with a five microliter internal sample loop. The JASCO syringe pump delivered an eluent of dichloromethane/ethanol/water (90/6/4) at a flow rate of eight microliters per minute. Samples were injected directly onto the silica column via a JASCO micro injector utilizing a 0.3 microliter volume.

The reversed phase micro LC system was identical to that reported previously. An eluent flow rate of 40 micro-

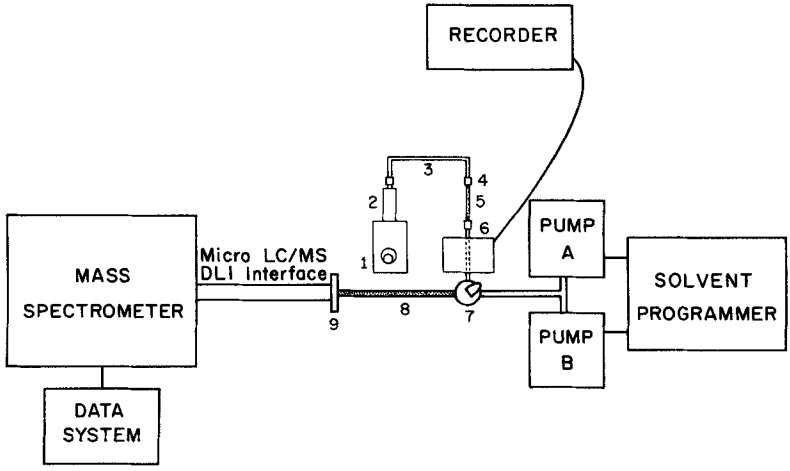


Figure 41. Schematic representation of multi-dimensional micro LC/MS system hardware.

liters per minute of 70/30 methanol/water affected chromatographic separation of the "heart cut" on 1 mm i.d. x 25 cm Whatman ODS-3 microbore column. The exit of the reversed phase column was connected directly to the micro LC/MS diaphragm probe interface described above.

An extract resulting from liquid-liquid extraction of equine urine was injected onto the JASCO silica LC column. The normal phase UV chromatogram resulting from this separation is shown in Figure 42. The endogenous hydrocortisone (peak 1) and administered 6-methylprednisolone (peak 2) are evident in this chromatogram, but they are co-eluting with high levels of other endogenous materials. Micro LC/MS on this separation above showed significant interference in the mass spectra of the components of interest.

When the UV chromatogram shown in Figure 42 is "heart cut" at the retention time of the 6-methylprednisolone the

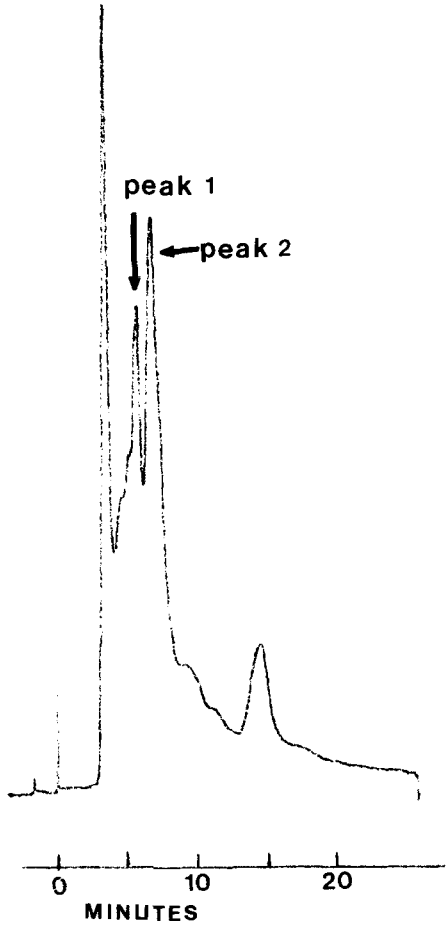


Figure 42. JASCO micro LC UV chromatogram of a crude equine urinary extract separated on a 0.5 mm X 200 mm silica micro LC column using dichloromethane/ethanol/water (90/6/4) flowing at 8 microliters min.

chromatographic resolving power of the reversed phase micro LC effectively separates the 6-methylprednisolone from the endogenous interferences. The micro LC/LC/MS ion current chromatogram and NCI mass spectrum for 6-methylprednisolone are shown in Figure 43. It is clear that the heart cut ef-

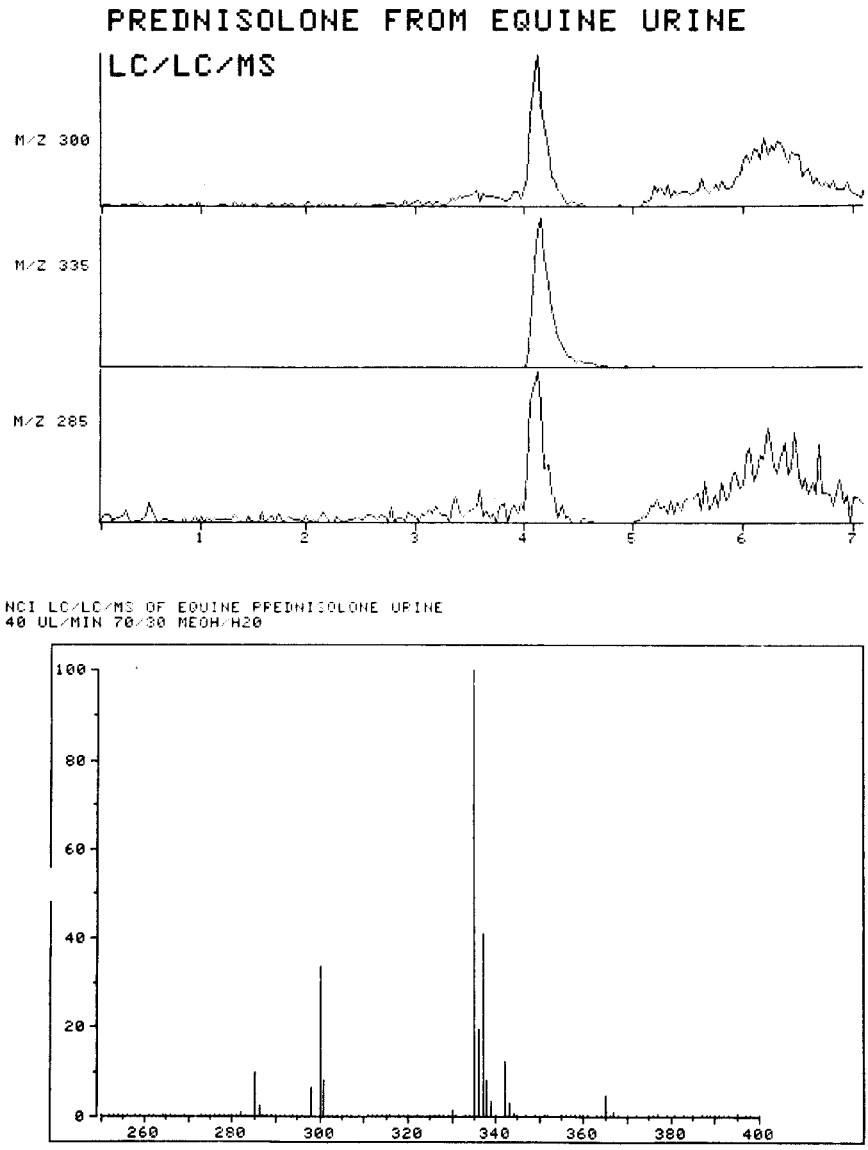


Figure 43. LC/LC/MS ion current profiles for methylprednisolone from an equine urine extract b. LC/LC/MS NCI mass spectrum of methylprednisolone from equine urine extract.

fectively "transferred" the desired 6-methylprednisolone to the second micro LC system and the DLI micro LC/MS interface quantitatively transferred this effluent into the mass spectrometer.

Unfortunately the poor miscibility of the dichloromethane normal phase eluent in the aqueous reversed phase eluent generates a purtubation of the MS ion current although it does not interfere with the results. In fact the the abundant m/z 335 ion in Figure 43 clearly reveals an unexpected chlorine isotope. This presumably results from a chloride attachment process due to the momentary excess of dichloromethane in the CI source at the time 6-methylprednisolone elutes. The NCI MS conditions utilized provide particular sensitivity to this process which effectively adds diagnostic utility to the analysis.

The practical usefulness of LC/LC/MS results only when chromatographic separation of a complex mixture cannot be attained. The added capability of normal phase chromatography in combination with reversed phase chromatography adds a very powerful dimension to the separation of complex mixtures. Since veterinary toxicology often involves dealing with complex biological mixtures, such multi-dimensional chromatography may be of value in certain instances.

Thermospray LC/MS

The discovery of thermospray LC/MS by Vestal (73,74) and others (76) appears to offer one of the most exciting developments towards a truly viable LC/MS interface. Histor-

ically, liquid chromatographers have been advised that LC/MS requires low or non aqueous eluent composition, restricted buffers or modifiers, and stable, relatively volatile compounds. Trace analysis (defined as low nanogram levels) of labile compounds on the moving belt (106) or heated concentrator wire (107) LC/MS interface appears limited in some instances. However, there have been some impressive applications using the transport interface and its use in various areas continues to be of interest. The direct liquid introduction (DLI) LC/MS interface reported by McLafferty (61) requires an unfavorable split of HPLC effluent which precludes routine full scan DLI LC/MS trace analysis. The determination of labile biological metabolites using DLI LC/MS appears preferred over the moving belt (108) but unless micro LC/MS techniques are utilized (70,71) the technique still does not provide trace analysis capability. These and other approaches have provided an increasingly viable means of accomplishing LC/MS, but routine senstivity in many instances has not been comparable to that afforded by GC/MS. Many researchers involved with environmental and toxicological studies require LC/MS detection limits better than has been commerically available. In addition, molecular weight and structural information from LC/MS mass spectra should be readily available from the fragile compounds that are so ideally suited for HPLC. Vestal and co-workers (74), have reported impressive thermospray applications including dramatic LC/MS sensitivity and the ability to handle high molecular weight

and labile compounds. These results were produced from similar but separate instruments utilizing the same design. The success of these results prompted us to implement the thermospray LC/MS concept into our existing LC/MS program.

We have constructed a new, dual purpose interface which provides both DLI and thermospray LC/MS capability from the same device (109). It is unique in that as a thermospray interface it is inserted or removed from the standard half-inch direct insertion probe inlet of a Hewlett-Packard 5985B GC/MS and also provides conventional DLI LC/MS. This device offers several new advantages which include: (a) dual purpose operation of either conventional or micro LC/ MS in the DLI mode or thermospray operation from one device, (b) a removeable thermospray interface which facilitates maintenance and conventional use of the solid probe inlet of the GC/MS, and (c) practical add-on to existing GC/MS instruments equipped with a chemical ionization (CI) source. These features combined with simplified construction offers an easy introduction to two of the most popular current approaches to practical LC/MS.

The new dual purpose DLI/thermospray LC/MS interface is shown in Figure 44 and its construction has been described in detail previously (109). Basically it consists of a central, microbore throughput capillary, a terminal heated copper vaporiser and a removeable end cap which houses a removeable pinhole diaphragm similar to that described above for the micro LC/MS probe. LC effluent passes through the central throughput capillary at flow rates ranging from

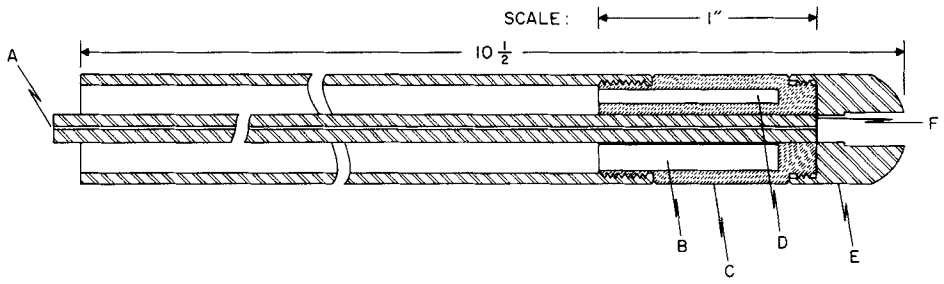


Figure 44. Dual purpose DLI/thermospray LC/MS probe interface. A. central micro-bore (0.004 in i.d.) throughput tube, B. 2-50 watt 120 V AC cartridge heaters, C. Heated copper vaporizer, D. thermocouple, E. removable end cap, F. stainless steel pinhole diaphragm.

0.1-1.0 ml/min and is rapidly heated prior to exiting the interface via the electrically heated copper vaporizer.

When the dual purpose DLI/thermospray LC/MS interface is inserted through the standard half inch solid probe inlet of the MS the excess volatilized LC effluent vapors are pumped away by additional pumping provided by modifications schematically shown in Figure 45. Basically the MS is modified to allow an auxillary 300 L/min roughpump to pump on the CI ion source via a half-inch line directly opposite the interface.

To evaluate the feasibility of this new LC/MS interface in the two different operational modes a variety of compounds of varying difficulty have been investigated. The mass spectrum shown in Figure 46A was obtained from the PCI thermospray LC/MS determination for five micrograms of the potent indole alkaloid tranquilizer, reserpine. This compound is very unstable to both heat and light, and is

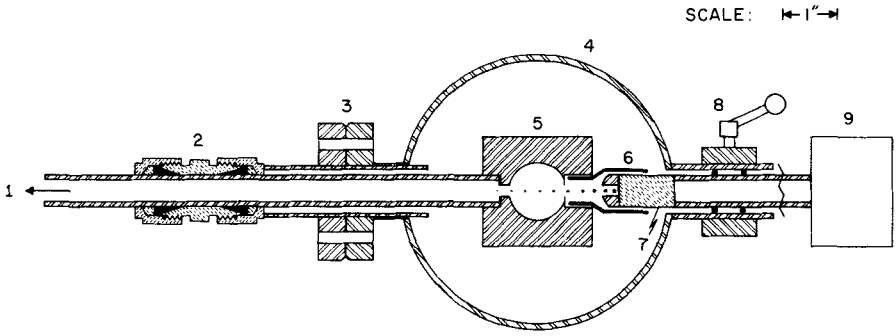


Figure 45. Schematic of DLI/thermospray inlet and pump out region of HP5985 GC/MS. A. to thermospray rotary pump. B. Swagelok union equipped with Teflon ferrules, C. outer tube welded to the high vacuum flange, D. high vacuum flange, E. half inch stainless steel slide tube, F. CI ion source, G. MS analyser housing, H. extended desolvation chamber, I. thermospray vaporizer, J. half-inch direct insertion probe isolation valve, K. DLI/thermospray LC/MS interface.

difficult to characterize by MS at trace levels in biological samples. Figure 46A shows that the $(M+1)^+$ ion at m/z 609 is the base peak which carries the majority of the total ion current in the experiment. Although there is no useful structural information in this mass spectrum, it is clear that thermospray ionization of reserpine is even more mild than PCI DLI LC/MS data reported earlier for this compound (76).

Figure 46B shows the thermospray PCI LC/MS mass spectrum of the important industrial dye, sulforhodamine B (Eastman Chemical Co., Rochester, NY). The structure of this compound is such that conventional direct insertion probe analysis does not provide useful mass spectral information for characterization of the intact molecule. Facile

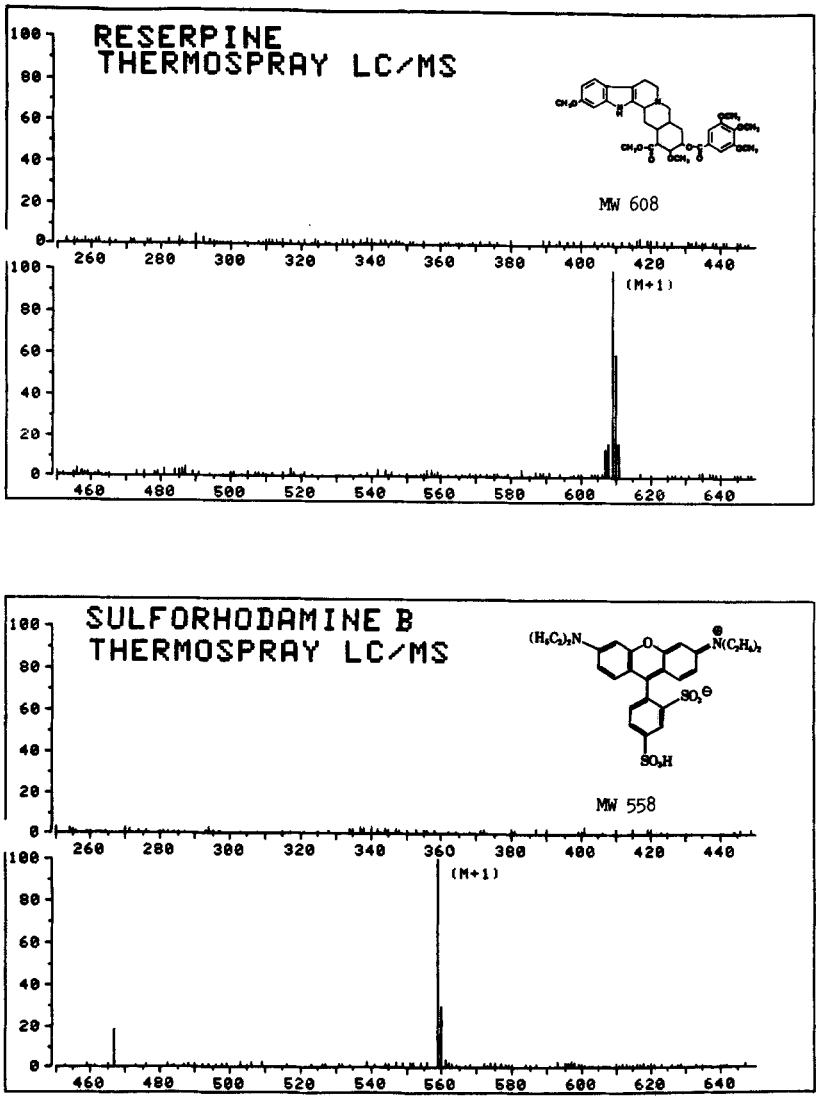


Figure 46. A. PCI FIA Thermospray LC/MS mass spectrum from 5 µg reserpine. B. PCI FIA thermospray LC/MS from 5 µg sulforhodamine B. The LC eluent conditions were 0.15 mL/min 50/50 CH_3OH/0.05 M NH_4OAC using a Brownlee 10 micron 005-222 reversed phase column.

expulsion of sulfur and nitrogen containing functional groups demands extremely mild ionization conditions in order to detect ions representive of the compound's molecular weight of 558. From the thermospray mass spectrum of sulforhodamine B shown in Figure 46B it is evident that the $(M+1)^+$ ion is the base peak with only a m/z 467 fragment ion pre sent in the remainder of the mass spectrum. These data again support the contention (73,74) that this "filamentless" mode of ionization imparts relatively little excess internal energy to the ionized molecules.

Finally, the PCI thermospray LC/MS data obtained from the potent pain killer, leucine enkephalin, are shown in Figure 47AB. Figure 47A shows two successive injections of a standard five microgram solution of leucine enkephalin. This extracted ion current profile for the $(M+1)^+$ ion at m/z 556 shows that a stable, abundant ion current signal was observed for intact the molecule. Figure 47B shows that some useful fragmentation ion current was observed in addition to the $(M+1)^+$ base peak. The relatively abundant $(M+23)^+$ ion is presumably due to the addition of a sodium atom to the parent leucine enkephalin. This natriazation has been observed by others (73) and may offer additional diagnostic information when better understood. These data also show that labile compounds such as this brain opiod peptide may be characterized by this new ionization technique. Traditionally, peptides of this type have been determined by field desorption (FD) MS, but required extensive work up of complex biological samples prior to MS analysis of the sample.

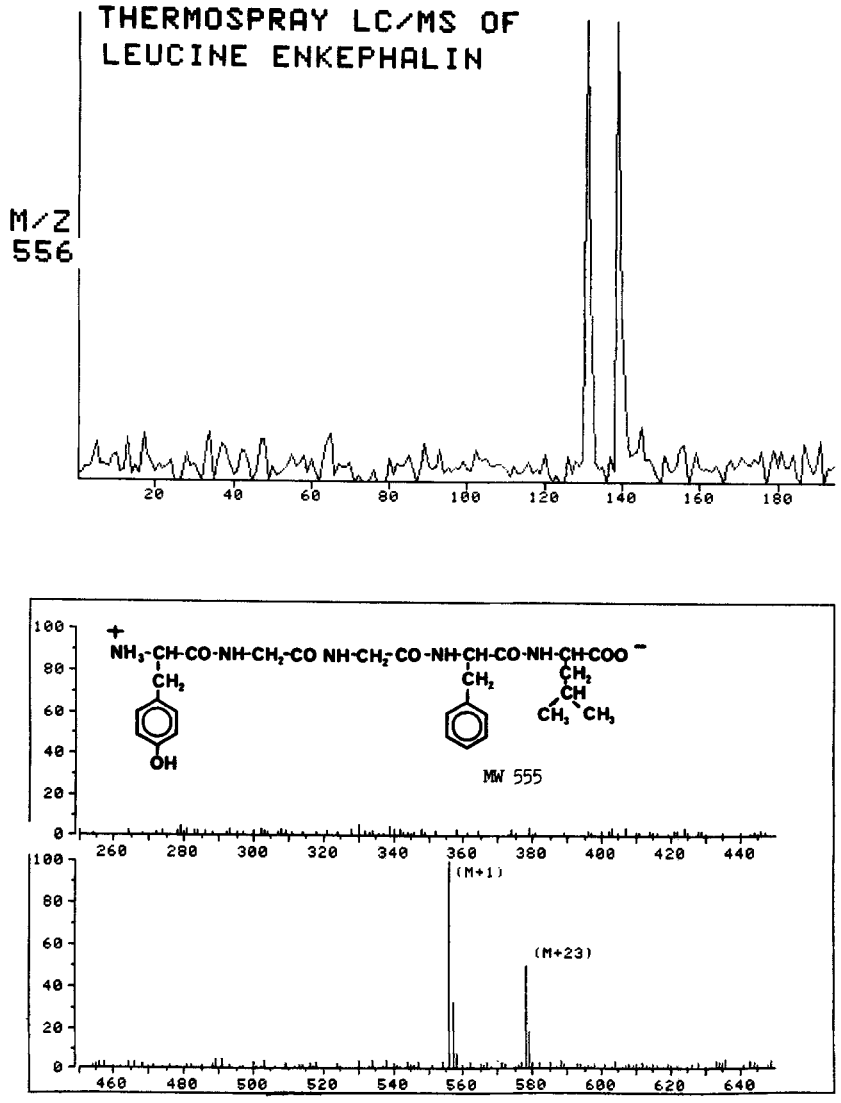


Figure 47. A. PCI FIA thermospray LC/MS ion current for the $(M+1)^+$ ion at m/z 556 from duplicate injections of 5 µg leucine enkephalin. The LC eluent was the same as the described for Figure 46. B. PCI thermospray LC/MS mass spectrum of leucine enkephalin taken from the first peak shown in A.

If thermospray ionization LC/MS can provide the powerful combination of on-line chromatographic separation with mild ionization the technique could be very useful in the area of biological trace analysis. If structual characterization could be accomplished by MS/MS techniques on the abundant $(M+1)^+$ ions such as those presented in this work and that reported by others (74,75), many challenging analytical problems could potentially be solved in a straightforward manner.

SUMMARY AND CONCLUSIONS

The advent of modern HPLC has greatly broadened the range of toxicants that can be analyzed in the veterinary toxicology laboratory. The technique has improved the sensitivity, specificity, and rapidity of many of the analyses which are required. Highly potent toxicants whose biological effects could be elicited at levels below the detection limit of earlier techniques now stand to be identified, quantified, and diagnosed as the cause of an intoxication through the use of HPLC.

The potential of HPLC for forensic analysis is far from being reached. In several classes of toxicants only a few applications have been reported. The vast majority of methods use reversed phase columns and isocratic elutions. A few of the multiresidue procedures
utilize gradient elutions in order to accommodate a wider range of analyte polarities. Extensive method development remains to be done in this important area of multiresidue

analysis so that samples can be rapidly analyzed for a wide range of compounds.

The use of the mass spectrometer as a detector for the HPLC has unexcelled potential for the identification of eluting peaks in toxicological analyses. Unfortunately, the high cost of the necessary equipment and expertise required to operate the equipment precludes its implementation by many diagnostic and forensic laboratories. However, the demonstrated fact that LC/MS can be routine and can solve problems that are not amenable to GC/MS techniques requires its availability to those who legitimately need it. These authors believe that the cost of the necessary equipment for LC/MS will come down dramatically in the near future so that LC/MS and (hopefully) LC/MS/MS equipment may become more affordable.

There is no doubt that LC/MS will become routine analytical technique to those interested in applying it to real world problem solving. However, it is and probably will remain essential that the personnel involved must be technically competent in both HPLC, MS, and the precision operation of each instrument. Aside from these restrictions the combined specificity and sensitivity available from LC/MS plus simplified sample work up such as solid phase extractions, multi-dimensional chromatography and short guard columns which protect analytical LC columns make LC/MS essentially the method of choice for many important forensic problems. When the technique of MS/MS is routinely available for such problems the detection and unequi-

vocal identification of toxic organic substances may be much more straightforward than it has been in the recent past.

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