JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 7 SUPPLEMENT 2 1984

Editor: DR. JACK CAZES
Associate Editor: DR. HALEEM J. ISSAQ

REVIEWS

CODEN: JLCHD8 7(S-2) i-iv, 205-432 (1984)
ISSN: 0148-3919

JOURNAL OF LIQUID CHROMATOGRAPHY

September 1984

Aims and Scope. The journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all modes of liquid chromatography.

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

• Analytical Abstracts • ASCA • BioSciences Information Service of Biological Abstracts (BIOSIS) • Chemical Abstracts • Current Awareness in Biological Sciences • Current Contents/Life Sciences • Current Contents/Physical and Chemical Sciences • Engineering Index • Excerpta Medica • Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR • Physikalische Berichte • Science Citation Index

Manuscript Preparation and Submission. See end of issue.

Subscription Information. *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (2 numbers), April, May (2 numbers), June, July (2 numbers), August, September, October (2 numbers), November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 7 (1984), containing fourteen numbers and two supplements, is $350.00 per volume (prepaid). The special discounted rate for individual professionals and students is $175.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $40.00 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

Mailing Address. *Please mail payment with order to:* Marcel Dekker Journals, P. O. Box 11305, Church Street Station, New York, New York 10249.

Contributions to this journal are published free of charge. Application to mail at second-class postage rates is pending at New York City, New York and additional mailing offices.

***THIS REFLECTS A 50% DISCOUNT GIVEN TO INDIVIDUAL SUBSCRIBERS.**

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 7, Supplement 2, 1984

CONTENTS

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 205-315 (1984)

High Performance Liquid Chromatography in Veterinary Toxicology

Thomas R. Covey and Jack D. Henion*
New York State College of Veterinary Medicine
Equine Drug Testing and Research Program
Cornell University
925 Warren Drive
Ithaca, NY 14850

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-

 0148-3919/84/07S2-0205$3.50/0

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-

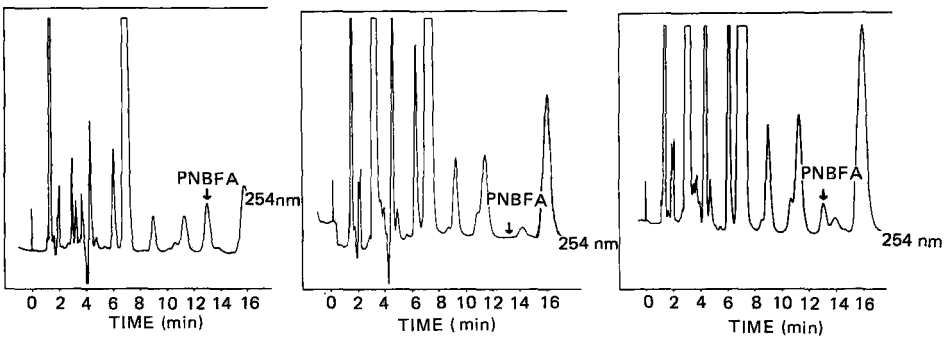


Figure 1. Typical chromatograms for HPLC analysis of 1080 as PNBFA in canine stomach contents: (left) 20 μg standard 1080: (middle) blank content: (right) content spiked with lppm 1080.

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 multi-dimensional 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-

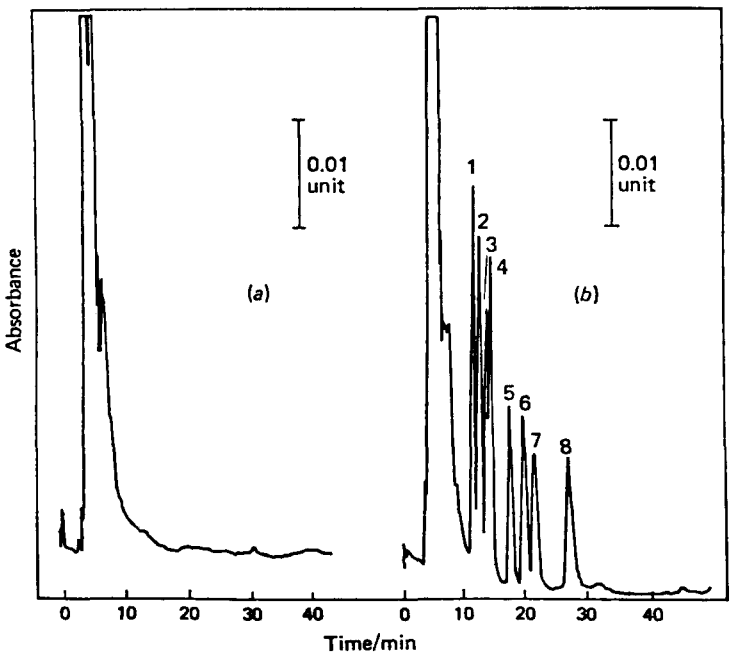


Figure 2. Typical chromatograms obtained from injections of wheat extracts: (a) unfortified, and (b) fortified with phenylurea herbicides at 2 mg/kg. 1, monuron; 2, monolinuron: 3, metabromuron; 4, chlorotoluron; 5, diuron; 6, linuron; 7, chlorbromuron; and 8, chloroxuron.

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 on-line by a heart cutting technique which traps the peak of interest from the first column in a loop injector and re-injects 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

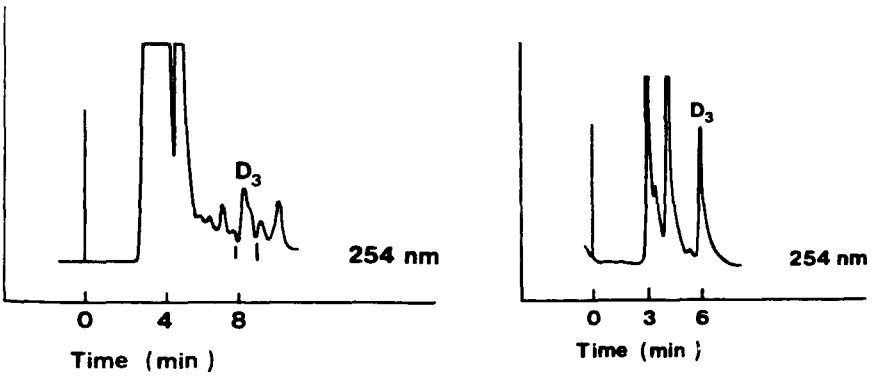


Figure 3. (A)Chromatogram of feed extract containing vitamin D_3 normal phase μPorasil column. Volume of eluate collected for reverse phase chromatography is indicated on chromatogram. (B)Chromatogram of fraction from normal phase column on a reversed phase μBondapak C_{18} column.

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:

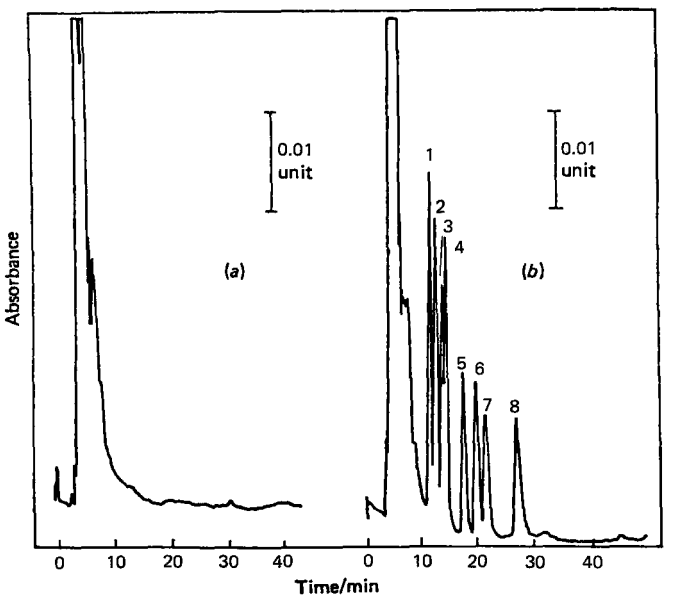


Figure 4. Typical chromatograms of beef liver extracts (TFA treated). Left = recovery sample (M_{2a}, B_{2a} G_{2a}=1ng/g; B_2, G_2 = 0.3 µg/g); Right = unspiked.

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-

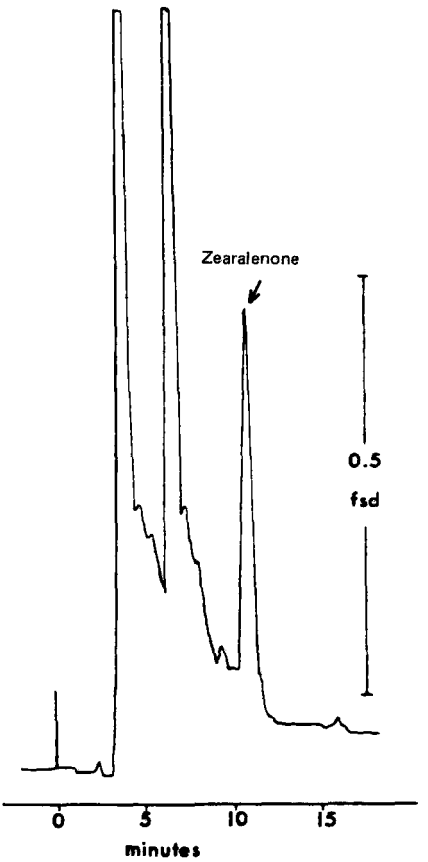


Figure 5. Chromatogram of animal feed sample with 0.01 ppm added zearalenone.

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

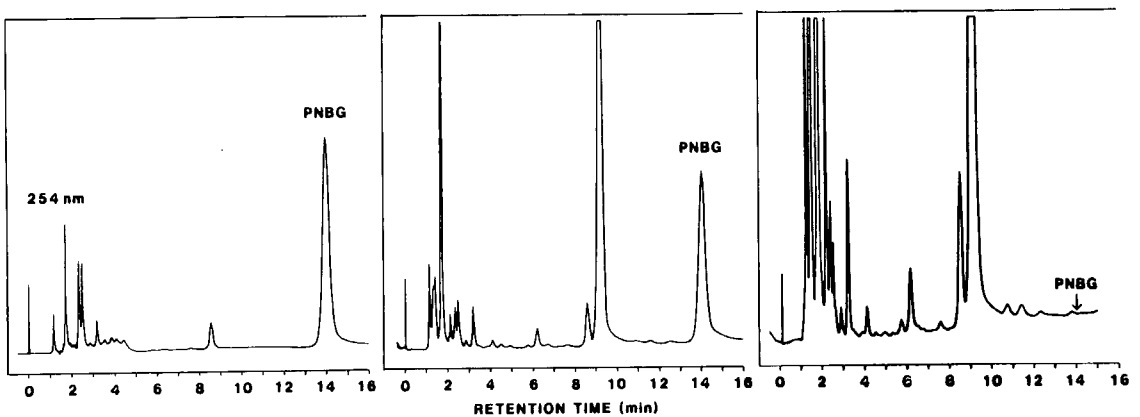


Figure 6. Typical chromatograms for HPLC analysis of glycolic acid as PNBG: (left) 1 mg standard glycolic acid; (middle) dog serum extract 3 hours after ethylene glycol administration; (right) control serum sample.

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.

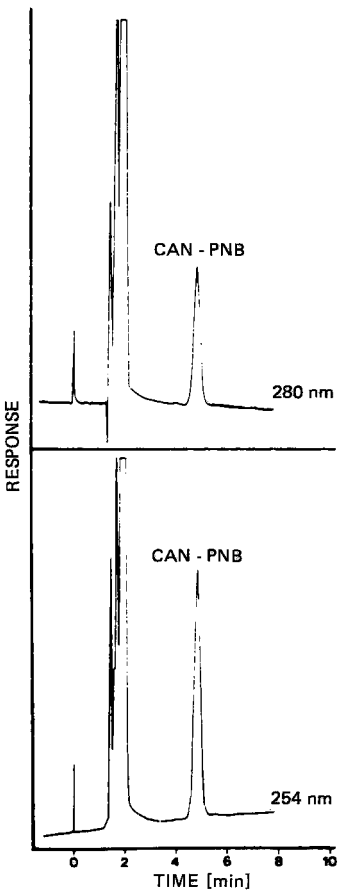


Figure 7. Chromatogram of derivatized extract of pony urine after feeding the animal with 5.3g of dried blister beetles. CAN-PNB = p-nitrobenzyloxime of cantharidin.

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

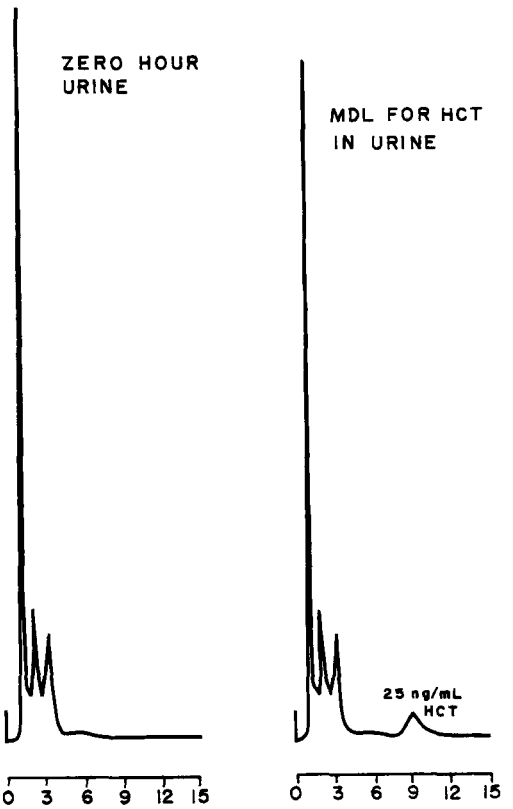


Figure 8. HPLC Chromatograms of extracts of a zero-hour equine urine (left) and a zero hour equine urine spiked with 25 ng/ml hydrochlorothiazide (right) This level of HCT in urine represents the practicle minimum detectable limit.

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-

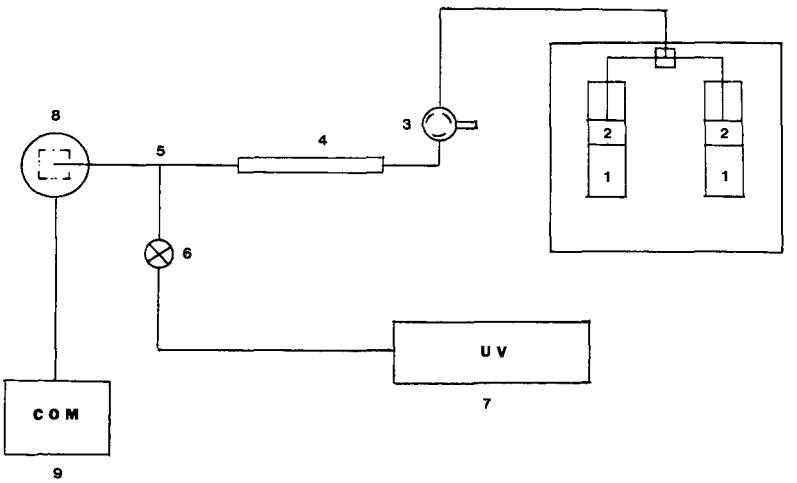


Figure 9. Schematic diagram of early DLI split effluent LC/MS system utilizing a glass capillary probe interface.

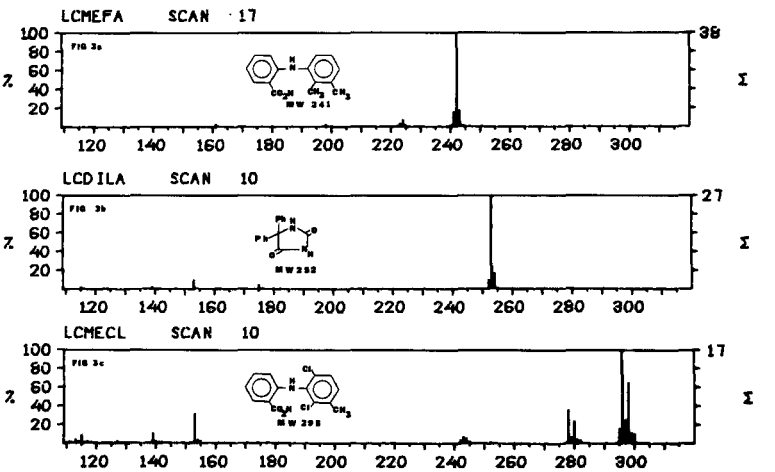


Figure 10. LC/MS chemical ionization mass spectra of (a) mefenamic acid, MW 241; (b) dilantin, MW 252; and (c) meclofenamic acid, MW 295, using 25/75 methanol/pentane as LC eluent and chemical ionization reagent gas.

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

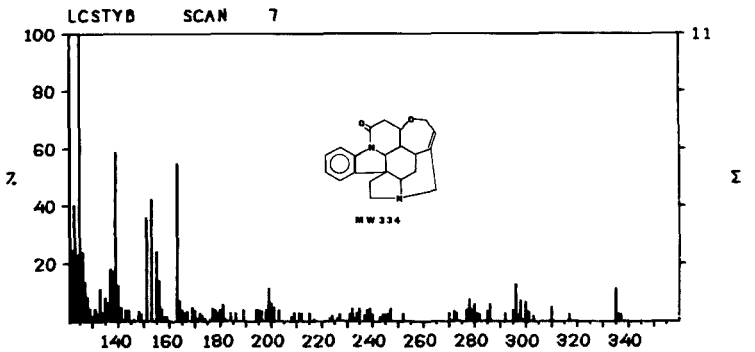


Figure 11. LC/MS mass spectrum of strychnine from the stomach contents of a poisoned dog.

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 10μm 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.

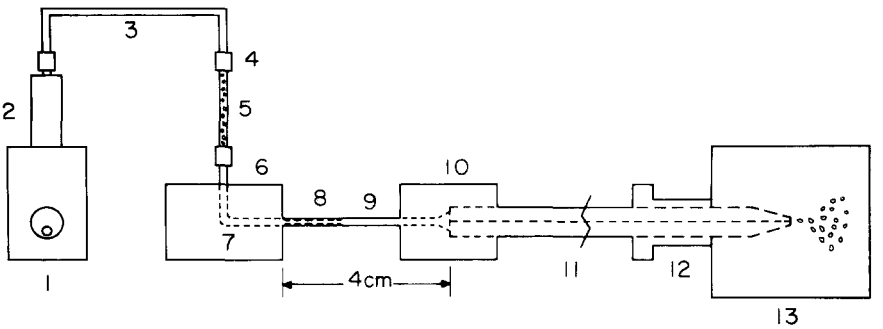


Figure 12. Schematic drawing of the JASCO micro LC/MS system: (1) pump: (2) 250- μL gas-tight syringe; (3) PTFE tubing, 0.5 mm i.d.; (4) sample inlet; (5) micro column; (6) UV variable wave-length detector; (7) micro UV cell; 0.3-μL volume; (8) stainless steel capillary; (9) PTFE tubing; (10) glass-to-teflon 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,

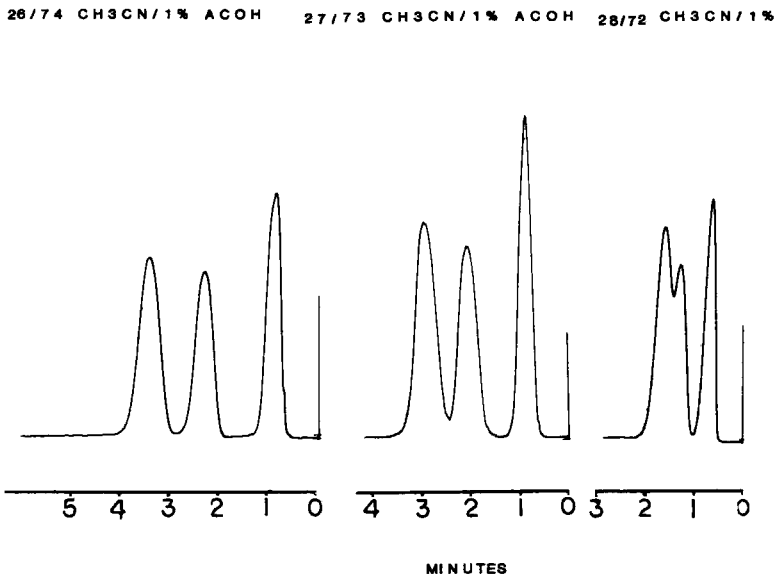


Figure 13. JASCO micro LC UV chromatograms showing the sensitvity to separation of 1% changes in eluent composition on three sulfa drugs. The eluent composition is shown above each chromatogram. The flow rate was 8 μL/min on a 0.5 mm i.d. X 10 cm SC-01 C_{18} column. The sulfa drugs were in elution order,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

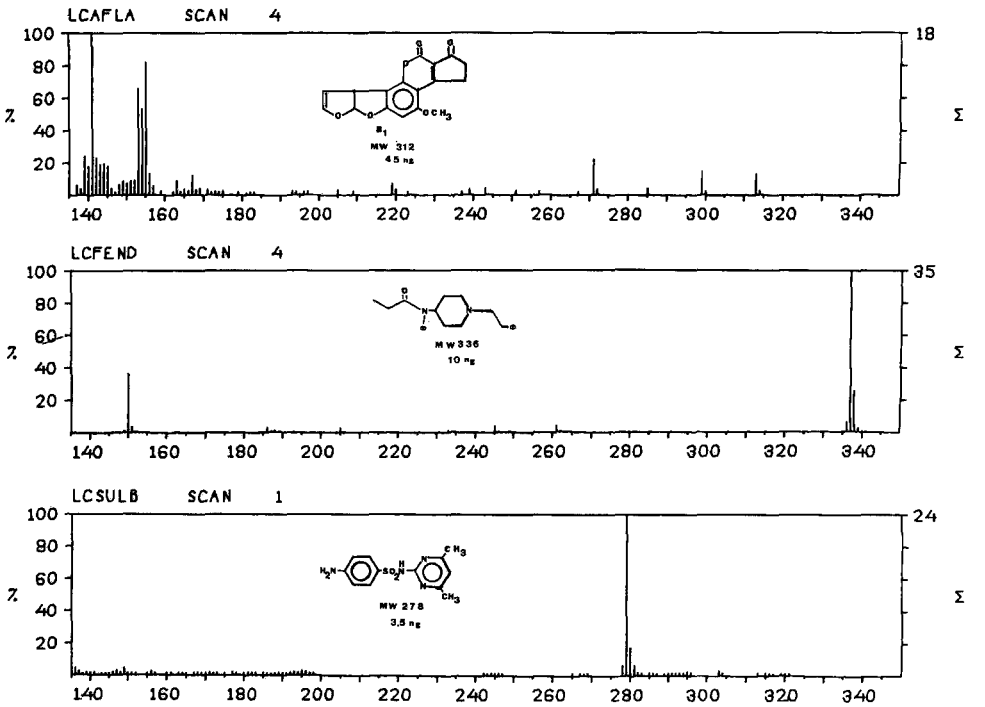


Figure 14. Micro LC/MS CI mass spectra of (a) 45 ng of aflatoxin B_1; (b) 10 ng fentanyl, and (c) 3.5 ng sulfamethazine. The micro LC eluent/CI reagent gas was 20/80 CH_3CN/H_2O for (a) and 40/60 $CH_3CN/-H_2O$ for (b) and (c).

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

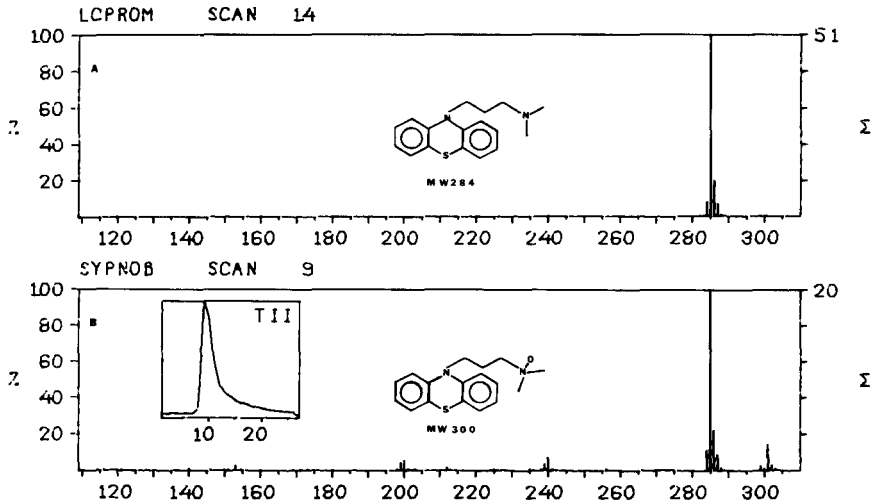


Figure 15. (a) CI/isobutane mass spectrum of promazine obtained via direct insertion probe sample introduction, and (b) micro LC/MS CI mass spectrum of the N-oxide equine urinary metabolite of administered promazine obtained using 50/50 CH_3CN/H_2O as micro LC eluent/CI reagent gas.

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°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

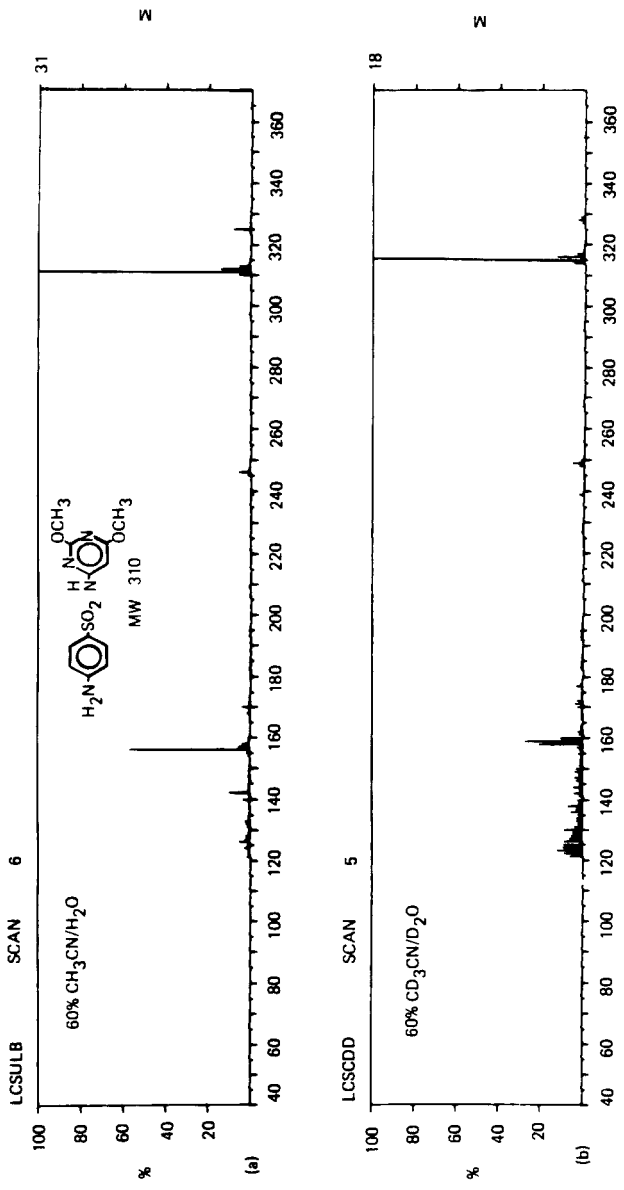


Figure 16. Micro LC/MS CI mass spectra for sulfadimethoxine using (a) 60/40 CH_3CN/H_2O as micro LC eluent reagent gas, and (b) 60/40 CD_3CN/D_2O as micro LC eluent/CI reagent gas. The SC-01 column was equilibrated with 60/40 CD_3CN/D_2O for 1 h prior to obtaining the data in (b).

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

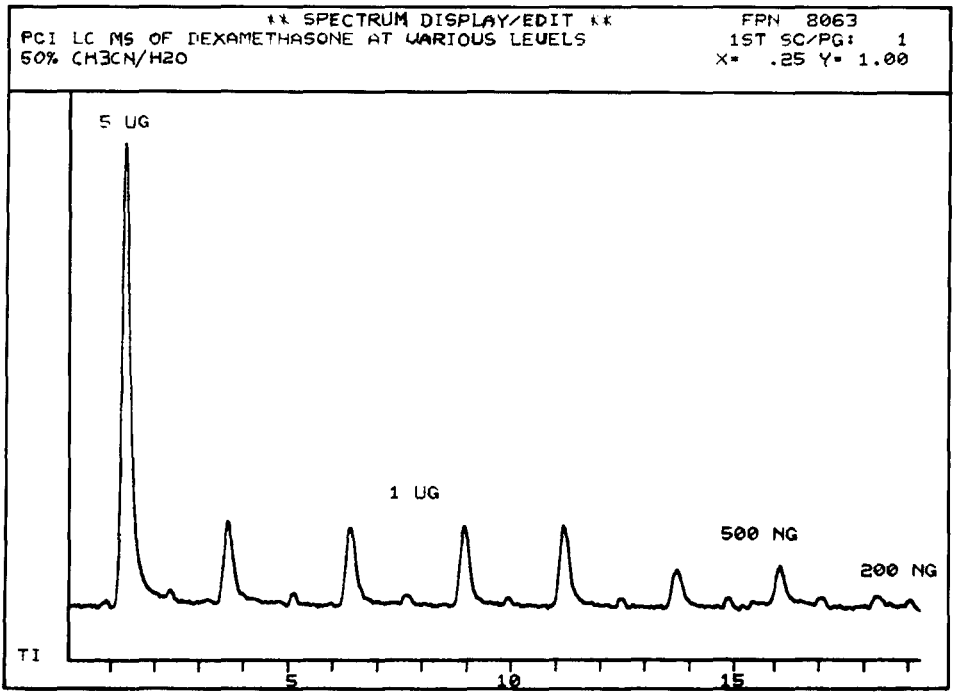


Figure 17. Diaphragm DLI LC/MS split effluent TICP for successive injections of dexamethazone at the 5-μg g, 1-μg, 500-ng, and 200-ng levels. These data were from full-scan (m/z 120-500) analysis of a 99/1 split and reveal the stability, reproducibility, and practical detection limit of this technique.

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

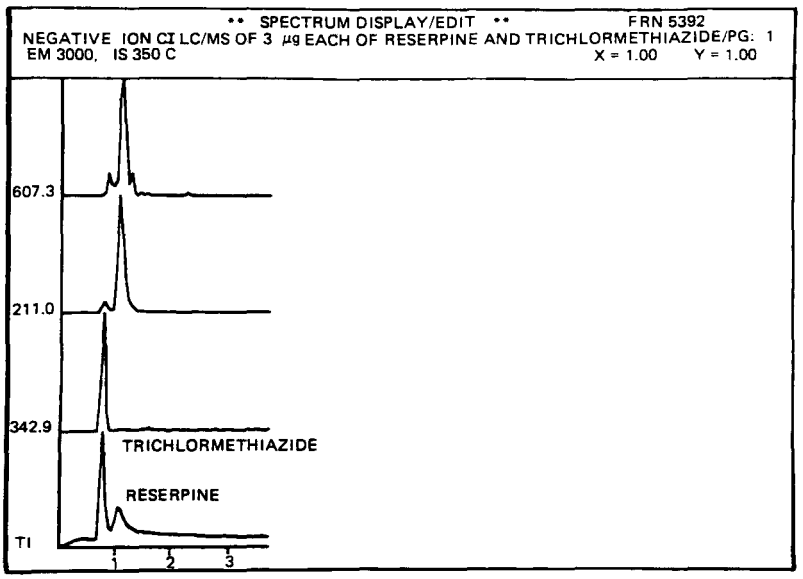


Figure 18. TICP and EICPs from the LC/MS analysis of 3 µg each of trichlormethiazide and reserpine using a conventional LC and the Hewlett-Packard DLI interface. LC eluent was 1 mL/min 95/5 CH_3CN/H_2O using a 2 mm i.d. X 5 cm C_{16} ODS column.

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

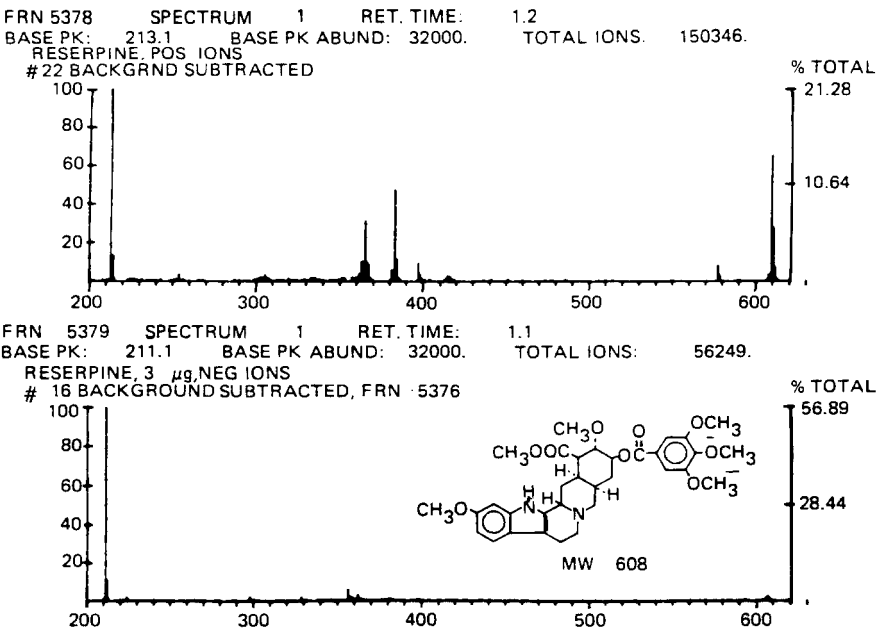


Figure 19. Positive ion CI (upper) and negative ion CI (lower) LC/MS mass spectra of 3.0 µg reserpine (MW 608).

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-

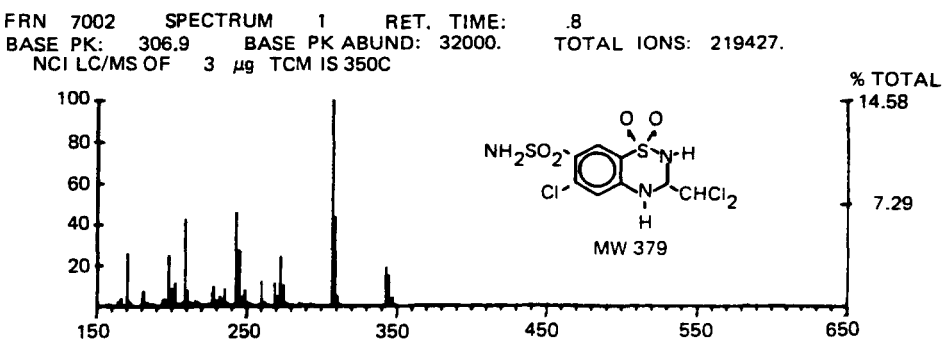


Figure 20. NCI LC/MS mass spectrum for a 3 µg trichlormethiazide using 95/5 CH_3CN/H_2O at a flow rate of 1 mL/min on a 2 mm i.d. X 5 cm C_{18} ODS column.

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.

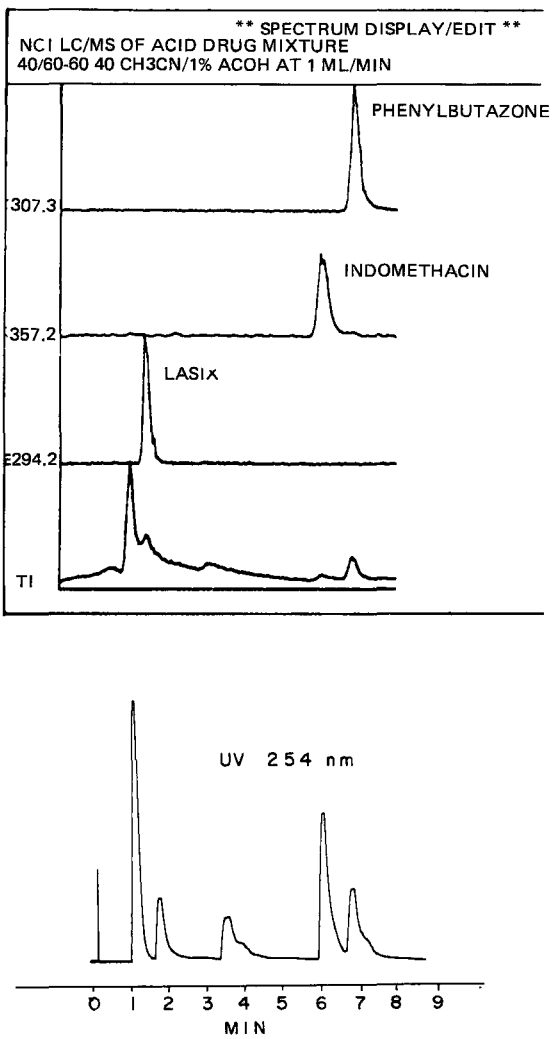


Figure 21. NCI LC/MS TICP and EICP (a) and UV chromatogram (b) for a mixture of five parent drugs, in elution order: sulfamethazine, furosemide, oxyphenbutazone, indomethacin, and phenylbutazone. The LC conditions were linear 10-min gradient from 40/60 - 60/40 CH_3CN/1% ACOH at 1 mL/min on a 2 mm i.d.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

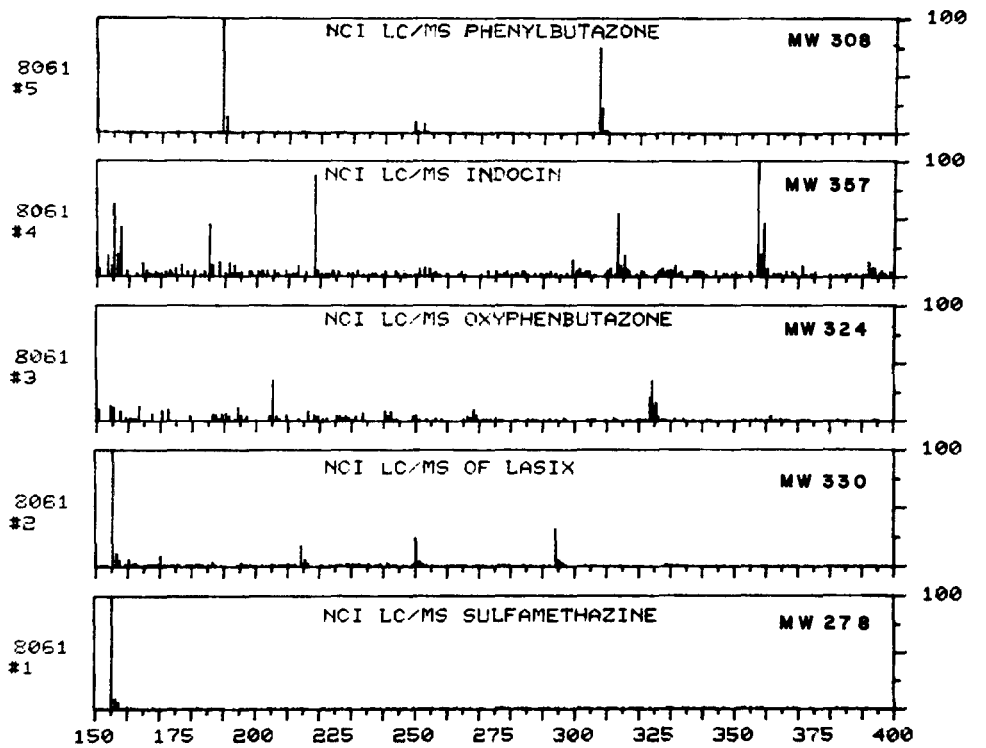


Figure 22. NCI LC/MS mass spectra of the five parent drugs shown in Fig. 21.

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

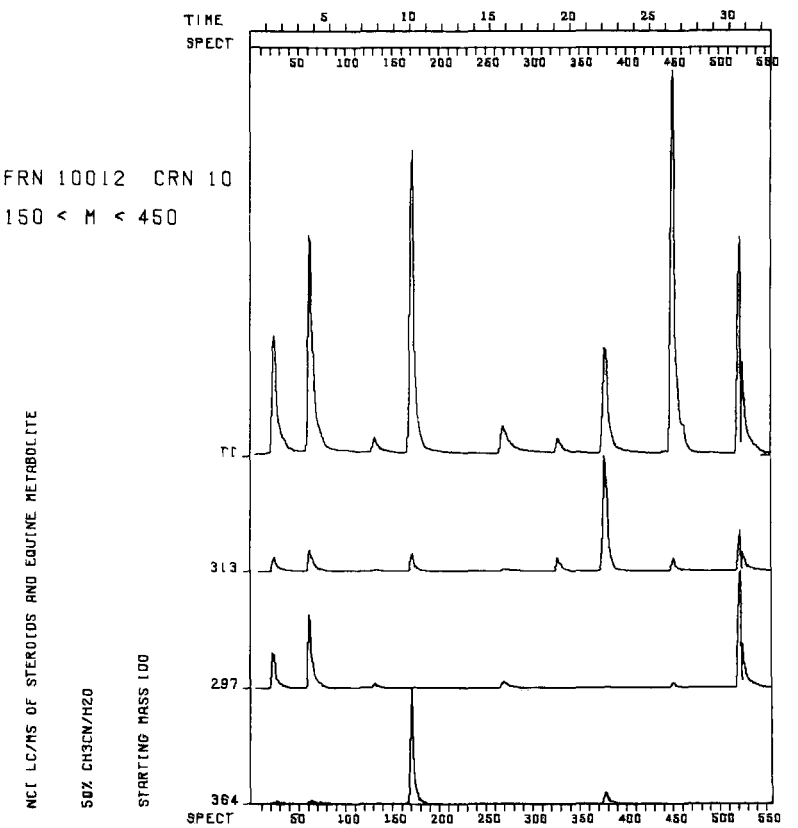
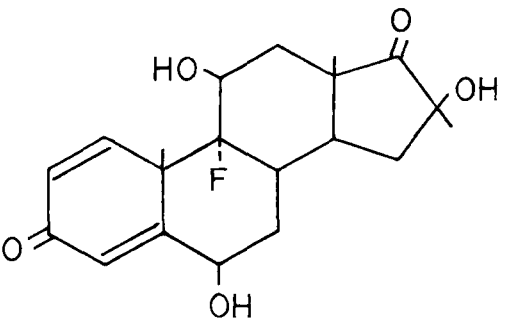


Figure 23. NCI LC/MS TICP and EICPs for several corticosteroids including dexamethasone and its major equine metabolite (peak 4). These data were obtained using 50/50 CH_3CN/H_2O at a flow rate of 1 mL/min on a 2 mm i.d. X 5 cm C_{18} ODS column.

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

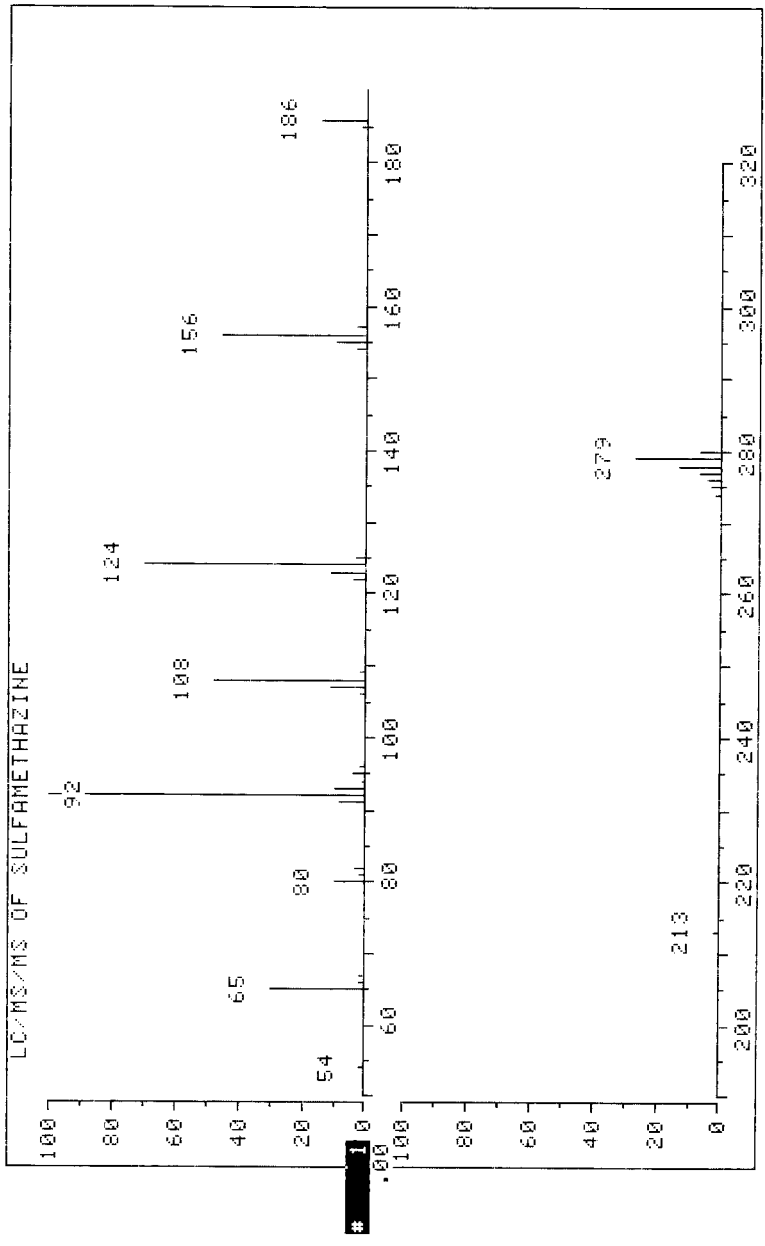


Figure 25. API LC/MS/MS mass spectrum of sulfamethazine $(M+1)^+$, m/z 279 ion. The sample was admitted via the probe using 50% CH_3CN/H_2O as CI reactant gas.

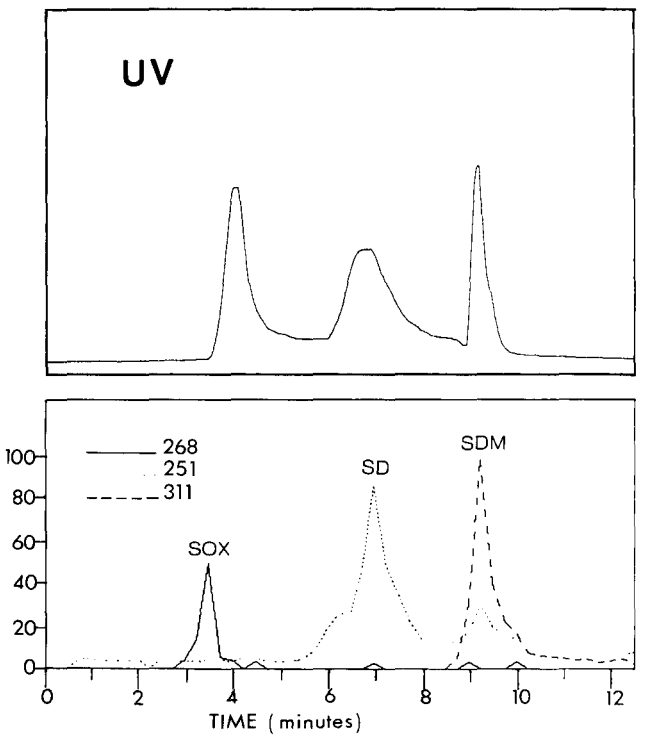


Figure 26. (a) UV liquid chromatogram at 254 nm of a standard mixture of 10 μ g levels of sulfisoxazole sulfadiazine, and sulfadimethoxine using a linear gradient of 10/90 to 90/10 CH_3CN/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PCS ODS column. (b) extracted ion current profiles for selected sulfa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

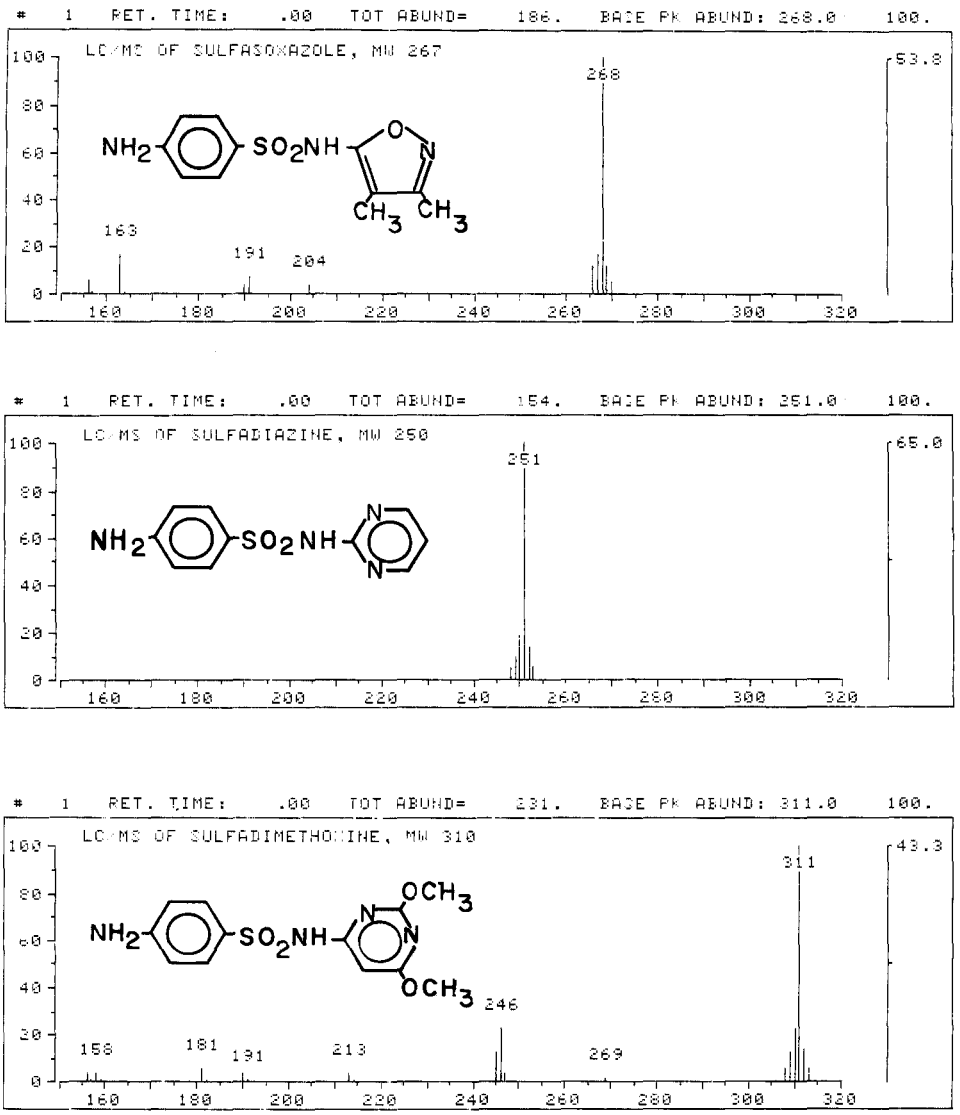
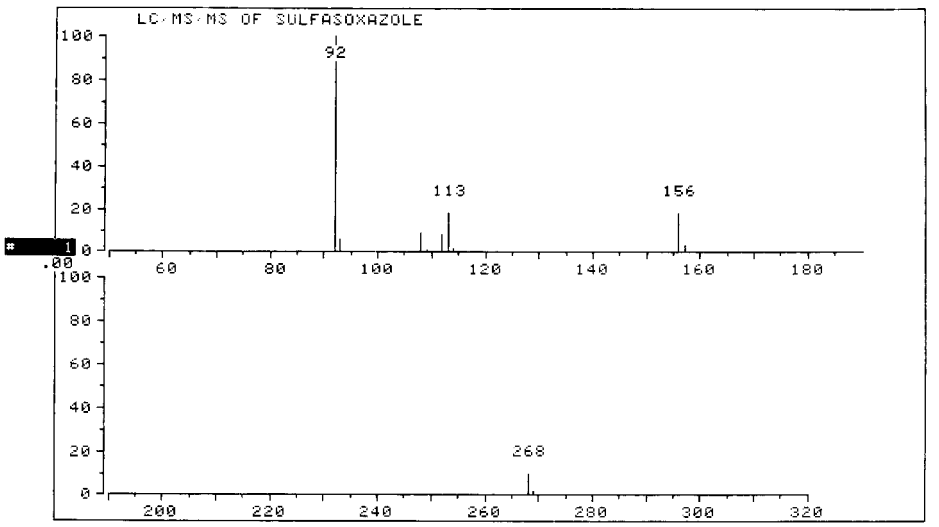
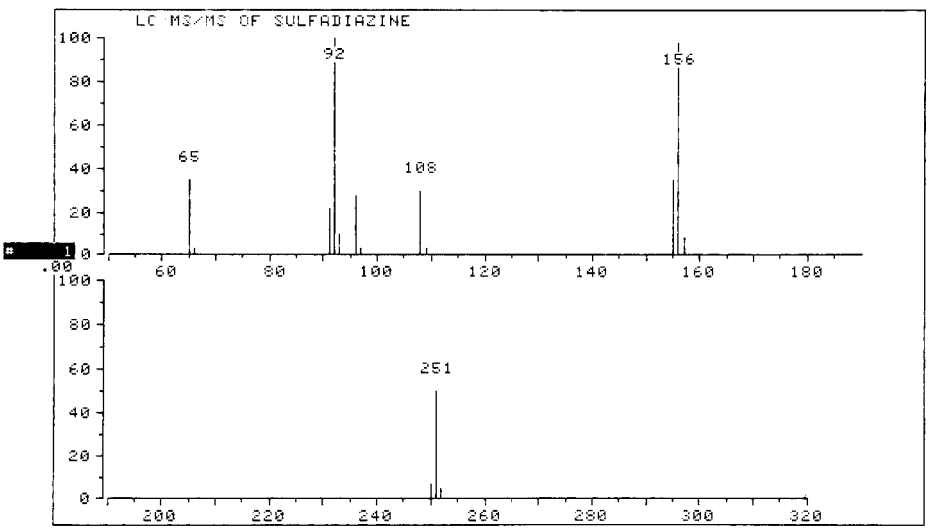


Figure 27. API LC/MS mass spectra for sulfisoxazole (a) sulfadiazine (b) and sulfadimethoxine (c) shown in Figure 26AB. The API reactant gas was aqueous acetonoitrile used as the LC eluent and the samples were admitted via the probe into the API source.


LC-MS-MS OF SULFASOXAZOLE
92
113
156
268


LC MS/MS OF SULFADIAZINE
92
156
65
108
251

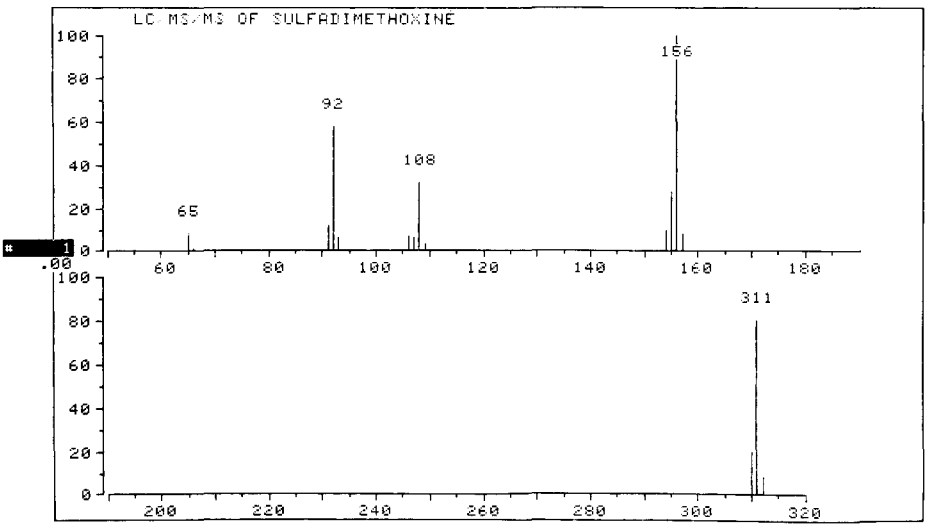


Figure 28. API LC/MS/MS mass spectra for the respective $(M+1)^+$ ions from sulfisoxazole (A), sulfadiazine (SDM) (B), and sulfadimethoxine (SM) (C) in the mixture shown in Figure 26 B.

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

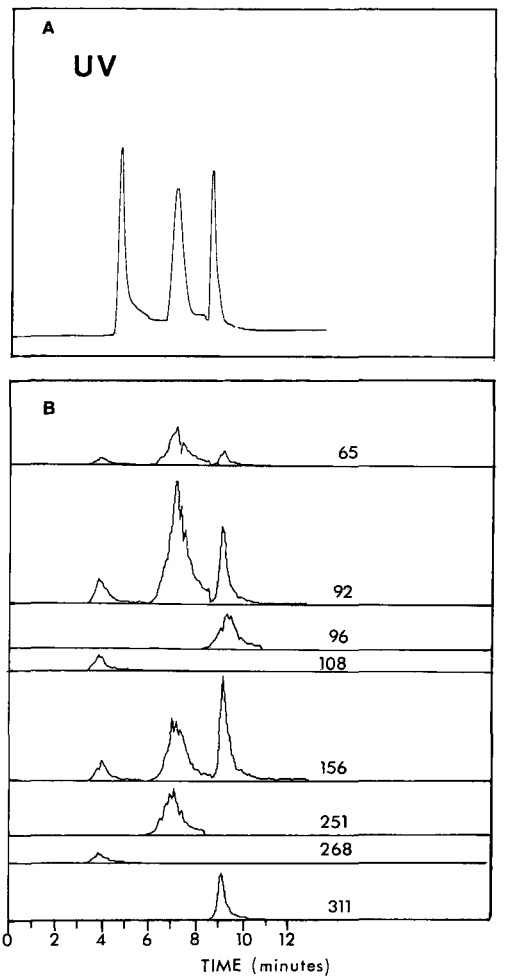


Figure 29. (A) UV liquid chromatogram at 254 nm of a standard mixture of 6 μ g levels of sulfisoxazole (SOX), sulfadiazine (SD) and sulfadimethoxine (SDM) using a linear gradient of 10/90 to 90/10 CH_3CN/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PXS ODS column. (B), Extracted ion current profiles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

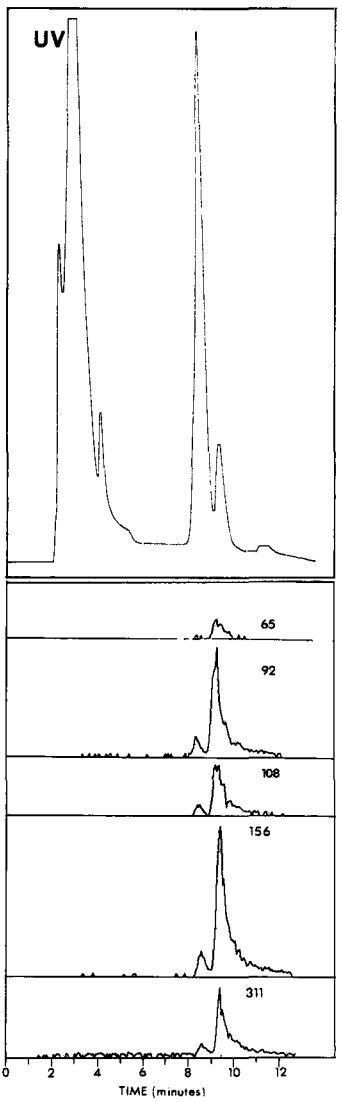


Figure 30. (A) UV liquid chromatogram at 254 nm of a hexane:dichloromethane:ether(1:1:1) extract of racehorse urine (pH 3.0) using a linear gradient of 10/90 to 90/10 CH_3CH/H_2O over 10 min at 1 mL/min on a Whatman 10/25 PXS ODS column. (B) API SIM LC/MS/MS traces for m/z 311,156 108,92 and 65 resulting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

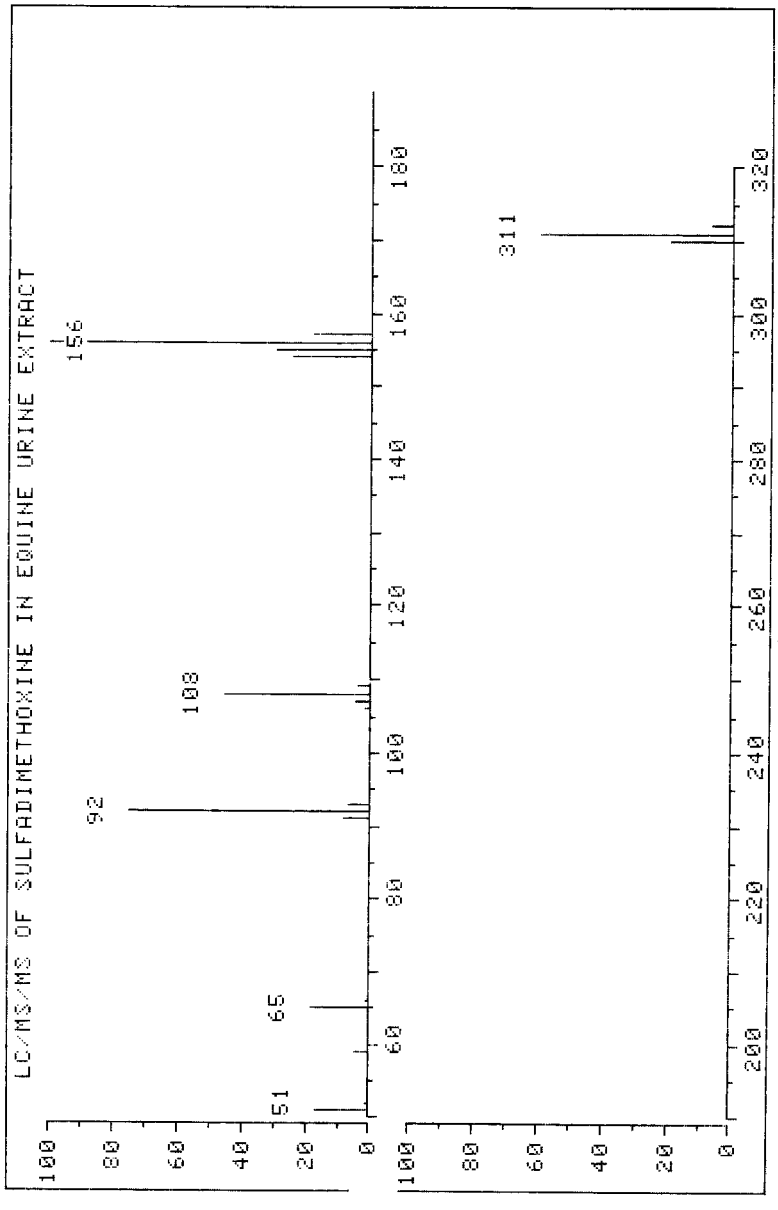


Figure 31. API LC/MS/MS mass spectrum for the 9.0 min retention time component shown in Figure 30A. This full scan LC/MS/MS mass spectrum agrees well with that of anthentic sulfadimethoxine obtained under identical conditions shown in Figure 28C.

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

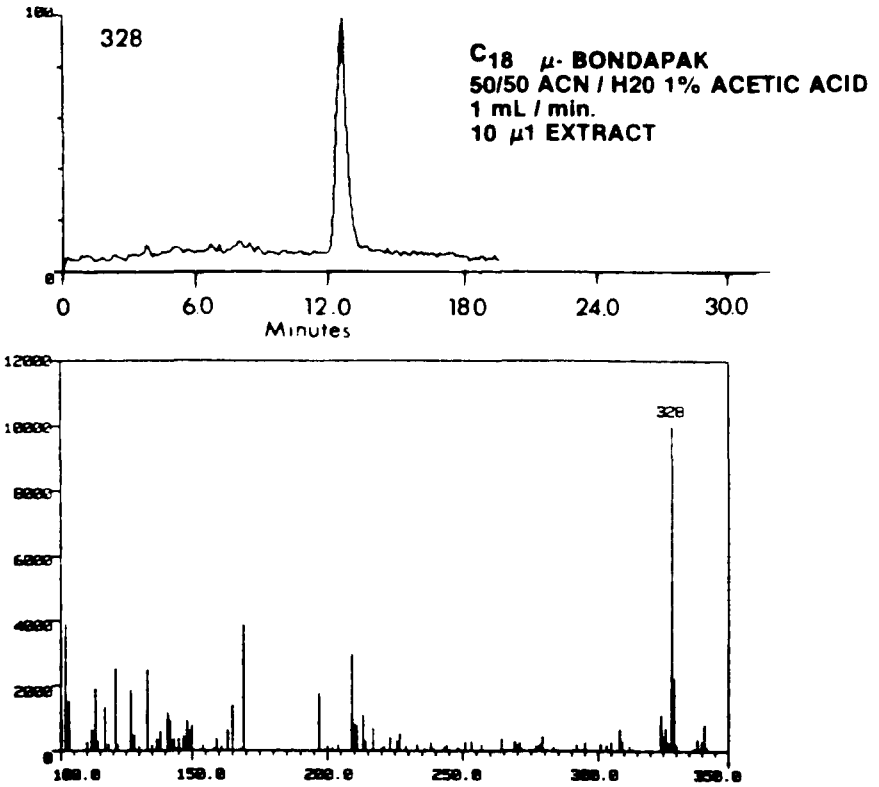


Figure 32. Full scan LC/MS of butorphanol in equine urine extract.

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

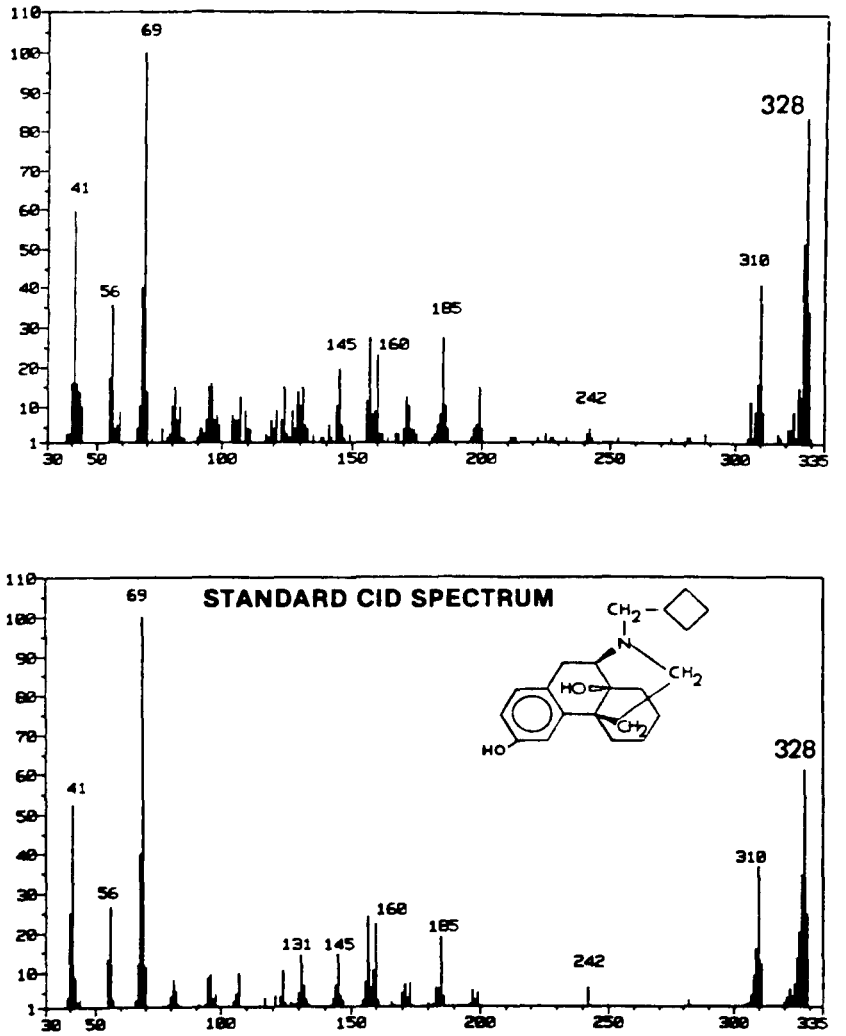


Figure 33. CID spectrum of m/z 328 from LC/MS/MS analysis of urine extract plus reference CID spectrum of butorphanol.

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

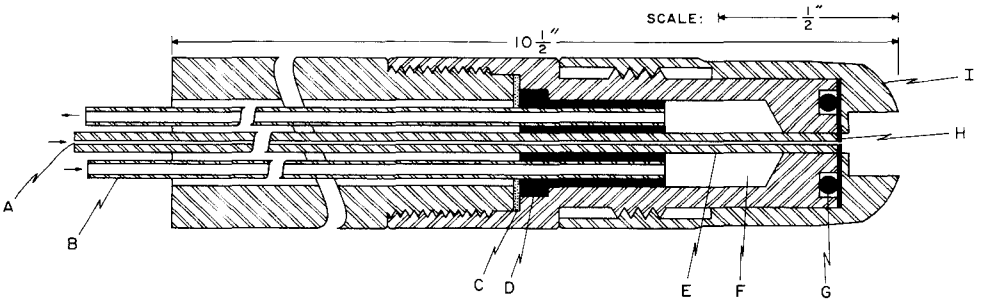


Figure 34. (A) micro LC effluent inlet line, (B) water cooling inlet tube. (C) Teflon washer for maintaining vacuum seal between probe tip/cooling chamber and probe shaft, (D) throughput tube collet, (E) 0.004 in. i.d. X 1/16 in. o.d. microbore tubing. (F) water cooling chamber, (G) Kalrez O-ring, (H) 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-ß-hydroxyprednisolone was accomplished on a 50 cm C_{18} HRSM microbore column using a flow rate of 34 µL 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-

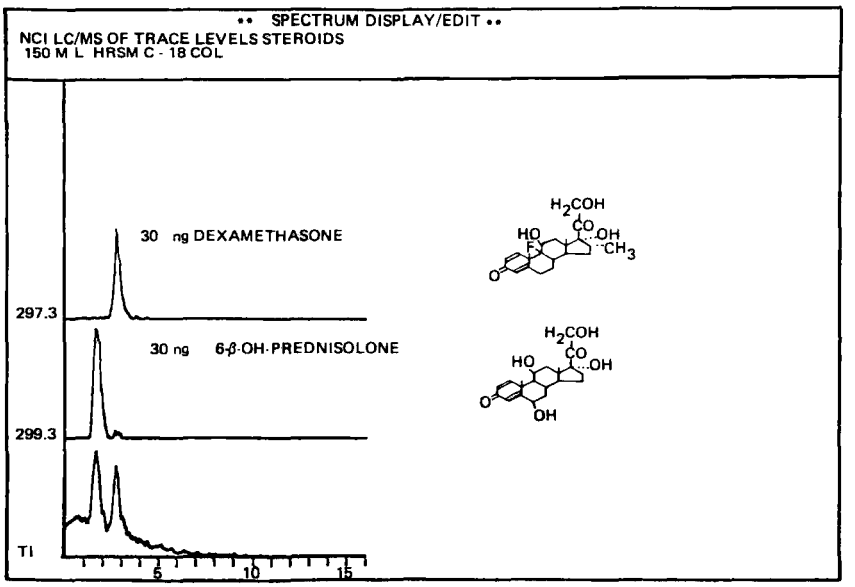


Figure 35. NCI micro LC/MS TICP and EICP for 30-ng levels of dexamethasone and 6-β-hydroxyprednisolone using 50% CH_3CN/H_2O at 34 μL/min as micro LC/MS eluent/CI reactant gas. The micro LC column was a C_{18} HRSM connected to an unchanged Waters ALC-202 pump and solvent programmer.

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

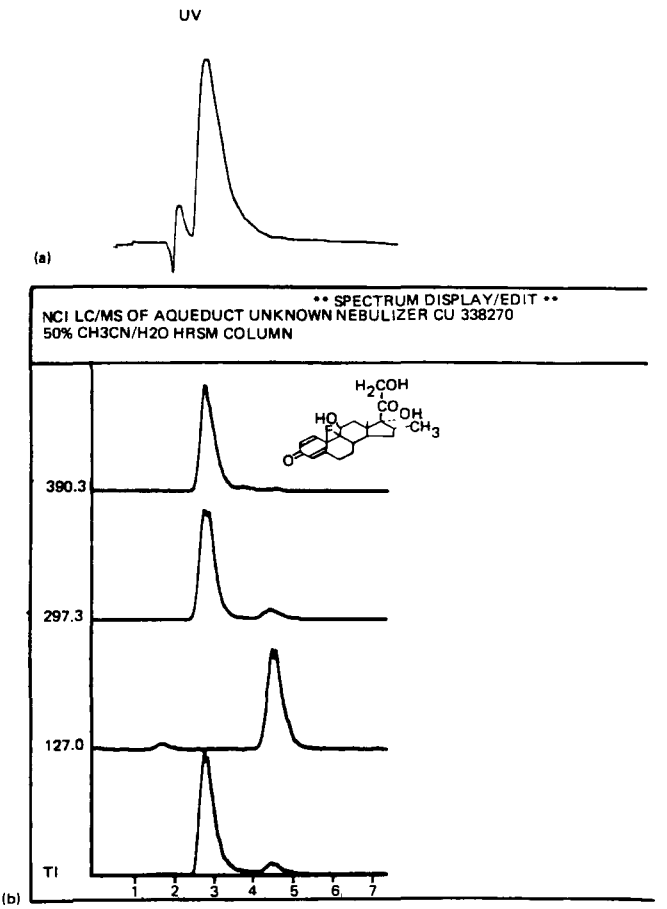


Figure 36 (A, upper) Micro LC UV trace for a TLC scrape of an unknown sample confiscated from a race track. The flow rate was 34 μ L/min 50% CH_3CN/H_2O on a C_{18} HRSM micro LC column and UV detection was 239 nm. (B, lower) NCI micro LC/MS TICP and EICP for the sample described in A. The major component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

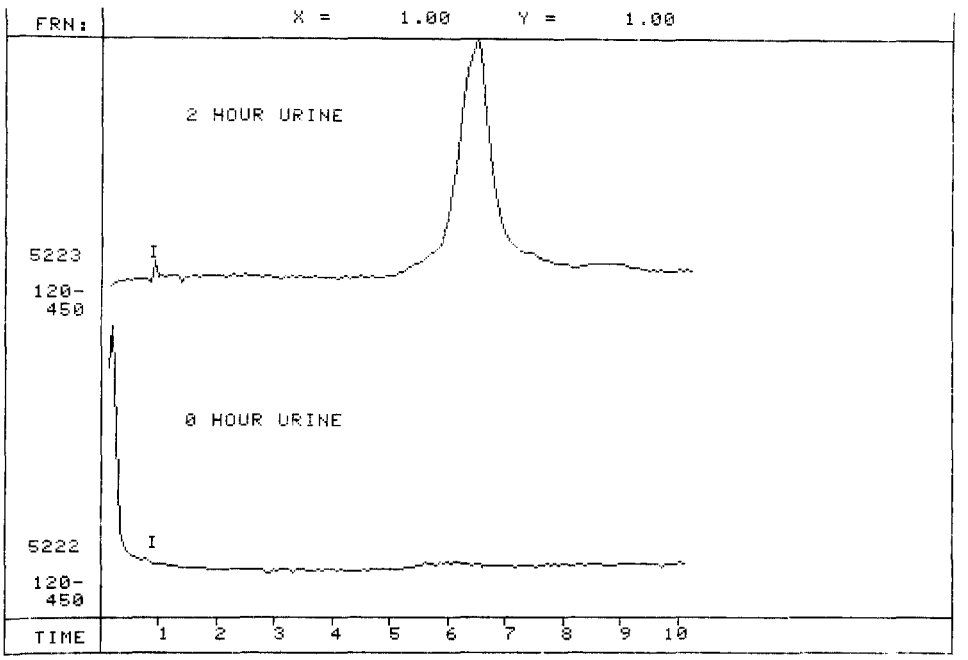


Figure 37. Negative-ion CI micro-LC/MS total ion current profiles of a TLC scrape from a zero-hour equine urine extract (lower), and an equine urine collected 2-h post oral administration of trichlormethiazide (upper).

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

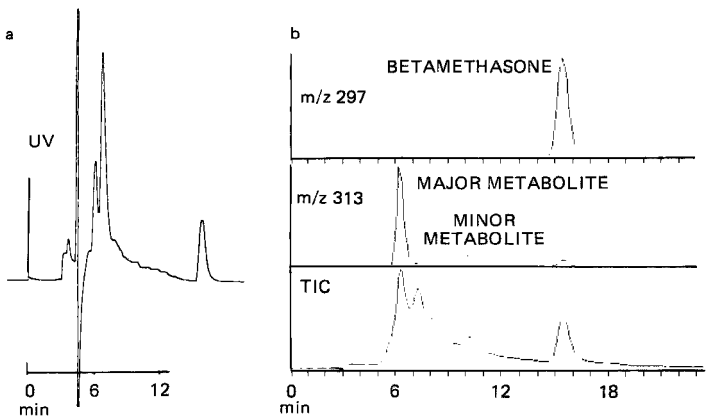


Figure 38. (a) micro LC UV chromatograms of a six hour post adminstration betamethasone equine urine extract utilizing 40 microliters per minute 70/30 methanol/water as eluent on an Alltech 1mm i.d. X 50 cm C_{18} reversed phase microbore column. (b) micro LC/MS ion current profiles of a six hour post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

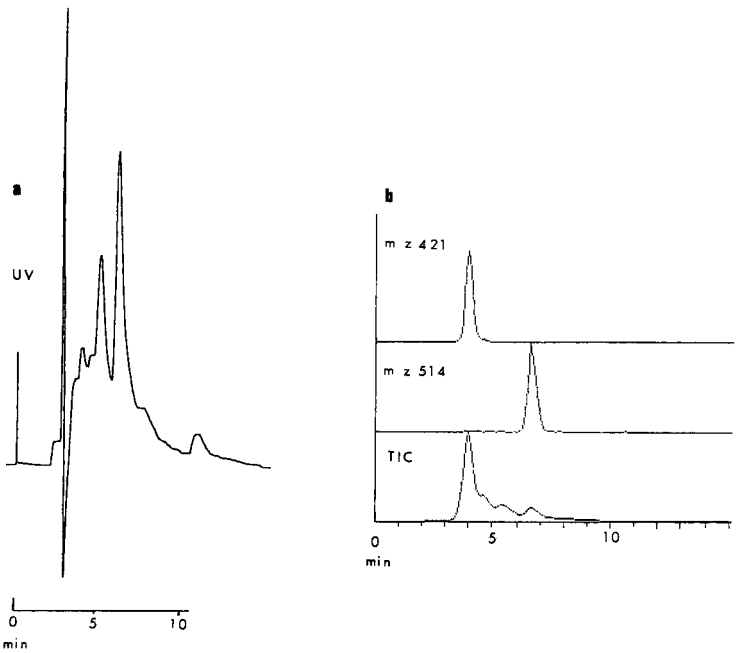


Figure 39. Micro LC UV chromatogram of a dichloromethane extract of a crude fermentation broth using aqueous methanol as eluent (b) micro LC/MS ion current profiles for antibiotics found in crude extract of fermentation broth.

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-

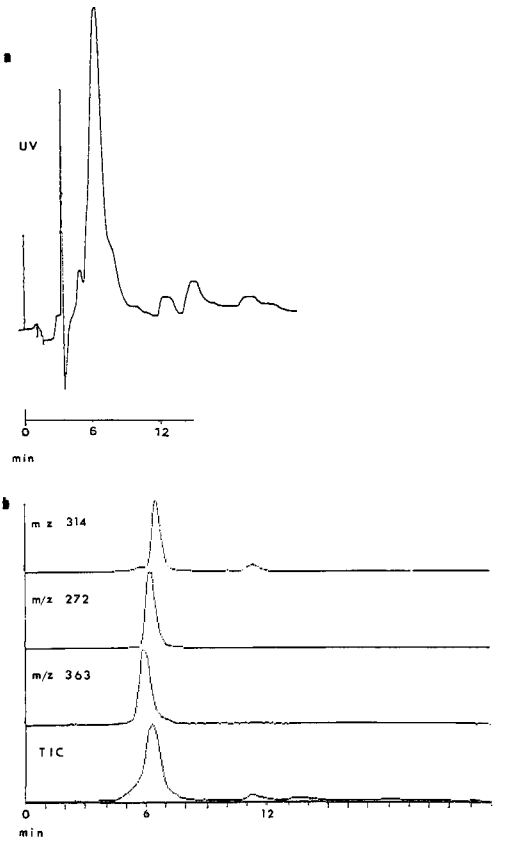


Figure 40. (a) micro LC UV chromatogram of preparative HPLC product from a synthetic mixture of Felodipine. (b) micro LC/MS ion current profiles indicating several different products detected in the preparative HPLC product.

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-

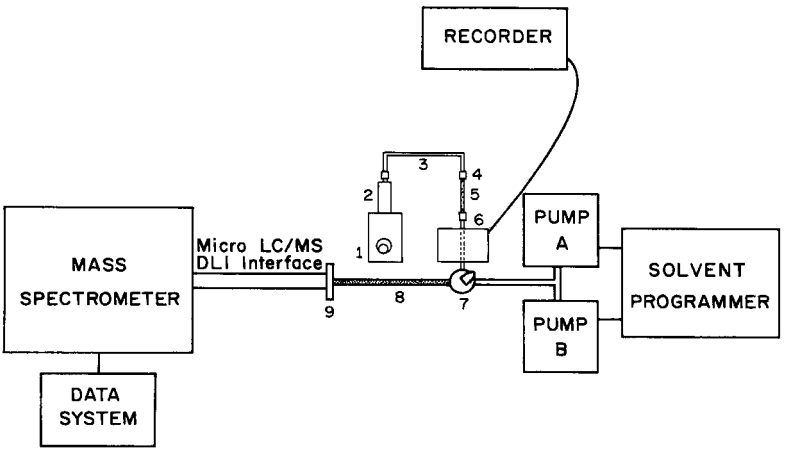


Figure 41. Schematic representation of multi-dimensional micro LC/MS system hardware.

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

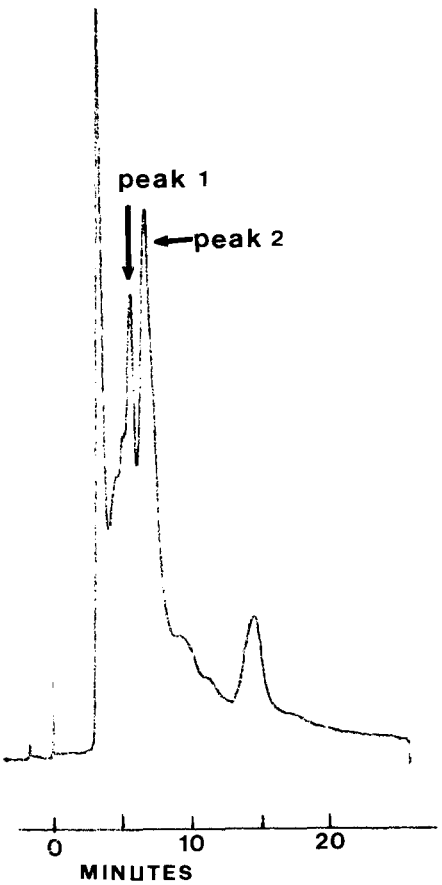


Figure 42. JASCO micro LC UV chromatogram of a crude equine urinary extract separated on a 0.5 mm X 200 mm silica micro LC column using dichloromethane/ethanol/water (90/6/4) flowing at 8 microliters min.

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-

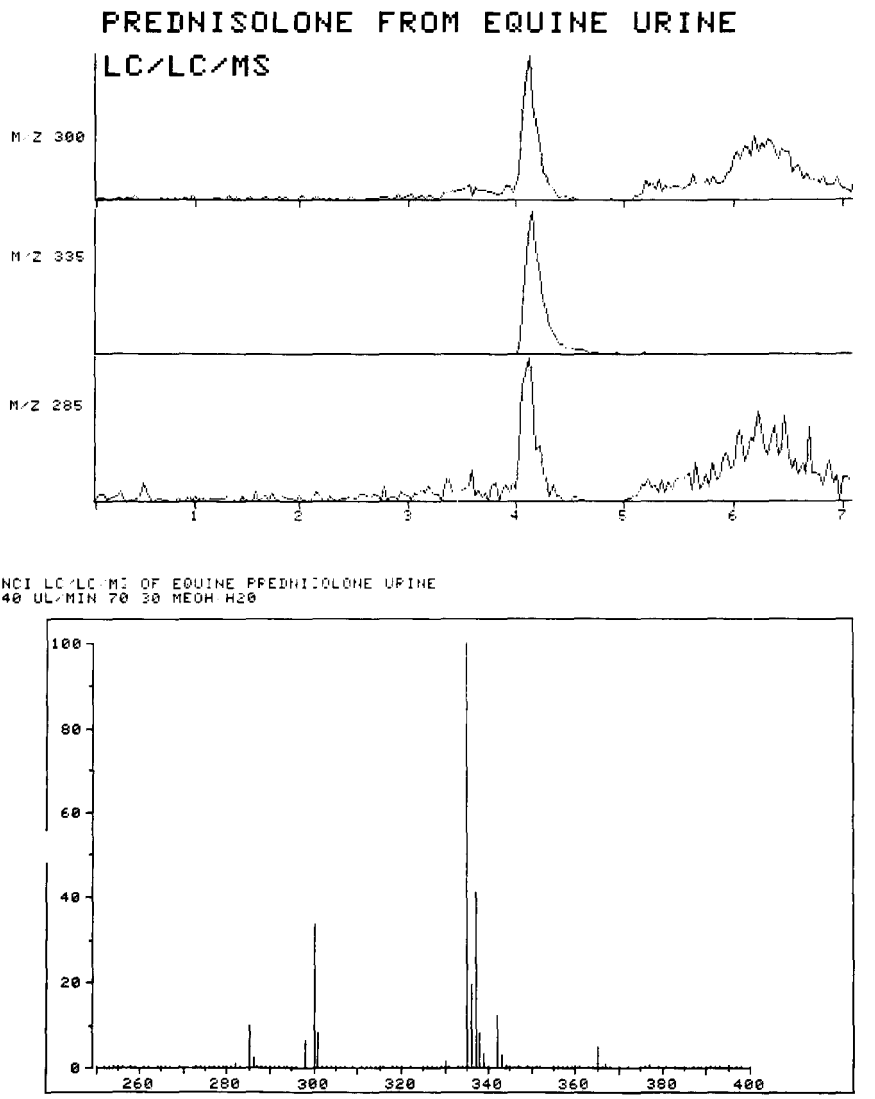


Figure 43. LC/LC/MS ion current profiles for methylprednisolone from an equine urine extract b. LC/LC/MS NCI mass spectrum of methylprednisolone from equine urine extract.

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

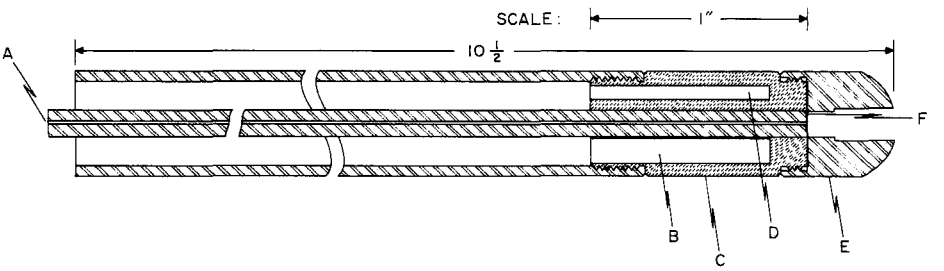


Figure 44. Dual purpose DLI/thermospray LC/MS probe interface. A. central micro-bore (0.004 in i.d.) throughput tube, B. 2-50 watt 120 V AC cartridge heaters, C. Heated copper vaporizer, D. thermocouple, E. removable end cap, F. stainless steel pinhole diaphragm.

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

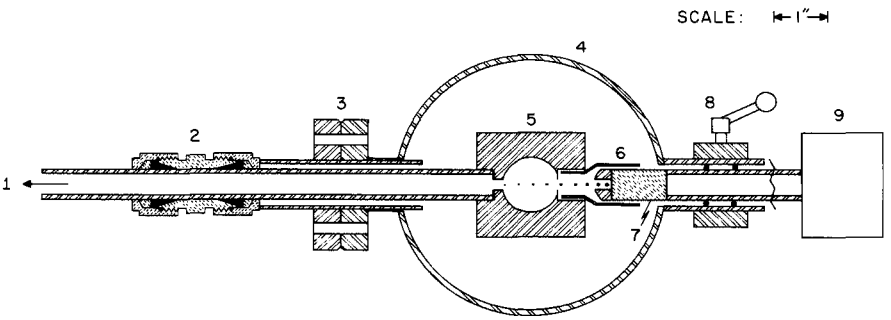


Figure 45. Schematic of DLI/thermospray inlet and pump out region of HP5985 GC/MS. A. to thermospray rotary pump. B. Swagelok union equipped with Teflon ferrules, C. outer tube welded to the high vacuum flange, D. high vacuum flange, E. half inch stainless steel slide tube, F. CI ion source, G. MS analyser 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

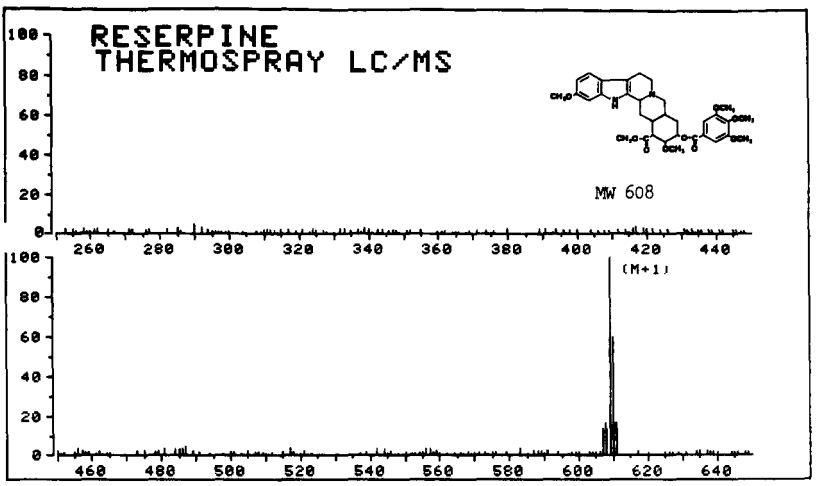


Figure 46. A. PCI FIA Thermospray LC/MS mass spectrum from 5 µg reserpine. B. PCI FIA thermospray LC/MS from 5 µg sulforhodamine B. The LC eluent conditions were 0.15 mL/min 50/50 CH_3OH/0.05 M NH_4OAC using a Brownlee 10 micron 005-222 reversed phase column.

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.

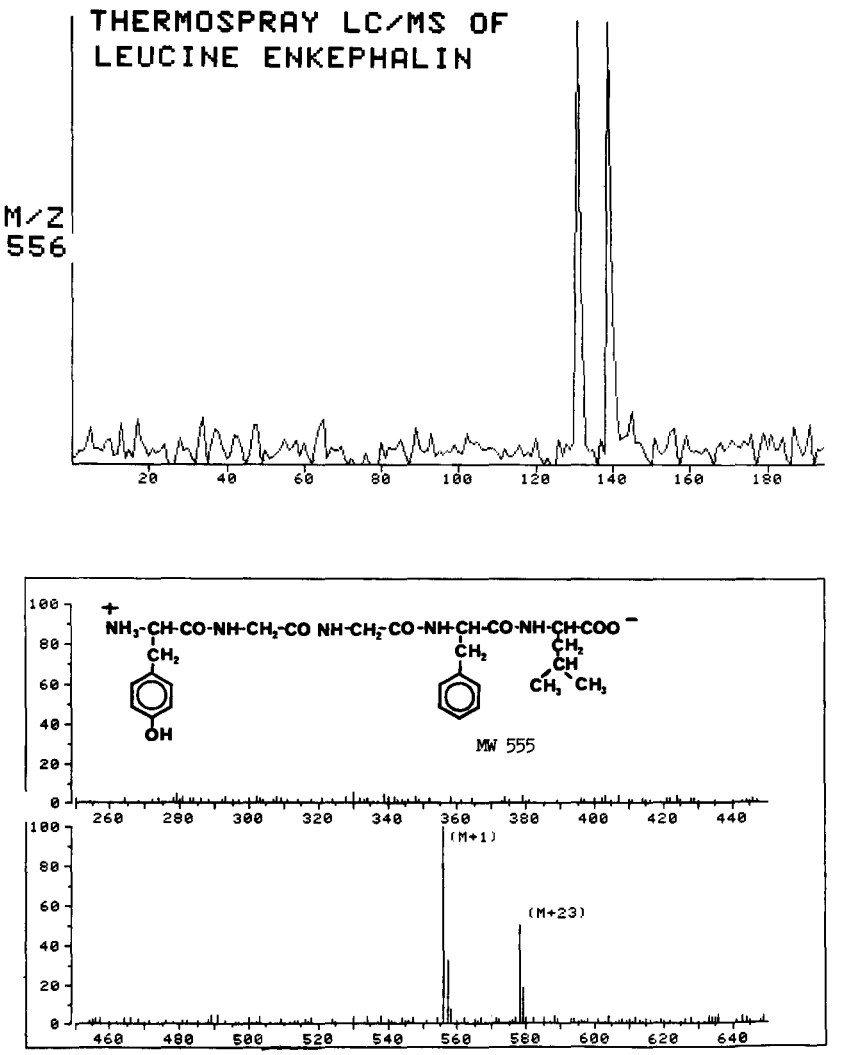


Figure 47. A. PCI FIA thermospray LC/MS ion current for the $(M+1)^+$ ion at m/z 556 from duplicate injections of 5 µg leucine enkephalin. The LC eluent was the same as the described for Figure 46. B. PCI thermospray LC/MS mass spectrum of leucine enkephalin taken from the first peak shown in A.

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.

REFERENCE

1. W.B. Buck, Poisons and the Veterinarian, 75th Annual Conference for Veterinarians, New York State College of Veterinary Medicine, Cornell University, Ithaca, N.Y. 1983.

2. A.M. Wilson and R.J. Bushway, High-performance liquid chromatographic determinatin of azinphosmethyl and azinphosmethyl oxon in fruits and vegetables, J. Chromatogr. 1981, 214, 140-147.

3. J.M. Lasker, K. Sivarajah, T.E. Eline, and M.B. Abov-Donia, High-performance liquid chromatography of neurotoxic phenylphosphonothioate esters and related compounds, Anal. Biochem., 1980, 109, 369-375.

4. R.T. Krause, Further characterization and refinement of an HPLC post-column fluorometric labeling technique for the determinatin of carbamate insecticides, J. Chromatogr. Sci., 1978, 16, 281-286.

5. M. DeBerardinis, W.A. Wargin, High-performance liquid chromatographic determination of carbaryl and 1-napthol in biological fluids, J. Chromatogr., 1982, 246, 89-94.

6. J.F. Lawrence, and R. Leduc, High pressure liquid chromatography with ultraviolet absorbance or fluorescence detection carboryl in potato and corn, J. Assoc. Off. Anal. Chem., 1978, 61, 872-876

7. Y.W. Lee, and N.D. Wescott, Direct analysis of carbofuran and 3-hydroxycarbofuran in rapeseed plants by reversed-phase high pressure liquid chromatography, J. Agric. Food Chem., 1980, 28, 719-722.

8. R.T. Krause, Multiresidue method for the determination of N-methylcarbamate insecticides in crops using high performance liquid chromatography, J. Assoc. Off. Anal. Chem., 1980, 63, 1114-1124.

9. A.C. Ray, L.O. Post, J.C. Reagor, High-pressure liquid chromatography determination of sodium fluoroacetate (Compound 1080) in canine gastric content, J. Assoc. Off. Anal. Chem., 1981, 64, 19-24.

10. D.M. Collins, J.P. Faucett, C.G. Rammel, Determination of sodium fluoroacetate (Compound 1080) in poison baits by HPLC, Bull. Envir. Contam. Tox. 1981, 26, 669-673.

11. R.A. Tasker, K. Nakatsu, Rapid, reliable and sensitive assay for warfarin using normal-phase high-performance liquid chromatography, J. Chromatogr., 1982, 346-349.

12. S.H. Lee, L.R. Field, W.N. Howald, W.F. Trager, High-performance liquid chromatographic separation and fluorescence detection of warfarin and its metabolites by postcolumn acid/base manipulation, Anal. Chem., 1981, 53, 467-471.

13. A.J. Kieboom, C.G. Rammell, Determination of brodifacoum in animal tissue by HPLC, Bull. Environ. Contaim. Toxicol., 1981, 26, 674-678.

14. J.D. Addison, Improved method for high pressure liquid chromatographic determination of chlorophacinone in in mouse tissue, J. Assoc. Off. Anal. Chem., 1982, 65, 1299-1301.

15. F.A. de Wolf, C.A.V. Tetteroo-Tempelman, P.M. Edelbrock, Determination of nanogram levels of the anticoagulant acenocoumarin in serum by high-performance liquid chromatography, J. Anal. Tox., 1980, 4, 156-159

16. J.X. DeVries, J. Harenberg, E. Walter, R. Zimmermann, M. Simon, Determination of the anticoagulant phenprocoumon in human plasma and urine by high-performance liquid chromatography, J. Chromatogr., 1982, 231, 83-92.

17. A.C. Ray, S.H. Tamulinas, J.C. Reagor, Applications of HPLC to Veterinary Toxicology, Amer. Assoc. Veter. Lab. Diagnos. , 1978, 21st Annual Proceedings, 185-194.

18. D.E. Mundy, A.F. Machin, The multiresidue determination of coumarin-based anticoagulant rodenticides in animal materials by high-performance liquid chromatography, J. Chromatogr., 1982, 234, 287-290.

19. J.D. Reynolds, A HPLC method for analysis of 8 anticoagulant rodenticides, Amer. Assoc. Vet. Lab. Diag. 1980, 23rd Annual Proceedings.

20. R.V. Slater, Determination of chlorsulfuron residue in grain, straw, and green plants of cereals by high performance liquid chromatography, J. Agric. Food Chem. 1983, 31, 113-117.

21. H.A. Maye, C.J. Miles, S.J. Scherer, A simplified high-performance liquid chromatographic residue procedure for determination of glyphosate herbicides and (amino methyl) phosphoric acid in fruits and vegetables em-

ploying post column fluorogenic labeling, J. Anal. Food Chem., 1983, 31, 69-72.

22. I. Ahmad, On-line trace enrichment of difenzoquat in water and its determination by HPLC, J. Environ. Sci. Health, 1983, 18, 207-219.

23. J. Osterloh, M. Lotti, S. Pond., Toxilogic studies in a fatal overdose of 2,4-D, MCPP, and chloropyritos., J. Anal. Tox., 1983, 7, 125, 129.

24. J.F. Lawrence, R. Leduc, High pressure liquid chromatographic analysis of Vrea herbicides in foods., J. Assoc Off. Anal. Chem., 1976, 61, 872.

25. D.S. Farrington, R.G. Hopkins, J.H.A. Ruzicha, Determination of residues of substituted phenylurea herbicides in grain, soil and river water by use of liquid chromatography. Analyst, 1977, 102, 377-381.

26. P. Carbras, P. Diana, M. Meloni, F.M. Pirisi., Reverse phase high performance liquid chromatography of pesticides. III Separation and quantitative determination of some rice field herbicides., J. Chromatogr., 1982, 234, 249-254.

27. M. Eichner, and R. Renner, Quick residue control of plant and animal food, tobacco, and tobacco-products by Sweep-Co analysis. 2. Herbicide residues, Z. Lebensm Unters Forsch, 1980, 170, 1-4.

28. M.L. Clark, D.G. Harvey and D.J. Hemphreys, Veterinary Toxicology, 1981, Macmillan Publishing Co. Inc., N.Y. 125.

29. A.Trenkle and W. Burroughs, Nutrition and Drug Interrelations, 1978, ed. J.D. Hathcock and J. Coon, Acad Press, N.Y., 577-611.

30. H.D. Stowe, B. Goelling, Experience with HPLC for diagnostic serum and tissue vitamin A assays. Amer Assoc. Veter. Lab. Diagnost., 1981, 24th Annual Proceedings, 385-396.

31. C.M. Stowe, T.D. Arendt, D. Barnes, J. Higbee, H.J. Kurtz, G. Nelson, G.R. Ruth, R.E. Werdin, and B. Yano, The frequency of animal intoxications in Minnesota: 1978, Amer. Assoc. Vet. Lab. Diag., 1978, 22nd Annual Proceedings, 423-434.

32. A.C. Ray, J.N. Dwyer, and J.C. Reagor, High pressure liquid chromatographic determination of vitamin D_3 in livestock feed supplements, J. Assoc. Off. Anal. Chem., 1977, 60, 1296.

33. G.L. Lookhart, Analysis of coumestrol, a plant estrogen in animal feeds by high-performance liquid chromatography, J. Agric. Food Chem., 1980, 28, 666-667.

34. W.A. Pons, High pressure liquid chromatographic determination of aflatoxins in corn, J. Assoc. Off. Anal. Chem., 1979, 62, 586-594.

35. R.M. Beebe, Reversed phase high pressure liquid chromatographic determination of aflatoxins in foods, J. Assoc. Off. Anal. Chem., 1978, 61, 1347-1352.

36. J.F. Gregory, and D.B. Manley, High performance liquid chromatographic quantitation of aflatoxin metabolites in animal tissues, J. Assoc. Off. Anal. Chem., 1982, 65, 869-875.

37. H. Cohen, M. Lapointe, High pressure liquid chromatographic determination and fluorescence detection of aflatoxins in corn and dairy feeds, J. Assoc. Off. Anal. Chem., 198164, 1372-1376.

38. G.D. Hanna, T.D. Phillips, L.F. Kubena, S.J. Cysewski, G.W. Ivie, N.D. Heidelbaugh, D.A. Witzel, and A.W. Hayes,High pressure liquid chromatographic determination of pencillic acid in chicken tissues, Poultry Sci., 1981, 60, 2246-2252.

39. G.H. Nelson, C.M. Christensen, and C.J. Mirocha, Fusarium and estrogenism in swine, J. Amer. Vet. Med. Assoc., 1973, 163, 1276-1277.

40. H. Cohen, and M.R. Lapointe, Sephadex-LH20 cleanup, high pressure liquid chromatographic assay and fluorescence detection of zearalenone in animal feeds, J. Assoc. Off. Anal. Chem., 1980, 63, 642-646.

41. L.J. James, L.G. McGirr, T.K. Smith, High pressure liquid chromatography of zearalenone and zearalenol in rat urine and liver, J. Assoc. Off. Anal. Chem. , 1982, 65, 8-13.

42. G.V. Turner, T.D. Philips, N.D. Heidelbough and L.H. Russel, High pressure liquid chromatographic determination of zearalenone in chicken tissues, J. Assoc. Off. Anal. Chem., 1983, 66, 102-104.

43. H.L. Trenholm, R.M. Warner, and R.E. Farnworth, High performance liquid chromatographic method using fluorescence detection for quantitative analysis of zearalenone and alpha-zearalenol in blood plasma, J. Assoc. Off. Anal. Chem., 1981, 64, 302-310.

44. M.E. Stack, and R.M. Eppley, High pressure liquid chromatographic determination of satratoxins Gand H in

cereal grains. J. Assoc. Off. Anal. Chem., 1980, 63, 1278-1281.

45. M.V. Howell and P.W. Taylor, Determination of alfatoxins, ochratoxin a, and zearalenone in mixed feeds with detection by thin layer chromatography and high-performance liquid chromatography, J. Assoc. Off. Anal. Chem., 1981, 64, 1356-1363.

46. D.E. Mundy, and A.F. Machin, Determination of pentachlorophenol and related compounds in animal materials by high performance liquid chromatography and gas chromatography, J. Chromatogr., 1981, 229-238.

47. B. Buttler and W.D. Horman, High-pressure liquid chromatographic determination of captan, captafol, and folpet residues in plant material, J. Agric. Food Chem., 1981, 29, 257-260.

48. J.E. Farrow, R.A. Hoodless, M. Sargent, and J.A. Sidwell, Determination of residues of post-harvest fungicides on citrus fruit by high-performance liquid chromatography, Analyst, 1977, 102, 752.

49. T.P. Hewlett, A.C. Ray, J.C. Reagor, Diagnosis of ethylene glycol (antifreeze) intoxication in dogs by determination of glycolic acid in serum and urine with high pressure liquid chromatography and gas chromatography-mass spectrometry, J. Assoc. Off. Anal. Chem., 1983, 66, 276-283.

50. A.C. Ray, S.H. Tamulinas, and J.C. Reagor, High pressure liquid chromatographic determination of cantharidin using a derivatization method in specimens from animal acutely poisoned by ingestion of blister beetles, Amer. J. Vet. Res., 1979, 40, 498-504.

51. A.C. Ray, L.O. Post and J.C. Reagor, GC/MS confirmation of cantharidin toxicosis due to ingestion of blister beetles, Vet. Hum. Tox., 1980, 22, 398-403.

52. A.C. Ray, J.N. Dwyer, G.W. Fambro and J.C. Reagor, Clinical signs and chemical confirmation of 4-amino pyridine poisoning in horses, Amer. J. Vet. Res., 1978, 39, 329-331.

53. R.W. Stringham, E.C. Mundell, and R.L. Smallidge, Use of post-column derivatization in liquid chromatographic determination of sulfamethazone and sulfathiazole in feeds and feed mixes, J. Assoc. Off. Anal Chem., 65, 823-827.

54. B.L. Cox, and L.F. Krzeminski, High pressure liquid chromatographic determination of sulfamethazine in pork tissue, J. Assoc. Off. Anal. Chem., 65, 1311-1315.

55. K.L. Eaves, B.M. Colvin, A.R. Hanks and R.J. Busnway, High pressure liquid chromatographic determination of sulfanitran and dinsed in medicated feeds and premixes, J. Assoc. Off. Anal. Chem., 60, 1064-1066.

56. C. Eckers, K.K. Cuddy, and J.D. Henion, Practical microbore column HPLC: System development and drug applications, J. Liq. Chrom. in press.

57. J.P. Sharma and R.F. Bevill, Improved high-performance liquid chromatograhic procedure for the determination of tetracyclines in plasma, urine, and tissues. J. Chromatogr., 1978, 166, 213-220.

58. M.C. Nahata and D.A. Powell, Simultaneous determination of chloramphenicol and its succinate ester by high-performance liquid chromatography, J. Chromatogr., 1981, 223, 247-251.

59. J.D. Henion and G.A. Maylin, Qualitative and quantitative analysis of hydrochlorothiazide in equine plasma and urine by high performance liquid chromatography, J. Anal. Tox., 1980, 4, 185-191.

60. V.L. Tal'rose, G.V. Karpov, I.G. Gordoetshii, and V.E. Skurat, Capillary system for the introduction of liquid mixtures into an analytical mass spectrometer. J. Phys. Chem. 1968; 40: 1658-1666.

61. M.A. Baldwin and F.W. McLafferty, Liquid chromatography mass spectrometry interface. Direct introduction of liquid solutions into a chemical ionization mass spectrometer, Org. Mass Spectrom., 1973; 7: 1111-1113

62. P.J. Arpino, B.G. Dawkins and F.W. McLafferty, Liquid chromatography mass spectrometry systems providing continuous monitoring with nanogram sensitivity, J. Chromatogr. Sci. 1974, 12, 574-579.

63. P. Arpino, M.A. Baldwin, and F.W. McLafferty, Liquid chromatography mass spectrometry, Continuous monitoring, Biomed. Mass Spectrom., 1974, 1, 80-83.

64. J. Serum and A. Melera, A new LC/MS system, presented at 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, Missouri, May 1978.

65. P.J. Arpino and P. Krien, Enrichment of the sample through a direct liquid inlet interface for on line LC/MS, presented at 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, Missouri, May 1978

66. P.J. Arpino, Progress in LC/MS interfacing based on the DLI concept, presented at 27th Annual Conference on Mass Spectrometry and Allied Topics, Seattle, Washington, June 1979.

67. A. Melera, Chemical applications of an LC/MS interface, presented at 27th Annual Conference on Mass Spectrometry and Allied Topics, Seattle, Washington, June 1979.

68. J.D. Henion, Continuous monitoring of total LC effluent by direct injection LC/MS/COM, presented at 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, Missouri, May 1978.

69. J.D. Henion, Drug analysis by continuously monitored liquid chromatography/mass spectrometry with a quadrupole mass spectrometer, Anal. Chem., 1978, 50, 1687-1693.

70. J.D Henion and T. Wachs, Micro liquid chromatography/mass spectrometry diaphragm probe interface, Anal. Chem. 1981, 53, 1963-1965.

71. C. Eckers, D.S. Skrabalak and J.D. Henion, On-line direct liquid introduction interface for micro-liquid chromatography/mass spectrometry: Application to drug analysis, Clin. Chem. 1982, 28, 1882-1886.

72. Hewlett-Packard Co. Palo Alto, CA 94304.

73. C.R. Blakely, J.J. Carmody and M.L. Vestal, An new soft ionization technique for mass spectrometry of complex molecules, J. Am. Chem. Soc; 1980, 102, 5931-5933.

74. C.R. Blakely and M.L. Vestal, Thermospray interface for liquid chromatography/mass spectrometry, Anal. Chem., 1983, 55, 750-754.

75. B.A. Thomson, J.V. Iribarne and P.J. Dziedzic, Anal. Chem. 1982, 54, 2219-2224.

76. W.H. McFadden, Liquid chromatography/mass spectrometry systems and applications. J. Chromatogr. Sci., 1980, 18, 97-115.

77. B.G. Dawkins and F.W. McLafferty, The mass spectrometer as a detector for high-performance liquid chromatography, in GLC and HPLC Analyis of Drugs (K. Tsiji and W. Morozowich, eds.), Dekker, New York, 1978.

78. P.J. Arpino and G. Guiochon, LC/MS Coupling, Anal. Chem., 1979, 51, 682A-701A.

79. Y. Hirata, M. Novotny, T. Tsuda, and D. Ishii, Packed micro capillary columns with different selectivities for liquid chromatography. Anal. Chem.,1979; 51, 1807-1809.

80. T. Tsuda, K. Hibi, T. Nakanishi, T. Takeuchi, and D. Ishi, Studies of open tubular micro capillary liquid chromatography, J. Chromatogr., 1978, 158, 227-232.

81. J.D. Henion and G.A. Maylin, Biomed. Drug analysis by direct liquid introduction micro liquid chromatography/mass spectrometry. Mass Spectrom., 1980, 7, 115-121.

82. J.D. Henion, Continuous monitoring of the total micro LC eluent by direct liquid introduction LC/MS. J. Chromatogr. Sci. 1981, 19, 57-64.

83. T. Takeuchi, Y. Hirata and Y. Okummura, On-line coupling of a micro liquid chromatograph and a mass spectrometer through a jet separator, Anal. Chem. 1978, 50, 659-660.

84. S. Tsuge, Y. Hirata, and T. Takeuchi, Vacuum nebulizing interface for direct coupling micro LC/MS, Anal. Chem., 1979, 51, 166-169.

85. Y. Hirata, R. Takeuchi, S. Tsuge and Y. Yashida, The application of a new sampling technique using an atomiser for CI MS. Org. Mass Spectrom., 1979, 14, 126-128.

86. S. Rottschaefer, L.B. Killmer, Jr., G.D. Robert, R.J. Warren, and J.E. Zarembro, Some applications of LC/CI/MS to pharmaceutical research, paper no 0129, 30th Pittsburgh Conference on ANlaytical Chemistry and Applied Spectroscopy, Cleveland, Ohio, March 1979.

87. R.G. Christensen and W.E. May, Detectors for liquid chromatographic analysis for polynuclear aromatic hydrocarbons, J. Liq. Chromatogr., 1978, 1, 385-399.

88. E. Dewey, G. Maylin, J. Ebel, and J. Henion, Metabolism of acetypromazine and promazine in the horse, J. Drug Metab. Dispos., 1980, 9, 30-36.

89. A.C. Cope and E.R. Trumbull, Olefins from amines: The Hofman elimination reaction and amine oxide pyrolysis, Org. React., 1960, 11, 317-493.

90. T.A. Bryce and J.R. Maxwell, Identification of the N-oxide group by mass spectrometry, J. Chem. Soc. Chem. Commun., 1965, 206-207.

91. W. Garland, B. Miwa, G. Weiss, G. Chen, R. Saperstern, and A. MacDonald, Determination of sulfadimethoxine in the liver and kidneys of swine and cattle by gas chromatography chemical ionization mass spectrometry and stable isotope dilution, Anal. Chem. 1980, 52, 842-846.

92. Y.Y. Lin and L.L. Smith, Active hydrogen determination by chemical ionization mass spectrometry, Biomed. Mass Spectrom., 1979, 6, 15-18. and references cited therein.

93. F.W. McLafferty, Tandem mass spectrometry (MS/MS): A promising new analytical technique for specific component determination in complex mixtures. Acc. Chem. Res., 1980, 13, 33-39.

94. J.D. Henion, B.A. Thomson and P.H. Dawson, Determination of sulfa drugs in biological fluids by liquid chromatography/mass spectrometry/mass spectrometry, Anal. Chem., 1982, 54, 451-456.

95. Sciex, Thornhill, Ontario, Canada.

96. B.A Thomson and L. Danylewych-May, Design and performance of an new total liquid introduction LC/MS interface, presented at 31st Annual Conference on Mass Spectrometry and Allied Topics, Boston, MA, May, 1983, Abstracts p. 852.

97. F.W. McLafferty and M.A. Baldwin, US Patent no. 3,997,298 (1976).

98. S. Tsuge, Y. Hirata, and T. Takeuchi, Vacuum nebulizing interface for direct coupling of micro-liquid chromatograph and mass spectrometer, Anal. Chem. 1979, 51, 166-168.

99. P.J. Arpino, H. Colin and G. Guiochon, On-line liquid chromatography, presented at 25th Annual Conference on Mass Spectrometry and Allied Topics, Washington, D.C., May 1977.

100. F.W. McLafferty and B.G. Dawkins, Continuous monitoring of liquid chromatography by mass spectrometry: Application on polypeptide sequencing, Biochem. Soc. Trans., 1975, 3, 856-860.

101. C. Eckers, J.D. Henion, G.A. Maylin, D.S. Skrabalak, J. Vessman, A.M. Tivert and J.C. Greenfield, Micro LC/MS applications: steroids, antibiotics and other biologically active compounds, Int. J. Mass Spectrom and Ion Phys., 1983, 46, 205-208.

102. K. Randerath, Thin Layer Chromatography. Academic Press, New York, 1965, pp. 51-53.

103. C. Horvath, The Practice of Gas Chromatography. L. Ettre and A. Zlatkis, eds., Interscience Publishers New York, 1967, pp. 226-232.

104. J. Sevcik and R.H. Gerner. Proceedings of the Third International Symposium on Glass Capillary Chromato-

graphy. R.E. Kaiser, ed., Institut of Chromatography, Bad Durkheim, West Germany, 1979, pp. 421-436.

105. G.C. Davis and P.T. Kissinger, Determination of serum dopamize-beta hydroxylase activity by reversed phase LC with column switching. Anal. Chem. 1979, 51, 1960-1965.

106. W.H. McFadden and H.L. Schwartz, Direct analysis of liquid chromatographic effluents, J. Chromatgr. 1976, 122, 389-396.

107. R.G. Christensen, H.S. Hertz, S. Meiselman, F. White V, Liquid chromatography/mass spectrometer interface with continuous sample preconcentration, Anal. Chem. 1981, 53, 171-174.

108. D. Games, G. Devant, D.J. Dixon, and L.E. Martin, 2nd Workshop on LC/MS and MS/MS, Montreux, Switzerland, Oct. 21-22, 1982.

109. T. Covey and J.D. Henion, A new direct liquid introduction/thermospray LC/MS interface, Anal. Chem., 1983, In press.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 317-351 (1984)

A REVIEW OF COMBINED LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

Dominic M. Desiderio and Genevieve H. Fridland
Department of Neurology and
Charles B. Stout Neuroscience Mass Spectrometry Laboratory
University of Tennessee Center for the Health Sciences
956 Court Avenue
Memphis, Tennessee 38163

ABSTRACT

The area of research involving the on-line combination of mass spectrometry and liquid chromatography has recently experienced a period of intense growth. A dependable LC-MS combination is eagerly awaited for by many researchers. The salient features of the combination aspect of LC-MS are analyzed in this review. LC-MS may operate in either the off-line or the on-line mode. The off-line mode is illustrated with the analytical measurement of biologically important peptides.

I. THE ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

A. INTRODUCTION

The on-line instrumental combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) is a natural combination that effectively couples the separation power of HPLC with MS, which is capable of achieving high levels of sensitivity and molecular specificity. The driving force for scientists to develop an on-line combination

 0148-3919/84/07S2-0317$3.50/0

of HPLC and MS is the need to decrease the number of experimental steps and manual interventions required to manipulate small amounts of precious endogenous biological materials, especially peptides. However, the coupling of these two very dissimilar instrumental methods is difficult to achieve and indeed, it is the authors' opinion that the rate of development of achieving an efficient and dependable on-line coupling for LC-MS is relatively slow, compared to the corresponding initial development of the combination of gas chromatography with mass spectrometry (1). Nonetheless, investigators are creatively approaching this interface problem and it is simply a matter of time until this combination technique will be in the hands of many investigators.

The literature pertaining to on-line LC-MS has been reviewed extensively (2-4).

B. BASIC CONSIDERATIONS

In principle, there are two different interfacing methods to achieve the on-line combination of HPLC with MS. On one hand, a direct liquid introduction (DLI) interface performs exactly what the name implies, that is, the liquid emerging from the HPLC column is introduced directly into the mass spectrometer. The solvent is removed by some appropriate process such as nebulization, thermospray, etc. On the other hand, the effluent from the HPLC column may be deposited upon a moving belt, which transports a chromatographic fraction from the external, high pressure region of the HPLC unit, through an interface, and into the high vacuum portion of the mass spectrometer where ionization is effected. These two separate interfacing techniques, DLI and moving belt, will be discussed in greater detail in the next section.

There are five operational parameters which must be thoughtfully analyzed and experimentally optimized for any particular type of on-line HPLC-MS development and

experimentation. These parameters include the use of a normal HPLC column versus a microbore column (5,6); the use of either a high voltage magnetic instrument or a low voltage quadrupole mass spectrometer; use of either the split or splitless mode for the LC-MS interface following the HPLC column; the use of either volatile or nonvolatile buffers for HPLC separations; and the selection of the ionization process, which plays a significant role in that either electron ionization (EI) or chemical ionization (CI) may be used to ionize the HPLC fraction, as opposed to a surface method such as secondary ion mass spectrometry (SIMS) or fast atom bombardment (FAB) mass spectrometry.

C. DIRECT LIQUID INTRODUCTION INTERFACE

One of the first sets of experiments to study the DLI of solutions into a mass spectrometer involved the use of CI mass spectrometry (7-11). The CI source for LC-MS followed the work with LC-MS in the EI mode. Chemical ionization LC-MS was developed to such a point whereby polypeptide sequencing was possible. For determining the amino acid sequence of a peptide, the authors felt that the information from a CI mass spectrum appeared to be at least as valuable as that information derived from EI. Direct solution introduction has the further advantage that the resulting direct CI mass spectrum allows sequencing of less volatile samples such as underivatized pentapeptides. In this study, the zwitterionic character of the peptide is eliminated by acetylation of the free amino group and esterification of the free carboxyl groups. The LC solvents used included acetonitrile and water. A capillary splitter interface is used in front of the HPLC UV detector and continuously introduces five to ten microliters of the LC effluent per minute into a CI mass spectrometer. Because only about one or two percent of

the peptide sample enters the mass spectrometer, the sensitivity of the method is decreased by a factor of 100. Mass spectral scans are cycled continuously during LC elution, and two or three corresponding mass spectra were obtained per HPLC peak. The HPLC solvent serves readily as the CI ionizing reagent although other reagents can be added concurrently to alter that type of ionization.

The critical instrumental parameters necessary for combining LC with a MS have been optimized. In one case, discussion centered on whether or not the solvent should be removed in the interface (12). It is stated that the basic philosophy in the development of the first LC-MS systems included:

1. introduction of the total HPLC column effluent into the separator;
2. selected removal of the chromatographic carrier;
3. vaporization of the neutral analyte;
4. ionization and mass analysis.

These initial developments led to transport systems which are based on the use of a moving belt or wire for conveying the solution, then the solute, through these four stages. In the cases of ionization, it was soon realized that preformed ions already existing in the solution could be directly vaporized and analyzed when enough energy is supplied to the liquid solution. Several methods are available for ionization and include electrospraying, electrohydrodynamic (EHD) ionization, field desorption (FD), thermospray ionization, and FAB mass spectrometry.

When interfacing a chromatographic column to a mass spectrometer, it is important to realize that the chromatographic column performs both as a separation and as a dilution unit (13). The problems involved with interfacing an HPLC unit to a mass spectrometer stem from the relative incompatibility between the HPLC effluent solution and the low pressure inside the source of the mass spectrometer (13). These two instrumental features make the coupling of the two instruments more difficult compared to the interfacing of a gas chromatograph to a mass spectrometer.

It is important to consider the main constraints which are introduced by the chromatographic process as well as to couple those constraints with the minimum requirements that the interface must possess. These considerations will accurately describe the degree of competitiveness that this interface would possess and will suggest compromises which are acceptable from the chromatographic point of view.

When combining an HPLC unit with a mass spectrometer, it is important to realize that, as this process occurs spontaneously (irreversibly) from a thermodynamic point of view, the decrease in entropy which arises from the chromatographic separarion is more than compensated for by the increase in entropy deriving from the dilution in the mobile phase. Whichever type of LC-MS interface that is selected for the on-line coupling, the use of a non-volatile HPLC buffer seems prone to considerable difficulties. Furthermore, although gradient HPLC elution is much talked about in LC research circles, that form of separation is relatively rarely used.

The chromatographer is always surprised by the low ionization yield (E) of the mass spectrometer. The factor E represents the number of ions collected on the mass spectrometer detector per number of molecules which is introduced into the ion source. It is important to review briefly the various sources of those losses (13):

1. To detect a signal at a given mass, and to calculate the location of the signal maximum, that is, the corresponding molecular weight, one needs approximately 100 ions at the detector entrance slit; practically all ions reaching this slit are detected.

2. The object and image slits in most mass spectrometers are of a rectangular cross-section. When a mass spectrometer is scanned, the product of these two (presumably identical) slits is a triangle. We require 200 ions to enter the analyzer. Losses in the analyzer itself are assumed to be negligible.

3. The extraction yield of ions from the source to the

analyzer across ion optics (ion-focusing) is about 10%. Therefore, we need 10 x 200, or 2,000 ions in the source.

4. For identification purposes, we need to obtain a complete scanned mass spectrum and the previous figure (2,000 ions) must be applied to those ions which account only for small peaks in the mass spectrum. Peaks that are 10% of the base peak must be detectable. Therefore, at least 2×10^{4} molecular ions must be formed during the time when the corresponding mass is scanned.

5. The ionization yields vary widely with both the ionization method used and for the particular compound being analyzed. While that efficiency may be nearly one for electron capture of haloaromatics, it may, on the other hand, be as low as 10^{-4}. If these two values are averaged to 10^{-3}, then 2×10^{7} molecules should be present during the scan.

6. The time to scan one mass in a spectrum is about one msec, and the introduction of sample molecules into the source must therefore proceed at a speed of approximately 2×10^{10} molecules per second.

The maximum concentration C_M of the Gaussian band of a solute of retention volume V_r and efficiency N is:

$$C_M = m\, N^{1/2} / V_r\, (2\pi)^{1/2} \qquad \text{eq. 1}$$

where m is the sample mass. If the column capacity is k' and the liquid cross-section of the column is s, then:

$$C_M = (m/s)\, (N^{1/2}) / [L\, (1 + k')\, (2\pi)^{1/2}] \qquad \text{eq. 2}$$

where L and u are the column length and the solvent velocity, respectively. The mass flow-rate of sample into the mass spectrometer source is then the product C_M F, where F (= Su) is the solvent flow-rate. With a splitting ratio r, the mass flow-rate of sample into the source is:

$$dm / dt = C_M Fr = (m \cdot u \cdot r \cdot N^{1/2}) / [L \cdot (1 + k') \cdot (2\pi)^{1/2}] \quad \text{eq. 3}$$

Comparing equation 3 with the condition (6) above;

$$N' \cdot (m \cdot u \cdot r \cdot N^{1/2}) / [M \cdot L \cdot (1 + k') \cdot (2\pi)^{1/2}] = 2 \times 10^{10} \quad \text{eq. 4}$$

where M is the molecular weight of the solute and N' is Avogadro's number. With L = 15 cm, N = 1.5 x 10^4 plates, u = 0.05 cm per second, r = 1, M = 500, and k' = 1, then m = 2x 10^{-10} = 0.2 nanograms = 200 picograms. This level of sensitivity is in general agreement with the specifications of modern mass spectrometer instruments which give the detection limit of 100 picograms of methyl stearate (M=298, m = 120 picograms). The latter specifications may not have been calculated with the rather favorable chromatographic conditions selected above: narrow peaks with small retention give large maximum concentration. The detection limit of a chromatographic detector is defined as the mass of a compound that generates a signal which is twice that of the noise.

On the other hand, if the mass spectrometer operates not in the scanning mode but rather as a true selected ion monitor, then a smaller amount of sample is necessary: with a one second time-constant, 1000 times less, or around one picogram would suffice for detection. The sensitivity values are similar for either a magnetic instrument or for a quadrupole. The only possibility of improving these detection limits in a significant fashion is whenever a very efficient ionization technique is utilized; a fact that explains why haloaromatics can be determined at the femotomole level with negative ions.

C.1. Controlled Desolvation Chamber

A high speed DLI interface is described (14). At the exit of the HPLC probe, a heated preevaporation chamber is provided, where the molecules have a low speed in a high

pressure region. Following that region is a short transition zone from which the molecules enter a high speed-low pressure region before entering the ion source. This latter region is the controlled desolvation chamber. The maximum velocity in the transition zone is sonic velocity.

A new desolvation chamber for droplet-focusing or Townsend discharge ionization is described as an interface for DLI into a mass spectrometer. This new desolvation chamber contains a conically-shaped volume and derives from considerations that utilize a slightly different concept because preliminary experiments show that the liquid droplets are highly positively-charged during nebulization, and that physical contacts with the walls of the desolvation chamber should in general be avoided. A positive potential on the walls therefore can be used to repel the droplets. On the other hand, by adoption of the appropriate conical geometry, droplets could either be focused along the drift tube axis or rather converge to an appropriate location. Other considerations of LC-MS interfacing have been published by this research group (15-17).

C.2. Thermospray

Thermospray is defined as the complete or partial vaporization of a liquid stream by heating it as it flows through a capillary (18). Thermospray has recently been demonstrated to be a versatile interface for combined LC-MS, where the heat is supplied electrically, and controlled by an electronic feedback system to maintain a constant level of vaporization. The optimal temperature for thermospray is a function of the solvent composition, flow rate, capillary dimensions and the sample to be analyzed. Thermospray interfaces were first heated by lasers, then by oxy-hydrogen torches, and most recently by cartridge heaters.

One of the first papers in this work describes a new soft ionization technique for the MS of complex molecules (19).

Briefly, the effluent from an HPLC column enters the vaporizer via a steel capillary tube which is partially immersed in a copper cylinder which is heated to about 1000°C by four small oxy-hydrogen flames. As a result of rapid heating, a jet of vapor and aerosol is produced near the exit of the stainless steel tube. The jet is further heated as it passes through the channel in the copper. It then undergoes an adiabatic expansion, and a portion passes through a skimmer to the ion source where the beam impinges on a nickel-plated copper probe which is electrically heated to about 250°C. While the ion source of the mass spectrometer is equipped with an electron gun for producing ions for normal CI operation, in the work described in this manuscript, that gun is turned off. When the charged particle undergoes a high energy impact with the heated probe, it is wholly or partially vaporized, and some of the resulting molecules are ionized. Spectra can be obtained for dinucleosides (CpG;ApU) and the pentapeptide leucine enkephalin (TyrGlyGlyPheLeu). Background ionization of the solvent is low and this fact, coupled with the reduced amount of fragmentation that is generally observed with this type of ion source, lead to the surprising result that the detection limit for the protonated molecular ion, $(M+H)^+$, of most of the nonvolatile substances investigated is substantially lower with the electron beam turned off!

Both positive and negative ion CI mass spectra were obtained for nonvolatile biologic samples in the one to ten nanogram range for full mass scans, and subnanogram quantities with selected ion monitoring (SIM) (19). It has been noted that vaporization is possible without pyrolysis because the sample spends a very short time in the high temperature region in the thermospray LC-MS interface. During that transit time, these samples are protected from overheating by the solvent. The liquid entering the hot region is heated from ambient temperature to the vaporization point in a few nsec., and the

vapor is expelled from the heat region within a msec. or less after it is formed. Experiments show that the detection limit (signal-to-noise-ratio of two) for arginine is 500 picograms and, for methionine, 2.5 nanograms. If the SIM measurement mode were used for only the intensity of $(M+H)^+$, the detection limits were predicted to be about one order of magnitude more sensitive. In this study, phosphate buffers cannot be used, but ammonium formate and ammonium acetate are used extensively with essentially no difficulties. The major limitation to the operation of the mass spectrometer is that the solvent vapor is also the CI reagent. This dual role of the same substance can cause difficulty in the simultaneous optimization of both the HPLC separation and the mass spectrometric detection.

As mentioned above, cartridge heaters have been used to replace the oxy-hydrogen torches in a thermospray interface readily adapted to quadrupole mass spectrometers (20). HPLC effluents are thermosprayed directly into the ion source, and the excess vapor is pumped away by the added mechanical pump which is directly coupled to the ion source through a port opposite the electrically-heated themospray vaporizer. When used with mobile phases containing a significant concentration of ions in solution (ca. 10^{-4} to 1M), no external ionizing source is required to achieve the detection of many nonvolatile solutes at the subnanogram level. The mass spectrum of the tridecapeptide renin substrate was shown and represents the largest molecule which has been successfully detected by using thermospray ionization. The $(M+H)^+$ of the renin substrate is 1,758 mass units.

A review of all the ionization techniques currently available for mass spectrometry for nonvolatile molecules has been published (21). A common feature of all of the ionization methods appears to be the direct production of ions from a condensed phase without formation of a neutral gas-phase molecule as an intermediate. An attempt is made to present a

unified ionization model which at least qualitatively accounts for the results obtained by the various techniques. A laser desorption mass spectrometer was interfaced to an HPLC unit using a moving stainless steel belt, where samples are sprayed online onto the belt under partial vacuum through a thermospray vaporizer. Laser desorption occurred with a laser. Two modes of operation are presented for the thermosprayer, one with (closed) and one without (open) the transfer line. Using an electrospray method for sample deposition, comparisons were made amongst the electrospray, closed, and open modes. It was assumed that the electrospray method has a 100% efficiency to transfer the sample; closed correlates to 40% and the open mode to 20% efficiency of covering the belt. A major limitation of the present moving belt system was found to be that the HPLC separations must be accomplished in less then nine minutes in order to prevent sample components from overlapping each other on that belt.

Triply and quadruply-charged molecules of nonderivatized glucagon (molecular weight equals 3,483 a.m.u.) are observed (22). In another experiment, a procedure is presented for peptide sequencing by utilizing an immobilized exopeptidase column which is directly coupled to a thermospray mass spectrometer (22). Amino acid sequence determination starting at the C-terminus is effected in this manner. This experimentation parallels that found previously for an immobilized enzyme (23). The underivatized peptide solutions are injected through a column containing immobilized carboxypeptidase Y and the amino acids were released, starting from the C-terminus of the peptide chain, and directly transported by a continuously flowing aqueous buffer into a thermospray mass spectrometer where the $(M+H)^+$ of each amino acid is determined (22). Temperature of enzymolysis is a factor because, at 22°, only two amino acids are found, whereas at 42°, five amino acids are determined from angiotensin.

C.3 Microbore LC-MS

The effluent from a microbore HPLC which operates at a flow rate of eight microliters min^{-1}, is introduced into a quadrupole mass spectrometer ion source operating in the CI mode (24). This paper studied evaporation of liquids from a capillary into a vacuum, where a jet was observed inside a glass envelope. The jet appeared to travel straight in air. However, when a vacuum was applied, that jet started to bend. This phenomenon is explained by considering the fact that both large and small droplets are formed and that the small droplets evaporate more rapidly than the larger droplets. If the small droplets are predominantly formed on one side of the jet, owing to the irregular shape of the orifice, the evaporating vapors from the small droplets push the large droplets away from the axis of the jet when a vacuum is applied.

Instrumental and analytical advantages have been taken of microbore HPLC coupled through a DLI interface to a CI mass spectrometer (25). The analytical capabilities of a microbore HPLC which is interfaced to an unchanged quadrupole mass spectrometer demonstrate continuous monitoring of the total HPLC effluent. Full scan CI mass spectra of drugs are obtained in the range of one to five nanograms. A microbore HPLC flow rate of eight microliters per minute is utilized and SIM provides a 20 picogram detection limit of a tranquilizer.

Conventional HPLC instruments generally operate at flow rates of 0.5 to two milliliters per minute. It is not practical to introduce the total HPLC effluent through a DLI interface. However, microbore packed columns operate at eluant flow rates of two to forty microliters per minute and are ideal for DLI. Because the entire effluent from a microbore HPLC can be directed into the ion source for mass spectral analysis, nearly 100-fold increases in sensitivity may be realized (26). Conventional HPLC systems yield peak volumes of 0.5-2.0 ml, where that range depends upon the flow rate and column efficiency. On the other

hand, utilizing microbore HPLC, peak volumes are found in the range of 10 to 30 microliters. The smaller volume in the latter case provides a much more concentrated solution passing through the detector, with a concomitant increase in the detection limits (27).

C.4. Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MS-MS)

In another study determining sulfa drugs in biologic fluids, it is noted that one limitation results from the DLI technique of LC-MS because the mass spectra are very simple and they lack sufficient specificity for elucidation. Futhermore, it is always possible that co-eluting compounds will appear superimposed in the mass spectrum, a process which renders the interpretation rather difficult. The novel technique of combined liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) offers the analytical capability of also providing collision activated dissociation (CAD) mass spectra of the characteristic LC-MS ions for each of the components of interest (28). In this study, the potential of the more open atmospheric pressure ionization (API) source design is utilized in combination with HPLC and mass spectrometry in a triple stage quadrupole (TSQ) instrument. Nitrogen is used as the collision gas with an effective target thickness of approximately 2×10^{14} molecules centimeter^{-2}. This study was prompted by the extreme paucity of fragmentation that occurs in the positive CI LC-MS mode.

C.5. Liquid Chromatography-Negative Chemical Ionization Mass Spectrometry

In another study utilizing HPLC, negative ion CI mass spectrometry was begun because of the need to analyze

mixtures of explosives (29). Post-explosion analysis of residues is noted to be very relevant in criminalistic bombing. A simplified construction of a new micro LC-MS probe is presented, where the volume of the HPLC microcell is calculated to be 1.2 microliter, and which maintains the one centimeter pathlength of the conventional cell, where the latter has a 15 microliter volume (30).

A desolvation chamber of new design sprays the HPLC effluent into droplets through a pinhole diaphragm (31). One of the most effective ways of controlling the desolvation of the solute droplets is to use a heated zone, usually called a desolvation chamber, in which the droplets acquire a high speed before entering the CI source. Three different desolvation chambers were studied and include the standard Hewlett-Packard, extended, and solvent-stripping models. A review of the micro LC-MS methodology used in drug analysis and metabolism studies has been published (32) and the quantitative analysis of betamethasone in equine plasma and urine by DLI micro-LC-MS is presented (33).

A new version of the thermospray LC-MS interface is described, where this version differs from the previously described interfaces in that it is a dual purpose probe-type interface which is introduced conveniently into the mass spectrometer via a standard direct insertion probe inlet (34). The device is a dual purpose LC-MS interface and it can provide conventional DLI LC-MS, or the copper vaporizer may be heated electrically to produce thermospray ionization. Detection limits are currently about 100 nanograms in the thermospray mode for nonvolatile, labile compounds using two mm i.d. microbore LC columns and a flow rate of about 150 microliters per minute.

C.6. Ultrasonic Interface

An ultrasonic spraying device was constructed to overcome difficulties in spraying aqueous solvents into the vacuum

system of a mass spectrometer. The ultrasonic vibration is achieved by means of a magnetoconstriction in the nickel inlet tube (35).

C.7. Segmented Wire Interface

It has been noted that the DLI technique has intrinsically simple implementation and that the moving belt interface has significant advantages in several parameters including solute concentration- a process that introduces a greater proportion of the sample into the mass spectrometer ion source (35,36). This group approached the interface problem by preconcentrating a liquid stream and introducing the effluent into the MS by means of DLI. The viewpoint is that the advantages of both ordinary DLI and the moving belt technique would be combined. The described interface device concentrates a liquid stream by allowing it to flow down a resistance-heated stationary wire having three successively-decreasing diameters of 0.8 millimeters, 0.6 millimeters and lastly, 0.3 millimeters, where each section has a length of 15, 7.5, and 1.5 centimeters, respectively. When a drop becomes too large, it flows either down the wire or along the outer surface of the DLI probe, and is lost. A light-emitting diode and photocell are fitted at the gap and sense the size of the drop. Electronic feedback from the photocell controls the current through the wire to hold the drop size constant. The instrumental parameters are met only with the wire that was not of homogeneous construction, and the three-segment wire design described above was arrived at empirically. A description is provided where the performance of the concentrator wire is tested in an effort to determine the maximun flow which could be accomodated and still maintain a 95% evaporation of the solvent, corresponding to a 230-fold increase in sample concentration.

C.8. Nebulization

The design of a DLI interface is described which uses a jet of helium gas to aid in the nebulization of the HPLC effluent and sample into the MS source (37). Interfaces used in coupling HPLC with MS fall into two basic categories: transport interfaces and DLI interfaces. A number of systems have been used in the DLI approach for introducing the LC effluent into the MS source and include formation of liquid jets through either viscous flow capillary or 1.5 micron diaphragms and vacuum nebulization techniques.

C.9. Supercritical Fluid Injection

Direct supercritical fluid injection interfaces have been designed for use with mass spectrometry (38). Direct fluid injection (DFI) mass spectrometry utilizes supercritical fluids for solvation and transfer of materials to a CI mass spectrometer source. Supercritical carbon dioxide with isobutane as the CI reagent gas has been used and DFI MS/MS is also illustrated for major ions in the isobutane CI mass spectrum of T2 toxin. More polar compounds may be analyzed using supercritical ammonia. This alternative LC-MS approach uses a supercritical fluid or "dense gas" for efficient transfer of material to the gas phase in the CI source. The DFI method allows mass spectra to be obtained for essentially any compound which is soluble in the supercritical fluid and hence allows a rapid qualitative evaluation of fluid phase solubility.

At high pressures and above the critical temperature, the resulting fluid or dense gas attains a density approaching that of a liquid which has relatively strong internal molecular interactions and therefore assumes some of the properties of a liquid. In describing the DFI process, the supercritical fluid exits from a 50 to 500 atmosphere pressure region through a

hole into another pressure region which contains a Mach disk, where initial clusters have a diameter of approximately 30 angstroms. The shock waves resulting after the Mach disk then transfer into a molecular spray which enters a CI region of a mass spectrometer at a pressure of approximately one torr. The Mach disk is characterized by two phemomena - the destruction of the highly-directed jet and the collisional energy transfer resulting in a redistribution of the directed kinetic energy of the jet among the various translational, vibrational, and rotational modes of the molecule.

The use of capillary column supercritical fluid chromatography (SFC)- MS can obviate the difficulities associated with previous interfaces and allows a simple interface readily adapted to existing GC-MS systems (39). The combination of SFC with mass spectrometry offers the following potential advantages relative to GC-MS or LC-MS methods:

1. high molecular weight, polymeric, polyfunctional, and thermally labile compounds can be separated, as well as more volatile species;

2. capillary SFC columns can provide greatly enhanced chromatographic efficiency relative to HPLC due to solute diffusivities which are about 100 times greater in the supercritical fluid than in the corresponding liquid phase and similar to the gas phase;

3. soluting power of the mobile phase can be readily controlled with pressure programming. Mixed mobile phases, gradient, and temperature programming are also feasible;

4. SFC using a capillary column provides low mobile phase flow rates which, when coupled with high mobile phase volatility, allows optimal interfacing of SFC and mass spectrometry.

Optimal mobile phase flow rates of five to 80 microliters per minute (supercritical fluid), depending on column diameter and pressure, may be obtained in this combination instrument. The SFC mobile phases used up to now in this work are isobutane and normal pentane. Initial evaluations of

the capillary SFC-MS interface demonstrate it to be mechanically simple and reliable. Polyaromatic hydrocarbon studies indicate that a detection limit of approximately one picogram is achieved.

C.10. Examples of LC-MS Analysis of Compound Types

Glucuronides are characterized using a thermospray LC-MS interface (40). Ten nanograms of the glucuronide injected onto a column suffice for characterization by a scanned mass spectrum. Unlike many of the other currently available LC-MS interfaces, the thermospray ionization interface allows 100% of an aqueous effluent to enter the mass spectrometer at flow rates up to 1.5 ml per minute.

Nanogram amounts of the peptides leucine enkephalin, methionine enkephalin (TyrGlyGlyPheMet), and alpha-amanitin are obtained by direct LC-MS (41). Both $(M+H)^+$ and $(M-H)^-$ ions are obtained in the positive and negative ion modes, respectively.

D. MOVING BELT INTERFACES

D.1. Introduction

The addition of a modified segmented flow extractor between an HPLC and a mass spectrometer permits the direct coupling of an HPLC operated in the reversed phase (RP) mode to a mass spectrometer, without compromising the operational characteristics of either instrument (42). Ion-pairing techniques were studied and demonstrate compatibility with on-line LC-MS (43).

Several approaches have been explored for the direct LC-MS combination:

1. A moving wire/belt system that transports the LC effluent through a series of vacuum locks for the solvent to be

evaporated. The sample is then introduced into the ion source for analysis by either EI or CI.

2. A 1% split of the LC effluent into the mass spectrometer, where the LC solvent is used as the CI reagent gas.

3. A vacuum nebulizing interface to introduce total effluent from a microbore HPLC into the ion source.

4. The system that converts the LC effluent into a molecular beam by forcing it through a nozzle restriction, followed by flash evaporation using a laser beam or sonic radiation, and then mass spectrometric analysis in either the EI or CI mode.

5. Direct evaporation of the total effluent into the mass spectrometer ion source followed by CI under API conditions.

6. A silicone membrane enrichment device which removes the solvent and permits preferential entry of the solute into the mass spectrometer.

The segmented-flow LC-MS interface desalts the organic phase with high efficiency. This fact is demonstrated by analyzing sodium ions and phosphate ions at the 0.3 ppm and 10 ppm levels, respectively.

In a further development of this type of LC-MS interface, a specially designed nebulizer was constructed for deposition of the effluent from the HPLC column onto a moving belt (44). Another aerosol liquid deposition device was described (45). Measurements of the solvent transfer efficiency to the belt were performed by first spraying a peak from the HPLC column onto the belt, and then comparing that peak area with the area obtained by depositing the same mass of sample onto the belt with a syringe, where the syringe was assumed to provide 100% efficiency in the transfer of the solvent onto the belt. A belt interface can be thought of as consisting of four basic steps, each of which may potentially effect chromatographic performance:

1. transfer or deposition step;
2. evaporation of the solvent remaining on the belt with an infrared heater;

3. passing of the solvent into the vacuum lock system;
4. desorption of the sample from the belt into the ion source.

The influence that the deposition step can have on the performance of the LC-MS interface is critical when comparing the conventional method of flowing the effluent onto the belt in a continuous stream versus spray deposition. Dispersion of the LC effluent into a fine mist can provide an efficient evaporation step. It is found that the dimensions of the orifice through which the liquid flows must be minimized to prevent liquid from accumulating on the glass tip, and also to allow formation of the smallest possible droplets. By using a 60° angle between the spray tip and belt, droplet formation on the belt behind the tip is minimized. Attention to these critical experimental steps is of primary importance in order to obtain good chromatographic fidelity including peak shape, variance, area, and reproducibility.

D.2. Peptide Studies

Using a quadrupole mass spectrometer outfitted with a moving belt interface, N-acetyl-N,O,S-permethylated oligopeptides were analyzed (46,47). Isobutane CI yields good intensity $(M+H)^+$ and N- and C-terminal ions. In addition, C-methylated peptides are separated by LC.

Permethylated peptides and peptide mixtures have been studied employing normal phase chromatography and a moving belt LC-MS interface (48). Eighteen different peptides, ranging in size from di- to heptapeptides, were studied and it was found that on-line LC-MS ammonia CI spectra produced complete or almost complete amino acid sequence determination information.

D.3. Analysis-of-Variance of System Components

Extra-column band-spreading on an HPLC-MS moving belt interface was analyzed by a numerical evaluation of the system variance (49). Spraying effects may be evaluated by considering the increase of the variance (the second moment of mass) of the chromatographic band. Variances are additive when contributions are independent.

D.4. Review of LC-MS Transport Devices

HPLC interfaces with transport devices were reviewed (50) and a comparison is made of moving belt interfaces for LC-MS (51). In the interfacing of the HPLC to a mass spectrometer, three fundamental problems must be overcome:

1. how to make the mass spectrometer, which can handle 20 cc per minute of gas if configured for CI, compatible with solvent flow rates of the order of one cc per minute which result in gas volumes in the range of 150 to 1200 cc per minute, depending on the solvent used;
2. introduction of the solute into the mass spectrometer so that mass spectral information can be obtained and the solute does not undergo thermal decomposition;
3. coupling of the HPLC with the mass spectrometer so that there can be no loss of the chromatographic performance.

It is noted that a further problem which occurs is that, with aqueous solvent systems containing more than 50% water, beading of the solvent on the belt causes pressure fluctuations in the ion source resulting in poor mass spectral data. Although microbore HPLC was initially utilized with the moving belt system, it has had little subsequent use.

A table in this review (50) collects the applications and corresponding references of HPLC using transport type

interfaces and includes the following compound types: aflatoxins, Amaryllidaceae alkaloids, antibiotics, aromatic acids, bile acids and their conjugates, carbamate pesticides, chinchona alkaloids, chlorinated phenols in urine, coal liquification products, dinitrophenyl hydrazones, drugs, effluent analysis, ergot alkaloids, glycosides, herbicides, lipids, liquid crystals, natural coumarins, nucleosides, peptides, pesticides, petroporphyrins, polychlorinated biphenyls and their metabolites, polynuclear aromatics, rotenoids, steroids, sugars, triglycerides, and waxes.

D.5. Ribbon Storage Device

A novel ribbon storage interface is described (52, 53). The distance between the mass spectrometer and the HPLC unit approximates five feet. This LC-MS interface is designed for use with SIMS and with conventional EI. The LC-MS interface includes a 120 cm region at atmospheric pressure. Aerosol deposition of the HPLC effluent allows the complete evaporation of the mobile phase before the first vacuum slit, and a 320 cm total length allows the storage of chromatographically-separated materials. Ten picograms of amino acids have been detected. The long ribbon also allows temporary storage of five to 60 minutes of HPLC separations on the ribbon for subsequent reanalysis by SIMS or EI.

A new method for ribbon cleaning using vapor deposition of a thin layer of silver is also described (52,53) which reduces background from contaminants and residues on the ribbon and is superior to heaters or solvent baths for ribbon cleaning. The liquid is deposited on the ribbon surface by an aerosol liquid deposition device. The heart of the interface is a ribbon (0.63 cm wide, 0.0087 cm thick, and 320 cm long) spot-welded to perform a continuous loop. Ribbons of high-purity nickel, molybdenum, and platinum are found to

have acceptable mechanical properties; most of the work is done with high-purity nickel.

E. IONIZATION METHODS FOR LC-MS

Ionization methods available for LC-MS are reviewed (54). Nine different methods are described and include desorption chemical ionization (DCI), laser desorption (LD), field desorption (FD), electrohydrodynamic ionization (EHI), ^{252}Californium plasma desorption (PD), SIMS, FAB, API and thermospray ionization technique.

F. CONCLUSIONS

At the present time, no one of the on-line interface devices described above has taken charge of the field to become universally useful in laboratories around the world. Each one of the proponents of the different on-line techniques can describe the advantages and disadvantages of each system. It is clear from the data and experience published in the literature that more developmental time is needed. That hesitation of employing the commercially available HPLC-MS interfaces notwithstanding, it is quite clear that this type of on-line LC-MS interface, once it has been appropriately developed and utilized, will significantly increase the use of mass spectrometry as the detector for HPLC for the measurement of endogenous compounds.

II. OFF-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY: EXAMPLES OF PEPTIDE MEASUREMENTS.

A. INTRODUCTION

While the first section reviewed the on-line combination of LC-MS, this section will focus on the off-line combination of

LC and MS, where a compound eluting at a known retention time is manually collected and is measured by MS techniques. Extensive analytical measurements have been made of endogenous peptides extracted from biological sources.

In this section, advantage is taken of the fast and facile production of $(M+H)^+$ ions of biologically important peptides by means of FAB mass spectrometry (55). Doubly-charged molecular ions of insulin have been observed in some instruments (56) and singly-charged ions of the proinsulin molecule in other instruments (57). However, because of the relatively high background produced by the matrix needed for FAB mass spectrometry, the resolution of this analytical system must be increased in one of two ways (58). On one hand, the mass resolution can be increased up to one part in ca. 20,000 to effectively isolate the accurate mass of $(M+H)^+$. However, especially in the case of peptides, it is known that even the $(M+H)^+$ of a peptide lacks sufficient molecular specificity for unambiguous quantification. On the other hand, structural resolution can be significantly increased by taking advantage of linked-field scanning MS techniques (59), especially in the B/E mode, where one selected product ion is selected from a precursor ion. In both cases, fragmentation can either be increased, or created, by the use of CAD processes.

Internal standards (60, 61) can be efficiently produced for measurement of peptides by incorporating a stable isotope such as ^{18}O into the peptide molecule (62, 63).

B. CONSTRUCTION OF CALIBRATION CURVE

The method of analysis employing the FAB-CAD-B/E-B'/E'-SIM-microcomputer measurement mode must demonstrate a linear response over the range of concentrations of endogenous peptides. The use of both the stable

isotope-incorporated peptide internal standard and this novel analytical measurement mode are important to demonstrate linearity and to overcome biologic matrix effects. Primary data from one of the accelerating voltage alternation experiments for leucine enkephalin versus ^{18}O leucine enkephalin were used to monitor the peak at mass 336 in the former case and 340 in the latter case. This selected pair of ions corresponds to the tripeptide sequence -GFL which derives from the CAD-B/E analysis mode (64). This amino acid sequence-determining ion is a product ion arising only from the $(M+H)^+$ ion of leucine enkephalin- a most significant experimental fact that rigidly maintains the molecular specificity of the analytical measurement.

The microcomputer interface is used to accept a large number of analog signals to optimize ion statistics (65). Integrated areas are calculated and the ratio of the known amounts of the ^{16}O and the ^{18}O species are plotted. The calibration curves for both the methionine enkephalin (ME) and leucine enkephalin (LE) experiments show that both curves intercept at, or very near, the origin and have correlation coefficients near unity. The statistical parameters for the two best-fit straight lines are, for ME: $y = 1.09x + 0.06$, $r^2 = 0.999$ and for LE: $y = 0.69x$, $r^2 = 0.995$.

C. HYPOTHALAMUS TISSUE EXTRACTS

The RP-HPLC chromatogram for canine hypothalamus tissue demonstrates that, at the UV wavelength being monitored (200 nm), the RP-HPLC chromatogram of a hypothalamic peptide-rich fraction is relatively clean of UV-absorbing material (66, 67). It is quite important to remember that, of course, a wealth of biologically active radioreceptorassayable, bioassayable, and/or radioimmunoassayable material may, and generally does, coelute on this chromatogram at the indicated retention times

and usually at other retention times (68, 69). However, it is experimentally observed that, at a UV detection sensitivity level of 0.1 AUFS, microgram amounts of peptides are detected (70) whereas radioreceptor assay (RRA), radioimmunoassay (RIA), and bioassay (BA) are capable of detecting ng and pg amounts, but with no structural information attached to those measurements.

While experimental experience indicates that the RP-HPLC resolution of the two enkephalin peaks may be increased by alterations of several experimental parameters including recycling and/or change of the buffer and/or organic modifier, flow rate, temperature, etc., it must be remembered that in the novel mode of off-line LC-MS analysis, the detector is not limited to only UV absorption, but rather, utilizes a unique amino acid sequence-determining ion which arises from a peptide eluting at one selected retention time. In this type of measurement mode, virtually all chromatographic and chemical background noise disappears. The plot of leucine enkephalin and the ^{18}O leucine enkephalin internal standard $(M+H)^+$ ratios for this hypothalamus data shows that the intersection of the signal and noise levels corresponds to 170 ng leucine enkephalin g^{-1} hypothalamus tissue (71).

D. THALAMUS TISSUE EXTRACTS

^{18}O-incorporated internal standards are used to determine endogenous amounts of enkephalin in canine thalamus tissue (72). The straight line intersects the abcissa at values corresponding to 62 ng LE and 125 ng ME g^{-1} thalamus, respectively.

E. PITUITARY

A number of canine pituitaries (70) is neuroanatomically separated into the anterior (1.9 g. total wet weight) and

posterior (0.44 g total wet weight) portions. The tissue is homogenized in acetic acid (1 M) and divided into three equal samples. In the anterior pituitary, 70 ng of LE and 2,950 ng of ME g^{-1} are found while 2 ng LE and 3760 ng ME g^{-1} are measured in the posterior pituitary extracts.

F. CAUDATE NUCLEUS

FDMS methods have been used to measure the endogenous amount of enkephalin in canine nucleus tissue extracts (71). The amount of endogenous leucine enkephalin in the canine caudate nucleus tissue extract is 1,500 ng leucine enkephalin g^{-1} tissue.

G. TOOTH PULP

Tooth pulp tissue is collected from four animals and pooled (four teeth from each animal; total = 16). Canine tooth pulp RP-HPLC chromatograms have been published (74). The endogenous amount of ME for pooled tooth pulp tissue is 3 micrograms g^{-1} tooth pulp tissue.

H. ELECTROSTIMULATED TOOTH PULP

In a study of nociceptive processes, levels of enkephalins are determined in canine tooth pulps which had been electrostimulated *in vivo* and compared to levels of these peptides in tooth pulps of control animals. The current working hypothesis underlying this type of physiologic study is that three peptidergic pathways (endorphinergic, dynorphinergic, and enkephalinergic) are available to a cell to maintain a dynamic homeostatic relationship and to deal with noxious stimuli. The three peptidergic pathways are composed of large

precursor peptides, the opioid oligopeptide, and metabolites. Noxious stimuli are hypothesized to activate the peptidergic pathways, and individual opioid peptides may have decreased concentrations following stimulation. Electrostimulation significantly decreases by 20% the amount of endogenous ME.

A general overall trend noted is that the two opioid pentapeptides ME and LE are altered upon electrostimulation (75,76). Electrostimulation is performed to elucidate those molecular mechanisms operating during a physiologically stressful situation. These preliminary data indicate that the three peptidergic pathways (dynorphinergic, endorphinergic, enkephalingeric) may be mobilized in the following sequence:

Large precursor ⟶ intermediate precursor(s) ⟶ pentapeptide(s) ⟶ inactive metabolites (77).

On one hand, there may be a naturally-occurring pool of pentapeptides which is electrostimulated towards metabolism or, on the other hand, the entire metabolic scheme noted above may be stimulated to produce a lowered endogenous amount of each constituent peptide. Other human and *in vivo* dynamic studies are needed to resolve that question.

I. CEREBROSPINAL FLUID

LE (44 ng ml^{-1}) was measured in canine CSF (71). This type of measurement is important in current clinical studies aimed towards the elucidation of molecular mechanisms involved in pain.

J. CONCLUSIONS

Several significant conclusions are derived from the experiments utilizing the off-line LC-MS measurement modes which are reported in this section.

A fast and facile method of tissue sample acquisition and procurement from the canine animal model is described (78).

This study demonstrates that rapid freezing of tissue is needed in a fashion similar to that described in the discovery of 19OH-PGE_2 compounds in human seminal fluid (79-81). Rapid freezing avoids, or at least minimizes, metabolic and chemical interconversions and also enhances the possibility of measuring only those endogenous target compounds and not artifacts or chemical/enzymic products. The need for an internal standard for an MS analytical measurement is demonstrated by previous workers (60, 61) to overcome the limitations imposed by the experimentally rather ill-defined, yet very real, biological matrix effects. Stable isotope-incorporated peptides are the most appropriate internal standards for measurement of endogenous peptides. Stable isotope-incorporated peptide internal standards also have hydrophobicity and MS behavioral characteristics similar to the endogenous peptides. An internal standard is added as soon as possible after tissue acquisition and before homogenization in the separation scheme as a means to accurately represent the endogenous amount of peptide, and also to provide sufficient time for equilibration (61) of the exogenous and endogenous peptides.

The most significant experimental parameter of any analytical measurement of a biological compound is the molecular specificity of that measurement; namely, is the compound one thinks is being measured the compound that is actually being measured? This concept of specificity is easy to state but experimentally rather difficult to prove unambiguously. One author calls this experimental phenomenon the "chromatographic uncertainty principle" (82). Many other assay methods are generally utilized because of their relative ease, low cost, high speed, high sensitivity, and putatively high molecular specificity. For example, chromatography, color reactions, enzymatic reactions, HPLC, BA, RRA, and RIA are some of the measurement procedures which are used in most laboratories around the world. However, one of the purposes of this review is to state unequivocally that the

molecular specificity of all of the above measurement methods is insufficient for unambiguous structural proof during an analytical measurement (78). Of course, the non-MS assay methods listed above will always be used, but investigators must at least be aware of and state the limitations of any statements made relating to structure. Only one measurement process, namely MS, offers unambiguous molecular specificity. On one hand, ability to produce the $(M+H)^+$ ion of a biologically important peptide is a signal advancement in the measurement of endogenous peptides. But even this parameter, $(M+H)^+$, while significantly increasing specificity, does not confer unambiguous molecular specificity to that measurement. The only analytical method currently available and which uses MS to provide maximum molecular specificity is to use an amino acid sequence-determining ion from the $(M+H)^+$ ion produced by FAB with either unimolecular or CAD processes, and then to collect by a linked-field scan only one unique amino acid sequence-determining ion. Furthermore, use of a stable isotope-incorporated internal standard, which is the same peptide as that being measured, additionally substantiates the molecular specificity of the analysis. In the study discussed above, the C-terminal tripeptide sequence ions -GFL from LE and -GFM from ME are selected for monitoring, and the analytic measurement of endogenous peptides is based upon those two ions and their corresponding ^{18}O-internal standards.

The detection sensitivity of the novel LC-MS process described above is quite encouraging for analytical measurement of endogenous peptides in most biological tissues and fluids. For example, enkephalin peptides in canine caudate nucleus tissue extracts are measured at the 200-400 ppb level. The current instrumental limitation corresponds to the 30 ppb level. It is significant to realize that several significant instrumental increases (10-100x) in the detection limits are forthcoming.

The peptide measurements include endogenous amounts of both ME and LE extracted from a variety of biological sources including hypothalamus, CSF, anterior and posterior pituitary, caudate nucleus, and tooth pulp (pooled and electrostimulated). It is important to undertake this type of analytical/physiological study within one laboratory to ensure quality control over all experimental manipulations which range from the live animal model through exsanguination, tissue procurement, homogenization, chromatography, MS, and data analysis. Inter-animal biologic variations are observed and it is possible to have one animal serve as it own control.

REFERENCES

1. J.T. Watson and K. Biemann, Anal. Chem., 36, 1135 (1964).

2. C.G. Edmonds, J.A. McCloskey and V.A. Edmonds, Biomed. Mass Spectrom. 10, 237 (1983).

3. P.J. Arpino, Trends Anal. Chem., 7, 154 (1982).

4. W.H. McFadden, J. Chromatogr. Sci., 19, 97 (1980).

5. C. Eckers, K.K. Cuddy and J.D. Henion, J.Liq. Chromatogr., 6, 2383 (1983).

6. C. Eckers, D.S. Skrabalak and J.D. Henion, Clin. Chem., 28, 1882 (1982).

7. B.G. Dawkins, P.J. Arpino and F.W. McLafferty, Biomed. Mass. Spectrom., 5, 1 (1978).

8. M.A. Baldwin and F.W. McLafferty, Org. Mass Spectrom., 7, 1111 (1973).

9. P.A. Arpino, B.G. Dawkins and F.W. McLafferty, J. Chromatogr. Sci., 12, 574 (1974).

10. F.W. McLafferty and B.G. Dawkins, Biochem. Soc.Trans. 3, 856 (1975).

11. B.G. Dawkins and F.W. McLafferty, GLC and HPLC Determination of Therapeutic Agents, Vol 1, Tsuji-Morozowich, (Eds), Marcel Dekker, 1978, p. 259.

12. P.J. Arpino and G. Guiochon, J. Chromatogr., 251, 153 (1982).

13. G. Guiochon and P.J. Arpino, J. Chromatogr., 271, 13 (1983).

14. M. Dedieu, C. Juin, P.J. Arpino, J.P. Bounine and G. Guiochon, J. Chromatogr., 251, 203 (1982).

15. P.J. Arpino, Liquid Chromatography Detectors, T.M. Vickrey (Ed.), Marcel Dekker, New York, 1983, p.243.

16. P.J. Arpino, J.P. Bounine and G. Guiochon, J. Chromatogr., 251, 203 (1982).

17. P.J. Arpino, J.P. Bounine, M. Dedieu and G. Guiochon, J. Chromatogr., 271, 43 (1983).

18. D. Pilosof, H.Y. Kim, D.F. Dyckes and M.L. Vestal, Anal. Chem., in press.

19. C.R. Blakley, J.C. Carmody and M.L. Vestal, Clin. Chem., 26, 1467 (1980).

20. C.R. Blakley and M.L. Vestal, Anal. Chem., 55, 447 (1983).

21. M.L. Vestal, Mass Spectrom. Rev., 2, 447 (1983).

22. D. Pilosof, H.Y. Kim, M.L. Vestal and D.F. Dyckes, Biomed. Mass Spectrom., in press.

23. H.E. May, F.S. Tanzer, G.H. Fridland, C. Wakelyn and D.M. Desiderio, J. Liq. Chromatogr., 5, 2135 (1982).

24. A.P. Bruins and B.F.H. Drenth, J. Chromatogr., 271, 71 (1983).

25. J.D. Henion and G.A. Maylin, Biomed. Mass Spectrom., 7, 115 (1980).

26. J.D. Henion, Adv. Mass Spectrom., 8, 1241 (1980).

27. J.D. Henion, J. Chromatogr. Sci., 19, 57 (1981).

28. J.D. Henion, B.A. Thomson and P.H. Dawson, Anal. Chem., 54, 451 (1982).

29. C.E. Parker, Y. Tondeur and J.R. Hass, J. Forens., Sci., 27, 495 (1982).

30. C. Eckers, D.S. Skrabalak and J. Henion, Clin. Chem., 28, 1882 (1982).

31. F.R. Sugnaux, D.S. Skrabalak and J.D. Henion, J. Chromatogr., 264, 357 (1983).

32. J. Henion, D. Skrabalak, E. Dewey and G. Maylin, Drug Metabol. Rev., 14, 961 (1983).

33. J. Henion and D.S. Skrabalak, Fifth International Symposium Equine Medication Control., G.H. Johnston and J.W. Martin (Eds), in press.

34. T. Covey and J. Henion, Anal. Chem., 55, 2275 (1983).

35. R.G. Christensen, H.S. Hertz, S. Meiselman and E. White, Anal. Chem., 53, 172 (1981).

36. E. White, H. S. Hertz and R.G. Christensen, US patent #4281246 (1981).

37. J.A. Apffel, U.A.Th. Brinkman and R. W. Frei, Anal. Chem., 55, 2280 (1983).

38. R.D. Smith and H.R. Udseth, Anal. Chem., 55, 2266 (1983).

39. R.D. Smith, W.D. Felix, J.C. Fjeldsted and M.L. Lee, Anal. Chem., 53, 1883 (1982).

40. D.J. Liberato, C.C. Fenselau, M.L. Vestal and A.L. Yergey, Anal. Chem., 55, 1741 (1983).

41. C.N. Kenyon, Biomed. Mass Spectrom., 10, 535 (1983).

42. B.L. Karger, D.P. Kirby, P. Vouros, R.L. Foltz and B. Hidy, Anal. Chem., 51, 2324 (1979).

43. D.P. Kirby, P. Vouros and B.L. Karger, Science, 209, 495 (1980).

44. M.J. Hayes, E.P. Lankmayer, P. Vouros, B.L. Karger and J.M. McGuire, Anal. Chem., 55, 1745 (1983).

45. R.D. Smith and A.L. Johnson, Anal. Chem., 53, 739 (1981).

46. T.J. Yu, H. Schwartz, R.W. Giese, B.L. Karger and P. Vouros, J. Chromatogr., 218, 519 (1981).

47. T.J. Yu, B.L. Karger and P. Vouros, Biomed. Mass Spectrom. 10, 633 (1983).

48. P. Roepstorff, M.A. McDowall, M.P.L. Games and D.E. Games, Int. J. Mass Spectrom. Ion Phys., 48, 197 (1983).

49. D.E. Games, M.J. Hewlins, S.A. Westwood and D.J. Morgan, J. Chromatogr., 250, 62 (1982).

50. N.J. Alcock, C.Eckers, D.E. Games, M.P.L. Games, M.S. Lant,

M.A. McDowall, M. Rossiter, R.W. Smith, S.A. Westwood and H.Y.Wong, J.Chromatogr., 251, 165 (1982).

51. D.E. Games, M.A. McDowall, K. Levsen, K.H. Shafer, P. Dobberstein and J.L. Gower, Biomed. Mass Spectrom., 11, 87 (1984).

52. R.D. Smith, J.E. Burger and A.L. Johnson, Anal. Chem., 53, 1603 (1981).

53. R.D. Smith and A.L. Johnson, Anal. Chem., 53, 1120 (1981).

54. N.M.M. Nibbering, J. Chromatogr., 251, 93 (1982).

55. M. Barber, R.S. Bordoli, G.V. Garner, D.B. Gordon, R.D. Sedgwick, L.W. Tetler and A.N. Tyler, Biochem. J., 197, 401 (1981).

56. D.M. Desiderio and I. Katakuse, Biomed. Mass Spectrom., (1984) in press.

57. M. Barber, R.S. Bordoli, G.J. Elliott, and N.J. Horoch, Biochem. Biophys. Res. Commun., 110, 753 (1983).

58. D.M. Desiderio, Adv. Chromatography Vol. 22, J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown (Eds). Marcel Dekker, N.Y., 1983 p. 1.

59. K.R. Jennings and R.S. Mason, Tandem Mass Spectrometry, F.W. McLafferty (Ed.), Wiley, N.Y., 1983, p. 197.

60. B.J. Millard, Quantitative Mass Spectrometry, Heyden, London, 1978.

61. A.M. Lawson, C.K. Lim, W. Richmond, D.M. Samson, K.D.R. Setchell and A.C.S. Thomas, Current Developments in the Clinical Applications of HPLC, GC and MS, A.M. Lawson, C.K. Lim and W. Richmond, (Eds), Academic Press, London, 1980, p. 135.

62. D.M. Desiderio and M. Kai, Biomed. Mass Spectrom., 10, 471 (1983).

63. D.M. Desiderio and M. Kai, Int. J. Mass Spectrom. Ion Phys., 48, 261 (1983).

64. D.M. Desiderio, I. Katakuse and M. Kai, Biomed. Mass Spectrom., 10, 426 (1983).

65. D.M. Desiderio, J. Laughter, M.Kai and J. Trimble, J. Com. Enchanc. Spectros., in press.

66. D.M. Desiderio, J.Z. Sabbatini and J.L. Stein, Adv. Mass Spectrom., 8, 1298 (1980).

67. D.M. Desiderio, J.L. Stein, M.D. Cunningham and J.Z. Sabbatini, J. Chromatogr., 195, 369 (1980).

68. F.S. Tanzer, D.M. Desiderio, C. Wakelyn and J. Walker, J. Dent. Res., submitted.

69. D.M Desiderio, H. Onishi, H. Takeshita, F.S. Tanzer, C. Wakelyn, J. Walker and G. Fridland, J. Neurochem., submitted.

70. D.M. Desiderio and M.D. Cunningham, J. Liq. Chromatogr., 4, 721 (1981).

71. D.M. Desiderio, M. Kai, F.S. Tanzer, J. Trimble and C. Wakelyn, J. Chromatogr., in press.

72. D.M. Desiderio and M. Kai, Biomed. Mass Spectrom., 10, 471 (1983).

73. S. Yamada and D.M. Desiderio, Anal. Biochem., 127, 213 (1982).

74. F.S. Tanzer, D.M. Desiderio and S. Yamada, Peptides: Synthesis-Structure-Function, D.H. Rich and E. Gross (Eds), Pierce Chem. Co., Rockford, Ill., 1981, p. 761.

75. T. Kudo, H.-L. Chang, S. Maeda, Y. Uchida, J. Kakamae and R. Inoki, Life Sci., 33, 677 (1983).

76. T. Kudo, S. Maeda, J. Nakamae, H.-L. Chang, Y. Uchida and R. Inoki, Life Sci., 33, 681 (1983).

77. J. Hughes, Brit. Med. Bull., 39, 17 (1983).

78. D.M. Desiderio, Peptide Measurements by HPLC and MS, Elsevier, Amsterdam, in press.

79. H.H. Jonsson, B.S. Middleditch and D.M. Desiderio, Science, 187, 1093 (1975).

80. H.T. Jonsson, B.S. Middleditch, M.A. Schexnayder and D.M. Desiderio, J. Lip. Res., 17, 1 (1976).

81. D.L. Perry and D.M. Desiderio, Prostaglandins, 14, 745 (1977).

82. M.F. Delaney, LC Magazine, 2, 85 (1984).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 353-376 (1984)

CHIRAL STATIONARY PHASES FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS: A MINI-REVIEW

Daniel W. Armstrong

Department of Chemistry, Texas Tech University,
Lubbock, TX 79409

ABSTRACT

There has been a proliferation of papers on the use of chiral stationary phases (CSP's) to separate optical isomers in high performance liquid chromatography. The chemistry, mechanism and stability of these CSP's can vary widely. Furthermore, the applicability, availability and cost of a CSP can mean the difference between its being of passing academic interest as opposed to a technique that could have a significant impact on science and technology. Six different classes of chiral stationary phases are examined and discussed including the new chiral cyclodextrin bonded phases. The separation mechanism, strengths and limitations of the CSP's are also considered whenever such information is available.

INTRODUCTION

In the last decade there has been a tremendous impetus to develop efficient liquid chromatographic techniques for the separation of racemates. There are several reasons for this. For example, an efficient method for determining optical purity would be highly beneficial in many scientific disciplines in-

 0148-3919/84/07S2-0353$3.50/0

cluding: organic and inorganic synthesis, kinetics, pharmacology, geochronology (i.e., using the degree of amino acid racemization to date organic articles of archaeological importance) and so forth. The pharmaceutical industry obviously needs effective analytical and preparative separations for a variety of enantiomeric compounds which are known to have different physiological activities (1-4). The fact that the traditional method of resolving racemic mixtures (i.e., fractional recrystallization of diastereomeric salts) is relatively difficult, inefficient and limited in applicability (5,6) has greatly increased the interest in alternative techniques. The efficiency, speed, wide applicability and reproducibility of the modern liquid chromatograph have made it the instrument of choice for most of the recently reported enantiomeric separations. Be this as it may, it is clear that recent HPLC techniques have evolved and/or benefited from classic column chromatographic methods (6). It also appears that the most interesting research in this area involves the development of new highly selective stationary phases. In this review the chemistry, applicability and limitations of six classes of chiral stationary phases will be examined. This work is not intended to be a comprehensive review of enantiomeric separations. For example, work on the use of chiral mobile phase additives and ligand exchange LC will not be covered. Readers interested in these particular areas are refered to the

the many fine reviews and papers that have recently been published (7-16). The six classes of chiral stationary to be considered in this work are: cyclodextrin bonded phases, π-complex/hydrogen bonding stationary phades, polymeric stationary phases, charge transfer stationary phases, protein bonded phases, and crown ether bonded phases.

I. Chiral Cyclodextrin Bonded Phases

Cyclodextrins are chiral, toroidal shaped molecules formed by the action of Bacillus macerans amylase on starch (see Figure 1)(17-19). These macrocyclic polymers contain from six to twelve glucose units bonded through α-(1,4)linkages. The three smallest homologs, α-cyclodextrin (cyclohexaamylose), β-cyclodextrin (cycloheptaamylose) and γ-cyclodextrin (cyclooctaamylase) are available commercially while larger homologues must be individually produced and isolated. Cyclodextrins have several structural features that make them highly useful in separations (Figure 1). First of all, the interior of the cyclodextrin cavity contains no hydroxyl groups and is relatively hydrophobic. Consequently they are able to complex a variety of water insoluble or sparingly soluble molecules, see Figure 2. This property led to their use as mobile phase modifiers in the TLC separation of a variety of structural isomers (20-22). Traditional column chromatography on polymerized cyclodextrin was investigated as well (23-25).

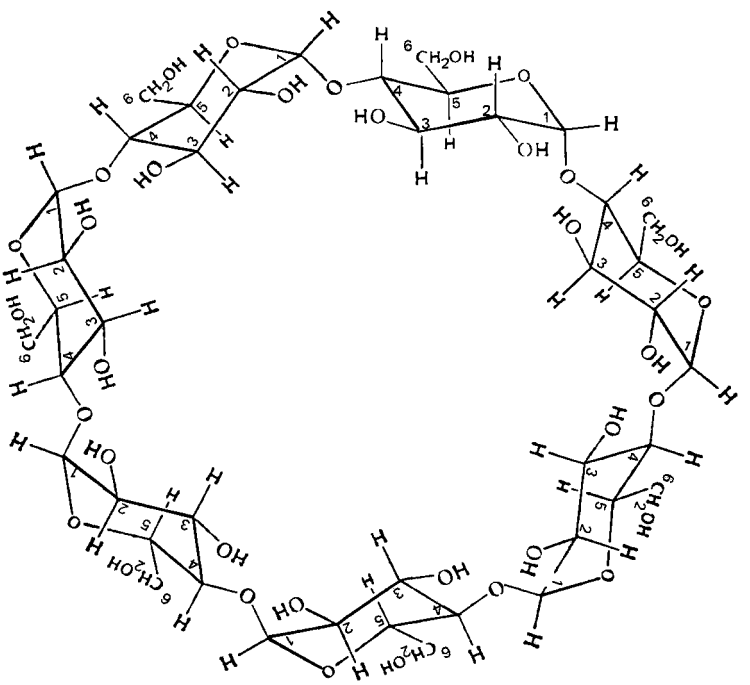


Figure 1. A schematic showing the structure of β-cyclodextrin. The cavity is hydrophobic and is 7 to 8 Å deep. Note that all glucose units are locked in a chair conformation and joined by a stable α-(1,4)glycosidic linkage. All hydroxyl groups are on the outer edges of the molecule with the primary 6-hydroxyls restricting the "bottom side" of the molecule.

Most importantly, each glucose unit contains five chiral atoms and the 2-hydroxyl groups at the entrance of the cyclodextrin cavity project in a clockwise direction (Figure 1). Chiral recognition has been shown to be optimal on a β-cyclodextrin column for compounds the size of biphenyl or a little larger (26,27). If, in addition, the chiral solute also contains a substituent that can hydrogen bond with the 2-hydroxyl

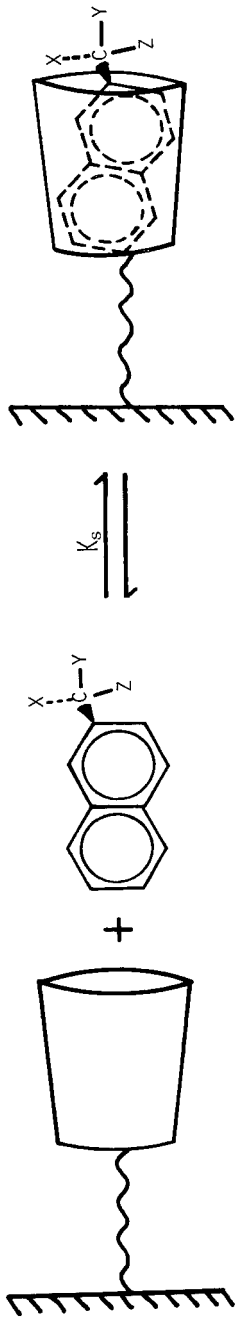


Figure 2. A schematic of cyclodextrin bonded to a silica gel support and reversibly forming an inclusion complex with a chiral molecule. Neither the linkage nor the cyclodextrin contain nitrogen (e.g., amines or amides) in any form.

groups at the mouth of cyclodextrin cavity, then enantiomeric separations are particularly efficient and predictable (see Figure 3). For example, the L-enantiomer of all dansyl or naphthyl amino acids is eluted first. Table I lists typical enantiomeric compounds that have been resolved on chiral cyclodextrin bonded phases.

It is interesting to consider whether or not chiral cyclodextrin inclusion complexes satisfy the three point chiral recognition model (29). Considering the simplified model shown in Figure 2, one could argue that there is a three point attachment via the hydrophobic group in the cavity and at least two of the groups projecting radially from the mouth of the cyclodextrin cavity. This seems to be likely for many of the compounds studied (Table I). One might also argue that there could be more than three points of interaction if the entire molecule is within the cyclodextrin. However small molecules that are completely enveloped by the cyclodextrin tend not to show enantioselectivity (a N-benzoyl amino acid in β-cyclodextrin for example). In these cases one must go to a smaller cyclodextrin (such as α-CD). A "tight fit" with part of an enantiomer extending out of the cyclodextrin cavity seems to produce the desired optical resolution in many cases. It may be possible that a one or two point attachment accompanied by steric restraints (as first suggested by Lochmüller (30, 31)) could in some cases be responsible for the observed

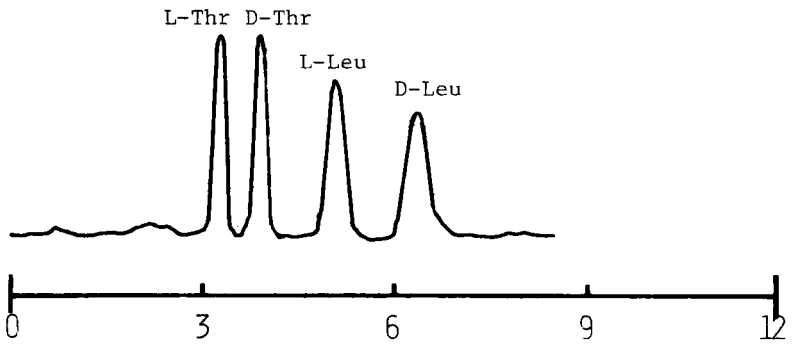


Figure 3. A chromatogram showing the separation of racemic dansyl threonine and leucine. Note that the L-enantiomer is always eluted first. Cromatographic conditions: column = 0.46 x 10 cm B-CD, solvent = 50% methanol + 50% water, flow = 1.0 ml/min, detection γ=254 nm.

enantioselectivity (see section IV). Because of its strict stereochemical requirements and the intimacy of the inclusion complex, cyclodextrin bonded phases may be a more useful means to assign absolute configuration than other CSP's. Certainly a good deal more work must be done in this area.

There are several advantages to chiral cyclodextrin packings. They are, for example, commercially available* and there are several different size cyclodextrins which allows one to separate a variety of different size enantiomers. This packing is water stable and is most often used with aqueous-methanol mobile phases (26-28). This is important since the presence of water is known to ruin most of the other chiral

*Advanced Separation Technologies, Inc. 37 Leslie Ct. P.O. Box 297, Whippany, NJ 07981.

Table I: A List of Typical Enantiomers Resolved on β-Cyclodextrin Columns Using Aqueous-Methanol Mobile Phases (26-28,32).

---

1. D,L-alanine β-naphthylamide
2. D,L-methionine β-naphthylamide
3. D,L-alanine β-naphyl ester
4. dansyl D,L-valine
5. dansyl D,L-threonine
6. dansyl D,L-norleucine
7. dansyl D,L-phenylalanine
8. dansyl D,L-leucine
9. dansyl D,L-methionine
10. dansyl D,L-tryptophan
11. dansyl D,L-serine
12. dansyl D,L-norvaline
13. dansyl D,L-α-amino-N-buteric acid
14. dansyl D,L-arginine
15. N-benzoyl-D,L-arginine β-naphthylamide
16. (+,-) hexabarbital
17. (+,-) mephobarbital
18. (+,-) usnic acid
19. (+,-) α-(1-naphthyl)ethylamine
20. (+,-) cyclohexylphenylacetic acid
21. D,L-propranolol
22. (+,-)2,3-O-isopropylidene-2,3-dihydroyx-1,4-bis(diphenylphosphino)butane [a.k.a. DIOP]
23. (+,-) trans α,α'-(2,2-dimethyl-1,3-dioxalane-4,5-biyl)-bis-(biphenylmethanol)

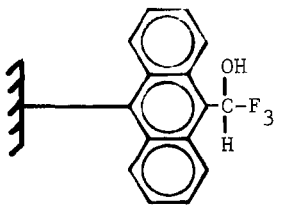
stationary phases. An added benefit of cyclodextrin bonded phases is that they are useful for the resolution of many nonenantiomeric compounds and have been found to be superior to conventional reverse phase packings for many of the more routine separations (27, 32). They can also be used as normal phase packings (e.g., with hexane-alcohol mobiles phases) although the separation is not due to inclusion complex formation but rather to hydrogen bonding and dipolar interactions

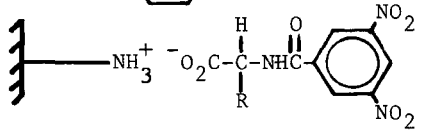
with surface hydroxyl groups (32). Lastly, these columns are currently less expensive than most routine normal and reverse phase columns.

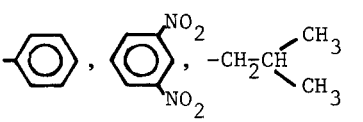
Cyclodextrin packings also have some shorcomings. For example they are generally about 80% as efficient as the best reverse phase columns. This is because the formation of strong inclusion complexes causes somewhat slower mass transfer which results in detectable band broadening. Fortunately, this relatively small decrease in efficiency is usually accompanied by a large increase in selectivity. Currently only β- and γ-cyclodextrin columns are available commercially, however an α-cyclodextrin packing for smaller molecules will undoubtedly be forthcoming. After extensive use (particularly when using mobile phases with a very high percentage of water) one finds that the efficiency and retention begins to decrease. This is because strongly retained impurities are occupying many of the cyclodextrin cavities. The column is easily regenerated by flushing with absolute methanol or ethanol.

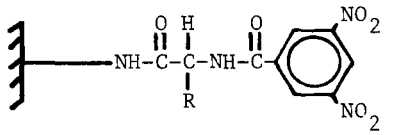
II. "π-Complex-Hydroden Bonding" Stationary Phases

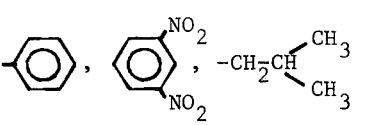
The three-point chiral recognition model has been used as a basis for the design of several chiral stationary phases. Pirkle and co-workers origionally designed CSP's "A" through "C" (see Figure 4) in view of this model and successfully resolved a series of enantiomeric sulfoxides and 3,5-dinitrobenzoyl deriva-

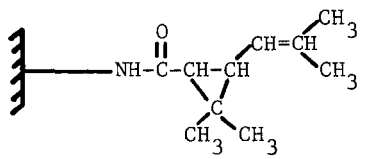
A 

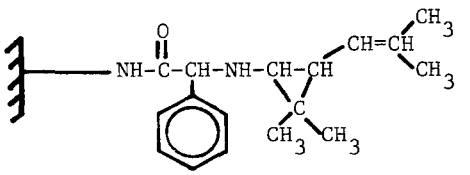
B 

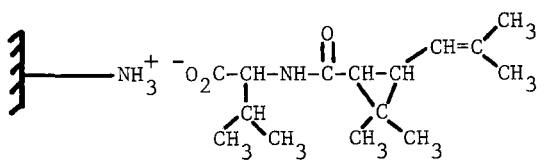
R = 

C 

R = 

D 

E 

F 

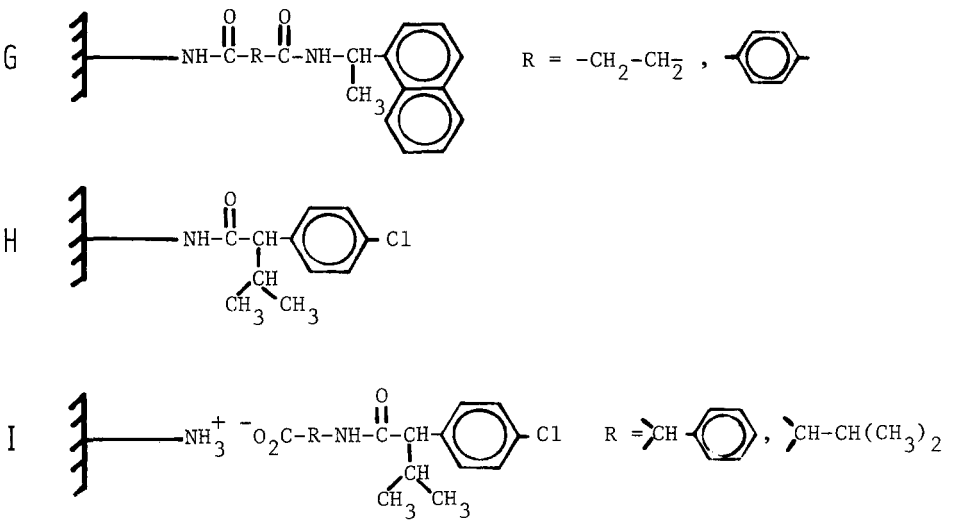


Figure 4. A schematic showing nine different class II chiral stationary phases. All are used under normal phase, conditions (e.g., hexane: isopropanol mobile phases). Packings A through C were developed by Pirkle and co-workers (33-35). Packings D through I were developed by Oi and co-workers (43-47). All packings except for "A" have the chiral molecule attached to the silica support via a γ-aminopropylsilane linkage.

tives of amines, alcohols, thiols, amino acids, amino alcohols and hydroxy acids (33-35). The necessary three point contact between the CSP and enantiomeric solute was thought to be maintained via two hydrogen bonds and a π-π donor-acceptor interaction (33-35). Subsequent work indicated that dipole-dipole stacking and Van der Waals interactions must also be considered in many cases (36). One important feature of these CSP's was that they seemed to be applicable to a broader range of compounds than most of the earlier reported chiral packings.

Recent research in this area involves the extension of applications, the development of a rational for predicting elution order and the developement of another generation of CSP's of this type. Wainer and co-workers, have largely been involved with the first two pursuits above, particularly in the separation of a variety of chiral compounds of pharmaceutical interest (36-40). Other research groups have evaluated enantiomeric separations of dihydroxy and tetrahydroxy polynuclear aromatic hydrocarbons (41) and a variety of acyclic alkyl carbinols and their derivatives (42). Oi and co-workers (43-46) have focused their attention on the developement of new and modified CSP's (see structures D through I, Figure 4). Their work has shown that there can be significant differences in selectivity for many of the related CSP's. In fact, it has been noted that there are selectivity differences in packings containing the same chiral base molecule but differing in whether they are attached covalently or ionically (structures "B" and "C" in Figure 4) (36). Undoubtedly these types of studies will continue to shed light on the separation mechanism.

There are several advantages to Type II CSP's. Firstly, CSP's "B" and "C" (Figure 4) are available commercially.* Currently, more enantiomeric seperations have been reported on this class of packing than on any other. As a result, the interested

*J. T. Baker, Phillipsburg, NJ

researcher has available a significant quantity of useful data which can be used to formulate future work. Indeed, a recent paper evaluated the role of mobile phase composition on enantiomeric selectivity and resolution (47).

A disadvantage of class II CSP's is that the presence of any water in the mobile phase tends to ruin both the separation and the packing. It is possible, however, to regenerate the ionically bonded packings provided a significant portion of the γ-aminopropylsilanized silica gel has not hydrolyzed. It has been noted that it is more difficult to separate ester derivatives of some enantiomers than the corresponding amides (36). It was also noted that there was a reversal in the elution order of an enantiomeric pair of compounds during the course of a study (36). It is not currently known how common this phenomenon is, but it could restrict the use of these CSP's for assigning absolute configurations.

III. Chiral Polymer Stationary Phases

There is an extensive literature on the use of chiral polymers for traditional column chromatography of enantiomers. Many but not all are naturally occuring polymers. Typical examples include: cellulose, microcrystalline cellulosetriacetate, starch, polymerized amino acids, cross linked polystyrene containing alkaloids, and isotactic (+)-poly(triphenylmethyl methacrylate)(6). The use of analogous stationary phases in

HPLC is much less extensive but will undoubtedly grow as a result of recent successful applications (48-50). There are presently two basic approaches in using chiral polymers as HPLC stationary phases. First, if the crosslinked polymer possess sufficient mechanical strength (to withstand the pressure) and can be obtained in particle sizes of appropriate dimensions (i.e., 5 to 10 μm) and distributions, then it can be used as is. A typical example of this approach is Lindner and Mannschreck's use of microcrystalline triacetylcellulose for the HPLC separation of several racemic compounds (48). Partial resolution of a cyclic allene hydrocarbon was also reported (48). A series of racemic 2,2'-disubstituted 1,1'-binaphthyls and racemic trans-disubstituted cyclic compounds were also resolved by HPLC using a finely ground insoluble (+)-poly(triphenylmethyl methacrylate) (50). A somewhat different approach is to adsorb the chiral polymer onto silica gel. This simple approach was shown to produce a highly effective chiral phase when a low molecular weight (+)-poly(triphenylmethyl methacrylate) was used (49). In addition, the selectivity is often different from that found for the analogous packing made from the "ground" or pulverized high molecular weight polymer. For example, the four stereoisomers of the insecticide phenothrin (i.e., (+) and (-), cis- and trans-3-phenoxybenzyl chrysanthemate) are resolved on the "coated" stationary phase but not on the "ground" type (49). One can expect many

additional enantiomeric separations in the near future as researchers adsorb different chiral polymers on a variety of silica gels. Certainly the use of polysaccharides, polyaminoacids and appropriate derivatives of these polymers will produce interesting results.

Initial experiments seem to indicate that the coated chiral polymer stationary phases may be more widely applicable than packings composed of pure polymer. Certainly one advantage with the "coated" material is that one can easily and inexpensively make a wide variety of different CSP's. A disadvantage is that use of mobile phases containing appreciable amounts of water will likely result in inferior separations and/or damage to the packing. The packing can always be recoated with polymer, however. Some polymer loss has even been noted when using nonaqueous solvents (49).

IV. Chiral Charge Transfer Stationary Phases

In 1955 it was demonstrated that hexahelicene could be resolved from a solution of chiral α-(2,4,5,7-tetranitro-9-fluorenylidenaminooxy) propionic acid (TAPA) by fractional recrystallization (51). By 1960 resolution was obtained by column chromatography with TAPA coated silicic acid (52). Mikes and co-workers extended this technique to HPLC, separating ten racemic helicenes and two double helicenes using a stationary phase of silica coated with TAPA (53). They also examined three

homologues of TAPA and found that the size of the substituent on the chiral carbon affected the selectivity. Alumina impregnated with (S)-(+)-TAPA has been used as well (54). TAPA and 2,2'-diylhydrogen phosphite XL has been bonded to silica gel (see Figure 5) and has shown similar success in the separation of helicenes (55, 56) as has silica gel coated with riboflavin (57). Lochmüller and Ryall utilized a small 2,4-dinitrophenyl group as the change transfer acceptor attached to a chiral atom, see Figure 5 (31). Although partial resolution of 1-aza[6] helicene and heptahelicene was achieved, the real significance of this work was in Lochmüller's conclusion as to the mechanism involved. It was postulated that the small 2,4-dinitrophenyl group could not have achieved the multiple overlaps with the helicenes as proposed for TAPA. Consequently it may be possible to achieve chiral discrimination in chromatography with only one (31) or two (30) strong interactions. It was indicated in previous gas chromatographic work that steric repulsion may be a possible substitute for positive interactions (30).

It is apparent that chiral charge transfer stationary phases are useful for the separation of racemic helicenes. Unfortunately it has not yet been demonstrated that they have any wider applicability. As these stationary phases are either coated on silica gel or bonded via an amide linkage through an aminopropylsilane, they should not be use with aqueous or aqueous-organic mobile phases.

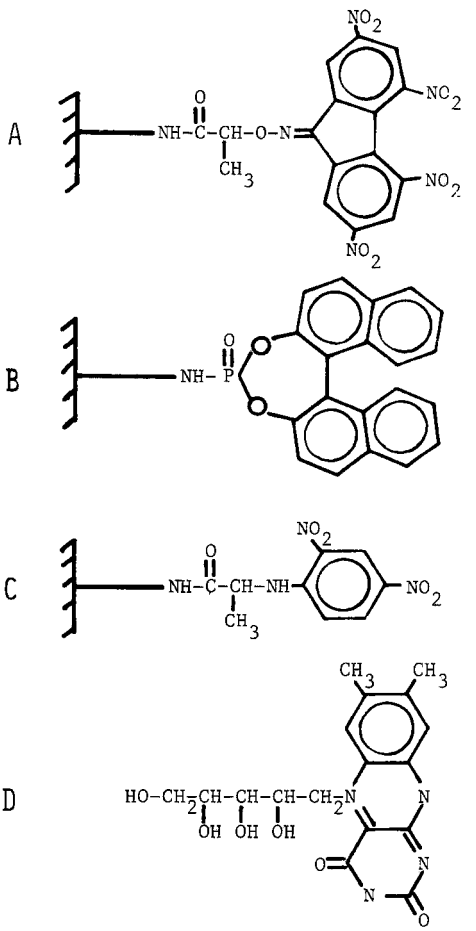


Figure 5. A schematic showing the structure of four different charge transfer adducts that have been bonded to or adsorbed on silica gel to create CSP's capable of separating a variety of helicenes. Again, the chiral molecule in all bonded packings was attached via a γ-aminopropylsilane linkage. Compound A = TAPA, B = 2,2'-diylhydrogen phosphite XL, C = N-2,4-dinitrophenylalanine, and D = riboflavin.

V. Protein Bonded Phases

For protein bonded phases, as for most of the previous chiral stationary phases, there was a legacy of successful applications by classic column chromatography. Generally bovine serum albumin (BSA) was linked to agarose (58), which could then resolve enantiomers of aromatic amino acids, some N-benzoyl-amino acids and aromatic suphinyl and sulphorximine compounds (59). More recently, Allenmark and co-workers successfully attached BSA to 10 μm silica and demonstrated its utility in HPLC (60). A series of ten different N-benzoyl and N-naphthoyl amino acids showed enantioselectivity on this packing.

Two of the advantages of this packing are that it can be used with aqueous or aqueous-organic mobile phases and that it is available commercially.* The only other packing that shares these advantages is the chiral cyclodextrin bonded phase (Part I). Currently it is not known whether or not this packing can separate any enantiomers other than N-aroyl D,L-amino acids. The stability, lifetime and cost of the packing as well as whether or not it can be regenerated is not presently known by this author.

VI. Chiral Crown Ether Bonded Phases

Cram and co-worker's research on the complexation of ions by crown ethers led to the development of cyclic compounds which

*Mackerey Nagel & Co., Duren, G. F. R.

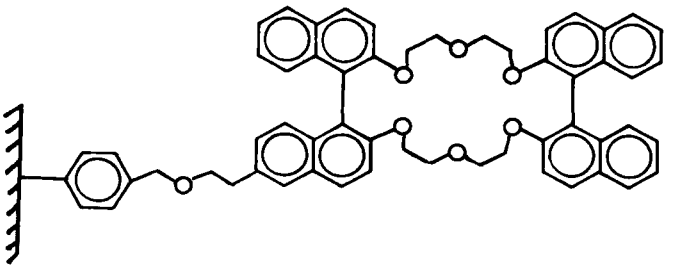


Figure 6. A schematic of a chiral crown ether which can complex protonated primary amines and in some cases show chiral recognition (61-63).

showed enantioselectivity (see Figure 6) (61-63). Crown ethers have the ability to complex a number of cations (e.g., Na^+, K^+, NH_4^+, etc.). The stability of the crown ether-ion complex depends, to a significant extent, on the fit of the ion in the crown ether cavity. For example, the larger crown ether, 18-crown-6, prefers to complex the larger potassium ion over the smaller sodium ion. For the smaller crown ether, 15-crown-5, the selectivity is the opposite. Ammonium ion is about the same size as potassium ion and fits well into the cavity of the crown ether shown in Figure 6. If the ammonium ion is attached to a chiral atom (as in a protonated amino acid) then the possibility exists for the separation of those enantiomers. Indeed, this was shown to be possible for a number of aromatic amino acids and analogous compounds (61-63).

Although these interesting stationary phases effectively separate some racemic compounds that contain primary ammonium

ions, their over all applicability is obviously limited (as are the chiral charge transfer stationary phases, for example). Judging from the current cost of many crown ethers the possibility exists that this packing may be prohibitively expensive for large scale use. It is well known that aqueous solvents and aqueous-organic mixtures tend to decrease the interaction between a crown ether and a guest ion (e.g., as opposed to organic solvents). It is not likely that the enantioselectivity observed in these systems (which utilized organic solvents) would remain in typical reverse phase mobile phases.

ACKNOWLEDGEMENT

The support of the Department of Energy (DE-A 505-84ER13159) is gratefully acknowledged.

LITERATURE CITED

(1) Barrett, A. M. and Cullum, V. A. Br. J. Pharmacol. 34, 43 (1968).

(2) Kawashima, K., Levy, A. and Spector, S. J. Pharmacol. Exp. Ther. 196, 517 (1976).

(3) Kaiser, D. G., Vangiessen, G. J., Reischer, R. J. and Wechter, W. J. J. Pharm. Sci. 65, 269 (1976).

(4) Goto, J., Goto, N. and Nambara, T. J. Chromatogr., 239, 559 (1982).

(5) Eliel, E. L. "Stereochemie der Kohlenstaffverbindungen" Verlag Chemie, Weinheim, 1966.

(6) Bloschke, G. Angew. Chem. Int. Ed. Engl. 19, 13 (1980) and references therein.

(7) Davankov, V. A. "Advances in Chromatography" Vol. 18, Eds. J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown. Marcel Dekker, New York, 1980, p. 139.

(8) Danvankov, V. A., Kurganov, A. A. and Bochkov, A. S. "Advances in Chromatography" Vol. 18, Eds. J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown. Marcel Dekker, New York, 1983, p. 71.

(9) Feibush, B., Cohen, M., Karger, B. L. J. Chromatogr. 282, 3 (1983).

(10) Hare, P. E. and Gil-Av, E. Science. 204, 1226 (1979).

(11) LePage, J. N., Linder, W., Davies, G., Seitz, D. E. and Karger, B. L. Anal. Chem. 51, 433 (1979).

(12) Lindner, W., LePage, J. N., Davies, G., Seitz, D. E. and Karger, B. L. J. Chromatogr. 185, 323 (1979).

(13) Gil-Av, E., Tishbee, A. and Hare, P. E. J. Am. Chem. Soc. 102, 5115 (1980).

(14) Gilen, C., Leshem, R. and Grushka, E. J. Chromatogr. 203, 365 (1981).

(15) Pavankiv, V. A., Bochkov, A. S. and Belov, Y. P. J. Chromatogr. 218, 547 (1981).

(16) Weinstein, S., Engel, M. and Hare, P. E. Anal. Biochem. 121, 370 (1982).

(17) Bender, M. L. and Komiyama, M. "Cyclodextrin Chemistry" Springer-Verlag, Berlin, 1978.

(18) Szejtli, J. "Cyclodextrins and Their Inclusion Complexes" Akademiai Kiado, Budapest, 1982.

(19) Hinze, W. L. "Separation and Purification Methods" Ed. C. J. van Oss, Vol. 10, p. 159, 1981.

(20) Armstrong, D. W. J. Liq. Chromatogr. 3, 895, (1980).

(21) Hinze, W. L. and Armstrong, D. W. Anal. Lett. 13, 1903 (1980).

(22) Burkert, W. G., Owensby, C. N. and Hinze, W. L. J. Liq. Chromatogr. 4, 1065 (1981).

(23) Widdenhof, N. Starke. 21, 164 (1969).

(24) Hoffman, J. L. Anal. Biochem. 33, 209 (1970)

(25) Harda, A., Furue, M. and Nozakura, S. I. J. Poly. Sci. 16, 189 (1978).

(26) Armstrong, D. W. Patents Pending, USA, Japan, Europe (1984).

(27) Armstrong, D. W. J. Chromatogr. Sci. in press, Sept. issue.

(28) Hinze, W. L., Riehl, T., Armstrong, D. W., DeMond, W., Ward, T., Alak, A. submitted (1984).

(29) Dalgliesh, E. E. J. Chem. Soc. 137, 3940 (1952).

(30) Lochmüller, C. H., Harris, J. M. and Souter, R. W. J. Chromatogr, 71, 405 (1972).

(31) Lochmüller, C. H. and Ryall, R. R. J. Chromatogr. 150 511 (1978).

(32) Armstrong, D. W. unpublished results (1984).

(33) Pirkle, W H., House, D. W. and Finn, J. M. J. Chromatogr. 192, 143 (1980).

(34) Pirkle, W. H. and Finn, J. M. J. Org. Chem. 46, 2935 (1981).

(35) Pirkle, W. H., Finn, J. M., Schreiner, J. L. and Hamper, B. C. J. Am. Chem. Soc. 103, 3964 (1981).

(36) Wainer, I. W. and Doyle, T. D. Liq. Chromatogr. 2, 88 (1984).

(37) Wainer, I. W., Doyle, T. D., Hamidzadeh, Z. and Aldridge, M. J. Chromatogr. 261, 123 (1983).

(38) Wainer, I. W., Doyle, T. D., Hamidzadeh, Z. and Aldridge, M. J. Chromatogr., 268, 107 (1983).

(39) Wainer, I. W. and Doyle, T. D. J. Chromatogr., 259, 465 (1983).

(40) Wainer, I. W., Doyle, T. D. and Breder, C. D. J. Liq. Chromatogr. 7, 4, 731 (1984).

(41) Weems, H. B. and Yang, S. K. Anal. Biochem. 125, 156 (1982).

(42) Kasai, M., Froussios, C., Ziffer, H. J. Org. Chem. 48, 459 (1983).

(43) Oi, N., Nagase, M. and Doi, T. J. Chromatogr. 257, 111 (1983).

(44) Oi, N., Nagase, M., Indo, Y. and Doi, T. J. Chromatogr. 259, 487 (1983).

(45) Oi, N., Nagase, M., Inda, Y. and Doi, T. J. Chromatogr. 265, 111 (1983).

(46) Oi, N. and Kitahara, H. J. Chromatogr. 265, 117 (1983).

(47) Zief, M., Crane, L. J. and Horvath, J. J. Liq. Chromatogr. 7, 4, 709 (1984).

(48) Lindner, K. R., Mannschreck, A. J. Chromatogr. 193, 308 (1980).

(49) Okamoto, Y., Honda, S., Okamoto, I. and Yuki, H. J. Am. Chem. Soc. 103, 6971 (1981).

(50) Okamoto, Y., Okamoto, I. and Yuki, H. Chem. Lett. 835, (1981).

(51) Newman, M. S., Lutz, W. B. and Lednicer, D. J. Am. Chem. Soc. 77, 3420 (1955).

(52) Klemm, L. H. and Reed, D. J. Chromatogr. 3, 364 (1960).

(53) Mikes, F., Boshart, G., Gil-Av, E. J. Chromatogr. 122, 205 (1976).

(54) Feringa, B. and Wynberg, W. Rec. Trav. Chim. Pays-Bas 97, 249 (1978).

(55) Mikes, F. and Boshart, G. J. Chromatogr. 149, 455 (1978).

(56) Mikes, F. and Boshart, G. Chem. Commun. 173 (1978).

(57) Kim, Y. H., Tishbec, A. and Gil-Av, E. J. Am. Chem. Soc. 102, 5915 (1980).

(58) Allenmark, S., Bomgren, B. and Boren, H. J. Chromatogr. 237, 473 (1982).

(59) Allenmark, S., Bomgren, B. J. Chromatogr. 252, 297 (1982).

(60) Allenmark, S., Bomgren, B. and Boren, H. J. Chromatogr. 264, 63 (1983).

(61) Sogah, G. D. Y. and Cram, D. J. J. Am. Chem. Soc. 98, 3038 (1976).

(62) Sogah, G. D. Y. and Cram, D. J. J. Am. Chem. Soc. 101, 3035 (1979).

(63) Peacock, S. S., Walba, D. M., Gaeta, F. C. A., Helgeson, R. C., Cram, D. J. J. Am. Chem. Soc. 102, 2043 (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 377-392 (1984)

RECENT CHROMATOGRAPHIC METHODS TO ISOLATE PYRROLIZIDINE ALKALOIDS

H. J. Segall
Department of Veterinary Pharmacology and Toxicology
University of California
Davis, California 95616

ABSTRACT

The application of recent chromatographic techniques to isolate various pyrrolizidine alkaloids are evaluated. Alkaloids which have been successfully separated include monoesters, diesters, and macrocyclic pyrrolizidine alkaloids exhibiting the retronecine base as well as the otonecine base. Both analytical and preparative high performance liquid chromatography methods are discussed.

INTRODUCTION

Pyrrolizidine alkaloid (PAs) are indigenous to a variety of plant species which are widely distributed throughout the world (Figs. 1 and 2). Plants containing these alkaloids include such diverse botanical families as the Compositae, Leguminosae, and Boraginaceae (1). The genera which exhibit the greatest toxicity to humans and livestock are Senecio, Crotalaria, Heliotropium, and Amsinckia (1,2). Pyrrolizidine alkaloids have been identified in a variety of human food sources [teas, medicinal herbs, milk, honey, cereals, and grains (3-11)] and have been identified as the source of injury (liver, lung) and death to livestock for many years (11). This contamination may be highly significant since PAs are responsible for numerous syndromes and are proven mutagens and carcinogens (12-14).

The difficulty in studying PAs has been the lack of available compounds to the investigator as only monocrotaline is sold commercially. Various isolation and extraction processes are

 0148-3919/84/07S2-0377$3.50/0

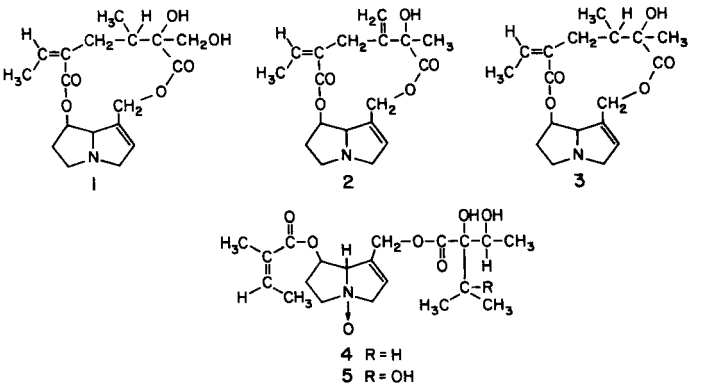


Figure 1. Examples of macrocyclic pyrrolizidine alkaloids exhibiting a retronecine base are: 1. retrorsine, 2. seneciphylline, 3. senecionine. Examples of diester pyrrolizidine alkaloids are: 4. symphytine-N-oxide, 5. echimidine-N-oxide.

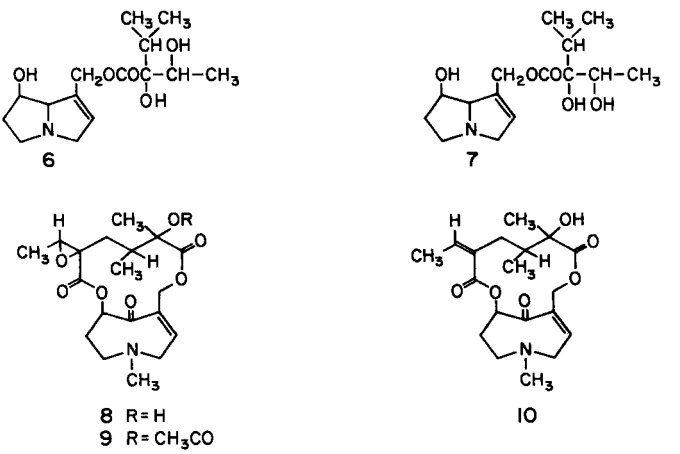


Figure 2. Examples of monoester pyrrolizidine alkaloids are: 6.intermedine and 7. lycopsamine. Examples of macrocyclic pyrrolizidine alkaloids exhibiting an otonecine base are: 8. petasitenine, 9. neopetasitenine, 10. senkirkine.

required prior to separating a mixture of PAs and have been summarized by numerous authors (15-17). To facilitate the initial extractions of PAs, some investigators have used large-scale apparati coupled with ion exchange columns while others have used smaller soxhlets (18,19). This paper will discuss recent chromatographic procedures for isolating PAs including analytical as well as preparative high performance liquid chromatography (HPLC).

DISCUSSION

My own laboratory expressed an interest in the rapid isolation of PAs in the mid 70s to aid our veterinary school with suspected PA poisonings. Two plants which contribute to livestock toxicities in California are Senecio vulgaris (common groundsel) and Amsinckia intermedia (fiddleneck).

A publication by Qualls and Segall was the first HPLC method to successfully separate PAs and used a single 10 μ Bondapak CN column (Waters Associates) and a THF/0.01 M ammonium carbonate (pH 7.8) solvent system (19). This procedure described the isolation of the macrocyclic PAs (retrorsine, seneciphylline, senecionine) from S. vulgaris (Fig. 3) and was also applied to two other Senecio species, S. longilobus (threadleaf groundsel) and S. jacobaea, commonly known as tansy ragwort (20,21). Senecio longilobus contains the same macrocyclic PAs as S. vulgaris plus an additional PA riddelline, which is structurally similar to retrorsine. Using this method the PAs derived from S. jacobaea were also isolated (20), Fig. 4. The procedure was further modified by the addition of another 10 μ CN Bondapak column in tandem as described in a later publication (22). The disadvantage of this chromatographic method was the relative high pH used (pH 7.8). Horvath et al. had stated that "the reproducibility and efficiency of these columns could be maintained as long as the mobile phase was kept at pH <7" (23). Reproducibility was not a problem, but maintaining the 10 μ CN Bondapak column efficiency for lengthy periods while operating at pH 7.8 was difficult (19-22).

Due to the above mentioned difficulties, a 10 μ C18 Bondapak column (3.9 mm x 30 cm) was utilized in conjunction with a 55:45

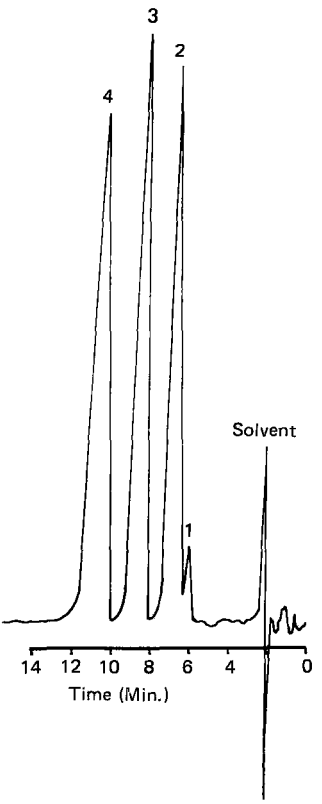


Figure 3. Isolation of pyrrolizidine alkaloids from Senecio vulgaris. Column 10 μ C18 Bondapak CN (Waters Associates), 3.9 mm x 30 cm. Solvent THF-0.01 M $(NH_4)_2CO_3$ (pH 7.8), linear gradient 13% → 26% THF over 30 min, flow rate 1.8 ml/min. Detector, SF770 (Kratos) at 235 nm. Peak 1 = Unknown, 2 = retrorsine, 3 = seneciphylline, 4 = senecionine.

methanol 0.01 M PO_4 (pH 6.3) solvent system (24). Excellent sensitivity was obtained and the lower pH was an important factor in extending column efficiency (Fig. 5). Another advantage of this technique was the low solvent costs, especially when this technique was later applied to semipreparative as well as preparative separations. Contrary to other reports (25), proper care of the

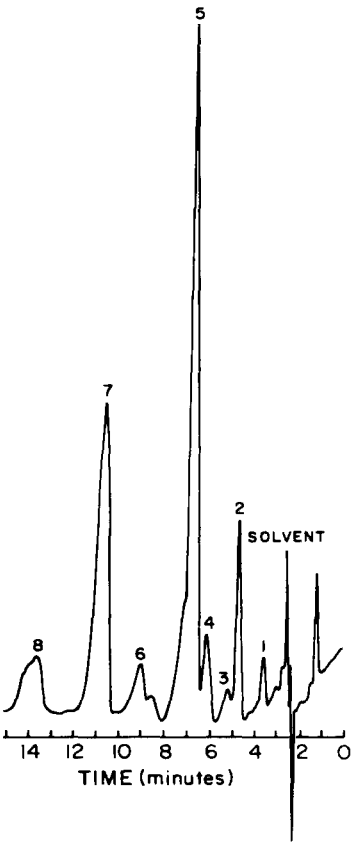


Figure 4. Isolation of pyrrolizidine alkaloids from Senecio jacobaea. Chromatography conditions as in Fig. 1. Peak 1 = jacoline, 2 = jacozine, 3 & 4 = jacobine and ? 5 = m/z 351 and m/z 385, 6 = jaconine, 7 = seneciphylline, 8 = senecionine.

reversed phase columns (flushing with water and programming to 100% MeOH at the end of each day) plus the advantage of using the low-cost solvents (water-methanol), make this a practical method.

Tittel et al. developed a quantative HPLC method to isolate the diester PAs (symphytine-N-oxide and echimidine-N-oxide) from Symphyti Radix (26). They used a 10 MN-Nucleosil® C18 column with solvents methanol/water (45:55). The methanol/water

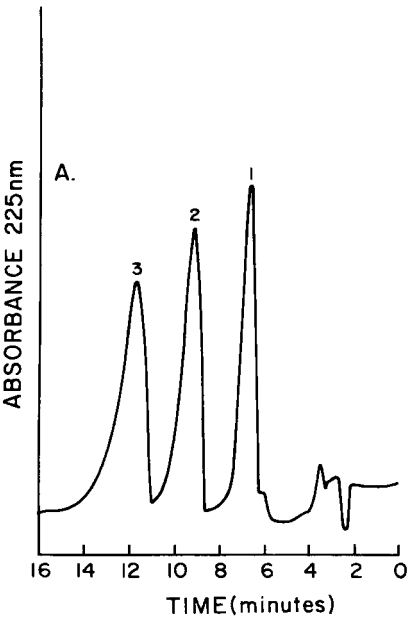


Figure 5. Separation of pyrrolizidine alkaloids from Senecio vulgaris. Column, 10 μ C18 Bondapak (Waters Associates), 3.9 mm x 30 cm. Solvent, MeOH/0.01 M KH_2PO_4 (pH 6.3), isocratic 55:45, flow rate 1.2 ml/min. Detector SF770 (Kratos) at 225 nm. Peak 1 = retrorsine, 2 = seneciphylline, 3 = senecionine.

solvent system was monitored at 220 nm which allowed a detectability of 40 ng of echimidine-N-oxide and 70 ng symphytine-N-oxide. In addition, the reduced PAs symphytine and echimidine were separated using a MN-Nucleosil®-NH_2 column with dichloromethane/propanol. The dichloromethane/propanol solvent system was monitored at 238 nm, which did not afford the sensitivity obtained when monitoring at 220 nm (26).

Huizing and Malingre (27) have successfully purified both monoester and diester PAs from the Boraginaceae using a polystyrene-divinylbenzene resin (XAD). The authors used a gradient elution of acidified methanol-water mixture to separate echimidine and symphytine, which are major alkaloids in the Symphytum species.

In addition, echimidine plus an unknown alkaloid fraction was isolated from Radix consolidae

Other investigators (25) have used a PRP-1 reversed phase resin column (HPLC) with a 10-30% acetonitrile and 0.1 M NH_4OH gradient in attempting to isolate the PAs from S. vulgaris and S. jacobaea (Fig. 6). The major disadvantage of this technique was the high pH (due to 0.1 M NH_4OH) as PAs are sensitive to alkaline pH.

Niwa and colleagues have recently developed a HPLC reversed phase system to separate the macrocyclic PAs of the otonecine type from the plant Petasites japonicus (29). P. japonicus is used as a folk medicine and food stuff in Japan and the PAs appear to be stable for at least one year at room temperature, according to Niwa et al. (28). Fortunately, it appears that the general population boil the young flower stalks of P. japonicus to remove the harsh taste (also known as processing) which removes slightly more than half of the PAs prior to use.

The authors were successful in separating the purified PAs neopetasitenine, senkirkine, and petasitenine as well as otosenine (otosenine is the stereoisomer of petasitenine with respect to the epoxy group). In addition, an alkaloidal mix from the ethanolic extracts of P. japonicus containing neopetasitenine, senkirkine, and petasitenine was successfully separated (Fig. 7). Previous HPLC methods which had been performed with PAs exhibiting the retronecine base (examples are senecionine and jacobine) were not applicable to the PAs exhibiting on otonecine base. The conditions used to separate the otonecine PAs are as follows: column, Cosmosil 5 pH (5μ, 15 cm x 4.6 mm), solvent system-isocratic methanol-0.02 M ammonium carbonate (45:55, pH 8.2) with a flow rate of 1.0 ml/min (Fig. 7). The relative high pH of 8.2 may lead to a premature destruction of the column according to the Cosmosil column bulletin, which advises maintaining a pH between 2 and 8 (29). A personal communication from Dr. Yamada stated that the pH of 8.2 did not appear to markedly decrease the efficiency of the Cosmosil 5 pH 5μ column.

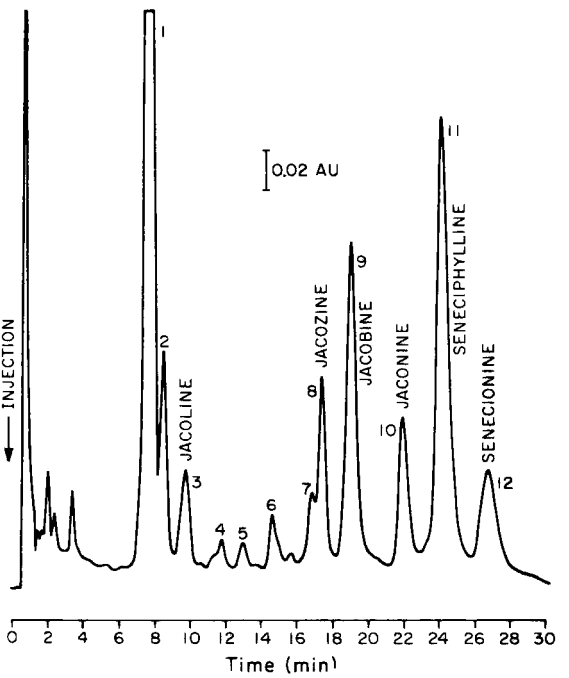


Figure 6. Separation of Senecio jacobaea extract. Column, PRP-1 (Hamilton), 4.1 mm x 15 cm. Solvent CH_3CN/0.01 M NH_4OH, linear gradient 10% to 30% CH_3CN over 20 min, flow rate 1 ml/min. Detector SF770 (Kratos) at 220 nm. Peaks labelled are jacoline, jacozine, jacobine, jaconine, seneciphylline, and senecionine.

Frahn et al. developed two preparative procedures to separate the diastereoisomers intermedine and lycopsamine which are found in the genera Amsinckia and Echium of the Boraginaceae and the genus Parsonsia of the Apocynaceae (30). Their procedures are based on the difference in the degree to which the vicinal glycol groups of different configurations (erythro and threo) complex with borate. The first method used a column of glass powder premoistened with a borax solution and eluted with chloroform (Fig. 8). The second method used a Bio-Rad AG 50 W-X2 resin (cation exchange) impregnated with 0.1 M borax and eluted with a 0.1 M borate solution

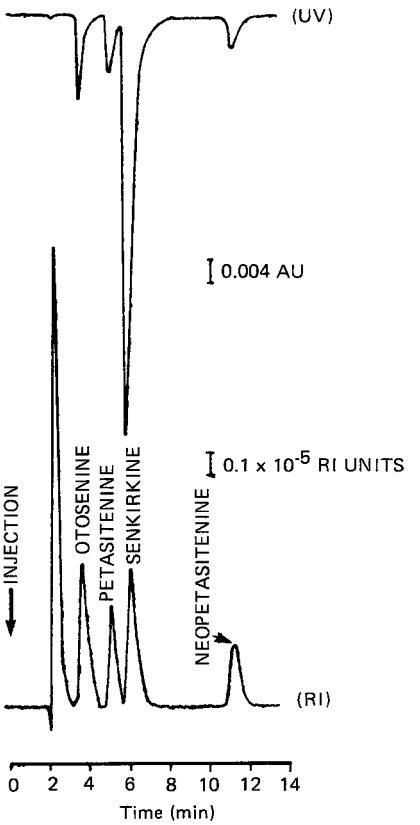


Figure 7. Separation of a mixture of otonecine pyrrolizidine alkaloids. Column, Cosmosil 5 Ph (Nakarai Chemicals), 4.6 mm x 15 cm. Solvent, MeOH/0.01 M $(NH_4)_2CO_3$ (pH 8.2), isocratic 45:55, flow rate 1 ml/min. Detectors, Uvidec-100-II UV spectrometer at 215 nm and Model SE^{-5}-II RI differential refractometer at sensitivity of 4 x 10^{-5} RI units. Peaks labelled are otosenine, petasitenine, senkirkine, and neopetasitenine.

(Fig. 9). Both methods may be used to isolate gram quantities of PAs, but aliquots must be taken from each tube, spotted on filter paper, and sprayed with manganese sulphate-potassium permanganate-sulphuric acid reagent to detect the alkaloid(s).

Mohanraj et al. (1982) proposed to separate diastereomeric PAs using alkalized silica gel (Silica gel C or G) with solvents

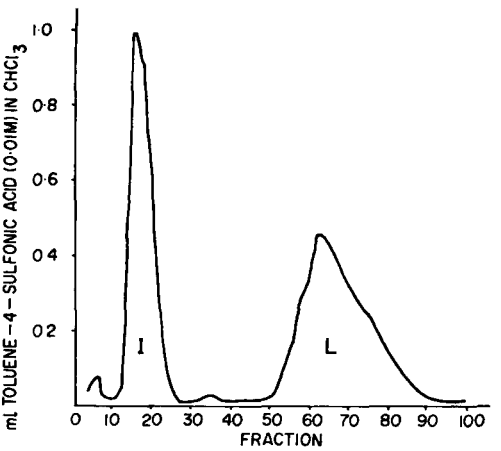


Figure 8. Elution pattern of intermedine (I) and lycopsamine (L) from borate partition column. Solvent, $CHCL_3$.

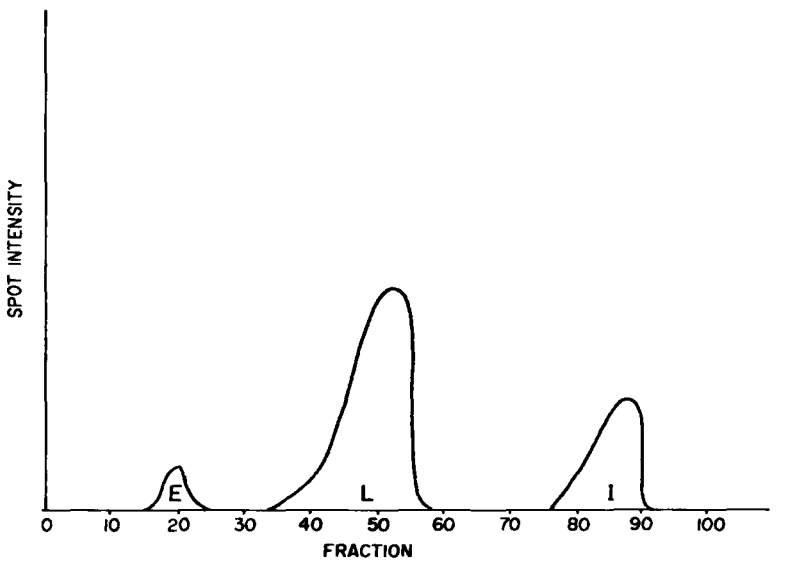


Figure 9. Elution pattern of echimidine (E), lycopsamine (L), and intermedine (I) from Bio-Rad AG 50W-X2 ion-exchange column. Solvent 0.1 M di-sodium tetraborate.

chloroform-methanol-25% ammonia (31). The authors used the PAs from Heliotropium curassavicum to separate curassavine, heliocurassavinine, coromandaline, heliocoromandaline, heliovicine, heliocurassavicine, and heliocurassavine. In addition to the required extraction of the PAs from H. curassavicum, PAs were fractionated on a column of neutral alumina prior to column chromatography on alkalized silica gel. Thin-layer chromatography was performed on silica gel C with the previously stated solvent system. Spots were visualized with iodine and/or Dragendorff's reagent.

Few investigators have successfully used HPLC to isolate large quantities of PAs. Our laboratory has used a reversed phase system which was based on our previously published analytical system (24). This method used a Waters Associates prep 500 system with a 0.005 M KH_2PO_4 pH 6.3/methanol solvent system (40:60) at 150 ml/min to separate retrorsine, seneciphylline, and senecionine from S. vulgaris (32).

To ensure that all three PAs were pure, recycling was used (Fig. 10). The advantage of this preparation were the low solvent costs (methanol/water), speed of separation, and the fact that no derivatives had to be made. The disadvantages were that not all investigators have access to a preparative system and the reverse phase columns required for this instrument are expensive. An adaptation of this method is to use the packing material found in the C18 prep 500 columns (Bondapak C18 50 → 125 μ particular packing material, Waters Associates) and bulkpack two 22.5 mm x 50 cm stainless steel columns, Whatman, Clifton, N.J. This method used a 0.01 M PO_4 pH 6.3/methanol (50:50) solvent system with a flow rate of 9 ml/min to isolate two PA metabolites from an in vitro hepatic microsomal mix (33). ^{14}C-labelled senecionine, ^{14}C-seneciphylline, and ^{14}C-retrorsine were isolated in part from S. vulgaris using a 7.8 mm x 300 cm column system, the Bondapak C18 50 → 125 μ particular packing material, and solvent system as previously described. (7).

Huizing et al. described the preparative ion-pair high performance liquid chromatography method to isolate the macrocyclic

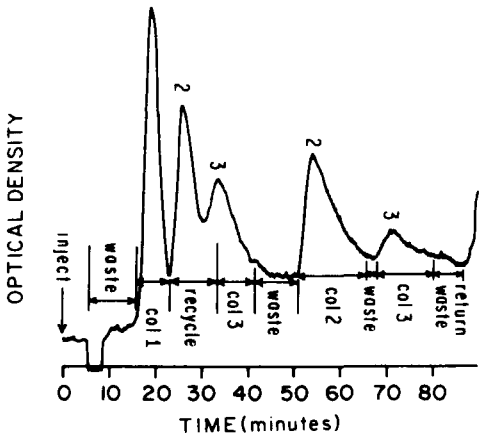


Figure 10. Preparative analysis of pyrrolizidine alkaloids derived from Senecio vulgaris. Columns, two prep-500/C18 reverse phase. Solvent, MeOH/0.005 M KH_2PO_4 (pH 6.3) isocratic (60:40), flow rate 150 ml/min. Detector RI (Waters Associates). Peak 1 = retrorsine, 2 = seneciphylline, 3 = senecionine.

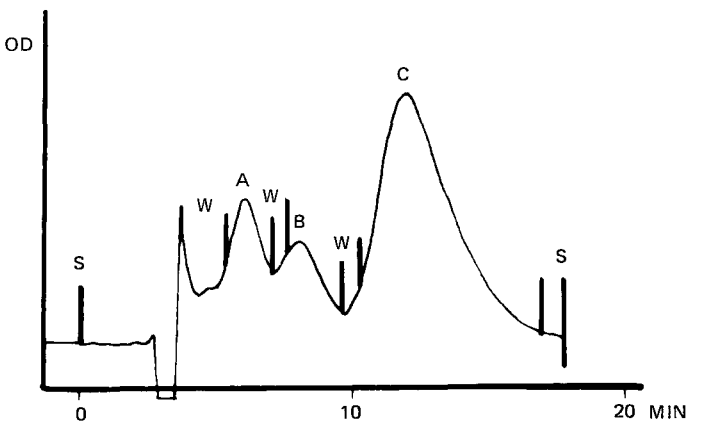


Figure 11. Preparative analysis of pyrrolizidine alkaloids from comfrey as ion pairs. Column, silica gel 60, 230-400 mesh, 2.5 cm x 53 cm stainless steel (Waters Associates). Solvent, .075 M lithium chloride in $CHCL_3$/MeOH isocratic (85:15), flow rate 50 ml/min. Detector RI (Waters Associates). Peak S = injection, W = waste, A = lycopsamine-intermedine, b = acetyllycopsamine + acetylintermedine, C = symphytine and/or isomer.

PAs from ground comfrey roots (34). A 25 mm x 53 cm stainless steel column (Waters Associates) was packed with dry silica gel 60 (230-400 mesh, Merck) connected to a Waters preparative LC/system 500A with solvent consisting of 0.075 M lithium chloride in chloroform/methanol (85:15) at a flow rate of 50 ml/min (Fig. 11). To reduce their solvent costs, the authors redistilled their solvents and added additional chloroform, or methanol to ensure a 85:15 solvent ratio.

An investigator who does not have access to HPLC can take comfort that many of the PAs were initially separated using silica gel columns and TLC techniques. Although more time consuming than "newer" chromatography techniques, they should not be overlooked.

ACKNOWLEDGMENT

The author gratefully acknowledges the excellent technical assistance of Crawford Brown and Thomas Krick. This work was supported by grants from the University of California (Faculty Research Grants, Livestock Disease Research Grants), the National Science Foundation (PFR-7806924), and the National Institute of Health (ES-03343).

Special thanks is extended to the publishers for permission to use the following figures and figure legends:

Figs. 3, 6, 7, 8, 9, & 11; J. Chromatogr., Elsevier Science Publishers.

Fig. 4.; Toxicol. Lett., Elsevier Science Publishers

Figs. 5 & 10.; J. Liquid Chromatogr., Marcel Dekker, Inc.

REFERENCES

1. Bull, L. B., Culvenor, C. C. J., and Dick, A. T., The pyrrolizidine alkaloids, North-Holland, Amsterdam, 1968.

2. McLean, E. K., The toxic actions of pyrrolizidine (Senecio) alkaloids, Pharm. Rev. 22, 429-483, 1970.

3. Hill, K. R., Rhodes, K., Stafford, J. L., and Aub, R., Liver diseases in Jamaican children (serous hepatosis), West Indian Med. J., 1, 49-63, 1951.

4. Huxtable, R. J., Herbal teas and toxins: Novel aspects of pyrrolizidine poisoning in the United States, Perspect. Biol. Med., 24, 1-4, 1980.

5. Culvenor, C. C. J., Clarke, J. A., Edgar, J. A., Frahn, J. L., Jago, M. V., Peterson, J. E. and Smith L. W., Structure and toxicity of the alkaloids of Russian comfrey (Symphytum and Uplandicum Nyman), a medicinal herb and item of human diet, Experientia, 15, 377-379, 1980.

6. Mattocks, A. R., Toxic pyrrolizidine alkaloids in comfrey, Lancet, 1136-1137, 1980.

7. Eastman, D. F., Dimenna, G. P., and Segall, H. J., Covalent binding of senecionine and seneciphylline to hepatic macromolecules and their distribution, excretion and transfer into milk of lactating mice, Drug Metab. Dispos., 10, 236-240, 1982.

8. Fowler, M. E., Pyrrolizidine alkaloid poisoning in calves, J. Am. Vet. Med. Assoc., 152, 1131-1137, 1968.

9. Deinzer, M. L., Thomson, P. A., Bergett, D. M., and Isaacson, D. L., Pyrrolizidine alkaloids: Their occurrence in honey from tansy ragwort (Senecio jacobaea L.). Science, 195, 497-499, 1977.

10. Mohabbat, O., Srivastava, R. M., Younos, M. S., Sediq, G. G., Merzad, A. A. and Aram, G. N., An outbreak of hepatic veno-occlusive disease in northwestern Afghanistan, Lancet, 2, 269-271, 1976.

11. Tandon, B. M., Tandon, R. K., Tandon, H. D., Narndranathan, M. and Joshi, Y. K., An epidemic of veno-occlusive disease of liver in central India, Lancet, 2, 271-272, 1976.

12. Harris, P. M. and Chen, K. K., Development of hepatic tumors in rats following ingestion of Senecio longilobus, Cancer Res., 30, 2881-2886, 1970.

13. Schoental, R., Toxicity and carcinogenic action of pyrrolizidine alkaloids, Cancer Res., 28, 2237-2246, 1968.

14. Yamanaka, H., Nagao, M., Sugimura, T., Furuya, T., Shirai, A., and Matsushima, T., Mutagenicity of pyrrolizidine alkaloids in the Salmonella/Mammalian microsome test, Mutation Res., 68, 211-216, 1979.

15. Koekemoer, M. J. and Warren, F. L., The Senecio alkaloids. Part VIII. The occurrence and preparation of the N-oxides. An improved method of extraction of the Senecio alkaloids, J. Chem. Soc. (Lond), 66-68, 1951.

16. Bradbury, R. B. and Culvenor, C. C. J., The alkaloids of Senecio jacobaea L. I. Isolation of the alkaloids and identification of jacodine as seneciphylline, Aust. J. Chem., 7, 378-383, 1954.

17. Mattocks, A. R., Extraction of heat-labile alkaloids from plants, Nature, 191, 1281-1282, 1961.

18. Deagen, J. T. and Deinzer, M. L., Improvements in the extraction of pyrrolizidine alkaloids, J. Natural Prod. (Lloydia), 40, 395-397, 1977.

19. Qualls, C. W. and Segall, H. J., Rapid isolation and identification of pyrrolizidine alkaloids (Senecio vulgaris) utilizing high pressure liquid chromatography, J. Chromatogr., 150, 202-206, 1978.

20. Segall, H. J., and Molyneux, R. J., Identification of pyrrolizidine alkaloids (Senecio longilobus), Res. Commun. Chem. Pathol. Pharm., 19, 545-548, 1978.

21. Segall, H. J., Pyrrolizidine alkaloids derived from Senecio jacobaea, Toxicol. Lett., 1, 279-284, 1978.

22. Segall, H. J. and Krick, T. P., Pyrrolizidine alkaloids organohalogen derivative isolated from Senecio jacobaea (tansy ragwort), Toxicol. Lett., 4, 193-198, 1979.

23. Horvath, C., Melander, W., and Molnár, I., Solvophobic interactions in liquid chromatography with nonpolar stationary phases. J. Chromatogr., 125, 129-156, 1976.

24. Segall, H. J., Reverse phase isolation of pyrrolizidine alkaloids, J. Liquid Chromatogr., 2, 424-436, 1979.

25. Ramsdell, H. S. and Buhler, D. R., High-performance liquid chromatographic analysis of pyrrolizidine (Senecio) alkaloids using a reversed-phase styrene-divinylbenzene resin column, J. Chromatogr. 210, 154-158, 1981.

26. Tittel, G., Hinz, H. and Wagner H., Quantitative bestimmung der pyrrolizidinalkaloide in Symphyti Radix durch HPLC, Planta Medica., 37, 1-8, 1979.

27. Huizing, H. J. and Malingre, T. M., Purification and separation of pyrrolizidine alkaloids from Boraginaceae on a polystyrene-divinylbenzene resin, J. Chromatogr., 176, 274-279, 1979.

28. Niwa, H., Ishiwata, H., and Yamada, K., Separation and determination of macrocyclic pyrrolizidine alkaloids of the otonecine type present in the edible plant Petasites

japonicus by reversed-phase high-performance liquid chromatography, J. Chromatogr., 257, 146-150, 1983.

29. Nakarai Chemical Bulletin, Specially Prepared Reagents, Cosmosil packed columns, Nakarai Chemicals, LTD, Nijyo Karasuma Nakagyo-Ku Kyoto, 1983.

30. Frahn, J. L., Culvenor, C. C. J., and Mills, J. A., Preparative separation of the pyrrolizidine alkaloids, intermedine and lycopsamine, as their borate complexes, J. Chromatogr, 195, 379-383, 1979.

31. Mohanraj, S., Herz, W. and Subramanian, P.S., Separation of diastereomeric pyrrolizidine alkaloids by chromatography on alkalized silica gel, J. Chromatogr., 238, 530-534, 1982.

32. Segall, H. J., Preparative isolation of pyrrolizidine alkaloids derived from Senecio vulgaris, J. Liquid Chromatogr., 2, 1319-1323, 1979.

33. Segall, H. J., Dallas, J. L. and Haddon, W. F., Two dihydropyrrolizine alkaloid metabolites isolated from mouse hepatic microsomes in vitro, Drug Metab. Dispos., 12, 68-71, 1984.

34. Huizing, H. J., DeBoer, F., and Malingre, Th. M., Preparative ion-pair high-performance liquid chromatography and gas chromatography of pyrrolizidine alkaloids from comfrey, J. Chromatogr., 214, 257-262, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(S-2), 393-431 (1984)

THEORETICAL FOUNDATIONS OF LIQUID ADSORPTION CHROMATOGRAPHY WITH MIXED ELUENT

M.Jaroniec and J.A.Jaroniec
Institute of Chemistry, M.Curie-Skłodowska University,
20031 Lublin, Poland

INTRODUCTION

One of the main advantages of liquid-solid (adsorption) chromatography (LSC) with mixed eluent is the possibility of modification of the capacity ratio over a wide range by changing the eluent composition [1].It means that the resolution and the analysis time may be optimized by correct choice of the eluent composition (isocratic LSC) or by eluent programing during the chromatographic process (gradient LSC).Optimization of the chromatographic process requires of the knowledge of dependence of the chromatographic quantities on the eluent composition [2-5]. Increasing interest in gradient LSC [2-13] has created a pressing need for the elucidation of the theoretical basis of LSC with mixed eluent.

Many attempts have been made to describe the dependence of the capacity ratio on the eluent composition [14-21]. The fundamental ideas in this field have been formulated by Snyder [14,21,22], Ościk [23-25] and Soczewiński [15,26],and developed theoretically by Jaroniec et al.[27-34].They pointed many factors determining the LSC process with mixed eluent : (a) competitive character of solute and solvent adsorption,

 0148-3919/84/07S2-0393$3.50/0

(b) non-specific and specific solute-solvent and solvent-solvent interactions in the mobile and surface phases,

(c) association or dissociation of components in the mobile and surface phases,

(d) differences in molecular sizes of solutes and solvents,

(e) multilayer character of the surface phase and contribution of the partition phenomena in the LSC process,

(f) orientation of solute and solvent molecules in the surface phase, and

(g) energetic heterogeneity of the adsorbent and topography of adsorption sites onto surface.

These factors determined mainly the adsorption effects in the LSC process.The other group of factors, such as : diffusion, porous structure of the adsorbent, parameters characterizing the eluent flow, etc., determines kinetics of the LSC process. The problem of their optimization is described in the many monographs devoted to chromatography.

In this chapter we shall present the theoretical studies dealing with the effects specified in the points (a) - (g). One of the first formulations of the LSC process with mixed eluent has been made by Ościk [23-25].Basing on the thermodynamics of conformal solutions [35], he derived an equation for the $R_{M,s}$ value of the s-th solute chromatographed in n-component eluent. This equation has been derived for energetically homogeneous solid surfaces.Its theoretical analysis is presented in the papers [25,27,16].The clasical Ościk's equation has been recently extended to energetically heterogeneous solid surfaces [36-38]. Moreover,it has been widely examined by using the thin-layer chromatographic (TLC) data [39-45].It is difficult to discuss the influence of the factors (a)-(g) on the LSC process in terms of the Ościk's formulation,because it is too much general and gives a scanty information about mechanism of the LSC process. Therefore,our discussion will be concentrated on the theoretical treatments assuming a definite model of the LSC process.

The most popular treatment of LSC with mixed eluent has been formulated by Snyder [14,22], which assumes the competitive character of solute and solvent adsorption. This treatment involves also energetic heterogeneity of the solid surface [14] and solute-solvent localization effects [46]. Recently, Snyder's treatment has been extended to LSC with multicomponent eluent [16,27,47-49]. The LSC model of Snyder [14,21] has been considerably enriched by Soczewiński studies [15,26]. On the other hand, Jaroniec et al. [16,27-34] described theoretically the LSC process by applying the general theory of adsorption from multicomponent liquid mixtures on solid surfaces and utilizing the fundamental studies of Snyder [14,22] and Soczewiński [15,26]. The treatment of Jaroniec et al. involves energetic heterogeneity of the solid surface [27-29,50-55], non-ideality of both phases [27,28,33], differences in molecular sizes of solute and solvents [29,31,56], and solute-solvent and solvent-solvent interactions in the mobile phase [30,31,57-60]. Recent studies of Jaroniec et al. [29,37,56,61] concern the correlation between adsorption and chromatographic parameters. An attempt of a global description of the LSC process with mixed eluent, involving different effects specified in the points (a)-(g), has been undertaken by M. Jaroniec and J.A. Jaroniec [31]. This chapter has been written basing on the paper [31]. We shall show that the majority of equations derived for the capacity ratio in LSC with mixed eluent may be obtained from our general treatment; they are : equations of Snyder [14], Snyder-Soczewiński [21,26], Slaats et al. [62].

GENERAL CONSIDERATIONS

One of the most important quantities in LSC is the capacity ratio k'_s, which is defined as the ratio of the number of moles of the s-th component in the surface phase to the number of moles of this component in the mobile phase. The other fundamental chromatographic quantities, such as retention volume, retention time, selectivity and resolution, may be expressed by means of the capacity

ratio.The capacity ratio k'_s is proportional to the distribution coefficient k_s :

$$k_s = x_s^s/x_s^l = C\, k'_s \qquad (1)$$

where C is a parameter which is assumed,to a first approximation, to be characteristic of a given adsorbent and independent of the nature of the eluent [14,33].In the above x_s^s and x_s^l are the total mole fractions of the s-th solute in the surface (s) and mobile (liquid) (l) phases,respectively.

The fundamental quantities employed in TLC are R_F and R_M .These values are related to the capacity ratio by the following equation [63] :

$$R_{M,s} = \log [(1-R_{F,s})/R_{F,s}] = \log k'_s \qquad (2)$$

Eq.(2) may be used for interconversion of equations applied in LSC and TLC.

Let us consider the LSC process for the s-th substance (solute) chromatographed in n-component eluent.The components of the mixed eluent are numbered successively beginning from the most efficient eluting solvent to the weakest solvent.Thus, 1-st solvent is the more efficient one,however, n-th component is the weakest solvent. One of the main assumptions of the proposed model is that concerning the competitive character of solute and solvent adsorption.

The adsorption process may be represented by the following reversible phase-exchange reactions [28] :

$$i^{(l)} + n^{(s)} \rightleftarrows i^{(s)} + n^{(l)} \quad \text{for } i = 1,2,\ldots,n-1 \qquad (3)$$

$$s^{(l)} + r\, i^{(s)} \rightleftarrows s^{(s)} + r\, i^{(l)} \quad \text{for } i = 1,2,\ldots,n \qquad (4)$$

where the subscripts (l) and (s) refer to the mobile and surface phases,respectively, "i" and "s" denote molecules of the i-th solvent and s-th solute, and r is the ratio of molecular sizes of the s-th solute and the i-th solvent.The reactions (3) and (4) have been written by assuming the equality of molecular sizes of all solvents, i.e.,

$$w_1 = w_2 = \cdots = w_n = w \tag{5}$$

and inequality of molecular sizes of solute and solvents :

$$w_s \neq w \tag{6}$$

Thus, the parameter r is defined as follows :

$$r = w_s/w \tag{7}$$

The next assumptions are following :

(a) the surface phase is assumed to be monolayer,

(b) the total number of moles of all solvents in the surface phase is constant and independent upon the presence of solute molecules, because the solute concentration is infinitely low, and

(c) molecules of solute and solvents have a spherical shape.

Further assumptions concern interactions in the surface and mobile phases and energetic heterogeneity of the adsorbent surface.

A MODEL INVOLVING NON-SPECIFIC INTERACTIONS BETWEEN MOLECULES OF SOLUTE AND SOLVENTS

Homogeneous solid surfaces

The non-specific interactions between molecules of solute and solvents in the mobile and surface phases are described in terms of the activity coefficients. The activities of the s-th solute and i-th solvent in the mobile and surface phases are defined as follows:

$$a_s^l = x_s^l f_s^l \tag{8a}$$

$$a_i^l = x_i^l f_i^l \quad \text{for } i=1,2,\ldots,n \tag{8b}$$

$$a_s^s = x_s^s f_s^s \tag{9a}$$

$$a_i^s = x_i^s f_i^s \quad \text{for } i=1,2,\ldots,n \tag{9b}$$

where f_s^s, f_i^s, f_s^l and f_i^l are the activity coefficients of the s-th solute and i-th solvent in the surface and mobile phases, respectively. The activity coefficients f_s^s and f_i^s are functions of the composition of the surface solution, whereas, f_s^l and f_i^l are functions of the composition of the bulk solution.

According to our previous studies [16,28,31] the reaction (4) for i=1 and the set of reactions (3) characterize synonymously the LSC process with mixed eluent.The thermodynamic equilibrium constants corresponding to these reactions may be written in the following form :

$$K_{s1}^{th} = \frac{a_s^s}{a_s^l}\left(\frac{a_1^l}{a_1^s}\right)^r = \frac{x_s^s}{x_s^l}\left(\frac{x_1^l}{x_1^s}\right)^r \cdot \beta_{s1} \qquad (10)$$

$$K_{in}^{th} = \frac{a_i^s}{a_i^l}\cdot\frac{a_n^l}{a_n^s} = \frac{x_i^s}{x_i^l}\cdot\frac{x_n^l}{x_n^s}\cdot\beta_{in} \quad \text{for } i= 1,2,\ldots,n-1 \qquad (11)$$

where

$$\beta_{s1} = \frac{f_s^s}{f_s^l}\left(\frac{f_1^l}{f_1^s}\right)^r \qquad (12)$$

$$\beta_{in} = \frac{f_i^s}{f_i^l}\cdot\frac{f_n^l}{f_n^s} \qquad \text{for } i= 1,2,\ldots,n-1 \qquad (13)$$

Combining eqs.(1) and (10) we obtain the most general equation for the capacity ratio of the s-th solute chromatographed in n-component eluent on an energetically homogeneous solid surface :

$$k_s' = K_{s1}^{th}\,(C\,\beta_{s1})^{-1}\,(x_1^s/x_1^l)^r \qquad (14)$$

or

$$\ln k_s' = \ln(K_{s1}^{th}/C) + r\,\ln(x_1^s/x_1^l) - \ln\beta_{s1} \qquad (15)$$

The mole fraction of 1-st solvent in the surface phase may be calculated according to the following expression :

$$x_1^s = \frac{(K_{1n}^{th}/\beta_{1n})\,x_1^l}{x_n^l + \sum_{i=1}^{n-1}(K_{in}^{th}/\beta_{in})\,x_1^l} \qquad (16)$$

Eq.(16) is one of the fundamental equations in the theory of adsorption from multicomponent liquid mixtures [64-68].This equation describes adsorption of 1-st solvent from n-component liquid mixture

on a homogeneous solid surface by assuming equality of molecular sizes of all solvents (eq.5) and non-ideality of the bulk and surface solutions ($\beta_{in} \neq 1$ for i=1,2,...,n-1).Similarly,eq.(14) has been derived by applying these same assumptions as in the case of eq.(16) and including an additional assumption (6).

Now,we shall consider the special cases of eq.(14).Assuming in eq.(14) ideality of the surface phase ($f_s^s = f_1^s = f_2^s = \ldots = f_n^s = 1$), non-ideality of the mobile phase ($f_s^l \neq f_1^l \neq f_2^l \neq \ldots \neq f_n^s \neq 1$) and $r \neq 1$ (difference in the molecular sizes of solute and solvents) we have :

$$k'_s = K_{s1}^{th} \, (f_s^l/C) \, (x_1^s/a_1^l)^r \tag{17}$$

and

$$x_1^s = K_{1n}^{th} \, a_1^l \, /(a_n^l + \sum_{i=1}^{n-1} K_{in}^{th} \, a_i^l) \tag{18}$$

If the elution strength of the 1-st solvent is considerably greater than the strengths of the other solvents,then the mole fraction x_1^s is practically equal to unity in the whole concentration region of x_1^l except at low concentrations of the 1-st solvent.Taking this fact into account in eq.(17) we get the relationship :

$$\ln k'_s = \ln(K_{s1}^{th}/C) - r \ln a_1^l + \ln f_s^l \tag{19}$$

The above relationship has been derived and examined experimentally by Slaats et al.[62].However,eq.(19) with r=1 and a regular mobile phase has been discussed by Jaroniec et al.[33].

Assuming both phases to be ideal and $r \neq 1$ in eq.(14) we have :

$$k'_s = (K_{s1}^{th}/C) \, (x_1^s/x_1^l)^r \tag{20}$$

where

$$x_1^s = K_{1n}^{th} \, x_1^l/(x_n^l + \sum_{i=1}^{n-1} K_{in}^{th} \, x_i^l) \tag{21}$$

Eq.(20) has been derived by Jaroniec et al.[56] and it was widely examined by using TLC data for binary eluents.For $x_1^s = 1$ (large

difference in elution strength between the 1-st and other solvents), eq.(20) gives :

$$\ln k'_s = \ln(K_{s1}^{th}/C) - r \ln x_1^1 \qquad (22)$$

Theoretical studies [27,33] show that the capacity ratio of the s-th solute in the 1-st pure solvent is connected with K_{s1}^{th} in the following way :

$$k'_{s1} = K_{s1}^{th}/C \qquad (23)$$

Then,eq.(22) assumes a more simpler form :

$$\ln k'_s = \ln k'_{s1} - r \ln x_1^1 \qquad (24)$$

Eq.(24) ,known as the Snyder-Soczewiński relationship [21,26], is one of the most popular equations in the theory of LSC.Jandera and Churacek [69] derived this equation by using Snyder's relationship for elution strength of a binary solvent [14,21].The correct analysis of Snyder's treatment [14] leads to eq.(24) with r=1 .The derivation of eq.(24) in terms of Snyder's treatment [14] by Jandera and Churacek [69] is slightly inconsistent [27,70].However,the full form of this equation results from theoretical considerations of Soczewiński [26] and our studies [16,29,31,56].Eq.(24) is widely used for characterizing LSC systems [71-86]; in reference [71] the chromatographic systems comply with this equation are discussed.

The very important relationship (25) may be obtained from eqs.(20),(21) and (23) for r = 1 ; it is

$$1/k'_s = \sum_{i=1}^{n-1} x_i^1/k'_{si} \qquad (25)$$

This relationship has been derived by Jaroniec et al.[27] in terms of the theory of adsorption from ideal multicomponent liquid mixtures on homogeneous solid surfaces.It may be rewritten in the equivalent form:

$$k'_s = \sum_{i=1}^{n-1} x_i^s \, k'_{si} \qquad (26)$$

Eq.(66) assumes the additivity of the capacity ratios k'_{si} for $i=1,2,\ldots,n$,and in this form it has been written by Snyder [14]. Eq.(25) for n=2 gives the following important relationship [70]:

$$1/k'_s = 1/k'_{s2} + (1/k'_{s1} - 1/k'_{s2}) x_1^1 \qquad (27)$$

The same type of dependence of the capacity ratio upon the mobile phase composition results from Scott and Kucera treatment [18].

Heterogeneous solid surfaces

Let us consider an energetically heterogeneous solid surface showing L types of adsorption sites.Let h_l be the ratio of the number of adsorption sites of the l-th type to the total number of adsorption sites.The ratios h_l for $l=1,2,\ldots,L$ fulfil the relationship :

$$\sum_{l=1}^{L} h_l = 1 \qquad (28)$$

The capacity ratio of the s-th solute on the entire heterogeneous solid surface $k'_{s,t}$ is defined as follows [14,16,34]:

$$k'_{s,t} = \sum_{l=1}^{L} h_l \, k'_{s,l} \qquad (29)$$

where $k'_{s,l}$ is the capacity ratio of the s-th solute for the l-th type of adsorption sites.The mathematical form of $k'_{s,l}$ is analogous to eq.(14).Since eq.(14) contains the activity coefficients of solute and solvents in the surface phase,which are functions of the surface phase composition,we consider their definitions by assuming two different distributions of adsorption sites on the surface.Theoretical considerations of adsorption models with non-ideal surface phase require always of an additional assumption about topography of adsorption sites on the surface [67,68].Usually two models of heterogeneous surfaces are considered.According to the first model the adsorption sites are distributed randomly onto surface,whereas, the second assumes the patchwise distribution of adsorption sites.

For the patchwise surfaces the activity coefficients are functions of composition of surface solution being on the l-th surface patch. However,for surfaces of random distribution of adsorption sites the activity coefficients are functions of the mole fractions of solute and solvents refering to the entire surface solution. The total mole fraction $x^s_{1,t}$,refering to the entire surface, is expressed as follows :

$$x^s_{1,t} = \sum_{l=1}^{L} h_l \, x^s_{1,l} \tag{30}$$

Taking into account the above discussion concerning the activity coefficients in the surface phase and eq.(14),we can write the following expressions for $k'_{s,l}$:

$$k'_{s,l} = K^{th}_{s1,l} \, (C \, \beta_{s1,l})^{-1} \, (x^s_{1,l}/x^l_1)^r \quad \text{for patchwise surfaces} \tag{31}$$

$$k'_{s,l} = K^{th}_{s1,l} \, (C \, \beta_{s1,t})^{-1} (x^s_{1,l}/x^l_1)^r \quad \text{for random surfaces} \tag{32}$$

Substituting eqs.(31) and (32) to eq.(29) we obtain the expressions for the capacity ratio on heterogeneous surfaces of patchwise and random distribution of adsorption sites.The most important equation is obtained for random surfaces; it is :

$$k'_{s,t} = C^{-1} \frac{f^l_s}{f^s_{s,t}} \left(\frac{f^s_{1,t}}{a^l_1} \right)^r \sum_{l=1}^{L} h_l \, K^{th}_{s1,l} \, (x^s_{1,l})^r \tag{33}$$

where $f^s_{s,t}$ and $f^s_{1,t}$ are functions $x^s_{1,t}$, $x^s_{2,t}$, ..., $x^s_{n,t}$.
It has been shown in the paper [34] that for adsorbents characterized by quasi-Gaussian energy distribution eq.(33) may be approximated as follows :

$$k'_{s,t} = C^{-1} \frac{f^l_s}{f^s_{s,t}} \left(\frac{f^s_{1,t}}{a^l_1} \right)^r \bar{K}^{th}_{s1} \, (x^s_{1,t})^{r/m} \tag{34}$$

where

$$x^s_{1,t} = (\bar{K}^{th}_{1n} \, x^l_1/\beta_{1n,t})^m \Big/ \left[(x^l_n)^m + \sum_{i=1}^{n-1} (\bar{K}^{th}_{in} \, x^l_i/\beta_{in,t})^m \right] \tag{35}$$

and m is the heterogeneity parameter relating to the width of the quasi-Gaussian distribution and $\bar{K}^{th}_{s1}$ is an average from $K^{th}_{s1,L}$ values.

Now,we consider the special cases of eq.(34) in a manner analogous to that adopted in the previous section.
For ideal surface phase and non-ideal mobile phase eqs.(34) and (35) assume the following form:

$$k'_{s,t} = C^{-1} f^1_s (a^1_1)^{-r} \bar{K}^{th}_{s1} (x^s_{1,t})^{r/m} \qquad (36)$$

and

$$x^s_{1,t} = (\bar{K}^{th}_{1n} a^1_1)^m / \left[(a^1_n)^m + \sum_{i=1}^{n-1} (\bar{K}^{th}_{in} a^1_i)^m \right] \qquad (37)$$

For m=1 eq.(37) reduces to eq.(18); however, for $x^s_{1,t} = 1$ it becomes eq.(19).According to eq.(36) ,for a large difference in elution strengths between the 1-st and other solvents,the energetic heterogeneity of the adsorbent surface does not influence the capacity ratio,because we obtain for $x^s_{1,t} = 1$ the same relationship as for homogeneous solid surfaces (cf.eq.19).Since eq.(36) has been obtained for ideal surface phase,it may be applied for heterogeneous surfaces showing quasi-Gaussian energy distribution and arbitrary topography of adsorption sites[28].

If both phases are ideal,eqs.(34) and (35) reduce to the following expressions :

$$k'_{s,t} = C^{-1} \bar{K}^{th}_{s1} (x^1_1)^{-r} (x^s_{1,t})^{r/m} \qquad (38)$$

where

$$x^s_{1,t} = (\bar{K}^{th}_{1n} x^1_1)^m / \left[(x^1_n)^m + \sum_{i=1}^{n-1} (\bar{K}^{th}_{in} x^1_i)^m \right] \qquad (39)$$

For $x^s_{1,t} = 1$ eq.(38) becomes the Snyder-Soczewiński relationship (24).

For r=1 (equal molecular sizes of solutes and solvents) eqs.(38) and (39) give the following relationship :

$$(1/k'_{s,t})^m = \sum_{i=1}^{n} (x_i^1/k'_{si,t})^m \qquad (40)$$

Eq.(40) defines the capacity ratio $k'_{s,t}$ by means of the capacity ratios $k'_{si,t}$ and the parameter m .This equation has been derived by Jaroniec et al.[34].This equation may be rewritten in the other form :

$$k'_{s,t} = \sum_{i=1}^{n} k'_{si,t} \, (x_{i,t}^s)^{1/m} \qquad (41)$$

which for m=1 reduces to the relationship (26).

Eq.(40) was examined by using HPLC data [34] and TLC data for binary [51] and ternary eluents [52,53].The chromatographic data for binary eluents were presented in the following linear form :

$$(k'_{s,t} \, x_1^1)^{-m} = (k'_{s1,t})^{-m} + (k'_{s2,t})^{-m} \, (x_2^1/x_1^1)^m \qquad (42)$$

In the case of TLC data eq.(42) may be rewritten as follows [51]:

$$(x_1^1 \, 10^{R_{M,s}})^{-m} = A_2 \, (x_2^1/x_1^1)^m + B_2 \qquad (43)$$

where

$$A_2 = 10^{-mR_{M,s2}} \quad ; \quad B_2 = 10^{-m \, R_{M,s1}} \qquad (44)$$

However,in the case of ternary eluents eq.(40) for TLC gives [52,53]:

$$F = (x_2^1 \, 10^{R_{M,s}})^{-m} = A_3 (x_1^1/x_2^1)^m + B_3 \qquad (45)$$

where

$$A_3 = 10^{-m \, R_{M,s1}} \qquad (46)$$

and

$$B_3 = 10^{-m \, R_{M,s2}} + (x_3^1/x_2^1)^m \, 10^{-m \, R_{M,s3}} \qquad (47)$$

If the experimental data $R_{M,s}$ are measured at $x_3^1/x_2^1 = \text{const}$, the dependence F vs. $(x_1^1/x_2^1)^m$ is linear.

For the purpose of illustration we presented the results of numerical

analysis of TLC data made by means of eq.(45).These results concern the TLC data for eight polycyclic hydrocarbons chromatographed in chloroform(1)/toluene(2)/benzene(3) and chloroform(1)/toluene(2)/carbon tetrachloride(3) eluents on silica gel at 25°C [52]. All measurements were carried out at $x_3/x_2=1.0$ .Fig.1 shows the linear dependence (45) for selected solutes.It follows from this figure that eq.(45) gives a good representation of the above TLC data.The parameters m , A_3 and B_3 ,found from the linear plots (45),are summarized in Table 1.In this table the R_M-values for polycyclic hydrocarbons chromatographed in single solvents, i.e., $R_{M,s1}$, $R_{M,s2}$, $R_{M,s3}$ are also given.These values have been used for prediction of the parameters A_3 and B_3 according to eqs.(46) and (47), and are denoted by A_3^o and B_3^o (c.f., Table 1).The parameters A_3^o and B_3^o,calculated directly from experimental values of $R_{M,s1}$, $R_{M,s2}$ and $R_{M,s3}$ according to eqs.(46) and (47),and those evaluated from TLC data for ternary eluents by means of eq.(45), are compared in Table 1 .It follows from this comparison that differences between A_3 and A_3^o , B_3 and B_3^o are rather small.Analysis of the parameter m shows that it is practically independent on the kind of polycyclic hydrocarbon ;it is characteristic for a given eluent and adsorbent. Similar results were also obtained for other TLC systems [34,51,87]. A good agreement between A_3 and A_3^o , B_3 and B_3^o ,and insignificant dependence of m on type of the solute create possibility for predicting the $R_{M,s}$-values for solutes chromatographed in ternary eluents by means of the experimental values of $R_{M,s1}$, $R_{M,s2}$, $R_{M,s3}$ and the parameter m .Such prediction is impossible for chromatographic systems,which show great differences in A_3 and A_3^o , B_3 and B_3^o ,and for which the parameter m depends strongly upon type of the solute.A great difference between A_3 and A_3^o , B_3 and B_3^o is observed for mobile phases of strong specific interactions between molecules of solute and solvents.In the next section we shall discuss the equations involving the specific solute-solvent and solvent-solvent interactions in the mobile phase.

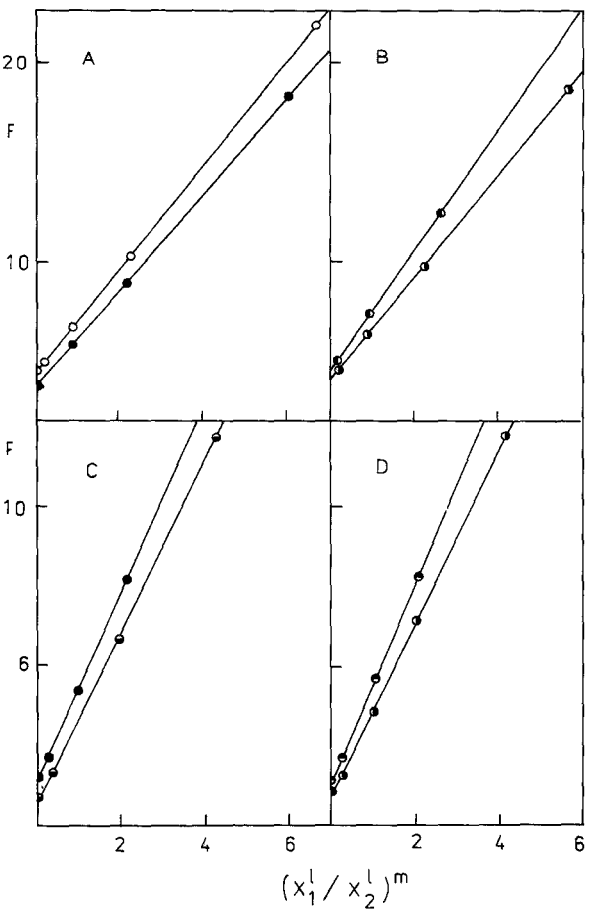


Figure 1. Linear dependences plotted according to Eq. 45 for selected polycyclic hydrocarbons in chloroform (1)/toluene (2)/ benzene (3) (parts A & B) and chloroform (1)/toluene (2)/carbon tetrachloride (3) (parts C & D).
● = pyrene; ◑ = benzopyrene; ○ = phenanthrene; ◒ = chrysene; ◒ = fluoranthene; ◐ = naphthalene. Figure taken from ref. [52], with permission of the copyright owner.

The mathematical properties of equations involving the adsorbent heterogeneity we shall illustrate by using the following expression :

$$R_M = -(1/m)\ \log[(x_1^l 10^{-R_{M,s1}})^m + (x_2^l 10^{-R_{M,s2}})^m] \qquad (48)$$

Eq.(48) has been obtained by combining eq.(40) for n=2 and eq.(2).

Table 1

Parameters Characterizing TLC Data of Polycyclic Hydrocarbons in Three-Component Mobile Phases on Silica Gel at 25°C [52]

Substance	$R_{M,s1}$	$R_{M,s2}$	$R_{M,s3}$	m	A_3	A_3°	B_3	B_3°
	chloroform(1)/toluene(2)/benzene(3)							
Pyrene	−0.43	−0.27	−0.25	0.98	2.44	2.65	3.62	3.58
Benzopyrene	−0.41	−0.25	−0.37	1.02	2.55	2.62	3.96	4.17
Phenanthrene	−0.41	−0.27	−0.39	1.04	2.60	2.66	4.30	4.44
Chrysene	−0.41	−0.25	−0.35	1.04	2.47	2.66	4.37	4.12
Fluoranthene	−0.41	−0.24	−0.36	1.06	2.53	2.72	4.27	4.19
Anthracene	−0.41	−0.23	−0.37	1.06	2.68	2.72	3.89	4.21
Diphenyl	−0.41	−0.28	−0.39	1.04	2.82	2.99	5.07	4.93
Naphthalene	−0.43	−0.27	−0.40	1.04	2.75	2.81	4.49	4.50
	chloroform(1)/toluene(2)/carbon tetrachloride (3)							
Pyrene	−0.43	−0.27	0.02	0.90	2.29	2.44	3.10	2.72
Benzopyrene	−0.41	−0.25	0.17	0.84	2.00	2.21	2.91	2.33
Phenanthrene	−0.41	−0.27	0.16	0.82	1.94	2.16	3.04	2.40
Chrysene	−0.41	−0.25	0.14	0.88	2.11	2.29	2.96	2.41
Fluoranthene	−0.41	−0.24	0.12	0.80	1.95	2.12	2.74	2.33
Anthracene	−0.41	−0.23	0.16	0.80	1.94	2.12	2.82	2.30
Diphenyl	−0.41	−0.28	0.11	0.80	1.89	2.13	3.03	2.49
Naphthalene	−0.43	−0.27	0.02	0.76	2.13	2.13	2.81	2.63

Fig.2 shows the dependence $R_{M,s}$ on x_1^1 calculated according to eq.(48) for $R_{M,s1}=0.5$, $R_{M,s2}=1.0$ (fig.2A) , $R_{M,s1}=-0.5$, $R_{M,s2}=1.0$ (fig.2B) and for different values of m .The dependence $R_{M,s}(x_1^1)$ calculated for m=1 (homogeneous solid surface) is a decreasing function. However,the curves $R_{M,s}(x_1^1)$ relate to m smaller than unity can show a minimum.This minimum is deeper when difference between $R_{M,s1}$ and $R_{M,s2}$ is small and the parameter m is close to zero.Thus,a strong heterogeneity of the adsorbent surface (m close to zero) may be the reason of minimum on the curve $R_{M,s}(x_1^1)$ [34].

A MODEL INVOLVING SOLUTE-SOLVENT AND SOLVENT-SOLVENT SPECIFIC INTERACTIONS IN THE MOBILE PHASE

Homogeneous solid surfaces

The specific interactions between molecules of solute and solvents in the mobile phase may be represented by suitable quasi-chemical reactions,the products of which are multimolecular complexes (associates).We assume that such associates form in the

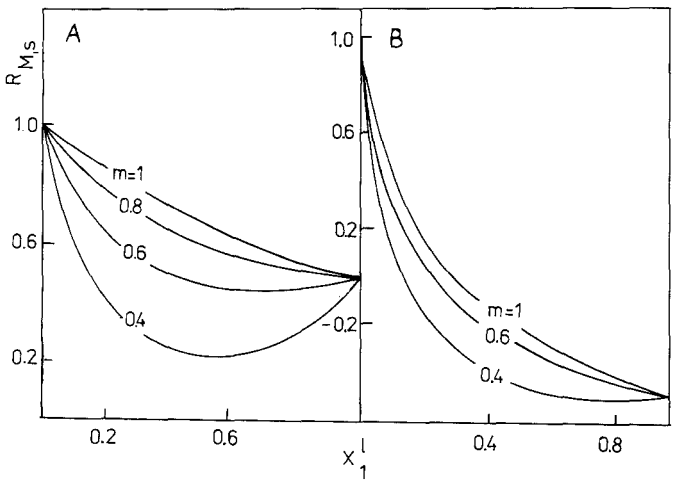


Figure 2. Theoretical functions $R_{M,s}(x_1^1)$ calculated according to Eq. 48 for $R_{M,s1}=0.5$; $R_{M,s2}=1.0$ (part A); $R_{M,s1}=-0.5$, $R_{M,s2}=1.0$ (part B) and different values of m. Figure taken from Ref. [51] with permission of the copyright owner.

mobile phase only [31].However,the silanol groups of the silica surface or other active groups in the case of other adsorbents can compete with complexes in the surface phase.Thus,stronger interactions of molecules of solute and solvents with the active groups and adsorption sites can preclude solute-solvent and solvent-solvent associates in the surface phase.Taking into account the possibility of destruction of solute-solvent and solvent-solvent associates in the surface phase by the active groups,we assume that these complexes form in the mobile phase only.

In n-component mobile phase the different types of multimolecular associates can be formed.Therefore,in theoretical considerations we assume formation of the most probable associates.Three types of associates play an important role in the chromatographic process. They are [30,31] :

(1) associates consisted from one molecule of solute and some molecules of the most polar solvent,

(2) associates consisted from molecules of the most polar solvent,and

(3) associates consisted from molecules of the most polar solvent and molecules of another solvent.

The chromatographic models assuming the formation of double associates of 1-st, 2-nd and 3-rd type in the mobile phase were discussed theoretically in the ref.[30].In [30] the three main equations for the capacity ratio have been derived by assuming that only one type of double associates can be formed.However,in [57] a model assuming the simultaneous formation of double associates of 1-st and 2-nd type in the mobile phase was discussed.A most general model has been considered in the ref.[31].According to this model molecules of solute and 1-st solvent form associates in the mobile phase.This process may be described by the following quasi-chemical reversible reactions :

$$s^{(l)} + q\ 1^{(l)} \rightleftharpoons (1_q s)^{(l)} \qquad (49)$$

$$p\ 1^{(1)} \rightleftharpoons (1_p)^{(1)} \qquad (50)$$

The first reaction represents the formation of (q+1)-molecular associates in the mobile phase,which consist from one molecule of solute and q molecules of 1-st solvent.However,the reaction (50) represents the formation of p-molecular associates by molecules of 1-st solvent only.The equilibrium constants of the reactions (49) and (50) are expressed as follows :

$$L_q = x_q/[\, x_s (x_1)^q] \qquad (51)$$

$$M_p = x_p/(x_1)^p \qquad (52)$$

In the above x_s and x_1 are the mole fractions of unassociated molecules of the s-th solute and 1-st solvent,respectively,however, x_q and x_p are mole fractions of (q+1)-molecular and p-molecular associates formed in the mobile phase according to the reactions (49) and (50).The mole fractions x_q and x_p are defined as the ratio of the number of associates to the total number of molecules in the mobile phase.The total mole fractions of the s-th solute and all solvents in the mobile phase are expressed as follows :

$$x_s^1 = x_s + x_q = x_s + L_q\, x_s (x_1)^q \tag{53}$$

$$x_1^1 = x_1 + q\, x_q + p\, x_p \approx x_1 + p\, M_p\, (x_1)^p \tag{54}$$

$$x_i^1 = x_i \quad \text{for } i=2,3,\ldots,n \tag{55}$$

Since, the value of the mole fraction x_q is limited by the mole fraction x_s, however, x_s^1 is infinitely low, the term $q\, x_q$ in eq.(54) is very small in comparison to x_1 and may be neglected. However, in the surface phases the association effects are neglected, i.e.,

$$x_s^s = y_s \quad \text{and} \quad x_i^s = y_i \quad \text{for } i=1,2,\ldots,n \tag{56}$$

where y_s and y_i are the mole fractions of unassociated molecules of the s-th solute and i-th solvent in the surface phase, respectively.

The solution of eq.(54) with respect to x_1 is a function of x_1^1, i.e.,

$$x_1 = X(x_1^1;\, p, M_p) \tag{57}$$

For double associates (p=2) the solution of eq.(54) is the following :

$$x_1 = X(x_1^1;\, 2, M_2) = [(1+8M_2 x_1^1)^{1/2} - 1]/(4M_2) \tag{58}$$

Analytical solutions of eq.(54) are also possible for three- and four-molecular complexes, i.e., p=3 and p=4. However, eq.(53) may be easily solved with respect to x_s :

$$x_s = x_s^1 /(1 + L_q x_1^q) \tag{59}$$

Next, we assume that in the phase-exchange reaction (4) the unassociated molecules take part only. Thus, the equlibrium constant K_{s1}^{th} is defined as follows (c.f. eq.10):

$$K_{s1}^{th} = (y_s/x_s)(x_1/y_1)^r \tag{60}$$

Taking into account eq.(56), i.e., $y_s = x_s^s$ and $y_1 = x_1^s$, and substituting eqs.(57) and (59) to eq.(60), we have

$$K_{s1}^{th} = (x_s^s/x_s^1)\, [\, X(x_1^1;p,M_p)/x_1^s\,]^r\, [1 + L_q X^q(x_1^1;p,M_p)] \tag{61}$$

Combining eqs.(61) and (1) we obtain a general equation for the capacity ratio involving the solute-solvent and solvent-solvent association :

$$k'_s = (K^{th}_{s1}/C)\,[\,x^s_1/X(x^1_1;p,M_p)]^r\,[1 + L_q X^q(x^1_1;p,M_p)]^{-1} \qquad (62)$$

For $x^1_1 = 1$ eq.(62) gives the expression for the capacity ratio k'_{s1} of the s-th solute in 1-st solvent :

$$k'_{s1} = (K^{th}_{s1}/C)\; D_p^{-r}\,[1 + L_q\, D_p^{\,q}\,]^{-1} \qquad (63)$$

where

$$D_p = X(1;p,M_p) \qquad (64)$$

Eq.(62) may be rewritten in the form,which contains k'_{s1} instead of K^{th}_{s1}. For this purpose the equilibrium constant K^{th}_{s1} may be evaluated from eq.(63) and substituted to eq.(62) :

$$k'_s = k'_{s1}\; D_p^{\,r}\,[1+L_q D_p^q]\,[x^s_1/X(x^1_1;p,M_p)]^r\,[1+L_q X^q(x^1_1;p,M_p)]^{-1} \qquad (65)$$

Now,we consider the special cases of eq.(62) or its other form, eq.(65).For $M_p = 0$ (then p=1) eq.(54) gives

$$x_1 = X(x^1_1;1,0) = x^1_1 \qquad (66)$$

Then,eq.(62) reduces to the following expression :

$$k'_s = (K^{th}_{s1}/C)\;(x^s_1/x^1_1)^r/[1+L_q(x^1_1)^q\,] \qquad (67)$$

Eq.(67) involves solute-solvent association in the mobile phase and difference in molecular sizes of solute and solvents.For $L_q=0$ (absence of solute-solvent association) eq.(67) gives the relationship (20),however, for $L_q=0$ and $x^s_1=1$ it becomes the Snyder-Soczewiński relationship (22).These relationships were discussed in the section devoted to non-specific interactions in LSC process. Similarly,for $L_q=0$ and r=1 eq.(67) gives eq.(25),which was discussed in the previous section.

Eq.(67) may be transformed to the following linear form :

$$(k'_s)^{-1}(x_1^s/x_1^1)^r = (K_{s1}^{th}/C)^{-1} + (L_qC/K_{s1}^{th})(x_1^1)^q \qquad (68)$$

Two special cases of eq.(68) are interesting for analysis of the chromatographic data.They are obtained from eq.(68) for r=1 :

$$x_1^s/(k'_s x_1^1) = (K_{s1}^{th}/C)^{-1} + (L_qC/K_{s1}^{th})\ (x_1^1)^q \qquad (69)$$

and for q=1 :

$$(k'_s)^{-1}(x_1^s/x_1^1)^r = (K_{s1}^{th}/C)^{-1} + (L_1C/K_{s1}^{th})\ x_1^1 \qquad (70)$$

The mole fraction x_1^s appearing in equations (69) and (70) may be evaluated by means of eq.(21).Assuming x_1^s=1 (this assumption is frequently used in LSC) eqs.(69) and (70) reduce to the very simple relationships :

$$1/(k'_s x_1^1) = (K_{s1}^{th}/C)^{-1} + (L_qC/K_{s1}^{th})(x_1^1)^q \qquad (71)$$

$$(k'_s)^{-1}(x_1^1)^{-r} = (K_{s1}^{th}/C)^{-1} + (L_1C/K_{s1}^{th})\ x_1^1 \qquad (72)$$

Eqs.(71) and (72) are especially convenient for interpretation of the chromatographic data,because they define in a simple way the capacity ratio.Eq.(71) for q=1 becomes the relationship obtained by Soczewiński [15], Jaroniec and Piotrowska [30]; it is

$$1/(k'_s\ x_1^1) = (K_{s1}^{th}/C)^{-1} + (L_1C/K_{s1}^{th})\ x_1^1 \qquad (73)$$

This relationship was widely examined by using HPLC data [58,59].

In [57] the following equation for the capacity ratio has been derived :

$$k'_s = (16\ K_{s1}^{th}\ M_2^2/C)\left[(1+8M_2x_1^1)^{1/2}-1\right]^{-1}\left[4M_2 + L_1(1+8M_2x_1^1)^{1/2}-L_1\right]^{-1} \qquad (74)$$

For small values of M_2 eq.(74) gives [57]:

$$k'_s = (K_{s1}^{th}/C)\ (x_1^1)^{-1}\left[1+(L_1-2M_2)x_1^1\right]^{-1} \qquad (75)$$

Eqs.(74) may be obtained from eq.(62) by assuming r=1 , q=1 and p=2 .For L_1=0 eq.(75) gives

$$1/(k'_s x_1^1) = (K_{s1}^{th}/C)^{-1} - (2M_2C/K_{s1}^{th})\, x_1^1 \qquad (76)$$

The dependence $1/(k'_s x_1^1)$ vs. x_1^1 is linear for two different models:

(1) model assuming formation of double associates (1s) in the mobile phase, and

(2) model assuming formation of double associates from molecules of 1-st solvent in the mobile phase.

The slope of eq.(73) is positive, whereas, the slope of eq.(76) is negative. This last difference can be very useful in interpretation of the experimental data. The positive slope of $(k'_s x_1^1)^{-1}$ vs. x_1^1 indicates that solute-solvent association dominate, however, the negative slope of this dependence means that solvent-solvent association play dominating role [30,88].

Now, we return to eq.(65). We shall discuss eq.(65) for r=1 and x_1^s smaller than unity. This last assumption means that the surface phase contains molecules of all solvents. Such situation is observed for solvents having similar adsorption energies. In the case of association of 1-st solvent in the bulk phase, the mole fraction x_1^s is given by the expression analogous to eq.(21):

$$x_1^s = [\, K_{1n}^{th}\, X(x_1^1;p,M_p)]/[\, x_n^1 + K_{1n}^{th}\, X(x_1^1;p,M_p) + \sum_{i=2}^{n-1} K_{in}^{th} x_i^1 \,] \qquad (77)$$

Combining eqs.(62) and (77) we have

$$k'_s = C^{-1} K_{s1}^{th}\, K_{1n}^{th} \left[x_n^1 + K_{1n}^{th}\, X(x_1^1;p,M_p) + \sum_{i=2}^{n-1} K_{in}^{th} x_i^1 \right]^{-1} \cdot$$

$$[1 + L_q X^q(x_1^1;p,M_p)]^{-1} \qquad (78)$$

For boundary-concentrations, i.e., $x_1^1 = 1$ and $x_i^1 = 0$ for $i=2,3,\ldots,n$, the capacity ratio k'_{s1} is defined by eq.(63) with r=1. The equilibrium constants K_{in}^{th}, K_{si}^{th} and K_{sn}^{th} fulfil the following condition:

$$K_{in}^{th} = K_{sn}^{th}/K_{si}^{th} \qquad (79)$$

where

$$k'_{si} = K^{th}_{si}/C \quad \text{for } i=2,3,\ldots,n \tag{80}$$

Since,1-st solvent forms p-molecular complexes in the bulk phase, the relationship between k'_{s1} and K^{th}_{s1} is more complex than that defining k'_{si} (i=2,3,...,n) by means of K^{th}_{si} .Taking into account eqs.(63) and (80) in eq.(78),we have :

$$k'_s = \left\{ X(x^1_1;p,M_p)/[k'_{s1}D_p(1+L_qD_p{}^q)] + \sum_{i=2}^{n}(x^1_i/k'_{si}) \right\}^{-1} \cdot$$

$$[\,1 + L_q\, X^q(x^1_1;p,M_p)]^{-1} \tag{81}$$

For M_p=0 eq.(81) gives :

$$k'_s = \left\{ x^1_1/[k'_{s1}(1+L_q)] + \sum_{i=2}^{n}(x^1_i/k'_{si}) \right\}^{-1} [1+L_q(x^1_1)^q]^{-1} \tag{82}$$

The influence of association constants on the dependence $k'_s(x^1_1)$ we shall discuss on the basis of eq.(81) for binary eluent, i.e.,

$$k'_s = \left\{ X(x^1_1;p,M_p)/[k'_{s1}D_p(1+L_qD_p{}^q)] + (x^1_2/k'_{s2}) \right\}^{-1} \cdot$$

$$[\,1+L_qX^q(x^1_1;p,M_p)]^{-1} \tag{83}$$

Figs.3-5 present the theoretical dependences $k'_s(x^1_1)$ calculated according to eq.(83) for different values of q , L_q , p and M_p . In all figures the part A shows the functions $k'_s(x^1_1)$ calculated for k'_{s1} = 1 and k'_{s2}=5 ,however,the part B relates to k'_{s1}=1 and k'_{s2}=100. Moreover,in figs.3-5, the dependences ln k'_s vs. x^1_1 are also presented.

Fig.3 shows the k'_s-curves calculated for different values of L_q . The parameter q was equal to unity ; it means that one molecule of 1-st solvent bounds one molecule of the solute.The k'_s-curves for $k'_{s2} \gg k'_{s1}$ lie above the Snyder-Soczewiński curve .The distances between the successive k'_s-curves increase gradually with increasing of L_q .The more complex behaviour of the k'_s-curves is observed for similar values of k'_{s1} and k'_{s2} (c.f.,fig.4A).This non-regular behaviour of k'_s-curves presented in fig.4A is caused by assuming

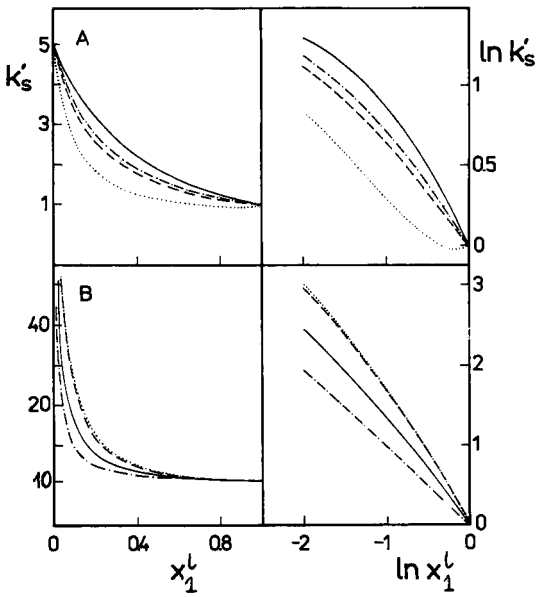


Figure 3. Theoretical functions, $k'_s(x_1^l)$ and $\ln k'_s(\ln x_1^l)$ calculated according to Eq. 83 for $k'_{s1}=1$, $k'_{s2}=5$ (part A); $k'_{s1}=1$, $k'_{s2}=100$ (part B); $M_p=0$, $q=1$, and $L_q=1$ (the solid line), 5 (the dashed line), and 10 (the dotted line). The Snyder-Soczewinski curve is denoted by the dashed line.

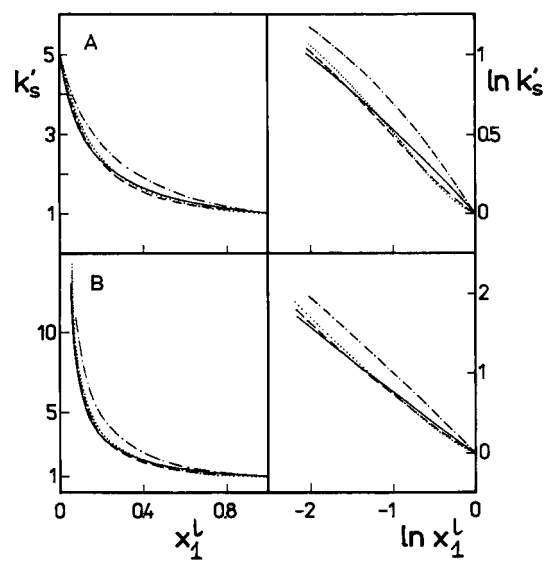


Figure 4. Functions as in Fig. 3 calculated for $L_q=0$, $M_p=1$ and $p=2$ (the solid line), 4 (the dashed line), and 6 (the dotted line). The other parameters as in Fig. 3.

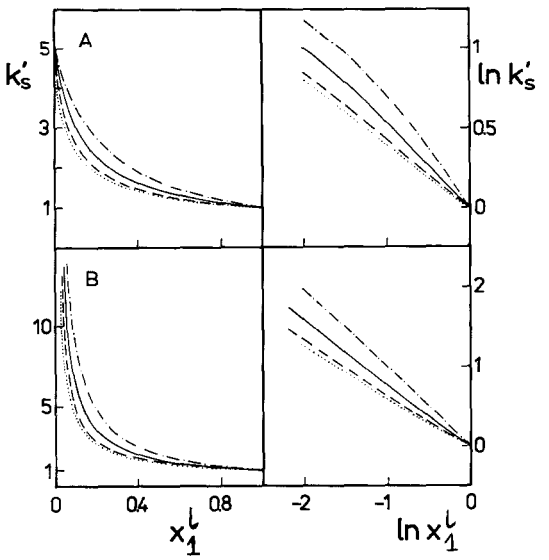


Figure 5. Functions as in Fig. 3 calculated for L_q=0, p=2, and M_p = 1 (the solid line), 5 (the dashed line), and 10 (the dotted line). The other parameters as in Fig. 3.

the constancy of k'_{s1} ; it means that the k'_s-curves are calculated at different values of K_{s1}^{th} (c.f., eq.63).A set of the k'_s-curves calculated for one value of K_{s1}^{th} shows a regular behaviour[60]

Fig. 4 shows the k'_s-curves calculated for L_q= 0 , M_p=1 and different values of p =2,4 and 6.However,fig.6 presents the k'_s-curves for L_q=0 , p=2 and M_p=1 , 5 and 10.These curves lie below the Synder-Soczewiński curve (the dashed-dotted line).Thus,association of molecules of 1-st solvent causes decrease of k'_s-values in comparison to those predicted by Snyder-Soczewiński model,however,association between molecules of solute and 1-st solvent gives opposite effect (c.f.,figs.4 and 6).

The k'_s-curves presented in figs.3-5 were also plotted in the logarithmic scale.In this scale the Snyder-Soczewiński curve (the dashed-dotted line) is linear.It follows from figs.3-5 that many curves $\ln k'_s$ vs. $\ln x_1^l$ may be approximated by the straight line

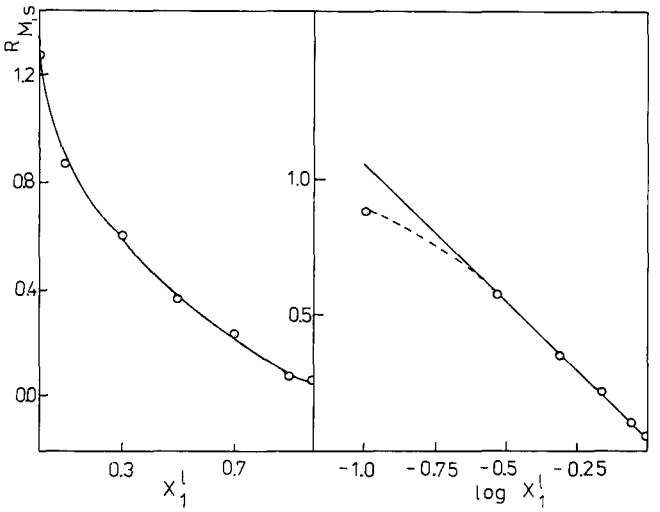


Figure 6. Experimental dependence $R_{M,S}$ vs. x_1^1 and the linear dependence (22) for o-nitrophenol in benzene/cyclohexane eluent on silica gel at 20°C. Benzene is denoted as 1st solvent.

in a wide concentration region. Although, these curves have been plotted for r=1, their slopes are not equal to those predicted by Snyder-Soczewiński relationship (22). Thus, the analysis of the chromatographic data by means of Snyder-Soczewiński relationship (22) creates a difficulty in physico-chemical interpretation of its slope, because a good linearity of the dependence of ln k'_S vs. ln x_1^1 is observed for different models of the LSC process. According to Snyder-Soczewiński model the ordinate of the linear plot (22) is equal to k'_{S1}. However, the ordinates of the linear segments of the ln k'_S -plots (c.f., figs. 3-5) are not equal to k'_{S1}. This fact may be very helpful during the interpretation of Snyder-Soczewiński plots.

Heterogeneous solid surfaces

The equations for the capacity ratio, discussed in the preceding section, have been derived by assuming association of solute and solvent molecules in the mobile phase and ideality of the surface

phase.Moreover,these equations have been discussed for energetically homogeneous adsorbents.Extension of these equations to heterogeneous solid surfaces do not require of an additional information about topography of adsorption sites onto surface,because we assume ideality of the surface phase.This problem was discussed in the section devoted to non-specific interactions in LSC process and is exactly explained in the references [28,67,68].Thus,the theoretical considerations presented in this section are valid for heterogeneous surfaces of different distributions of adsorption sites onto surface.

The total capacity ratio $k'_{s,t}$ for the LSC process with association effects in the mobile phase is defined by eq.(29) in which $k'_{s,1}$ is expressed by equation analogous to eq.(62) :

$$k'_{s,1} = (K^{th}_{s1,1}/C) \left[x^s_{1,1}/X(x^1_1;p,M_p)\right]^r \left[1+L_q X^q(x^1_1;p,M_p)\right]^{-1} \qquad (84)$$

Eqs.(29) and (62) give :

$$k'_{s,t} = C^{-1}\left[X(x^1_1;p,M_p)\right]^{-r} \left[1+L_q X^q(x^1_1;p,M_p)\right]^{-1} \sum_{l=1}^{L} h_l K^{th}_{s1,l}(x^s_{1,l})^r \qquad (85)$$

Eq.(85) is an extension of eq.(62) to energetically heterogeneous solid surfaces.For $M_p=0$ (neglect of association of 1-st solvent molecules) eq.(85) reduces to the following expression :

$$k'_{s,t} = C^{-1} (x^1_1)^{-r} \left[1+L_q(x^1_1)^q\right]^{-1} \sum_{l=1}^{L} h_l K^{th}_{s1,l} (x^s_{1,l})^r \qquad (86)$$

Similarly as in the case of eq.(34),for quasi-gaussian energy distribution eqs.(85) and (86) may be approximated by the following expressions :

$$k'_{s,t} = C^{-1} \bar{K}^{th}_{s1} (x^s_{1,t})^{r/m} \left[X(x^1_1;p,M_p)\right]^{-r} \left[1+L_q X^q(x^1_1;p,M_p)\right]^{-1} \qquad (87)$$

$$k'_{s,t} = C^{-1} \bar{K}^{th}_{s1} (x^s_{1,t})^{r/m} (x^1_1)^{-r} \left[1+L_q(x^1_1)^q\right]^{-1} \qquad (88)$$

Eqs.(87) and (88) for m=1 reduce to eqs.(62) and (67),respectively, which have been derived for homogeneous solid surfaces.However, for M_p=0 and L_q=0 (neglect of association effects in the mobile phase) eqs.(87) and (88) give the relationship (38),which relates to the LSC model with ideal both phases on a heterogeneous solid of quasi-gaussian energy distribution.However,assuming $x_1^s = 1$ in eqs.(87) and (88) we obtain :

$$k'_s = C^{-1} K_{s1}^{th} [X(x_1^l;p,M_p)]^{-r} [1+L_q X^q(x_1^l;p,M_p)]^{-1} \quad (89)$$

$$k'_s = C^{-1} K_{s1}^{th} (x_1^l)^{-r} [1+L_q (x_1^l)^q]^{-1} \quad (90)$$

where

$$\bar{K}_{s1}^{th} = K_{s1}^{th} \quad \text{for } m=1 \quad (91)$$

Eqs.(89) and (90) may be also obtained from eqs.(62) and (67) in which x_1^s is assumed to be unity.Substituting in equations derived for heterogeneous surfaces $x_1^s = 1$ we neglected the heterogeneity effects automatically.

An important equation is obtained from eq.(87) for r=1 :

$$k'_{s,t} = C^{-1} \bar{K}_{s1}^{th} (x_{1,t}^s)^{1/m} [X(x_1^l;p,M_p)]^{-1} [1+L_q X^q(x_1^l;p,M_p)]^{-1} \quad (92)$$

The mole fraction $x_{1,t}^s$ is given by the equation analogous to eq.(39):

$$x_{1,t}^s = [\bar{K}_{1n}^{th} X(x_1^l;p,M_p)]^m \Big/ \left\{ (x_n^l)^m + [\bar{K}_{1n}^{th} X(x_1^l;p,M_p)]^m + \sum_{i=2}^{n-1} (\bar{K}_{in}^{th} x_i^l)^m \right\} \quad (93)$$

Eq.(93) may be also applied to calculate $x_{1,t}^s$ in eqs.(87) and (88). Combining eqs.(92) and (93) ,we obtain

$$k'_{s,t} = C^{-1} \bar{K}_{s1}^{th} \bar{K}_{1n}^{th} \left\{ (x_n^l)^m + [\bar{K}_{1n}^{th} X(x_1^l;p,M_p)]^m + \sum_{i=2}^{n-1} (\bar{K}_{in}^{th} x_i^l)^m \right\}^{-1/m} \cdot$$

$$[1+L_q X^q(x_1^l;p,M_p)]^{-1} \quad (94)$$

For $x_1^l = 1$ and x_i^l=0 (i=2,3,...,n) eq.(94) gives :

$$k'_{s1} = (\bar{K}_{s1}^{th}/C)[D_p(1+L_q D_p^q)]^{-1} \quad (95)$$

Taking into account the relationship (79) and eq.(95) in eq.(94), we have :

$$k'_{s,t} = \left\{ [X(x_1^1;p,M_p)(k'_{s1}D_p)^{-1}(1+L_qD_p^q)^{-1}]^m + \sum_{i=2}^{n}(x_i^1/k'_{si})^m \right\}^{-1/m} \cdot$$

$$[1 + L_qX^q(x_1^1;p,M_p)]^{-1} \qquad (96)$$

For M_p=0 eq.(96) gives :

$$k'_{s,t} = \left\{ [x_1^1(k'_{s1})^{-1}(1+L_q)^{-1}]^m + \sum_{i=2}^{n}(x_i^1/k'_{si})^m \right\}^{-1/m} \cdot [1+ L_q(x_1^1)^q]^{-1} \quad (97)$$

However,for m=1 eqs.(96) and (97) give the relationship derived for homogeneous surfaces (c.f.,eqs.81 and 82).

In this section we discussed many equations for the capacity ratio,which can be obtained from the general relationship (87). This relationship involves main factors determining the LSC process:

- competitive character of solute and solvent adsorption,
- differences in molecular sizes of solute and solvents,
- solute-solvent association in the mobile phase,
- association of molecules of the most polar solvent in the mobile phase,and
- energetic heterogeneity of the adsorbent.

Applying a similar procedure to that described above we can derive the further equations for the capacity ratio by assuming that the mobile phase contains some types of associates of 1-st solvent and they can bound one molecule of the solute.These equations contain many unknown parameters,therefore,they are less useful for analysing the HPLC and TLC data.

CORRELATION BETWEEN ADSORPTION FROM MULTICOMPONENT LIQUID MIXTURES AND LIQUID ADSORPTION CHROMATOGRAPHY WITH MIXED ELUENT

Ościk [23,24], Jaroniec et al.[27,28,60], Riedo and Kováts[89] pointed on a great similarity of the LSC process with mixed eluent and adsorption from multicomponent liquid mixtures.Theoretical studies of Riedo and Kováts [89] concentrate on derivation of relationships

determining correlation between adsorption and chromatographic fundamental quantities.The studies of Ościk [23,24] and Jaroniec et al.[27,28,60] base on the fundamental definition (1),which determines the dependence between the mole fraction of the s-th solute in the surface phase (the typical adsorption quantity) and the capacity ratio (the typical chromatographic quantity),and tend for deriving the relationships describing adsorption and chromatographic multicomponent systems.

The LSC process with one solute and n-component eluent is very similar to the liquid adsorption process from (n+1)-component mixtures.In the LSC process concentration of a given solute is assumed to be infinitely low ; this process relates to adsorption of a solute from dilute n-component liquid mixtures [90].According to the theory of single-solute adsorption from dilute solutions on homogeneous solid surfaces,the mole fraction of the s-th solute in the surface phase is given by the following equation [90]:

$$x_s^s = K_{sn}^{th}\, x_s^l \,/(x_n^l + \sum_{i=1}^{n-1} K_{in}^{th}\, x_i^l) \qquad (98)$$

Eq.(98) has been derived for ideal both phases.Combining this equation with the definition (1) ,we obtain

$$k_s' = C^{-1}\, K_{sn}^{th}/(x_n^l + \sum_{i=1}^{n-1} K_{in}^{th}\, x_i^l) \qquad (99)$$

Combining eqs.(99),(79) and (80) we obtain the relationship (25). Applying for x_s^s different equations known in the theory of single-solute adsorption from dilute solutions on solid surfaces,we can derive by means of eq.(1) different equations for the capacity ratio. The procedure basing on eq.(1) and that basing on eq.(14) lead to the same equations for k_s' ,although the procedure basing on eq.(14) is more universal because involves differences in molecular sizes of solute and solvents.The procedure basing on eq.(1),introduced in the paper [27],was developed by Borówko et al.[55,60].
In [55] non-ideality and surface heterogeneity effects in LSC process

were investigated,however,the paper [60] is devoted to the association effects in LSC.

The capacity ratio for different LSC models is a function of the mole fractions of solvents in the mobile and surface phases. Of course, the mole fraction of a solvent in the surface phase is the function of the mobile phase composition.In all equations for the capacity ratio,discussed in the preceding sections,we calculated the mole fraction of 1-st solvent in the surface phase by using the equations known from the theory of adsorption from multicomponent liquid mixtures.Now,we sygnalize the other possibility of calculation of x_1^s ; it may be evaluated directly from the experimental excess adsorption isotherm n_1^e by using the following relationship [68]:

$$x_1^s = n_1^e/n^s + x_1^l \qquad (100)$$

where n_1^e is the adsorption excess of 1-st component from n-component liquid mixture,which is measured for eluent/adsorbent system independently on the chromatographic measurements,and n^s is the total number of moles of all solvents in the surface phase. The parameter n^s may be determined directly from the excess adsorption isotherm n_1^e [91,92].Thus,in all equations for the capacity ratio,discussed in the previous sections,the quantities x_1^s and $x_{1,t}^s$ may be replaced by the expression (100).Substituting eq.(100) for x_1^s ,we eliminate the parameters appearing in equations for x_1^s and introduce the parameter n^s ,which may be evaluated from the excess adsorption isotherm.The studies of LSC process ,in which eq.(100) is utilized,were presented in series of the papers [29,36, 38,56,61].Now,we shall present some results illustrating a great utility of the excess adsorption data in interpretation of HPLC and TLC data .

In [56,61] the TLC data for o-nitrophenol and some dichlorophenols chromatographed in benzene/cyclohexane, acetone/benzene and ethyl acetate/carbon tetrachloride eluents on silica gel at 20°C

were investigated. Application of Snyder-Soczewiński relationship (22) for describing these data gives deviations at low concentrations of x_1^1 ; it results from the fact that eq.(22) gives non-physical value of k'_s at $x_1^1 = 0$.

Fig. 6 shows the experimental dependence $R_{M,s}$ vs. x_1^1 for o-nitrophenol and the dependence $R_{M,s}$ vs. log x_1^1 (c.f., eq.22), which is linear for the concentrations x_1^1 greater than 0.3 . In Fig. 7 the excess adsorption isotherms of benzene from cyclohexane, acetone from benzene and ethyl acetate from carbon tetrachloride on silica gel at 20°C are presented. These excess adsorption data have been applied to calculate the mole fraction x_1^s according to eq.(100). Substituting eq.(100) to eq.(20) and transforming eq.(20) to TLC, we obtain [56]:

$$R_{M,s} = R_{M,s1} + r \log [n_1^e/(n^s x_1^1) + 1] \qquad (101)$$

It follows from eq.(101) that $R_{M,s}$ is a linear function of $\log[n_1^e/(n^s x_1^1) +1]$. In [56,61] we showed that the linear relationship (101), associating the excess adsorption data and TLC data, is fulfiled for many chromatographic systems. For the purpose of illustration Fig. 8 shows the linear dependence (101) for o-nitrophenol chromatographed in three different mobile phases, for which the excess adsorption isotherms are presented in Fig. 7 . The experimental points (circles) lie on the straight lines even at low concentrations x_1^1 , whereas, for eq.(22) we observe deviations from linearity at low values of x_1^1.

Similarly, a good results have been obtained for TLC data interpreted by eq.(38) associated with eq.(100); for TLC with binary eluent eqs.(38) and (100) give [29]:

$$R_{M,s} = R_{M,s1} + (r/m) \log [(n_1^e/n^s)(x_1^1)^{-m} + (x_1^1)^{1-m}] \qquad (102)$$

The parameters n^s and m were evaluated by means of the following equation [29]:

$$\log[x_{1,t}^s/(1-x_{1,t}^s)] = m \log \overline{K}_{12}^{th} + m \log (x_1^1/x_2^1) \qquad (103)$$

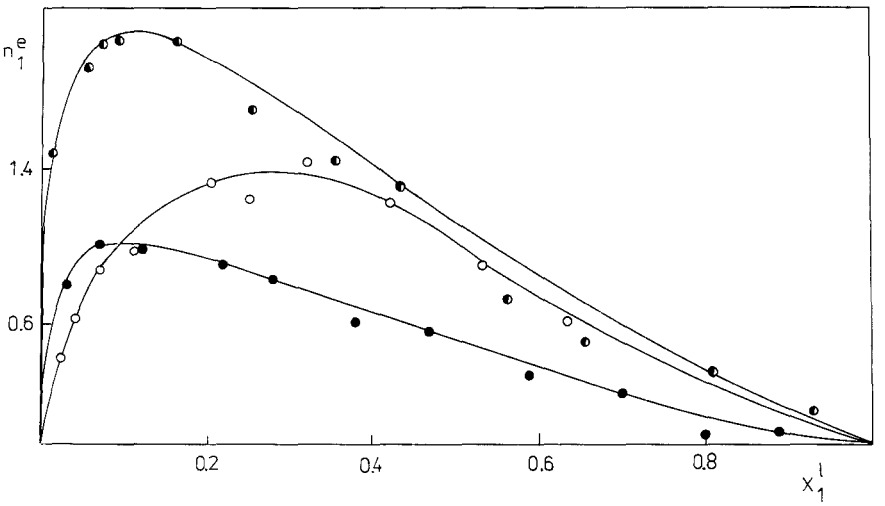


Figure 7. Excess adsorption isotherms for benzene (2)/acetone(1) (◐), cyclohexane(2)/benzene(1) (O) and carbon tetrachloride(2)/ ethylacetate(1) (●) on silica gel at 20°C. Figure taken from Ref [61] with permission of the copyright owner.

where $x^s_{1,t}$ is given by eq.(100).Eq.(103) is linear form of eq.(39) for n=2.

Recent studies in this problem concern the application of the excess adsorption data for characterization of the chromatographic systems showing association effects in the mobile phase.Such studies have been made by means of eq.(69) and equation analogous to eqs. (75) and (76) [88]:

$$G(x_1^l) = x_1^s/(x_1^l\, k_s') = (C/K_{s1}^{th}) + [(L_1-2M_2)C/K_{s1}^{th}]\, x_1^l \qquad (104)$$

Combining eqs.(104) and (100),we have

$$G(x_1^l) = n_1^e/(n^s x_1^l k_s') + 1/k_s' = (C/K_{s1}^{th}) + [(L_1-2M_2)C/K_{s1}^{th}]x_1^l \qquad (105)$$

Eq.(105) describes the LSC process with mobile phase,which contains the mixed double solute-solvent associates and the pure associates consisting from two molecules of 1-st solvent.The other assumptions are following :

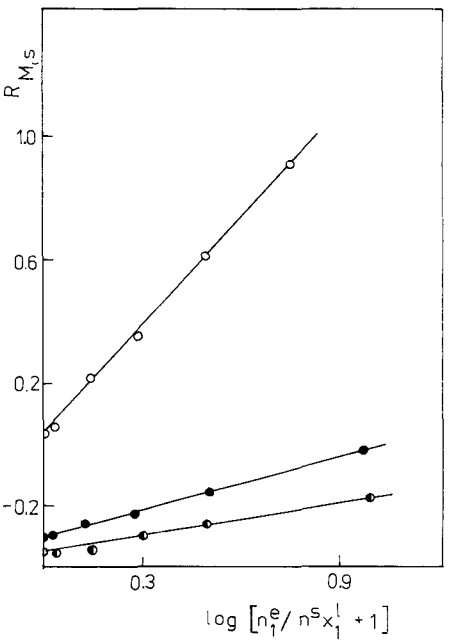


Figure 8. Dependence $\log(n_1^e/n^s x_1^l + 1)$ vs. $R_{M,S}$ for o-nitrophenol chromatographed in cyclohexane/benzene (○), carbon tetrachloride/ethylacetate (●) and benzene/acetone (◐) on silica gel at 20°C. Figure taken from Ref. [61] with permission of the copyright owner.

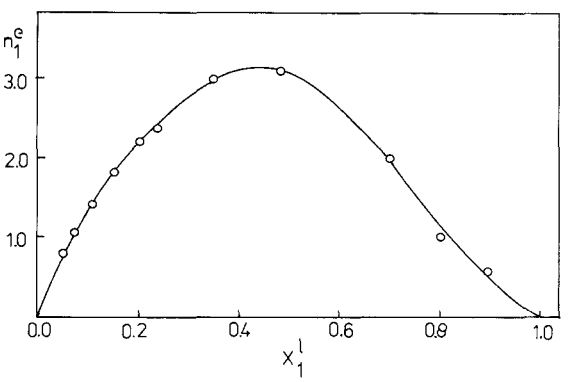


Figure 9. Excess adsorption isotherm for methanol from acetone on silica gel at 20°C. Figure taken from Ref. [88] with permission of the copyright owner.

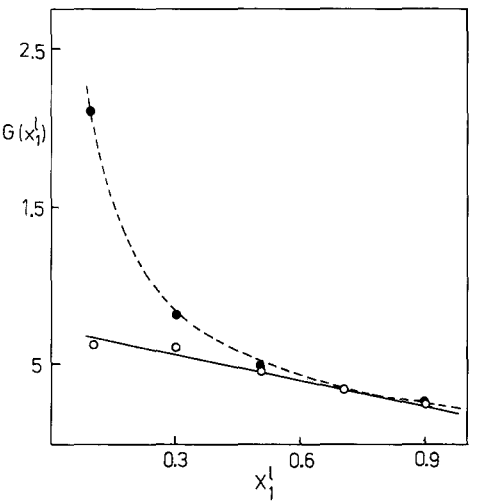


Figure 10. Dependence $G(x_1^1)$ vs. x_1^1 for 2,3-dihydroxynaphthalene in methanol/acetone on silica gel at 20°C. The solid line denotes $x_1^s/(k'_s x_1^1)$ vs. x_1^1, whereas the dashed line denotes $1/(k'_s x_1^1)$ vs. x_1^1.

- ideal surface phase,
- association in the mobile phase,
- surface phase contains molecules of all solvents ($x_1^s < 1$),
- molecular sizes of solute and solvents are identical, and
- adsorbent is assumed to homogeneous.

In [88] the TLC data were measured for naphthalene derivatives in methanol/acetone eluent on silica gel at 20°C. Eqs. (22) and (102) do not fulfil of the above data. Fig. 9 presents the excess adsorption isotherm of methanol from acetone on silica gel at 20°C. However, Fig. 10 shows the dependence $(k'_s x_1^1)^{-1}$ vs. x_1^1 (the dashed line) and the dependence $x_1^s/(k'_s x_1^1)$ vs. x_1^1 (the solid line) for one selected solute. It follows from fig. 10 that a reasonable interpretation of TLC data for naphthalene derivatives chromatographed in methanol/acetone on silica gel requires of equations involving association effects in the mobile phase and changeability of the surface phase composition during the chromatographic process.

REFERENCES

1. L.R.Snyder and D.L.Saunders, J.Chromatogr.Sci., 7 (1969) 195.
2. M.Borówko, M.Jaroniec, J.Narkiewicz, A.Patrykiejew and W.Rudziński, J.Chromatogr., 153 (1978) 309.
3. M.Jaroniec, M.Borówko, J.Narkiewicz, A.Patrykiejew and W.Gołkiewicz, Chromatographia, 12 (1979) 29.
4. L.R.Snyder, in "HPLC :Advances and Perspectives",(ed.,C.Horvath), Academic Press, New York, vol.1 ,1980.
5. P.Jandera and J.Churacek, in "Advances in Chromatography", Marcel Dekker, New York, vol.19 , 1980 p.125 , (eds.J.C.Giddings, E.Grushka, J.Cazes and P.R.Brown).
6. M.Borówko, M.Jaroniec, J.Narkiewicz and A.Patrykiejew, J.Chromatogr., 153 (1978) 321.
7. P.J.Schoenmakers, H.A.H.Billiet, R.Tijssenaud and L.Galan, J.Chromatogr., 149 (1978) 519.
8. W.Gołkiewicz and M.Jaroniec, Journal HRC & CC, 1 (1978) 245.
9. W.Gołkiewicz and E.Soczewiński, Chromatographia, 11 (1978) 454.
10. W.Gołkiewicz, Chromatographia, 14 (1981) 411.
11. W.Gołkiewicz, Chromatographia, 14 (1981) 629.
12. G.Guiochon, in "HPLC :Advances and Perspectives",(ed.,C.Horvath), Academic Press, New York, vol.2 ,1980.
13. H.J.Issaq, J.R.Klose, K.L.McNitt, J.E.Haky and G.M.Muschik, J.Liquid Chromatogr., 4 (1981) 2091.
14. L.R.Snyder, "Principles of Adsorption Chromatography", Marcel Dekker, New York, 1968.
15. E.Soczewiński, Anal.Chem., 41 (1969) 179.
16. M.Jaroniec and J.Ościk, Journal HRC & CC, 5 (1982) 3.
17. R.P.W.Scott and P.Kucera, J.Chromatogr., 149 (1978) 93.
18. R.P.W.Scott and P.Kucera, J.Chromatogr., 112 (1975) 425.
19. R.E.Boehm and D.E.Martire, J.Phys.Chem., 84 (1980) 3620.
20. D.E.Martire and R.E.Boehm, J.Liquid Chromatogr.,3 (1980) 753.
21. L.R.Snyder and H.Poppe, J.Chromatogr., 184 (1980) 363.
22. L.R.Snyder, Anal.Chem., 46 (1974) 1384.

23. J.Ościk, Przem.Chem., 44 (1965) 129.
24. J.Ościk, in"Physical Adsorption from Multicomponent Phases" (in Russian), M.M.Dubinin and V.V.Serpinsky eds.,Nauka, Moscow,1972,pp.138-147.
25. J.Ościk and J.K.Różyło, Chromatographia, 4 (1971) 518.
26. E.Soczewiński, J.Chromatogr., 130 (1977) 23.
27. M.Jaroniec, J.Narkiewicz and M.Borówko, Chromatographia, 11 (1978) 581.
28. M.Jaroniec and A.Patrykiejew, J.Chem.Soc.Faraday I, 76 (1980)2486
29. M.Jaroniec and B.Ościk-Mendyk, J.Chem.Soc.Faraday I, 77 (1981) 1277.
30. M.Jaroniec and J.Piotrowska, Journal HRC & CC, 3 (1980) 257.
31. M.Jaroniec and J.A.Jaroniec, J.Liquid Chromatogr.,4 (1981) 2121.
32. M.Jaroniec, Annales M.Curie-Skłodowska Univ.,Sec.AA, 36 (1981) 73.
33. M.Jaroniec, J.Narkiewicz and B.Klepacka, J.Chromatogr., 177 (1979) 299.
34. M.Jaroniec, J.K.Różyło and W.Gołkiewicz, J.Chromatogr., 178 (1979) 27.
35. H.Buchowski, Thesis, University of Warsaw, 1963.
36. J.Ościk, J.K.Różyło, B.Ościk-Mendyk and M.Jaroniec, Chromatographia, 14 (1981) 95.
37. J.K.Różyło, J.Ościk, B.Ościk-Mendyk and M.Jaroniec, Journal HRC & CC, 4 (1981) 17.
38. J.K.Różyło, M.Jaroniec and B.Ościk-Mendyk, Chem.Anal., 26 (1981) 215.
39. J.Ościk and G.Chojnacka, Chromatographia, 11 (1978) 731.
40. J.Ościk and G.Chojnacka, J.Chromatogr., 93 (1974) 167.
41. J.K.Różyło, M.Jaroniec, J.A.Jaroniec and H.Kołodziejczyk, Journal HRC & CC , 2 (1979) 524.
42. J.K.Różyło, Chromatographia, 9 (1976) 74.
43. J.K.Różyło, J.Chromatogr., 116 (1976) 125.
44. J.K.Różyło, G.Chojnacka and I.Malinowska, Chromatographia, 13 (1980) 215.

45. J.K.Różyło, I.Malinowska and J.Gross, Journal HRC & CC, 3 (1980) 79.
46. L.R.Snyder and J.L.Glajch, J.Chromatogr., 214 (1981) 1 .
47. E.Paanakker, J.C.Kraak and H.Poppe, J.Chromatogr.,149 (1978)111.
48. J.L.Glajch and L.R.Snyder, J.Chromatogr., 214 (1981) 21.
49. L.R.Snyder, J.L.Glajch and J.J.Kirkland, J.Chromatogr.218(1981)299.
50. M.Jaroniec, J.K.Różyło, J.A.Jaroniec and B.Ościk-Mendyk, J.Chromatogr., 188 (1980) 27.
51. J.K.Różyło, J.A.Jaroniec, H.Kołodziejczyk and M.Jaroniec, Journal HRC & CC, 2 (1979) 236.
52. J.K.Różyło, M.Jaroniec, H.Kołodziejczyk, J.A.Jaroniec and J.Gross, Journal HRC & CC, 3 (1980) 180.
53. J.K.Różyło, H.Kołodziejczyk, J.A.Jaroniec and M.Jaroniec, J.Liquid Chromatogr., 2 (1979) 799.
54. M.Jaroniec, B.Ościk-Mendyk, A.Dąbrowski and H.Kołodziejczyk, J.Liquid Chromatogr., 4 (1981) 227.
55. M.Borówko and M.Jaroniec, Chromatographia, 12 (1979) 672.
56. M.Jaroniec, J.K.Różyło and B.Ościk-Mendyk, J.Chromatogr., 179 (1979) 237.
57. M.Jaroniec and J.A.Jaroniec, J.Chromatogr., 210 (1981) 130.
58. M.Jaroniec, J.A.Jaroniec and W.Gołkiewicz, Journal HRC & CC, 4 (1981) 89.
59. M.Jaroniec, J.K.Różyło and J.A.Jaroniec, Chem.Anal., 26 (1981).
60. M.Borówko and M.Jaroniec, J.Chem.Soc.Faraday I, in press.
61. B.Ościk-Mendyk, J.K.Różyło and M.Jaroniec, Journal HRC & CC, 4 (1981) 74.
62. E.H.Slaats, J.C.Kraak, W.J.T.Brugman and H.Poppe, J.Chromatogr., 149 (1978) 255.
63. E.C.Bate-Smith and R.G.Westall, Biochem.Biophys.Acta,4 (1950)427.
64. C.Minka and A.L.Myers, AIChE,19 (1973) 453.
65. M.Jaroniec, J.Res.Inst.Catalysis,Hokkaido Univ., 26 (1978) 155.
66. M.Borówko, M.Jaroniec and W.Rudziński, Monatsh.Chem., 112 (1981) 59.

67. M.Jaroniec, Thin Solid Films, 71 (1980) 273.
68. M.Jaroniec, A.Patrykiejew and M.Borówko, Progress in Surface and Membrane Sci., 14 (1981) pp.1-68.
69. P.Jandera and J.Churacek, J.Chromatogr.,91 (1974) 207.
70. J.Narkiewicz, M.Jaroniec, M.Borówko and A.Patrykiejew, J.Chromatogr., 157 (1978) 1.
71. E.Soczewiński and J.Jusiak, Chromatographia, 14 (1981) 23.
72. E.Soczewiński and W.Gołkiewicz, Chromatographia,5 (1972) 431.
73. E.Soczewiński and W.Gołkiewicz, Chromatographia,4 (1971) 501.
74. W.Gołkiewicz and E.Soczewiński,Chromatographia, 5 (1972) 594.
75. E.Soczewiński and W.Gołkiewicz, Chromatographia, 6 (1973) 269.
76. E.Soczewiński, W.Gołkiewicz and W.Markowski, Chromatographia, 8 (1975) 13.
77. W.Gołkiewicz, Chromatographia, 9 (1976) 113.
78. E.Soczewiński, W.Gołkiewicz and T.Dzido, Chromatographia, 10 (1977) 221.
79. E.Soczewiński, T.Dzido and W.Gołkiewicz, Chromatographia, 10 (1977) 98.
80. E.Soczewiński and J.Kuczmierczyk, J.Chromatogr., 150 (1978) 53.
81. T.Wawrzynowicz and T.Dzido, Chromatographia, 11 (1978) 335.
82. S.Hara, J.Chromatogr., 137 (1977) 41.
83. S.Hara, Y.Fujii, M.Hirasawa and S.Miyamoto, J.Chromatogr., 149 (1978) 143.
84. M.D.Palamareva and B.J.Kurtev, J.Chromatogr., 178 (1979) 139.
85. S.Hara, K.Kunihiro, H.Yamaquchi and E.Soczewiński, J.Chromatogr., 239 (1982) 687.
86. J.A.Perry, J.Chromatogr., 163 (1979) 117.
87. J.K.Różyło, I.Malinowska and M.Jaroniec, Journal HRC & CC, 3 (1980) 29.
88. M.Jaroniec, J.K.Różyło, B.Ościk-Mendyk and J.AJaroniec, J.Liquid Chromatogr., in press.
89. F.Riedo and E.Kováts, J.Chromatogr., 239 (1982) 1.
90. M.Jaroniec, Advances in Colloid Interface Sci., in press.

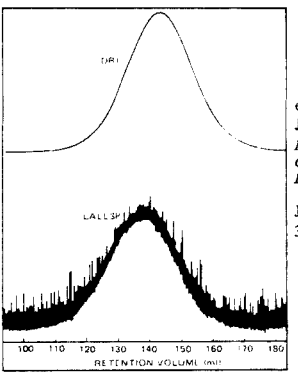
91. J.Ošcik, Adsorption, E.Horwood Publ.,1982.

92. G.Schay and L.Nagy, J.Chim.Phys., 58 (1961) 149.

Providing state-of-the-art understanding of . . .

Steric Exclusion Liquid Chromatography of Polymers

(Chromatographic Science Series, Volume 25)



edited by
JOSEF JANČA
Institute of Analytical Chemistry
Czechoslovak Academy of Sciences
Brno, Czechoslovakia

January, 1984
352 pages, illustrated

". . . Dr. Janča has succeeded in bringing together several of the most innovative giants in this field. Each has contributed significantly. In this monograph, we are able to look over their shoulders to see where future advances will lie."
–Dr. JACK CAZES
Fairfield, Connecticut *(from the Foreword)*

WITH ITS UNSURPASSED capacity to determine a polymer's molecular weight distribution and other properties, it is easy to recognize the vast importance of steric exclusion chromatography– *AND the value of this unparalleled single-source reference!*

Certainly, no other work can match *Steric Exclusion Liquid Chromatography of Polymers* for its up-to-date, fully detailed coverage–provided by leading international experts–with features that include

- in-depth examination of separation mechanisms and secondary interactions and their correlation with the structure and behavior of macromolecules
- refinements in calibration and data evaluation techniques
- complete information on solvent and column packing, correction of zone dispersion, and experimental variables
- practical applications involving molecular weight distribution, polymer branching, chemical composition, chain growth and degradation mechanisms, and others

Complete with illustrative experimental data, this state-of-the-art volume leads the way to future innovations for a wide range of scientists. Furthermore, this volume serves as an excellent reference for a number of graduate-level chemistry courses.

Readership: Liquid Chromatographers; Analytical, Polymer, Organic, and Industrial Chemists; Physicists; Polymer Technologists; Polymer Processors; and Graduate Chemistry Students.

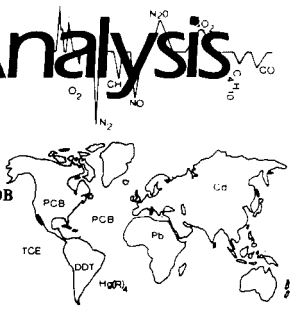
CONTENTS

ISBN: 0–8247–7065–X

MARCEL DEKKER, INC.
270 MADISON AVENUE, NEW YORK, N.Y. 10016
(212) 696-9000

improving your ability to conduct environmental analyses . . .

Chromatographic Analysis of the Environment



Second Edition, Revised and Expanded

edited by **ROBERT L. GROB**
Villanova University
Villanova, Pennsylvania

September, 1983
736 pages, illustrated

praise for the First Edition from both sides of the Atlantic . . .

". . . the editor, contributors and the publisher have combined to produce a splendid and valuable compendium; it should be made readily accessible to all those who are concerned with environmental monitoring."
–Alan T. Bull, *International Biodeterioration Bulletin*

"The unique organization of the book allows the reader to locate and examine information in his area of interest with a minimum of difficulty. . . ."
–Joseph H. Caro, *Soil Science*

"Professor Grob and his team of coauthors set out to provide a 'comprehensive work of chromatographic techniques of practical value to consultants, engineers and chemists'. . . . the book certainly provides a full and reasonably critical perspective of the practical applications of chromatography to environmental problems. . . ."
–H. Egan, *Chemistry in Britain*

". . . since it is a compendium prepared by 'users for users' it has real value to microbiologists who frequently need advice on analytical methods for traces of organic and inorganic compounds. . . ."
–D. Perlman, *ASM News*

Completely revised, expanded, and updated, this instructive work authoritatively examines the latest chromatographic techniques used to determine toxic substances in the atmosphere, water, waste effluents, and soil. With this comprehensive volume, environmental scientists have a single-source guide to the selection and performance of the most effective chromatographic analyses.

Organized for easy reference, *Chromatographic Analysis of the Environment:*

- **offers essential background material covering the theory and practice of chromatography**
- **provides a new chapter examining sample preparation techniques–an essential topic in analysis**
- **presents numerous time-saving references and a detailed subject index**
- **expands coverage of ion exchange and paper chromatography techniques–approaches undergoing a rebirth of interest**

With its concentration on environmental considerations, this important reference serves as a unique resource for environmental scientists, analytical chemists, chromatographers, and government environmental protection officials. In addition, the logical format of *Chromatographic Analysis of the Environment* makes it an ideal text for graduate-level courses and professional seminars examining topics in environmental analysis.

CONTENTS

ISBN: 0–8247–1803–8

MARCEL DEKKER, INC.
270 MADISON AVENUE, NEW YORK, N.Y. 10016
(212) 696-9000

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

Journal of Liquid Chromatography is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

Directions for Submission

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

Reprints

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

Format of Manuscript

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

8. Each page of manuscript should be numbered lightly at the bottom of the sheet with a light blue pencil.

9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

Typing Instructions

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

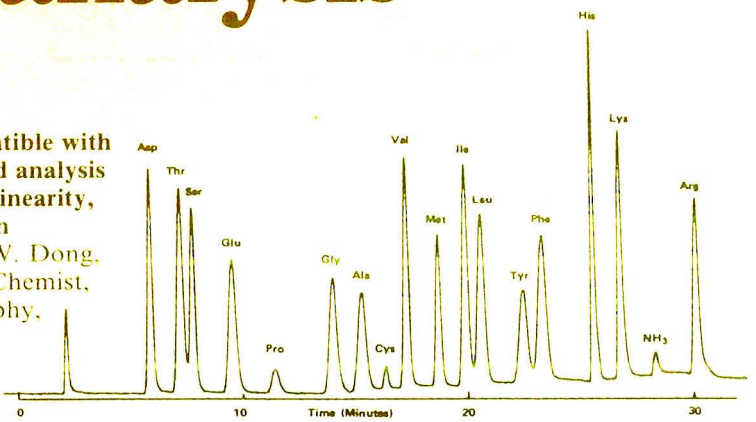
4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.

Buffēlute™: high performance buffers for amino acid analysis

"Buffēlutes are compatible with high-speed amino acid analysis with good precision, linearity, resolution and column lifetime..." Michael W. Dong, Senior Applications Chemist, Liquid Chromatography, Perkin-Elmer.



Your HPLC can resolve amino acids like this with Pierce's new Buffēlute High Performance Buffers and High Speed Ion-Exchange Column. Buffēlutes are sodium based, non-citrate buffers designed for high sensitivity with minimal baseline noise. No other commercial buffers can match the performance of Buffēlutes.

The High Speed Ion-Exchange Column is packed with a special sulfonated polystyrene divinylbenzene copolymer. The column, the Buffēlute buffers and Fluoraldehyde™ Reagent Solution (*o*-phthalaldehyde) are designed to work in combination for the analyst who requires high speed and high resolution of amino acids.

For more information on these products call or write Product Manager Steve Seely. Ask about our **Special Introductory Offer** on these products: 800-435-2960 (Illinois residents call 815-968-0747 collect)

800-8-PIERCE
(direct line to order desk)

27615 Buffēlute Hydrolyzate Buffer Kit
25132 High Speed Ion-Exchange Column
26025 Fluoraldehyde OPA Reagent Solution

PIERCE CHEMICAL COMPANY
P.O. Box 117, Rockford, IL U.S.A. 61105

PIERCE

Circle Reader Service Card No. 105