The Simultaneous Determination of Lidocaine and its Metabolites in Serum by High Performance Liquid Chromatography

by

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ABSTRACT

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Lidocaine is a heart drug used widely in the management of ventricular arrhythmias. The deethylated metabolites of lidocaine (i.e., monoethylglycinexylidide and glycinexylidide) have been shown to have antiarrhythmic potencies and central nervous system toxicities similar to those for lidocaine. In some patients receiving lidocaine there is evidence that these metabolites may accumulate to significant levels. Therefore it would be advantageous to analyze for monethylglycinexylidide and glycinexylidide simultaneously with lidocaine for the proper clinical evaluation of lidocaine therapy. The development of a high performance liquid chromatographic method suitable for this purpose is the subject of this report.

A reverse phase adsorption liquid chromatographic system was used in this study to resolve and quantitate lidocaine and its metabolites. This system consisted of a Waters U Bondapak C-18 column stationary phase in conjunction with a phosphate-buffered acetonitrile moving phase of pH 6.1.

A charcoal adsorption technique was employed to extract lidocaine and its metabolites from serum. The average

extraction efficiency of this procedure was 72.3% for lidocaine, 64.3% for monoethylglycinexylidide and 49.8% for glycinexylidide. Partition extraction techniques using organic solvents immiscible with serum were also tested, but were found to have lower extraction efficiencies and to be more susceptible to interferences. Prilocaine was found to be a suitable internal standard for the assay procedure.

A spectrophotometer was used as the detector for the chromatographic system. A detection wavelength of 205 nm was used as this is near the absorption maximum of lidocaine and its metabolites. The limit of detection for lidocaine, monoethylglycinexylidide and glycinexylidide in serum was 0.4 ug/ml. Standard curves for all three substances were prepared and were found to be linear over the ranges tested; i.e., 0.4-16.0 ug/ml for lidocaine and 0.4-8.0 ug/ml for monoethylglycinexylidide and glycinexylidide.

Four serum samples from three patients receiving lidocaine were analyzed. Three of the four samples were identified by retention time as having lidocaine concentrations of 2.9, 3.5 and 9.4 ug/ml. The first sample listed also had monoethylglycinexylidide and glycinexylidide concentrations of 0.5 ug/ml each. Monoethylglycinexylidide was also indicated in another sample, but could not be quantifed due to an interfering peak.

Interference studies were performed which established that the drugs procainamide, ethosuximide, hydrochlorithiazide and primidone have elution times which interfer with the assay system used in this study.

For future work the use of a dual-wavelength detection system is strongly recommended as it would provide a greater degree of certainty in the identification of chromatographic peaks. Also the development of an alternative column separation procedure, e. g., ion-exchange, would provide another approach to providing greater certainty in peak identification when used in conjunction with the method described in this study.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the Chemistry Department Faculty for their advice and encouragement. My special thanks go to Dr. Francis W. Smith for his support throughout the duration of this investigation. Thanks are also expressed to Dr. Sylvan M. Sax of West Pennsylvania Hospital and William A. Coughlin of Timken Mercy Hospital for supplying the samples of serum from patients receiving lidocaine.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS
^{o}C	Temperature	degrees Celsius
cm	Length	centimeter
mm	Length	millimeter
nm	Length	nanometer
mg	Mass	milligram
µg	Mass	microgram
ng	Mass	nanogram
ml	Volume	milliliter
µl	Volume	microliter
min	Time	minutes
rpm	Rotational speed	revolutions per minute
psi	Pressure	pounds per square inch
M	Molar Concentration	moles per liter
N	Normality	equivalents per liter
pH	Negative logarithm of hydrogen ion activity	—
pKa	Negative logarithm of an acid dissociation constant	—
a_1	Slope constant of a linear equation	variable
a_o	Intercept constant of a linear equation	variable
MEGX	Monoethylglycinexylidide	—
GX	Glycinexylidide	—
LC	Liquid Chromatography	—
HPLC	High Performance Liquid Chromatography	—

SYMBOL	DEFINITION	UNITS
GLC	Gas Liquid Chromatography	—
PIC	Paired-Ion Chromatography	
EMIT	Enzyme Multiple Immunoassay Technique	

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CHAPTER 1

INTRODUCTION

Medicinal Uses of Lidocaine

As an Anesthetic Agent

Lidocaine was first synthesized in 1943 and was promptly employed in medicine as a local anesthetic(1). Lidocaine may be used as a local anesthetic in the form of: an ointment, for application to the skin; a spray, to provide tracheal and bronchial anesthesia and as a solution, for regional intramuscular injection. Lidocaine has also been used to a small extent as a general anesthetic usually in conjunction with other agents, in the form of a solution for intravenous infusion. As an anesthetic agent it is thought to act by stabilizing the neuronal membrane, thus preventing the initiation and transmission of nerve impulses(2).

As an Antiarrhythmic Agent

Although still used as an anesthetic agent, lidocaine's primary use at this time is as a heart antiarrhythmic drug. The first use of lidocaine as a antiarrhythmic drug was in 1950(3), and since that time has been widely used in the management of ventricular arrhythmias such as those occuring during acute myocardial infarctions or with digitalis intoxication(4,5,6). Lidocaine is not effective against arrhyth-

mias of non-ventricular origin or against ventricular fibrillation once it has occured.

Lidocaine is reported to exert its antiarrhythmic effect by increasing the electrical stimulation threshold of the ventricle during diastole(2,4). Lidocaine, when administered properly, has the advantage over some other antiarrhythmic drugs such as procaine and procaineamide of not causing a reduction of cardiac function as measured by the blood pressure, cardiac output, absolute refractory period or myocardial contractility(2,4).

Therapeutic and Toxic Lidocaine Concentration Range

The therapeutic serum (or plasma) concentration range for lidocaine is defined as 1.2 to 5.0 µg/ml at which concentration 80% of those patients with ventricular premature beats or other ventricular ectopies have been observed to respond (5,6). For any single patient a dose response relation can be seen in which ventricular arrhythmias were suppressed as the patient's lidocaine blood level increased(5).

The toxic serum (plasma) concentration range for lidocaine has been defined as greater than 5.0 µg/ml, with signs of toxicity being manifested in some patients at concentrations of from 5.0 to 9.0 µg/ml and in most patients at concentrations of 9.0 µg/ml or greater(5,6). Central nervous system toxicity is the most common side effect of lidocaine administration and may be excitatory and/or depressant in nature. The milder signs of toxicity include dizziness,

drowsiness, disorientation, agitation, double vision, twitching and diminished hearing. More severe side effects include hallucinations, seizures and respiratory arrest(2,5,7,8). Cardiovascular reactions to overdoses of lidocaine are depression of cardiac function and may be characterized by hypotension (low blood pressure), bradycardia (slow heart beat) and cardiac arrest(2).

Form of Administration as an Antiarrhythmic

For treatment of cardiac arrhythmias lidocaine is usually administered as a intramuscular or intravenous bolus injection in conjunction with a continuous intravenous infusion (5,6,9). A combination of bolus injection and infusion is necessary as an infusion alone would require several hours to attain a therapeutic lidocaine blood level, whereas a bolus injection alone would provide a therapeutic blood level rapidly but would last for only about 15 or 20 minutes due to the rapid clearance of lidocaine from the blood(5,6). Using a combination of bolus injection with infusion results in the rapid attainment of a therapeutic blood level which drops to a minimum after about 30 minutes followed by a gradual rise to a steady state concentration(5).

Orally administered lidocaine has been shown to be ineffective in attaining therapeutic blood concentrations of lidocaine. Metabolism and inactivation of lidocaine in the liver after absorption from the gastrointestinal tract has been cited as the probable cause(6).

Lidocaine consists of an aromatic group, 2-6 xylidine, to which is coupled diethylglycine by an amide bond to form a tertiary amine whose chemical name is 2-(diethylamine)-N-(2,6-dimethylphenyl) acetamide. For medicinal purposes the hydrochloride salt of lidocaine is most commonly administered, as the salt has a much greater water solubility than the free base(10). Lidocaine is a weak base with a pKa of 7.85 (6) and at normal blood pH is predominantly in its ionized form as a quaternary amine, see Figure 1. In addition lidocaine in the blood stream is protein bound (i.e., 55% bound at a concentration of 5 μg/ml)(11). The free un-ionized form of lidocaine is highly lipid soluble and rapidly equilibrates, within a minute after an intravenous injection, with the tissues(11).

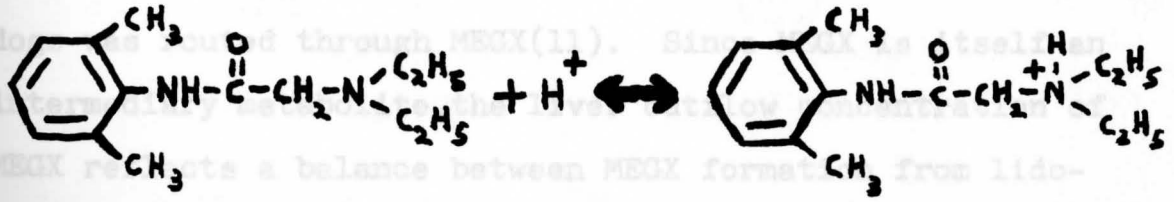


Fig. 1.-- Structure of lidocaine as a un-ionized tertiary amine (left) and as an ionized quaternary amine (right).

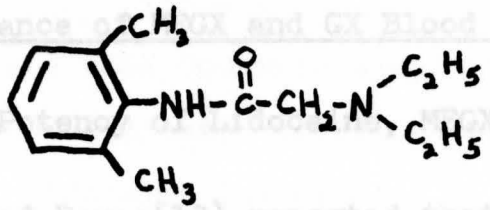
Metabolism and Elimination

The liver has been consistently found to be the active site of lidocaine metabolism both in vitro and in vivo studies(11). Metabolism of lidocaine in the liver, in fact, is the primary pathway for the inactivation and subsequent elimination of lidocaine from the body. Direct excretion of

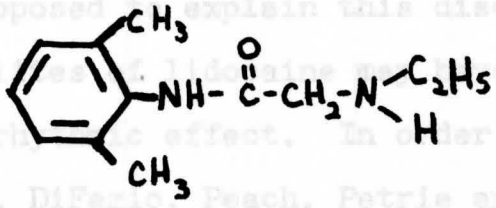
lidocaine by the urinary or biliary routes occurs only to a small extent, 2-11% of administered dose, where as the bulk of the lidocaine is eliminated in the form of simpler metabolites which are produced in the liver and excreted in the urine(11,12).

The first and most important step in the liver metabolism of lidocaine, which is shown schematically in Figure 2, is the deethylation of lidocaine to the secondary amine monoethylglycinexylidide(MEGX). The importance of this step lies in the fact that the amidase enzymes, which hydrolyze MEGX to the simpler compounds which are eventually excreted by the kidney, do not act to an appreciable extent on lidocaine itself(11). The principal route for lidocaine metabolism, therefore, is seen to be through MEGX; one study showed that 76% of the administered lidocaine eliminated from dogs was routed through MEGX(11). Since MEGX is itself an intermediary metabolite,the liver outflow concentration of MEGX reflects a balance between MEGX formation from lidocaine and further degradation to the lidocaine excretion products.

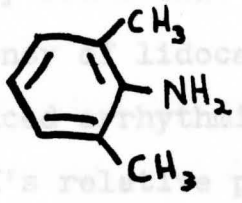
The primary amine glycinexylidide(GX) is also formed as a metabolite of lidocaine, as shown in Figure 2, but not as part of the main pathway from MEGX to the urinary excretion products. GX is resistant to further metabolism and is excreted in the urine unchanged, thus accounting for a small amount of the administered lidocaine dose (i.e., less than 10%)(11).



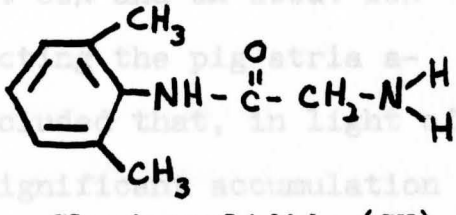
Lidocaine



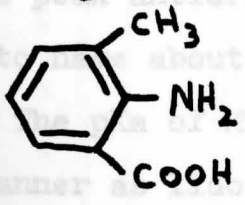
Monoethylglycinexylidide (MEGX)



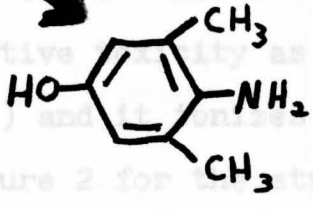
2, 6-Xylidine



Glycinexylidide (GX)



2-amino-3-methyl benzoic acid



4-hydroxy-2, 6 Xylidine
(main excretion product)

Fig. 3. The major metabolic pathway of lidocaine degradation (liver). Some minor branching pathways not shown

Source: C.A. DiFazio, "Biotransformation of Lidocaine," International Anesthesiology Clinics 18(4), 21 1975

Significance of MEGX and GX Blood Levels

Relative Potency of Lidocaine, MEGX and GX

Smith, Duce and Boyes(13) reported that the peak antiarrhythmic effect of orally administered lidocaine occurred after the peak plasma levels of lidocaine were achieved. One explanation proposed to explain this discrepancy was that one or more metabolites of lidocaine may have been exerting a significant antiarrhythmic effect. In order to test this possibility Burney, DiFazio, Peach, Petrie and Silvester(14) determined the relative potencies of lidocaine, MEGX and GX for suppressing digitalis-induced arrhythmias in guinea pig atria. They found that MEGX had about 80% and GX about 10% of the potency of lidocaine for protecting the pig atria against induced arrhythmias. They concluded that, in light of MEGX and GX's relative potencies, a significant accumulation of these metabolites, particularly MEGX, could account for the discrepancy between the peak concentration of lidocaine and the peak antiarrhythmic effect. MEGX has also been shown to have about the same relative toxicity as lidocaine (15). The pKa of MEGX is 8.07(12) and it ionizes in the same manner as lidocaine; see Figure 2 for the structure of MEGX.

Observed Plasma Lidocaine, MEGX and GX Concentrations in Relation to the Presence of Toxicity Signs

Tables 1 and 2 show the plasma lidocaine, MEGX and, in the case of Table 2, the GX levels observed in plasma from

TABLE 1

Lidocaine and MEGX Concentrations in Plasma Samples from Patients Receiving Continuous Lidocaine Infusions

	Lidocaine µg/ml	MEGX µg/ml	GX µg/ml	CNS Toxicity
Patient # 1	4.2	0.58	-	No
" " 2	3.8	0.83	-	No
" " 3	2.4	0.35	-	No
" " 4	1.9	0.31	-	No
" " 5	6.5	0.52	-	Yes
" " 6	5.2	0.60	-	Yes
" " 7	4.4	2.6	-	Yes

Source: J. M. Strong and A. J. Atkinson Jr., Anal. Chem., 44(14), 2289 (1972).

TABLE 2

Lidocaine and MEGX Concentrations in Plasma Samples from Patients Receiving Continuous Lidocaine Infusions

	Lidocaine µg/ml	MEGX µg/ml	GX µg/ml	CNS Toxicity
Patient # 1	2.8	2.4	2.7	Yes
" " 2	3.6	1.5	1.5	No
" " 3	15.0	1.1	1.0	Yes
" " 4	8.2	0.3	Below 1.0	Yes
" " 5	1.2	0.3	" "	No
" " 6	2.3	0.6	" "	No
" " 7	1.4	0.3	" "	No
" " 8	1.6	0.2	" "	No

Source: J. M. Strong, M. Parker and A. J. Atkinson Jr., Clin. Pharmacol. Ther., 14, 67 (1973).

patients receiving lidocaine infusions as determined in the studies by Strong and Atkinson(16), Table 1, and Strong, Parker and Atkinson(17), Table 2. Analysis in both studies was by gas liquid chromatography. In addition to the lidocaine, MEGX and GX plasma concentrations, Tables 1 and 2 show whether signs of central nervous system toxicity were manifested in the patients at the time their blood was collected.

Three patterns of lidocaine and MEGX concentration in relation to toxicity can be seen from these results. The pattern seen for most of the patients was a lidocaine concentration within its therapeutic range (1.2-5.0 μg/ml) and MEGX and GX concentrations approximately one-sixth that of the lidocaine concentration with no signs of toxicity present.

A second pattern can be seen for patients 5 and 6 of Table 1 and patients 3 and 4 of Table 2. These four patients show a pattern of a lidocaine concentration greater than the therapeutic range with signs of central nervous system toxicity apparent. The MEGX concentration in these patients was about one-tenth that of their lidocaine concentrations and approximately equal to the MEGX concentrations in the toxic free group of patients.

A third pattern of lidocaine and MEGX concentration in relation to the presence of toxicity can be seen for patient 1 of Table 1 and patient 7 of Table 2. These two patients showed signs of central nervous system toxicity even though their lidocaine levels are within the therapeutic range. The MEGX and GX levels were considerably higher than those deter-

mined for any of the other patients and approached one-half to two-thirds the concentration of lidocaine. The sum of the lidocaine and MEGX concentrations for these two patients was equal to a toxic concentration of lidocaine alone and indicates that the toxicity in these patients was probably due to the accumulative action of both lidocaine and MEGX. In addition GX might also be contributing to a small extent to the overall toxicity reaction; note that the GX concentration usually approximates MEGX's concentration in Table 2.

These data would suggest that patients showing toxicity when treated with lidocaine may have different patterns of altered lidocaine metabolism. In some patients toxicity may be caused exclusively by lidocaine whereas in others by both lidocaine and its active metabolites, particularly MEGX.

Conditions Resulting in MEGX Accumulation

As was mentioned previously conversion to MEGX is the necessary first step in the liver metabolism of lidocaine. If for some reason the lidocaine metabolic pathway bogs-down after conversion to MEGX then an accumulation of MEGX would be expected to result. This has been suggested as occuring in some patients with primary or secondary liver problems or with genetic deficiencies(17).

Congestive heart failure, with reduced blood flow to the liver, is known to result in decreased lidocaine clearance from the blood(5,6) and may be involved in metabolite build-up. A decrease in cardiac output is seen to some degree in most cases of acute myocardial infarction(5,6). In addition,

patients with normal liver function have been observed to recover from symptoms of lidocaine toxicity quicker than those with abnormal liver function, which has been interpreted as resulting from the latter's inability to completely metabolize the lidocaine(8).

It is evident from the above summary that a method for the simultaneous analysis of lidocaine and MEGX would be advantageous in the clinical evaluation of lidocaine therapy. The development of a high performance liquid chromatographic method capable of such an analysis is the purpose of this study.

Published Methods of Analysis for Lidocaine

History

In the 1950's a colorimetric method for the analysis of lidocaine in solution was published by Sung and Truant(18). This method had the disadvantage of having a very low specificity, and would produce a color reaction not only with lidocaine but with other amino-containing conpounds as well, including, of course, MEGX and GX. Due to the development of other more effective assay techniques this method has fallen into disuse.

In the mid 1960's gas liquid chromatographic techniques were developed and became the standard methods of analysis for lidocaine in blood(19,20). In the early 1970's GLC methods were published which allowed for the simultaneous assay of MEGX and GX as well as lidocaine in blood(16,17,21).

Recently a high performance liquid chromatography (HPLC) method for the analysis of lidocaine in serum has been published(27). In addition Syva Incorporated is reportedly about to release a enzyme multiple immunoassay technique (EMIT) test kit for the analysis of lidocaine in plasma.

Summary of the General Extraction Techniques Used in the GLC Methods

The published papers describing GLC methods for the analysis of lidocaine and/or its metabolites in serum or plasma utilize variations of either a single or double organic extraction technique. A sample of serum or plasma is alkalized to shift lidocaine, MEGX and GX into their non-ionic forms; refer to Figure 1. An organic solvent immiscible with water is then added to the alkalized serum or plasma and the contents mixed in some fashion. Lidocaine, MEGX and GX in their non-ionic forms are much more soluble in the non-polar organic phase than in the polar aqueous environment of serum, therefore, alkalizing the serum maximizes the extraction of these substances into the organic phase.

In the single extraction GLC procedures, the organic layer is separated from the serum or plasma layer and either an aliquot is injected into a chromatograph for analysis or in most cases the entire organic phase is evaporated to dryness. The residue of the organic phase is dissolved in a small volume of a volatile organic solvent, an aliquot of which is removed for injection into a GLC for analysis.

The evaporation and redissolving of the extract in a smaller volume of fluid has the effect of concentrating the solutes into a volume small enough to be injected into a chromatograph.

In the double organic extraction techniques an extraction from alkalized serum or plasma into an immiscible organic solvent is performed as described for the single extraction techniques. After separating the organic phase from the serum phase, however, the organic extract is added to a dilute aqueous solution of acid, which shifts the lidocaine, MEGX and GX into their ionic forms, and a back extraction is performed. Since the ionic forms of these substances are more soluble in the aqueous phase, the organic phase, after mixing and centrifuging, is separated and discarded. The aqueous phase is then made basic to shift the substances back into their non-ionic forms again and is reextracted with a fresh aliquot of organic solvent. The organic phase is separated and evaporated to a small volume which is then directly injected into a chromatograph for analysis. The double organic extraction techniques involve more steps and therefore take longer to perform than the single organic extraction techniques, but have the advantage of providing a cleaner (less denatured protein) sample.

Summary of Specific GLC Methods

In view of the confusion in the literature as to the best procedure for extracting lidocaine from blood, in the summary below the various extraction procedures used by different work-

ers are described in some detail.

In 1968 Keenahan(19) published a paper describing the GLC analysis of lidocaine in whole blood. In this work 5 ml of benzene was used to extract lidocaine from 2.5 ml of heparinized whole blood which had been alkalinized with 0.2 ml of 5N sodium hydroxide. After mixing and centrifuging, the organic phase was transferred to another tube and evaporated to dryness at 50°C. The residue was then dissolved in 50 µl of benzene and a aliquot was removed by syringe and injected directly into a GLC equipped with a flame ionization detector.

The average extraction efficiency for lidocaine of this procedure was reported as 98% with a limit of detection of less than 0.5 µg/ml. No interferences were reportedly observed in fresh blood samples; some interferences were reported in stored samples.

Reynolds and Beckett(20) described a double organic extraction procedure for the analysis of lidocaine and other local anesthetics in blood. In this paper 2.5 ml of ether was added to a mixture containing 2 ml of water, 1 ml of an aqueous internal standard solution, 2.5 ml of 5N sodium hydroxide and 2 ml of blood. After mixing and centrifuging, the ether phase was removed and transferred to another tube, and the extraction repeated three times. The four ether extracts were combined and back extracted into 0.1N hydrochloric acid. The separated aqueous layer was made alkaline and extracted four times with ether. The four ether extracts were combined and evaporated at 42°C until the ether ceased to moisten the ground glass neck of the centrifuge tube. At this point the

tube was removed from the 42°C bath, stoppered and inserted into an ice bath to condense the remaining ether vapors. The evaporation - condensation cycle was repeated until only about 20 ml of condensate remained, 2 µl of which was removed by syringe and injected into a GLC equipped with a flame ionization detector.

The extraction efficiency was reported as 98.5% with a limit of detection of 0.04 µg/ml; no interferences were observed. This procedure is not very practical for routine clinical use as it is very time consuming, due to the repetitive extractions involved.

In 1971 DiFazio and Brown(21) published the first method which included the simultaneous anaylsis for MEGX and GX as well as lidocaine. The procedure was to use 10 ml of chloroform to extract 2 ml of blood, plus an internal standard, which had been alkalized with concentrated ammonium hydroxide. After shaking and centrifuging,the organic phase was removed and the aqueous layer was reextracted with another aliquot of chloroform. The two chloroform extracts were combined and evaporated to dryness at 45°C. The residue was dissolved in 25 ml of chloroform, 5 ml of which was removed and injected into a flame-ionization-detector-equipped GLC.

The extraction efficiencies reported were 92.1% for lidocaine, 101.1% for MEGX and 72.1% for GX. No detection limit information was provided; nor were any results from patients receiving lidocaine reported. The procedure was reported to be free of interferences.

In 1972 Strong and Atkinson(16) described a method for the simultaneous assay of lidocaine and MEGX in blood using the technique of mass fragmatography. This technique involved the use of a mass spectrometer as the detector of a gas liquid chromatograph. Since the amount of material injected into the chromatograph was not sufficient to obtain a complete mass spectrum the intensities of selected mass spectral ions were measured. This kind of approach constitutes the technique of mass fragmatography and, used in conjunction with chromatographic retention time data, provides a much greater degree of certainty in identifying peaks than retention time alone.

While this technique provides high sensitivity and selectivity, it also requires expensive and sophisticated equipment and highly trained personnel to operate it and, therefore, is not practical for use in most general clinical laboratories.

The extraction procedure used in Strong and Atkinson's paper was of the single extraction variety using 5 ml of benzene to extract 1 ml of plasma alkalized with 0.2 ml of 5N sodium hydroxide. After mixing and centrifuging, the organic phase was separated from the aqueous phase and evaporated to dryness at 25°C. The residue was dissolved in 50 ml of benzene, 2 ml of which was injected into the chromatograph.

The extraction efficiency was reported as greater than 90% for both lidocaine and MEGX. No detection limit or interference information was provided. The plasma lidocaine and

MEGX concentrations for 7 patients receiving continuous lidocaine infusions were reported and are reproduced in Table 1.

Strong, Parker and Atkinson(17) also used the mass fragmatographic technique to analyze for GX in urine and plasma in addition to lidocaine and MEGX in plasma. The plasma extraction procedure was the same as was described for Strong and Atkinson's paper above with the exception that the aqueous mixture was extracted twice with benzene.

Urine samples were analyzed by adjusting 25 ml of urine to a pH greater than 10 with 2 ml of 5 N sodium hydroxide, followed by extraction with 25 ml of benzene. The extraction was repeated a second time and the benzene extracts combined and centrifuged to remove traces of urine. The extracts were then evaporated to dryness at 25°C and the residue redissolved in 2.1 ml of benzene, 2 µl of which was injected into the gas chromatograph.

The minimum measurable plasma concentration of GX was 1.0 µg/ml. No extraction efficiencies were reported. The plasma concentrations of lidocaine, MEGX and GX of eight patients receiving continuous lidocaine infusions were reported in this paper and are reproduced in Table 2. The GX urine concentrations of these patients were also reported.

In 1973 Berowitz and Rowland(22) described a GLC method for the analysis of lidocaine in blood and tissues. A sample of heparinized blood or plasma was placed into a centrifuge tube and its volume was adjusted to 2.0 ml by adding an internal standard solution made in pH 7.4 phosphate butter. This mixture was shaken with 7 ml of ether which after mixing and

centrifuging was transferred to a specially made nipple-bottomed centrifuge tube containing 0.2 ml of 0.1 N hydrochloric acid. The contents were shaken and centrifuged and the ether layer was then discarded. The aqueous layer was air dried to remove any residual ether. After making the layer alkaline 50 ul of distilled carbon disulfide was added and the contents mixed and centrifuged. A clear bubble of carbon disulfide was left at the bottom of the tube's nipple and 3 to 5 µl of this was removed by syringe and injected into a GLC equipped with a flame ionization detector.

Tissue samples were analyzed by homogenizing the tissue in a blender and allowing the homogenate to solublize in a solution of sodium hydroxide. Samples of the solublized homogenate were processed in a manner similar to that for the blood samples with additional acid or base added as needed to control the pH of the various extraction steps.

The limit of detection for this method was reported as 0.01 µg/ml; no extraction efficiency information was provided. Nor was there any information concerning interferences.

In 1976 Nation, Triggs and Selig(23) described a method for the simultaneous analysis of lidocaine and MEGX in blood by GLC. The extraction procedure was very similar to that used by Reynolds and Beckett(20) discussed earlier. The extraction procedure employed in both papers was of a double organic type, using ether as the organic solvent. The only significant difference was that Nation et al. did not employ repetitive extractions at each extraction stage as was done by Reynolds and Beckett. For this reason the procedure of

Nation et al. is much more simpler and faster than Reynolds and Beckett's procedure, and therefore more practicable in a clinical laboratory. No information on extraction efficiency or limit of detection was reported.

Ingens, Henderson and Shelver(24) used an alkaline flame ionization detector for their GLC method. This type of detector is an element-specific detector useful for the analysis of compounds containing nitrogen, such as lidocaine and MEGX.

The procedure involved using 5 ml of benzene, containing an internal standard, to extract 2.5 ml of plasma which had been alkalized with 0.2 ml of 5 N sodium hydroxide. After mixing and centrifuging,the organic phase was transferred to a pear-shaped tube and evaporated to dryness at 50°C. The residue was dissolved in 50 µl of benzene from which 5 µl was removed by syringe for injection into the gas chromatograph.

The extraction efficiencies reported were 91.9% for lidocaine and 88.7% for MEGX. The reported limit of detection for both compounds was 0.1 µg/ml. No results for patients receiving lidocaine were reported.

Hucker and Stauffer(25) used a nitrogen-phosphrous sensitive detector to measure lidocaine in blood. The advantages claimed by the authors for this type of detector over the more common flame ionization detectors are: increased sensitivity, rapidity and specificity.

Their procedure involved adding 1 ml of plasma, an aqueous internal standard solution, 1 ml of 0.5 N sodium hydroxide and 1 ml of benzene to a glass-stoppered centrifuge tube.

After shaking and centrifuging, 5 µl of the benzene phase was removed by syringe and injected directly into a gas chromatograph. Note that no evaporation step was used in this procedure. The extraction efficiency was reported as 103.2% with a limit of detection of 10 µg/ml.

Caille, LeLorier, Latour and Besner(26) described a GLC method for lidocaine analysis in blood. This paper provided information on possible drug interferences not included in previous studies.

Their procedure used 10 ml of methylene chloride to extract 2 ml of plasma which had been diluted with 2 ml of water and alkalized with 0.5 ml of 5N sodium hydroxide. After mixing and centrifuging, the organic layer was separated from the aqueous layer which was extracted with another 10 ml of methylene chloride. The organic phases were combined, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 0.5 ml of chloroform transferred to another tube and evaporated to dryness. This residue was dissolved in 50 µl of chloroform, small aliquots of which were injected into a GLC for analysis.

The reported extraction efficiency was 99.0%. Of 23 drugs commonly given in conjunction with lidocaine and tested in this paper, none was found to interfere with the analysis.

Summary of Published HPLC Assay Method for Lidocaine

During the early stages of the present investigation a HPLC procedure for the analysis of lidocaine in serum was discovered in the literature(27). The paper was by Adams,

Vandemark and Schmidt and involved a charcoal adsorption method for isolating lidocaine from blood.

The assay protocol used by Adams et al. was to add 1.0 ml of serum, 10µl of an internal standard solution in methanol, 2 ml of 2% ammonium hydroxide and 8 mg of charcoal (Norit A) to a culture tube. The contents were mixed by vortexing for 15 seconds followed by centrifuging for 1 minute at 2,500 rpm, which caused the charcoal to form a button on the bottom of the tube, The liquid phase was aspirated to waste leaving the charcoal button behind. To desorb the lidocaine off the charcoal's surface 1 ml of dichloromethane (methylene chloride) was added to the tube, and the contents were mixed and centrifuged as before. The dichloremethane was then decanted into another tube and evaporated to dryness at 60°C. The residue was dissolved in 20 µl of methanol, 4 ml of which was removed by syringe and injected into a HPLC equipped with a UV spectrophotometer detector set at a detection wavelength of 205 nm.

The average extraction efficiency reported was 66.5% with a limit of detection of 0.1 µg/ml. To assess the accuracy of this method 15 serums from subjects on lidocaine therapy were analyzed for lidocaine by both an accepted GLC method and by the HPLC method, the agreement was excellent. This paper contained no information concerning the metabolites of lidocaine.

Discussion of HPLC

General Characteristics of Liquid Chromatography

All types of chromatography are based on the phenomenon that each component in a mixture ordinarily interacts with its environment differently from all other components under the same conditions. In liquid chromatography (LC) a dilute solution of the sample is passed through a tube or column packed with solid particles, which may or may not be coated with another immiscible liquid. With proper choice of solvent, operating conditions and stationary phase the components in the sample will travel through the column at different rates, resulting in the desired separation of the components.

In gas liquid chromatography all the components to be analyzed for must have an appreciable vapor pressure(volatility) so as to be picked-up and dispersed in the carrier gas. If a compound is not volatile, two approaches may be employed to make it volatile. First, since a substance's vapor pressure increases with its temperature, simply operating the GLC at an elevated temperature up to 300^{o}C, may provide the necessary volatility. Some compounds however may decompose before they become sufficiently volatile. The second approach is to make and analyze for a volatile derivative of the non-volatile compound of interest(28).

The need for high operating temperatures or the making of volatile derivatives and the problems these operations

entail are not encountered in LC. This is because the fundamental requirement of liquid chromatography is only that all of the components be in solution. Since the components in LC do not have to be volatile, LC has a much more general applicability than GLC although the latter is still considered the method of choice for the analysis of compounds which can be volatilized(29).

Until recent years liquid chromatography had fallen into disuse and was eclipsed by GLC techniques in spite of the former's greater applicability. This was due to the slowness and low resolution of the open column gravity-fed techniques of classical LC. With the creation in the last decade of new column packing materials and equipment, making possible faster liquid phase flow rates through high efficiency columns, the new high performance form of liquid chromatography (HPLC) has come into its own with capabilities equal to or better than those of other types of chromatography.

In addition to being nearly universal in application HPLC also has the advantage of being a more versatile technique than GLC. HPLC can be performed in several different modes, which may be used singly or in combination to achieve the desired separation and analysis. These modes represent 4 types of moving and stationary phase interactions with the components to be resolved and include the following mechanisms: adsorption (liquid-solid chromatography), partition (liquid-liquid chromatography), ion exchange and selective exclusion according to molecular size (gel permeation chroma-

tography)(28). The adsorption type will be the only one discussed here as this was the HPLC mode used in this study.

Adsorption Chromatography

Adsorption chromatography is commonly applied to the separation of polar, but not ionic, organic compounds. The stationary phase in adsorption chromatography consists of active sites on the adsorbent's surface which are themselves polar organic groups (i.e., Si-OH). Adsorption chromatography depends, therefore, on the interaction between the polar groups of the stationary phase and the polar or polarizable groups of the compounds to be analyzed(28).

The function of the adsorption mode of HPLC is greatly influenced by the selection of the moving phase solvent used in conjunction with a particular stationary phase. Solvents which are themselves strongly adsorbed onto the stationary phase effectively displace sample molecules from that surface. The solvent strength (dielectric constant) of the moving phase therefore is the primary factor in the control of the elution characteristics of any given set of components on any given adsorption column, but solvent strength is not the only factor involved. The criterion of solvent strength takes into account only interactions between the solvent and the adsorbent, whereas selectivity in adsorption chromatography is also significantly affected by sample and solvent interactions. These secondary solvent effects cannot be readily predicted(28).

Analyses by adsorption chromatography are normally performed with a polar stationary phase and a non-polar noving

phase. The alternative technique of reverse phase adsorption chromatography was introduced in the 1950's and involves the use of a non-polar stationary phase with a polar moving phase (30).

Both normal and reverse phase adsorption chromatography have developed very rapidly since the appearance of bonded stationary phases in the late 1960's and early 1970's. Prior to the introduction of bonded stationary phase column packings the stationary phase was held to the support particles only by absorptive or non-covalent bonding forces. In bonded stationary phase packings the stationary phase (functional groups) is chemically bonded to the support particles by covalent bonds. It is now possible to obtain commercially prepared chromatographic columns with chemically bonded stationary phases of either polar groups for normal phase work or non-polar groups for reverse phase adsorption chromatography (30).

The covalently-bonded stationary phases now available have several important advantages over the non-bonded stationary phases which helped make possible the development of HPLC. Some of these advantages are: One, the moving and stationary phases should always be immiscible, which is never completely the case when using non-bonded phases but is insured when bonded phases are used. Two, large shear forces are generated in the narrow-bore HPLC columns at high moving-phase velocities. These forces tend to strip-off non-bonded stationary phases from their support particles thus limiting their use to low flow rates. This does not occur to a signi-

ficant extent with bonded stationary phases, thereby allowing fast flow rates and shorter analysis times with less band spreading. Three, the bonded phases are more versatile than the non-bonded phases, making possible changes in solvent composition during a run (i.e., gradient elution) or the running at elevated temperatures.

Column Efficiency

Another factor in the revival of liquid chromatography in the form of HPLC has been the development of high efficiency column packings, which have great resolving capablities. Column efficiency is dependent on the particle size and shape of the column packing material and on the internal diameter of the column. Theoretically columns should be packed with the smallest possible size and most regular spherical shaped support particles(29). One of the reasons for this is that in classical liquid chromatography the support particle is porous throughout, thus resulted in some of the moving liquid phase getting into the particle's deep pores and stagnating there for a time. This causes band spreading and significantly reduces the column's resolving capablity (efficiency). Using a small particle size reduces the particle's deep pore volume in relation to its overall surface area thereby reducing the band spreading effect. The disadvantages of using smaller particle sizes are the need for special packing techniques (i.e., slurry packing) and a greater pump pressure to force the moving phase through the column at any given flow rate.

Another approach to reducing the deep pore volume and thus increase the column efficiency, without reducing the particle size, is to use a support particle which has a solid non-porous core with the stationary phase bonded on the surface layers of the particle. Solid core stationary phase packings have the advantages of being able to be dry packed and require lower pump pressures to maintain any given flow rate, however they are neither as efficient nor as fast as an comparable totally porous microparticle packings. In addition since the solid-core packings have less relative stationary phase surface area than the porous microparticles they do not have as great a sample capacity as the microparticles do, which is significant in preparative-scale work. A totally porous microparticle reverse phase adsorption column was used in this study.

Statement of Purpose

High performance liquid chromatography is rapidly becoming a common clinical laboratory technique and is being used more and more in conjunction with or instead of gas liquid chromatography. The purpose of the present study, therefore, is to develope a HPLC method for the analysis of MEGX and GX, which would be of use in a clinical laboratory having a HPLC. The present investigation provides the first reported simultaneous analysis for lidocaine, MEGX and GX by HPLC.

CHAPTER II

APPARATUS AND MATERIALS

Chromatographic Apparatus

The components of a high performance liquid chromatograph consists essentially of: (1) a solvent delivery system, (2) a sample injection system, (3) a stationary phase column and (4) a detector and recorder system.

Solvent Delivery System

The solvent delivery system consists of the solvent holding tank and the high pressure pump required to achieve fast moving phase velocities in the small-bore HPLC columns. There are two classes of HPLC pumps in general use at the present time: (1) the continuous displacement variety (i.e., gas displacement, gas amplier and syringe pumps) and (2) the intermittent displacement types (i.e., peristaltic, diaphragm and reciprocating piston pumps)(28).

Continuous displacement pumps have the advantage of delivering a smooth, pulseless flow of solvent, and the disadvantages of having a limited solvent capacity and the inablity to change the solvent composition during operation. Intermittent displacement pumps, by contrast, operate from an open, and hence unlimited, solvent reservoir at ambient pressure. Solvent reservoir refill and composition changes during pump operation are possible with intermittent displacement pumps.

The main disadvantage of these types of pumps is the pulsating nature of the outputs which can generate a significant amount of detector noise. To smooth-out the output of intermittent displacement pumps a dampening or filtering system is usually incorporated into these pumps.

The type of pump incorporated into the HPLC unit used in this study is a multihead reciprocating piston intermittent displacement pump, Waters model M6000A. This pump has two pump heads which operate in such a way that while one piston is on its return stroke the other is on its compression stroke, thus, upon combining the outputs of the two pump heads a roughly pulse-free flow is obtained. The final pump output is smoothed-out by built-in filters. The M6000A pump is electronically controlled and capable of providing liquid flow rates of from 0.1 to 9.9 ml/min in 0.1 ml increments and at a pump pressure of up to 6000 psi.

Sample Injection System

By comparison with gas chromatography, diffusion in the mobile phase is negligible in HPLC, which makes a stop-flow injection technique possible. As the name implies this technique involves stopping the flow of the solvent, injecting the sample and restarting the solvent flow. Stop-flow injection systems are cumbersome to use but are inexpensive and reliable(28).

Septum injection techniques involving the injection of a sample directly into the flowing solvent stream via a syringe are also in general use. Since the syringe in these types of

injection systems is exposed to the full pressure of the solvent stream, special pressure syringes must be used, and even with the use of these special syringes the pump pressure, and therefore the moving phase flow rate, must be kept at relatively low levels.

The most versatile and expensive injection system used in HPLC at this time is the loop and valve type, such as the Waters model UGK used in this study. This type of injector consists of a length of pressure tubing (the loop) and a valve which can be set so as to either incorporate the loop into the solvent stream or bypass it. In the bypass position the loop, which is filled with solvent, can be vented to the atmosphere and a syringe inserted through a plug into the loop and a sample injected at ambient pressure. The volume of sample injected will cause a equal volume of solvent to be displaced from the vent end of the loop. After closing the vent the injector valve can be switched to incorporate the loop into the moving solvent stream and the solvent in the loop along with the sample will be swept away downstream to the column.

HPLC Column

As was mentioned in Chapter I there are several modes of HPLC separation, i.e., adsorption, partition, ion exchange and gel permeation, each of which requires a particular type of stationary phase. The stationary phase used in this study was a prepacked Waters μBonadapak C-18 column which is a reverse phase adsorption type of column.

This column was packed with totally porous microparticles

(10 micron or less particle diameter) to which was bonded a monomolecular layer of octadecyltrichlorosilane(C-18) groups. The C-18 groups were bonded to the support particles via silica-carbon bonds which are hydrolytically stable between pH 1 to 8 and thermally stable up to 300°C. The dimensions of the column were 30 cm long, with an outside diameter of 6.4 mm and an inside diameter of 3.9 mm(31).

HPLC Detector

One of the major reasons for the rejuvenation of liquid chromatography, along with the creations of high pressure pumps and bonded columns, has been the development of detectors capable of the continuous monitoring of column effluent(32). Most HPLC detectors utilize a flow cell as opposed to a scanning device and therefore measure a physical quantity of the effluent which depends on the effluent composition.

The most common single type of HPLC detector is the ultraviolet(UV) absorbance photometric detector. Most commercial HPLC detectors of this type are essentially double-beam photometers filled with flow cells. The high sensitivity, approximately 5×10^{-10} g/ml, and low cost of these detectors result from the use of a low-pressure mercury arc light source. The mercury lamp puts out 90% or more of its energy in a narrow band centered at 254 nm. Other wavelengths can be obtained from this light source by interposing a phosphor between the lamp and the cell(28).

The second most common HPLC detector is the refractive index(RI) detector. Most RI detectors are of the differential

type and have a double-beam and double flow cell arrangement where the refractive index of both the elutent stream and a reference stream of pure moving phase solvent are continuously monitored and compared. RI detectors have the advantage over UV absorption detectors of being nearly universal in application, however they have the disadvantage of being less sensitive than UV detectors, approximately 5×10^{-7} g/ml(28).

The UV and RI detection techniques are both sensitive to changes in the moving phase flow rate and, therefore, require some kind pulse dampening or filtering of the pump output. Other types of detection techniques have been adopted for use in HPLC such as fluorescence, flame ionization, thermal and conductance detectors, but compared to use of UV and RI detectors are quite uncommon.

The Waters ALC/GPL model number 202 HPLC unit used in this study was equipped with both a UV absorption detector, which operated at a fixed detection wavelength of 254 nm or with the insertion of a phosphor at 280 nm, and a differential RI detector. Both of these detectors proved to be inadequate for the analysis of lidocaine and its metabolites at the concentrations at which they are found in blood. The RI detector because of its generally low sensitivity and the UV detector because lidocaine, MEGX and GX do not absorb well at the wavelengths at which the detector was capable of detecting. Figures 19, 20 and 21 in Chapter III, pages 54, 55 and 56. show the absorption spectra of lidocaine, MEGX and GX dissolved in the moving phase solvent used in this study. These spectra show that all three compounds have absorption maxima

at a around 200 nm and practically no absorption at 254 or 280 nm.

Adaption of Spectrophotometer as the Detector

In order to detect lidocaine, MEGX and GX at their therapeutic concentrations on the equipment available, an attempt was made to find a solvent which would shift their absorption spectra in such a way that they would have a significant adsorbance at the wavelengths at which the UV detector was capable of detecting. The UV spectra of lidocaine in several different reverse phase solvents and aqueous buffer systems were obtained,however none was found to cause lidocaine to have an appreciable absorbance at a wavelength of 254 or 280 nm. These absorbance spectra are shown in the appendix beginning on page 115.

In order to detect at a wavelength at which lidocaine, MEGX and GX had an appreciable absorbance,a Beckman model number 26 spectrophotometer was adapted as the detector for the HPLC system used in this study. The Beckman 26 can be set to detect at any wavelength between 190 and 900nm and may be operated in either a double beam mode, as was the case when the unit was used to make the absorbance spectra shown in this study, or in a single beam mode, as was the case when it was used as the HPLC detector. In the single beam mode the baseline absorbance of the spectrophotometer was controlled by a manual photomultiplier tube gain control.

The flow cell assembly that was part of the UV detector built into the Waters HPLC unit was removed. This assembly

consisted of two 1 cm long by 1 mm internal diameter flow cells incorporated into one cylindrical housing. In the HPLC unit one flow cell had air pumped through it and acted as a reference to which the absorbance of the effluent stream flowing through the sample flow cell could be compared. The reference flow cell of the assembly was covered by tape at both ends so light could not pass through while the sample flow cell windows were left uncovered.

The standard cell holders in the Beckman spectrophotometer were removed and a cradle was designed and built to hold the HPLC flow cell assembly in position in the sample light path of the spectrophotometer. Figures 3, 4 and 5 show pictures of the flow cell assembly in its cradle, the cradle and flow cell assembly in the Beckman spectrophotometer and the overall HPLC and spectrophotometer arrangement. The position of the flow cell assembly in the spectrophotometer light path was optimized by trial and error.

The Beckman spectrophotometer was equipped with a single pen recorder which was used to record all chromatograms at a chart speed of 0.5 inches per minute and at a full-scale recorder span of either 0.10, 0.25, 0.50, 1.00 or 2.00 absorbance units. For comparison the most sensitive full-scale recorder span of the Waters ALC/GPL 202's built in UV detector was 0.02 absorption units; almost 5 times more sensitive than the most sensitive Beckman 26 setting.

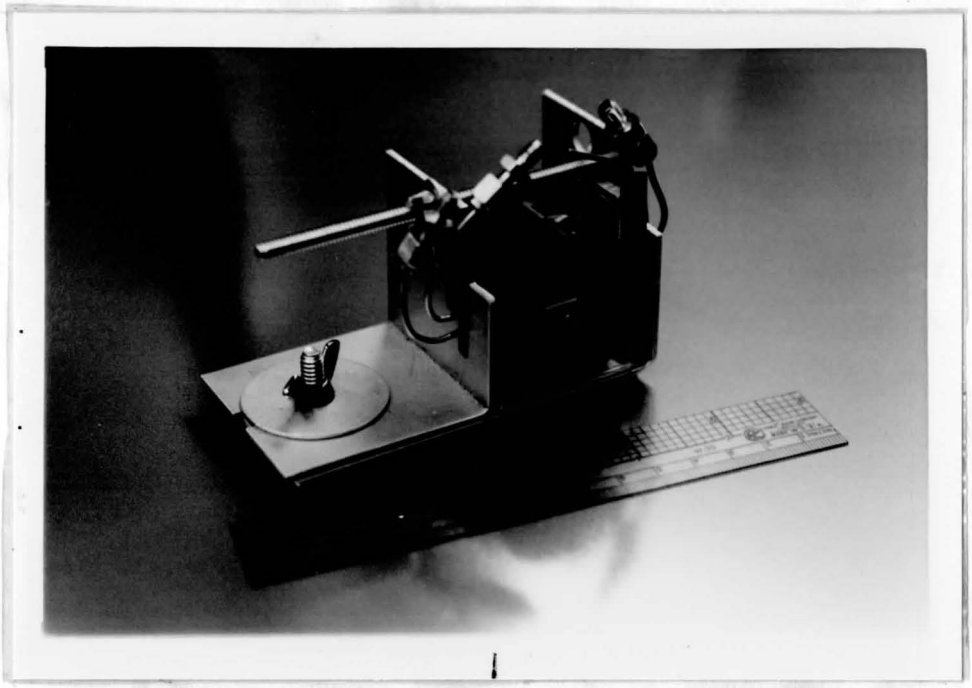


Fig. 3. -- The flow cell assembly(black cylinder) in its cradle. Note the alignment shims to the side and underneath the flow cell assembly.

Fig. 4. -- The flow cell assembly and cradle installed in the sphectrophotometer detector's sample compartment.

Fig. 5. -- The overall HPLC and Spectrophotometer arrangement. The HPLC is shown to the left and the spectrophotometer to the right. Note, the HPLC column shown in the upper right hand corner of the HPLC unit.

Materials

Pure samples of the following drugs and the pharmaceutical companies from which they were obtained are listed below:

Lidocaine hydrochloride -- Astra Inc.

Monoethylglycinexylidide hydrochloride -- Astra Inc.

Glycinexylidide hydrochloride -- Astra Inc.

Prilocaine -- Astra Inc.

Procaine hydrochloride -- Sigma Chemical Co.

Procainamide hydrochloride -- ICN - K and K Laboratories

Digoxin -- Zenith Laboratories Inc.

Atropine sulfate -- Lilly Research Laboratories

Isoproterenol sulfate -- Abbott Laboratories

Propranolol hydrochloride -- Ayerst Laboratories

Diazepam -- Hoffmann - LaRoche Inc.

Ethosuximide -- Parke, Davis and Company

Diphenhydramine -- Parke, Davis and Company

Diphenylhydantoin -- Parke, Davis and Company

Propoxyphene hydrochloride -- Lederle Laboratories

Hydrochlorothiazide -- Abbott Laboratories

Glutethimide -- USV Laboratories

All the standard laboratory reagents and solvents used in this study were reagent grade with the exception of acetonitrile which was of spectro grade from Eastman Chemical Company. All the water used to make the aqueous moving phases employed in this study was obtained from a Corning Mega-pure distillation apparatus.

CHAPTER III

EXPERIMENTAL

Resolution

For the initial trials in this study, the Applications Department at Waters Associates suggested an acetonitrile-water mixture, buffered in the range pH 3 - 5, as a moving phase in conjunction with a Waters μBondapak C-18 column stationary phase. This system is similar to that described by Adams et al.(27) for lidocaine by HPLC, whose paper appeared in Chemical Abstracts during the early stages of this project.

In an attempt to optimize the resolution of lidocaine, MEGX and GX from one another the various system parameters were investigated as follows.

Flow Rate

Trying the simplest possibility first the system was checked as to the effect on resolution of varing the moving phase flow rate. Figures 6 and 7 show the chromatograms of the same lidocaine, MEGX and GX solution chromatographed at a moving phase flow rate of 2.0 ml/min (Figure 6) and 3.0 ml/min (Figure 7) all other conditions being the same. As can be seen from these figures at the slower moving phase flow rate the elution times and band-width of the component peaks are increased as compared to the faster flow rate

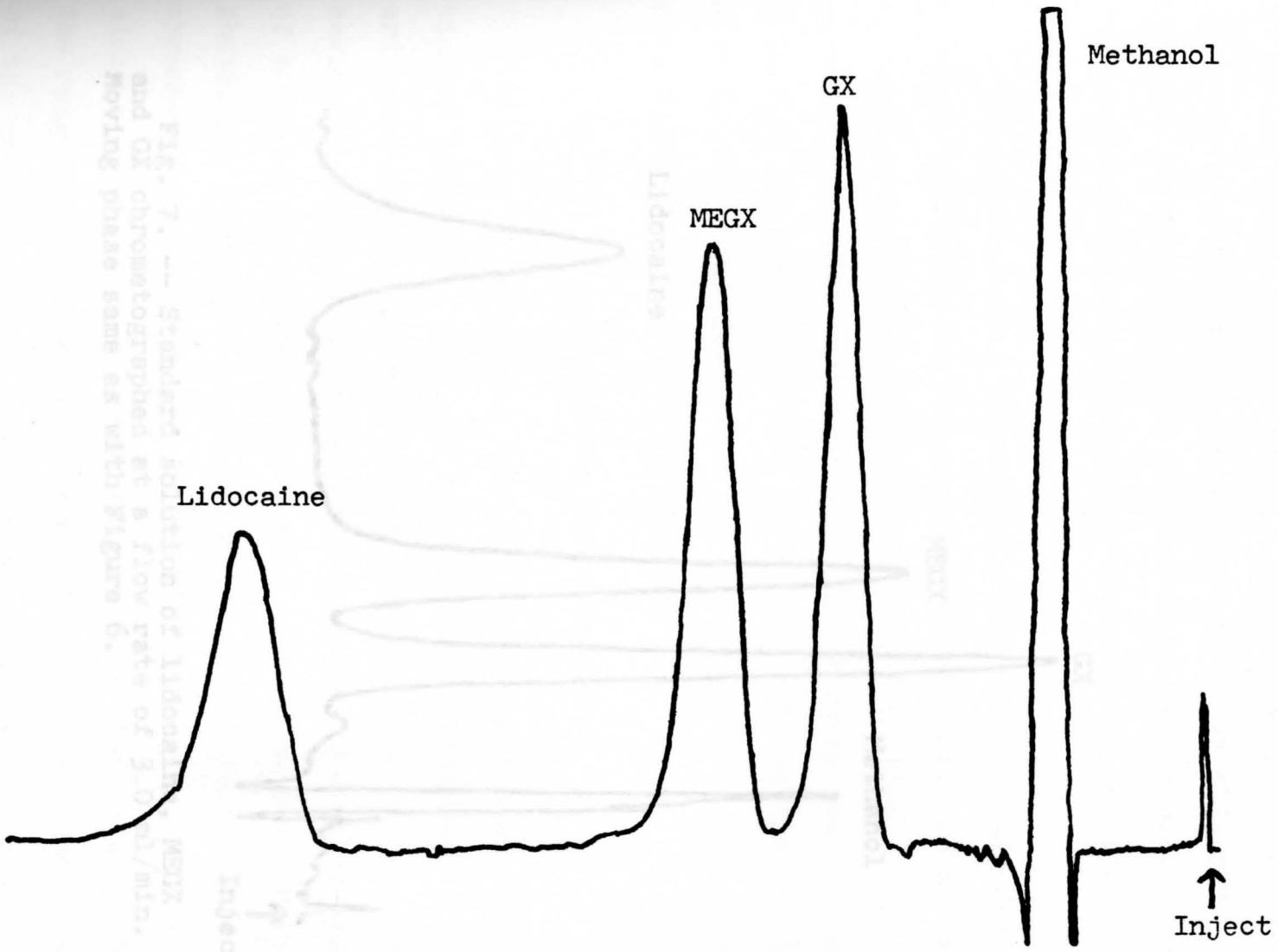


Fig. 6. -- Standard solution of lidocaine, MEGX and GX chromatographed at a flow rate of 2.0 ml/min (actual size). Moving phase; phosphate-buffered acetonitrile pH 6.1, acetonitrile to buffer volume ratio 1:9.

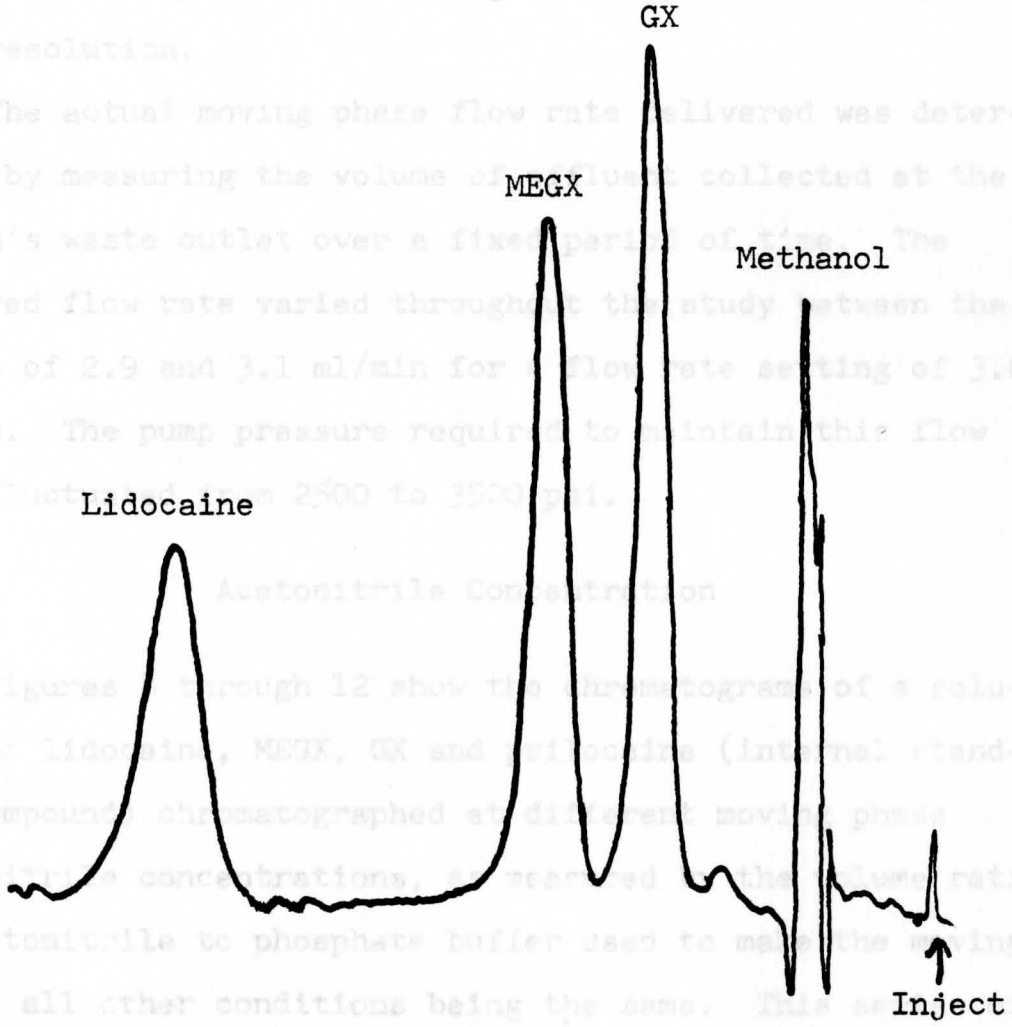


Fig. 7. -- Standard solution of lidocaine, MEGX and GX chromatographed at a flow rate of 3.0 ml/min. Moving phase same as with Figure 6.

chromatogram in such a way that the resolution of the peaks is not significantly improved. A flow rate of 3.0 ml/min was considered practical for use in this study as this flow rate provided conveniently fast elution times for lidocaine, MEGX and GX with only minimal tailing of the peaks and adequate peak resolution.

The actual moving phase flow rate delivered was determined by measuring the volume of effluent collected at the system's waste outlet over a fixed period of time. The measured flow rate varied throughout the study between the values of 2.9 and 3.1 ml/min for a flow rate setting of 3.0 ml/min. The pump pressure required to maintain this flow rate fluctuated from 2500 to 3500 psi.

Acetonitrile Concentration

Figures 8 through 12 show the chromatograms of a solution of lidocaine, MEGX, GX and prilocaine (internal standard compound) chromatographed at different moving phase acetonitrile concentrations, as measured by the volume ratio of acetonitrile to phosphate buffer used to make the moving phase, all other conditions being the same. This series of chromatograms confirms two facts. First, the order of peak elution is unaffected by the acetonitrile concentration over the range tested. Second, the elution times and band-widths of the chromatographic peaks increase with a decrease in acetonitrile concentration.

HPLC columns vary from one another in their efficiencies (i.e., effective number of theoretical plates). Adjusting the

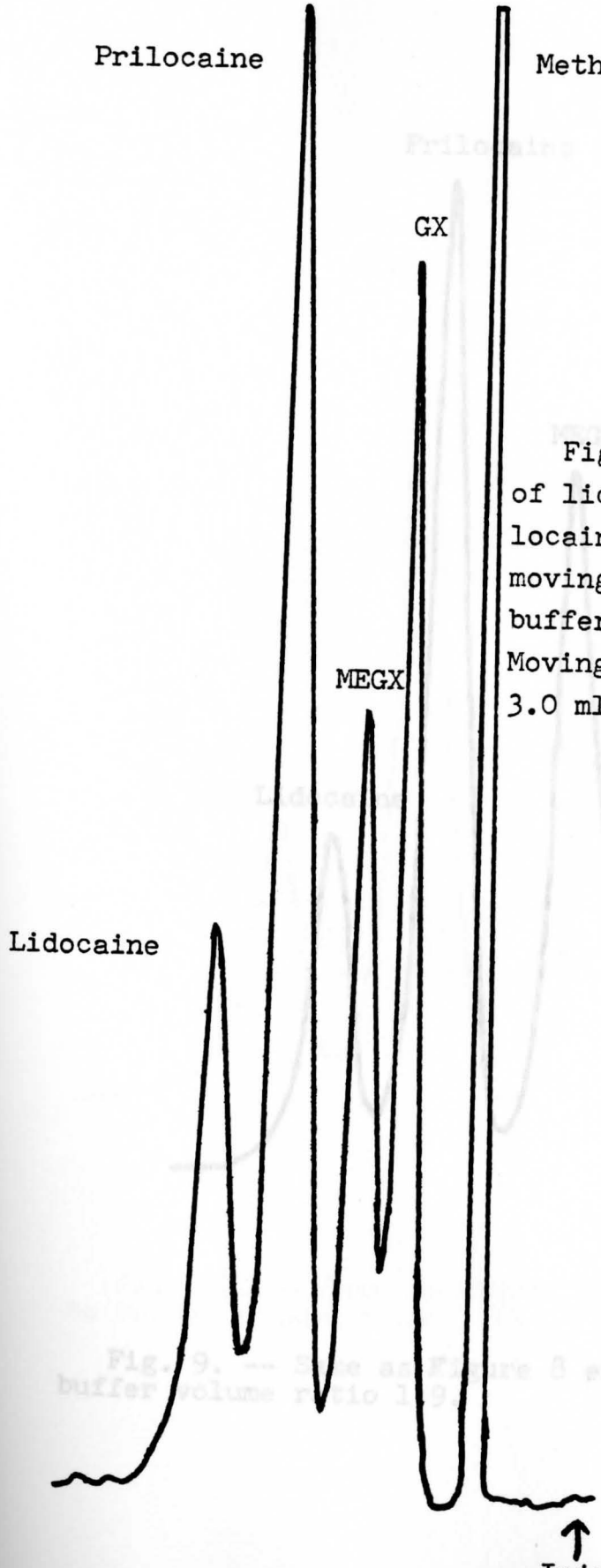


Fig. 8. -- Standard solution of lidocaine, MEGX, GX and prilocaine chromatographed with a moving phase acetonitrile to buffer volume ratio of 1:6. Moving phase pH 6.1, flow rate 3.0 ml/min.

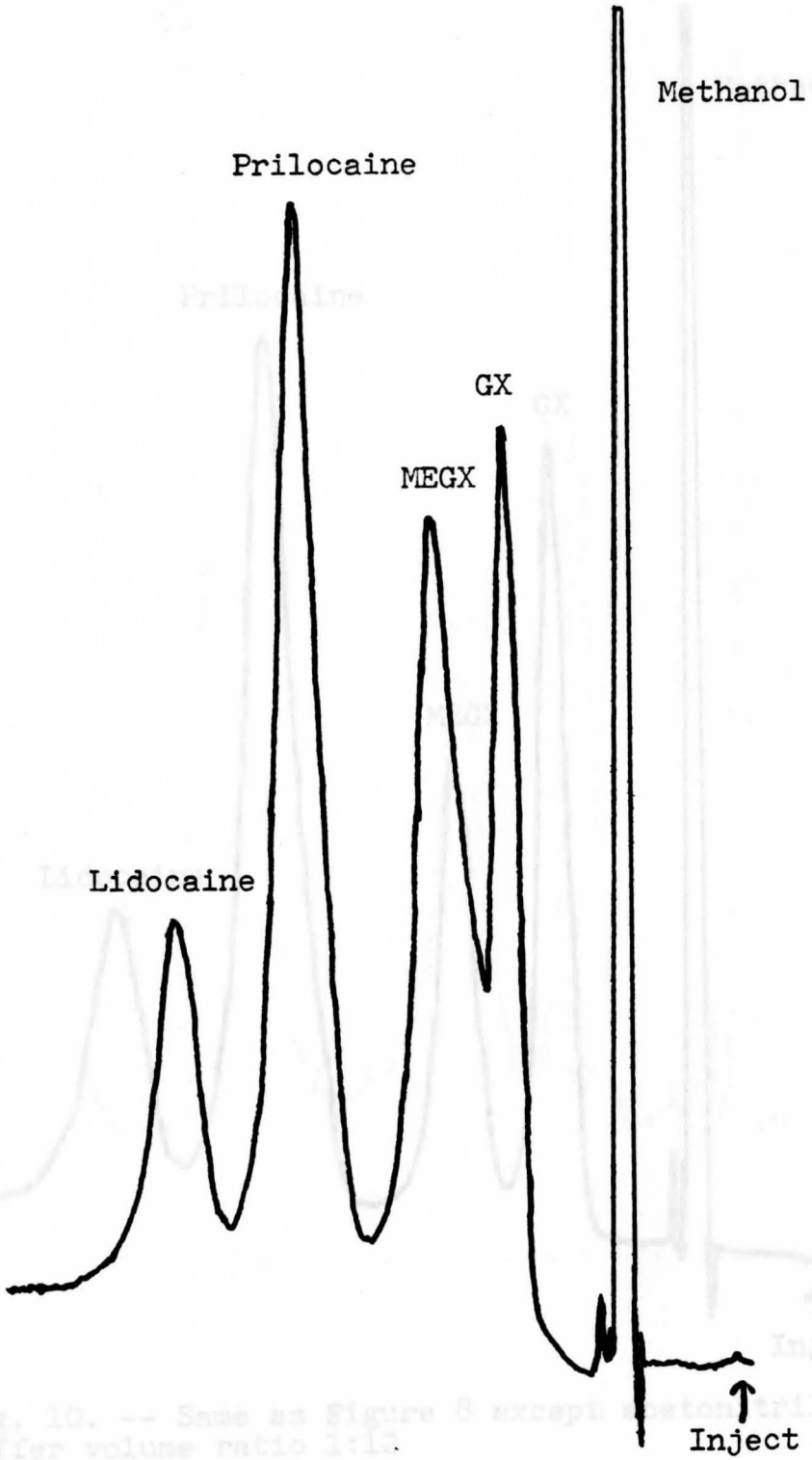


Fig. 9. -- Same as Figure 8 except acetonitrile to buffer volume ratio 1:9.

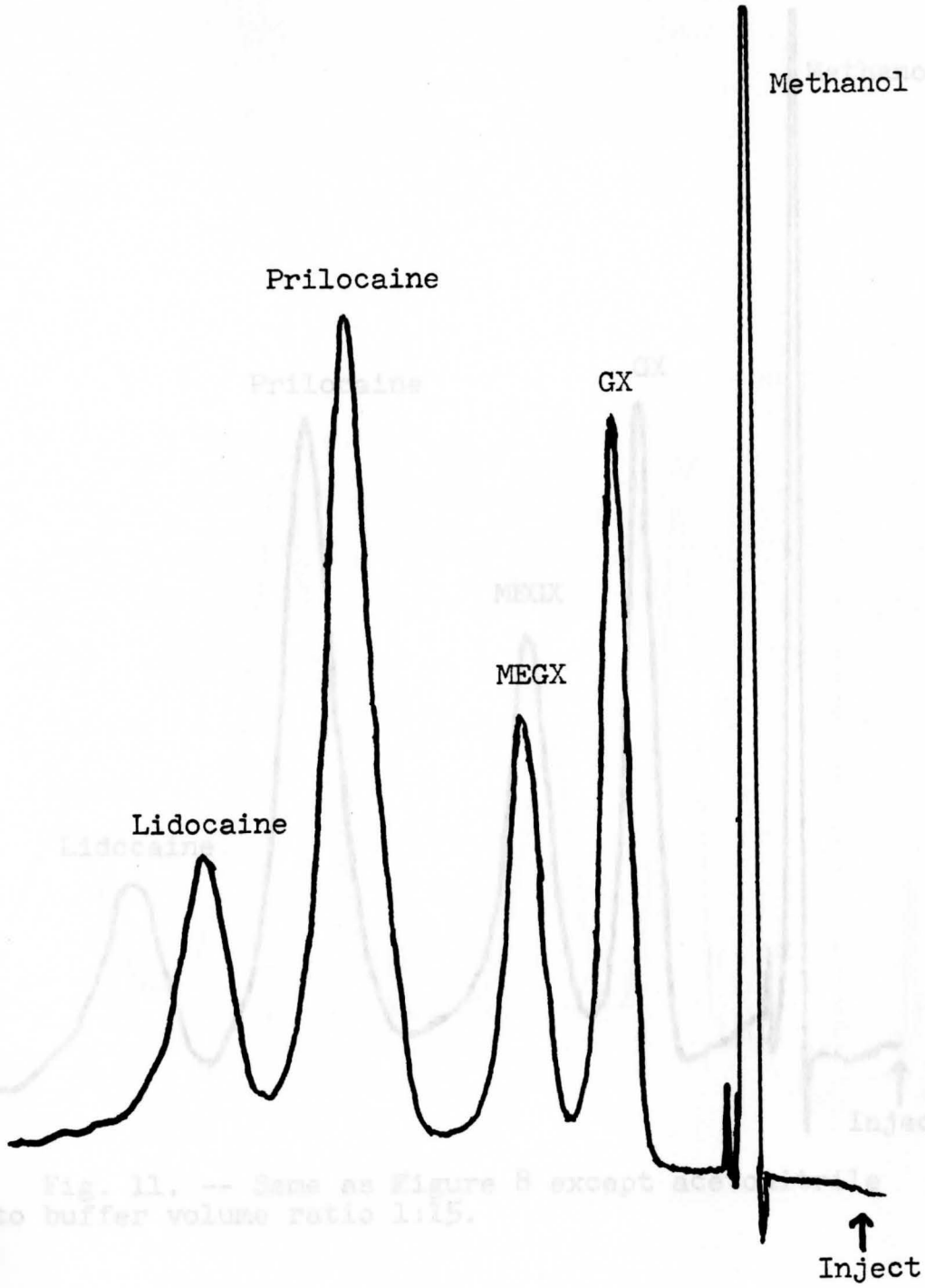


Fig. 10. -- Same as Figure 8 except acetonitrile to buffer volume ratio 1:12

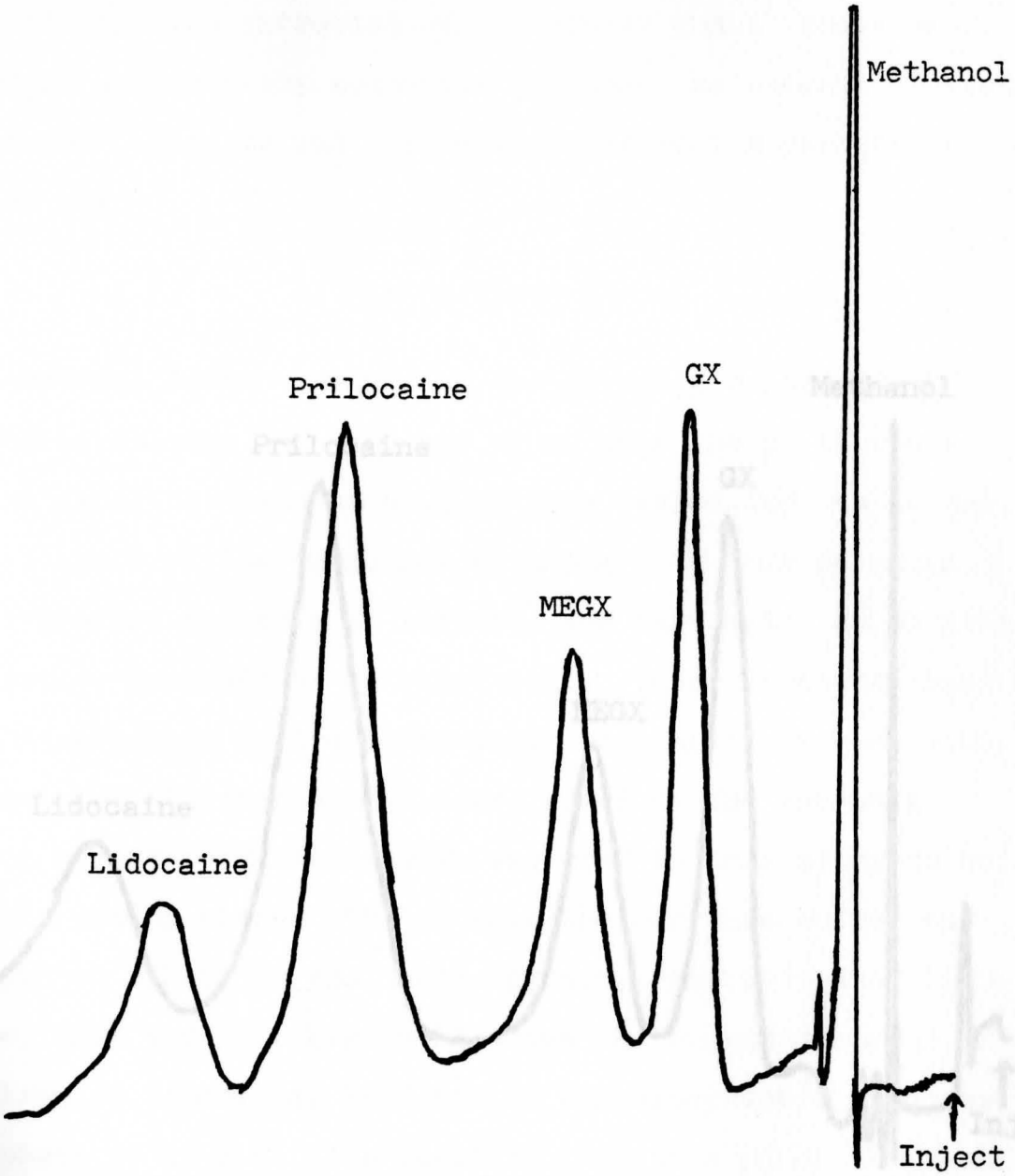


Fig. 11. -- Same as Figure 8 except acetonitrile to buffer volume ratio 1:15.

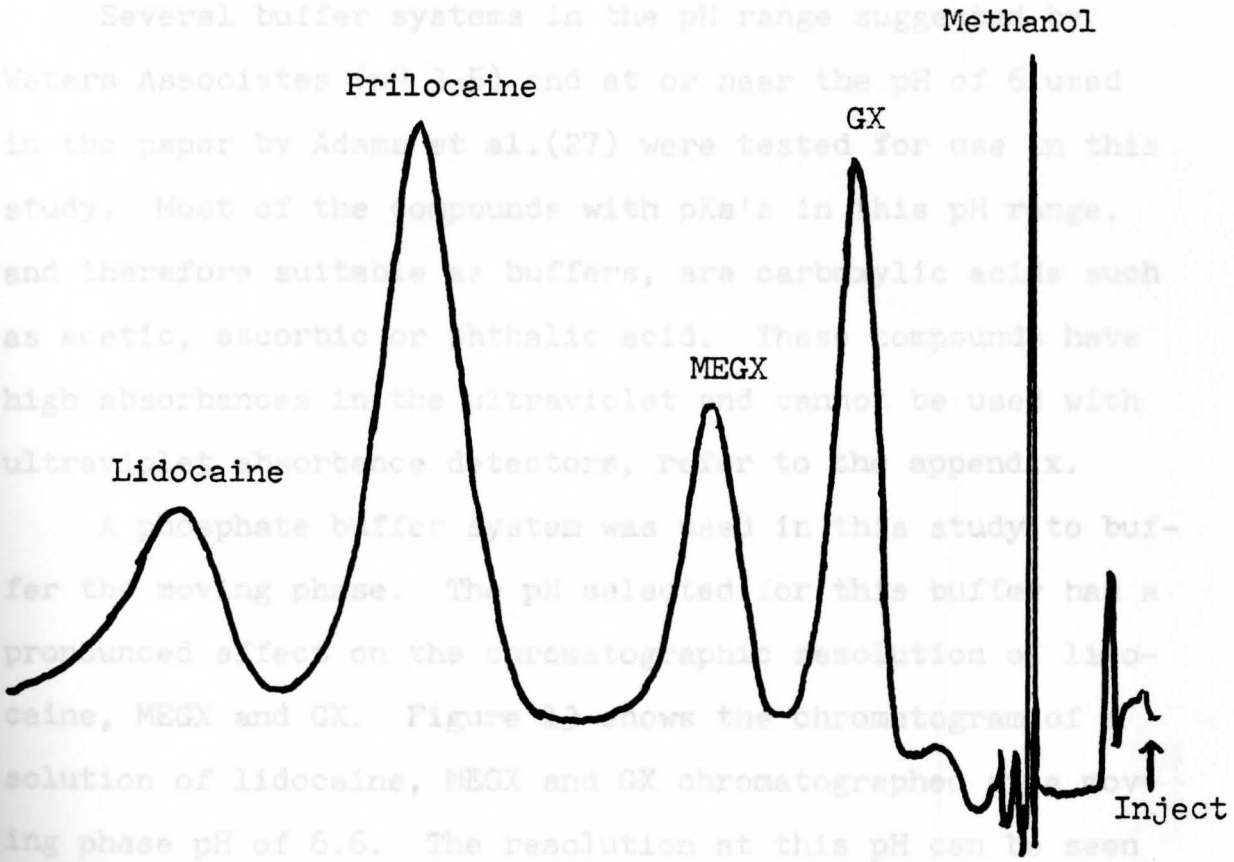


Fig. 12. -- Same as Figure 8 except acetonitrile to buffer volume ratio 1:18.

acetonitrile concentration of the moving phase, therefore, provides a convenient means for adopting the overall elution times for lidocaine and its metabolites to the particular column used.

Moving Phase pH

Several buffer systems in the pH range suggested by Waters Associates (pH 3-5) and at or near the pH of 6 used in the paper by Adams et al.(27) were tested for use in this study. Most of the compounds with pKa's in this pH range, and therefore suitable as buffers, are carboxylic acids such as acetic, ascorbic or phthalic acid. These compounds have high absorbances in the ultraviolet and cannot be used with ultraviolet absorbance detectors, refer to the appendix.

A phosphate buffer system was used in this study to buffer the moving phase. The pH selected for this buffer had a pronounced effect on the chromatographic resolution of lidocaine, MEGX and GX. Figure 13 shows the chromatogram of a solution of lidocaine, MEGX and GX chromatographed at a moving phase pH of 6.6. The resolution at this pH can be seen to be greater than the resolution for the chromatogram shown in Figure 7 which was chromatographed at a pH of 6.1. The flow rate was 3.0 ml/min and the acetonitrile to buffer ratio was 1:9 for both chromatograms.

Although increasing the pH of the moving phase improved the resolution of lidocaine, MEGX and GX this advantage was offset by the fact that the absorbance of the phosphate buffer of the moving phase was also greater, thereby reducing

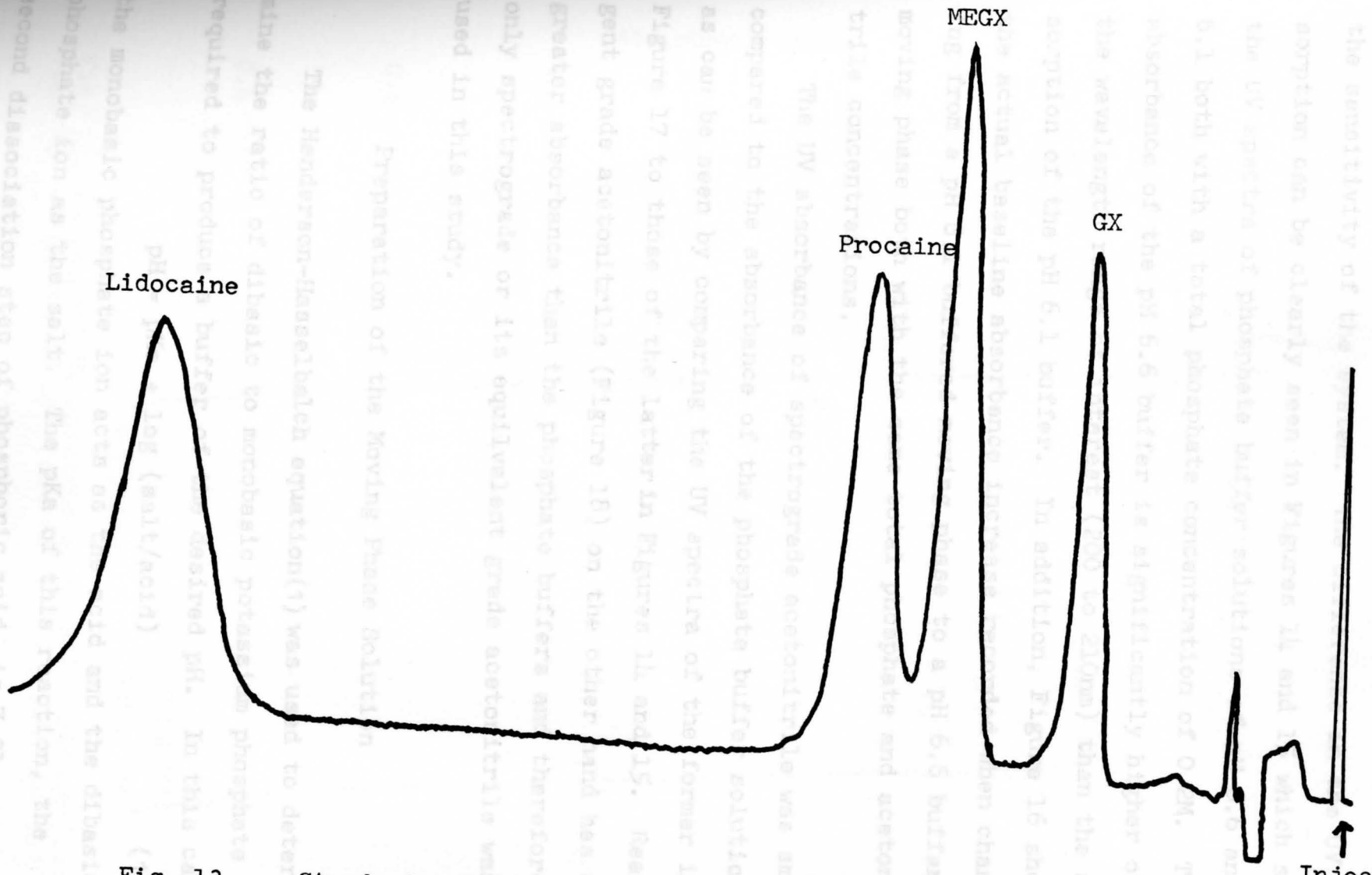


Fig. 13. -- Standard solution of lidocaine, MEGX, GX and procaine chromatographed at a moving phase pH at 6.6. Moving phase acetonitrile to buffer ratio 1:9, flow rate 3.0 ml/min.

the sensitivity of the system. The difference in the UV absorption can be clearly seen in Figures 14 and 15 which show the UV spectra of phosphate buffer solutions of pH 6.6 and 6.1 both with a total phosphate concentration of 0.2M. The absorbance of the pH 6.6 buffer is significantly higher over the wavelength range of interest (200 to 210nm) than the absorption of the pH 6.1 buffer. In addition, Figure 16 shows the actual baseline absorbance increase recorded when changing from a pH 6.1 buffered moving phase to a pH 6.6 buffered moving phase both with the same total phosphate and acetonitrile concentrations.

The UV absorbance of spectrograde acetonitrile was small compared to the absorbance of the phosphate buffer solutions as can be seen by comparing the UV spectra of the former in Figure 17 to those of the latter in Figures 14 and 15. Reagent grade acetonitrile (Figure 18) on the other hand has a greater absorbance than the phosphate buffers and therefore only spectrograde or its equilvalent grade acetonitrile was used in this study.

Preparation of the Moving Phase Solution

The Henderson-Hasselbalch equation(1) was used to determine the ratio of dibasic to monobasic potassium phosphate required to produce a buffer of the desired pH. In this case

$$pH = pKa + \log (salt/acid) \qquad (1)$$

the monobasic phosphate ion acts as the acid and the dibasic phosphate ion as the salt. The pKa of this reaction, the second dissociation step of phosphoric acid, is 7.21.

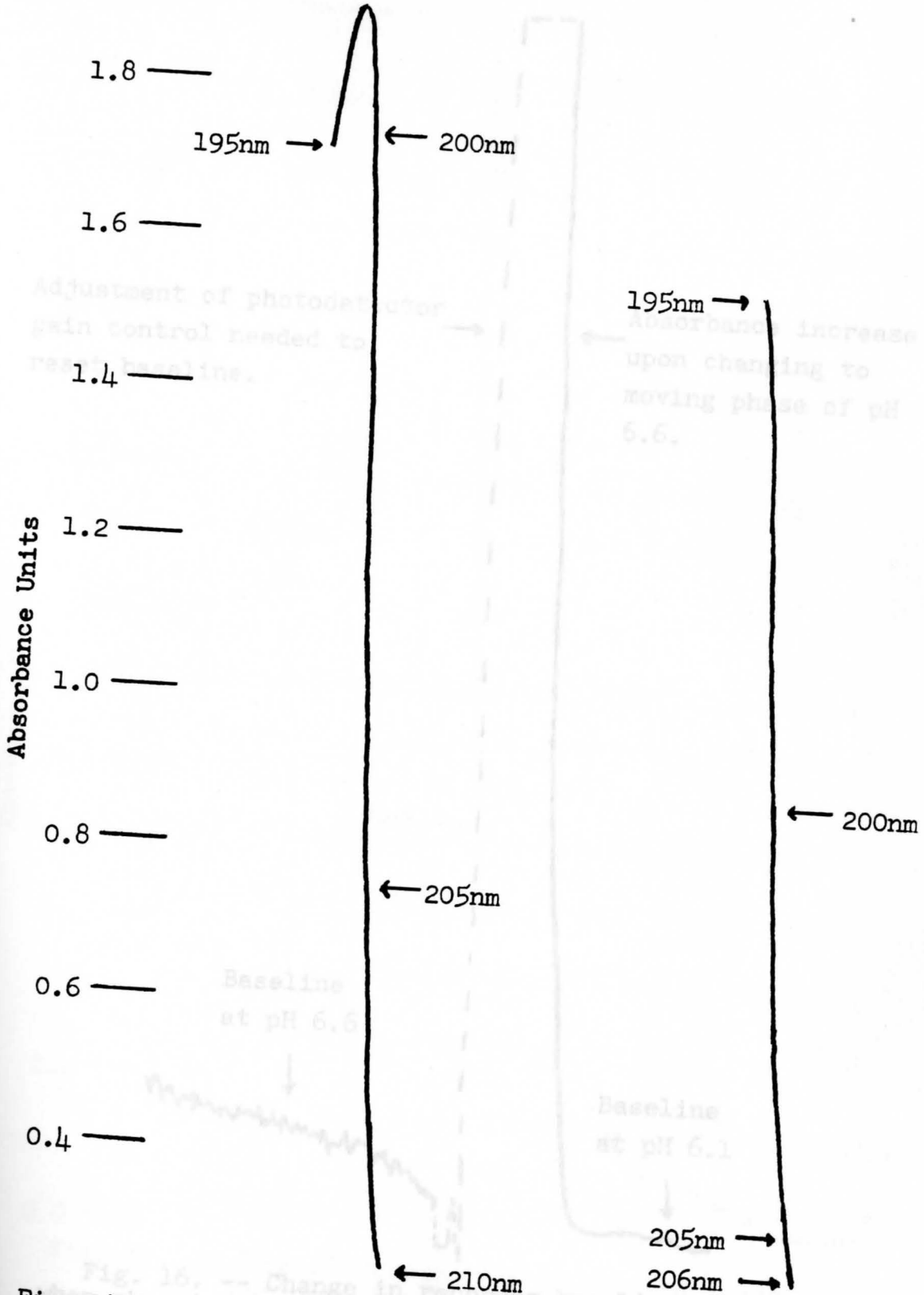


Fig. 14. -- (Right) UV spectrum of pH 6.1 phosphate buffer
Fig. 15. -- (Left) UV spectrum of pH 6.6 phosphate buffer

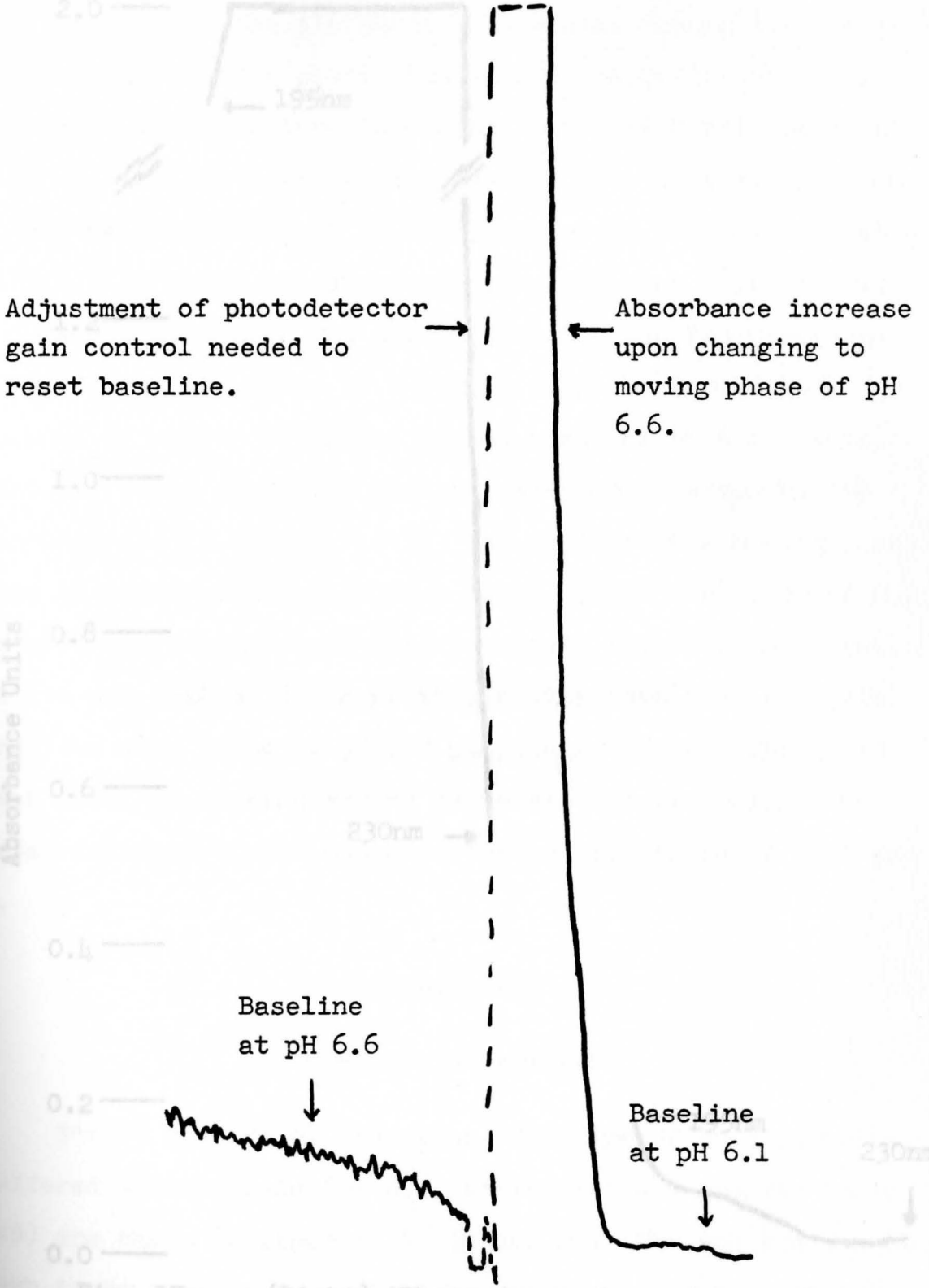
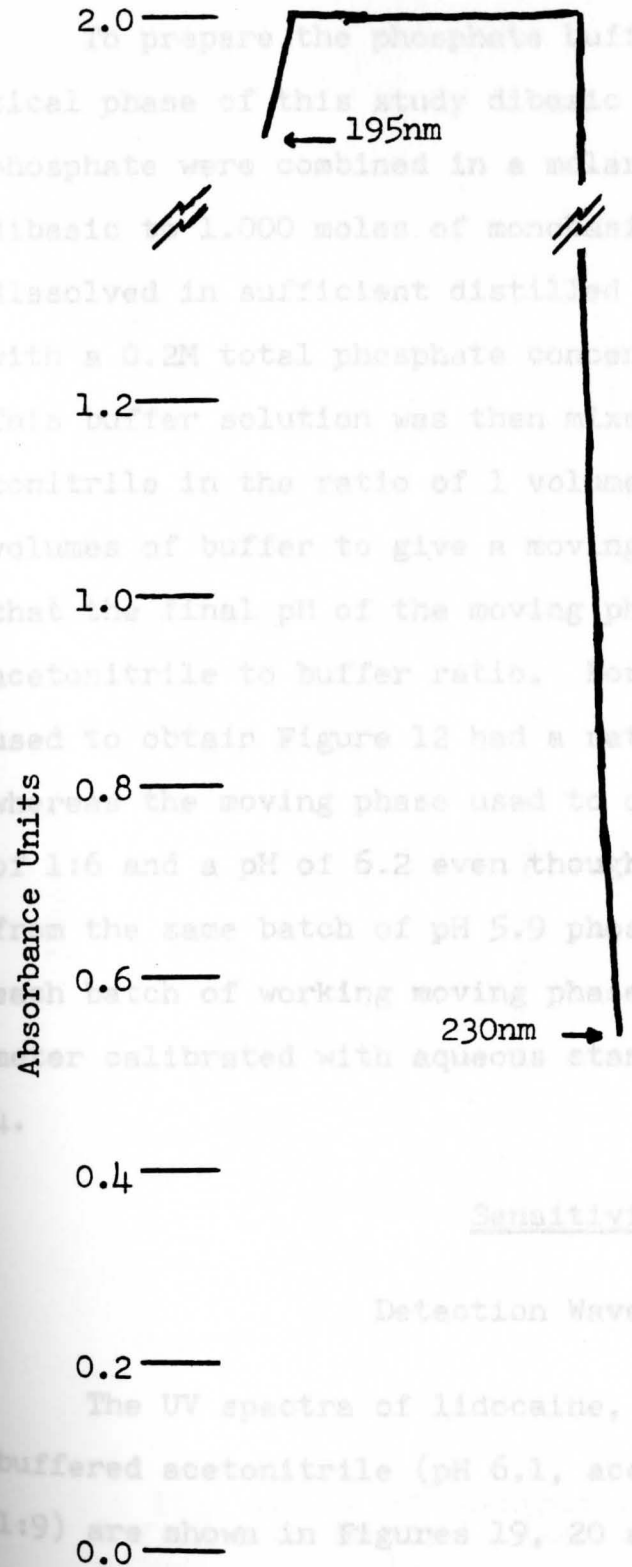


Fig. 16. -- Change in recorder baseline absorbance when changing from and moving phase buffered at pH 6.1 to a moving phase buffered at pH 6.6. Detection wavelength 205 nm.



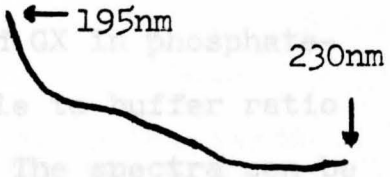


Fig. 17. -- (Right) UV spectrum of spectro grade acetonitrile
Fig. 18. -- (Left) UV spectrum of reagent grade acetonitrile

To prepare the phosphate buffer used during the analytical phase of this study dibasic and monobasic potassium phosphate were combined in a molar ratio of 0.0617 moles of dibasic to 1.000 moles of monobasic. This mixture was then dissolved in sufficient distilled water to produce a solution with a 0.2M total phosphate concentration and a pH of 5.9. This buffer solution was then mixed with spectrograde acetonitrile in the ratio of 1 volume of acetonitrile to 9 volumes of buffer to give a moving phase of pH 6.1. Note, that the final pH of the moving phase was affected by the acetonitrile to buffer ratio. For instance the moving phase used to obtain Figure 12 had a ratio of 1:18 and a pH of 6.0 whereas the moving phase used to obtain Figure 8 had a ratio of 1:6 and a pH of 6.2 even though they both had been made from the same batch of pH 5.9 phosphate buffer. The pH of each batch of working moving phase was checked using a pH meter calibrated with aqueous standard solutions of pH 7 and 4.

Sensitivity

Detection Wavelength

The UV spectra of lidocaine, MEGX and GX in phosphate-buffered acetonitrile (pH 6.1, acetonitrile to buffer ratio 1:9) are shown in Figures 19, 20 and 21. The spectra can be seen to be similar for all three substances with an absorption maximum in each case at about 200nm. This wavelength would be the obvious choice as the detection wavelength for

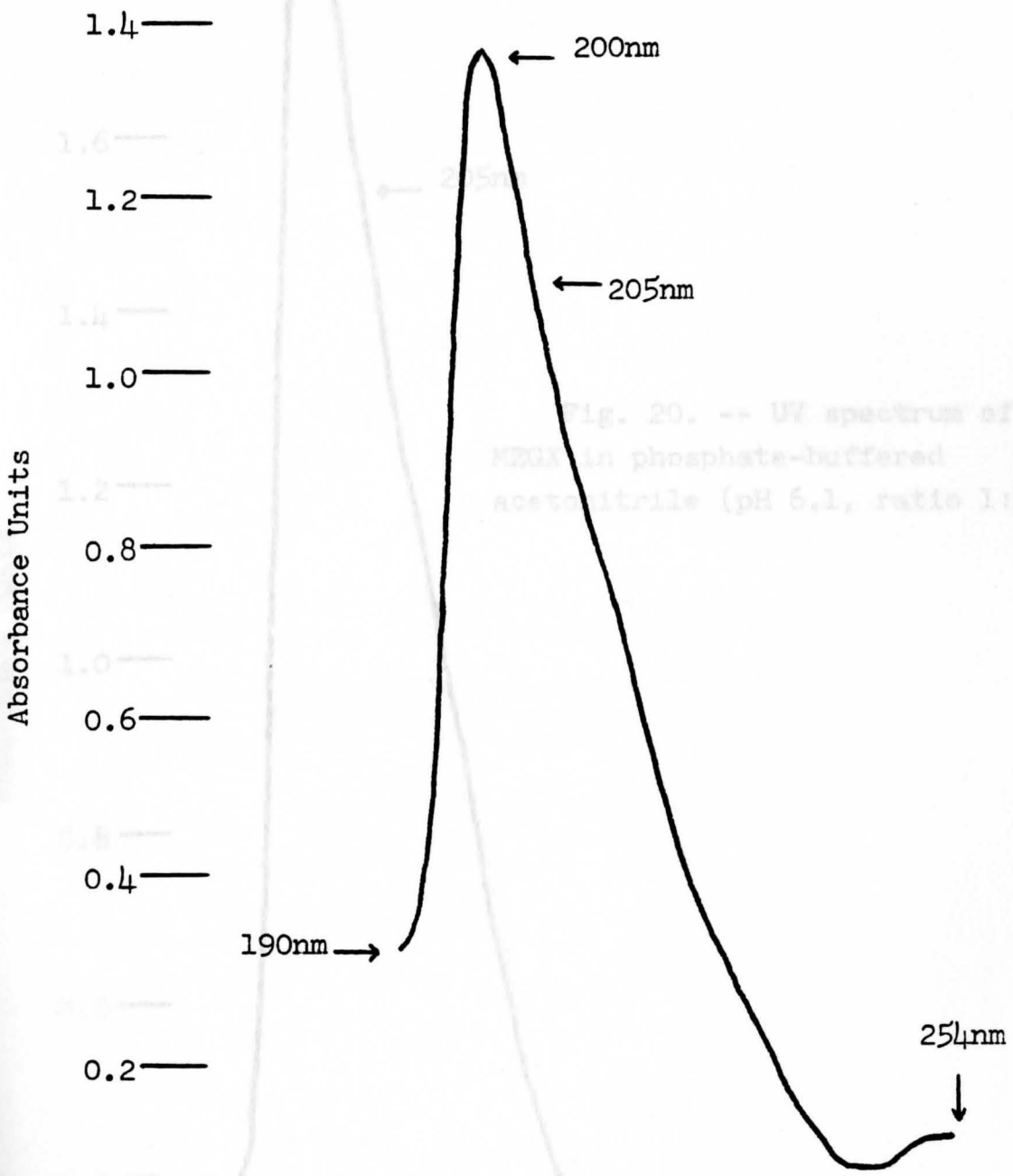


Fig. 19. -- UV spectrum of lidocaine in phosphate-buffered acetonitrile (pH 6.1, acetonitrile to buffer volume ratio 1:9).

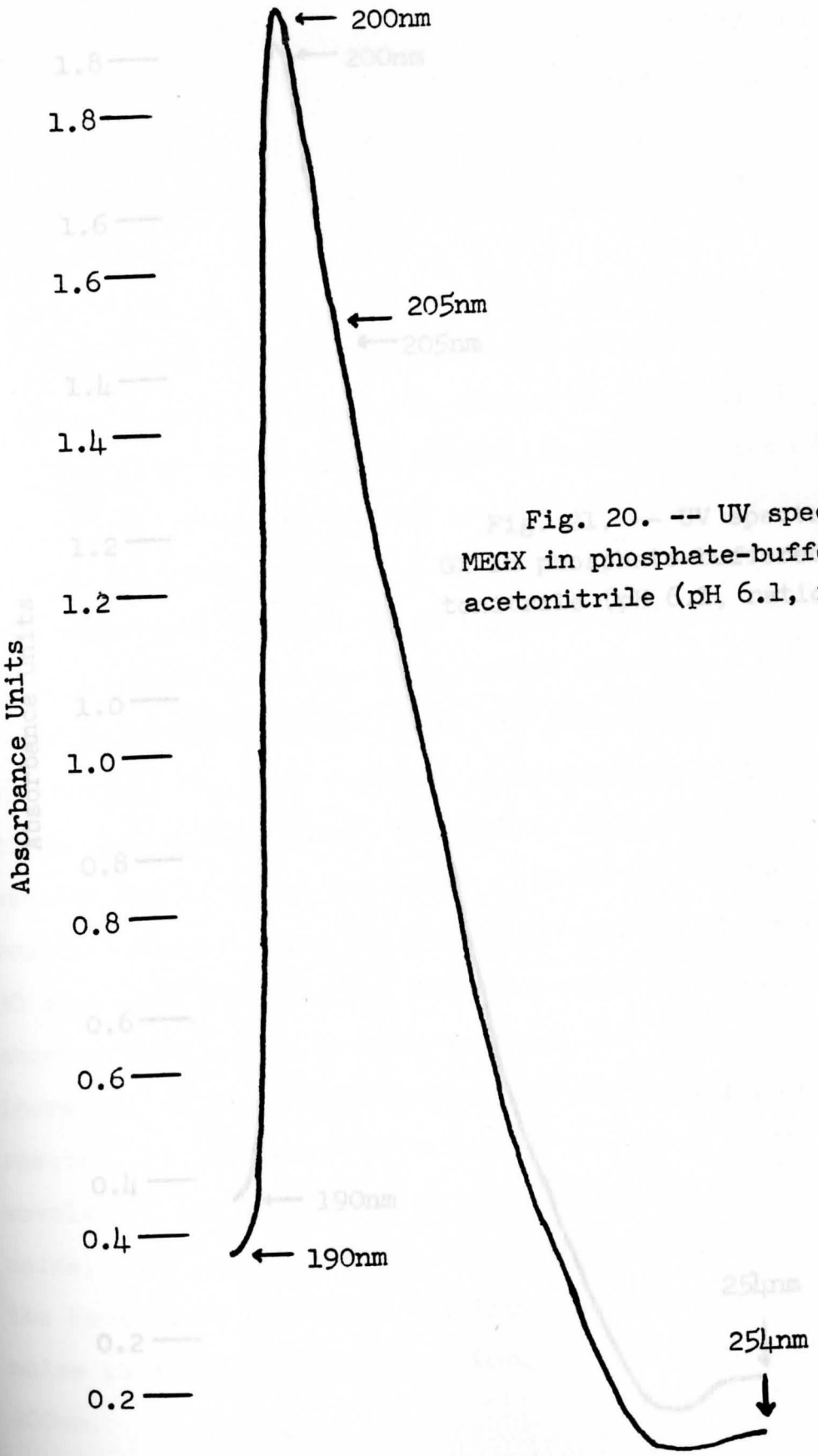


Fig. 20. -- UV spectrum of MEGX in phosphate-buffered acetonitrile (pH 6.1, ratio 1:9).

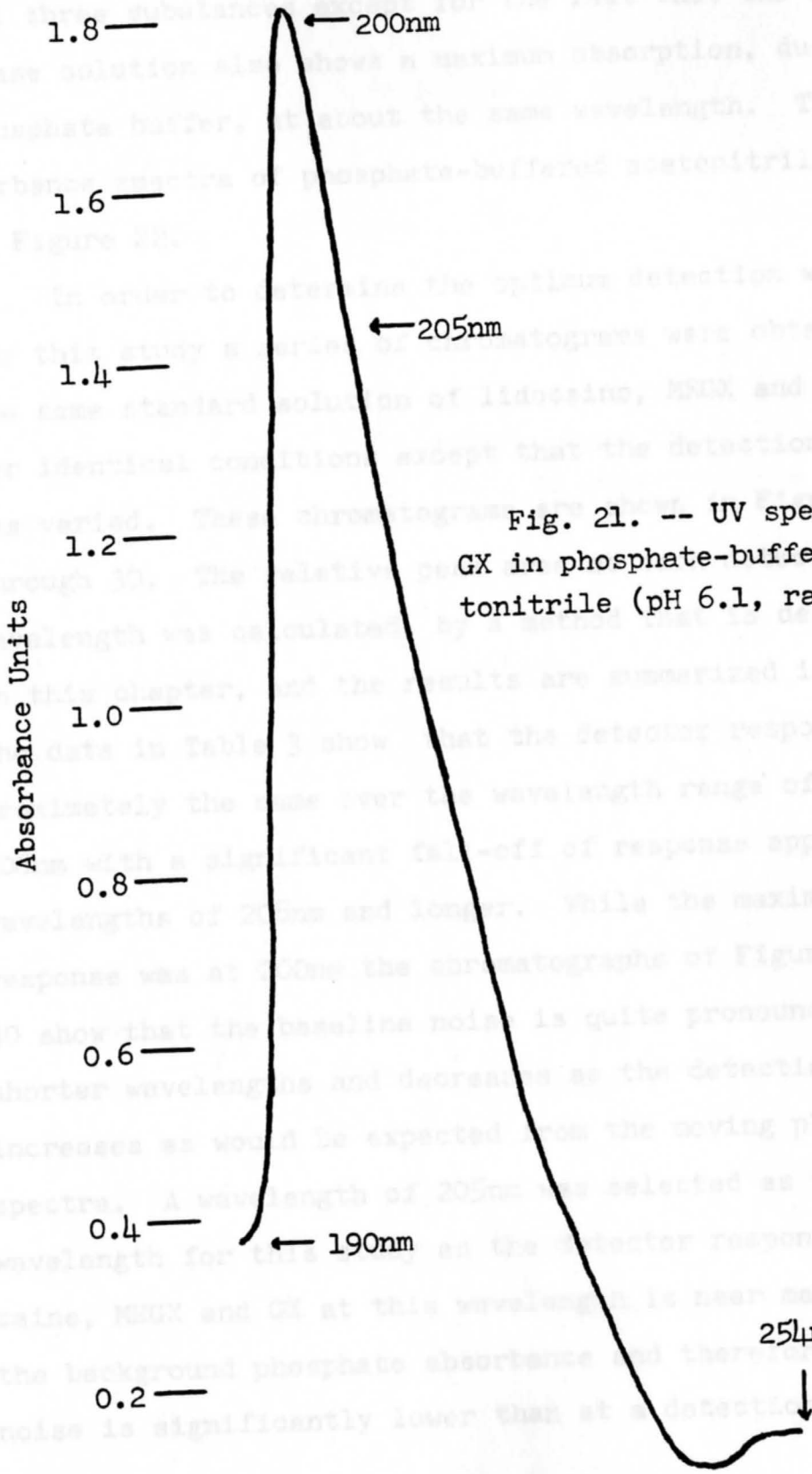


Fig. 21. -- UV spectrum of GX in phosphate-buffered acetonitrile (pH 6.1, ratio 1:9).

all three substances except for the fact that the moving phase solution also shows a maximum absorption, due to the phosphate buffer, at about the same wavelength. The UV absorbance spectra of phosphate-buffered acetonitrile is shown in Figure 22.

In order to determine the optimum detection wavelength for this study a series of chromatograms were obtained for the same standard solution of lidocaine, MEGX and GX run under identical conditions except that the detection wavelength was varied. These chromatograms are shown in Figures 23 through 30. The relative peak area at each detection wavelength was calculated, by a method that is described later in this chapter, and the results are summarized in Table 3. The data in Table 3 show that the detector response was approximately the same over the wavelength range of from 200 to 206nm with a significant fall-off of response apparent at wavelengths of 208nm and longer. While the maximal detector response was at 200nm the chromatographs of Figures 23 through 30 show that the baseline noise is quite pronounced at the shorter wavelengths and decreases as the detection wavelength increases as would be expected from the moving phase's UV spectra. A wavelength of 205nm was selected as the detection wavelength for this study as the detector response for lidocaine, MEGX and GX at this wavelength is near maximum while the background phosphate absorbance and therefore the baseline noise is significantly lower than at a detection wavelength of 200nm.

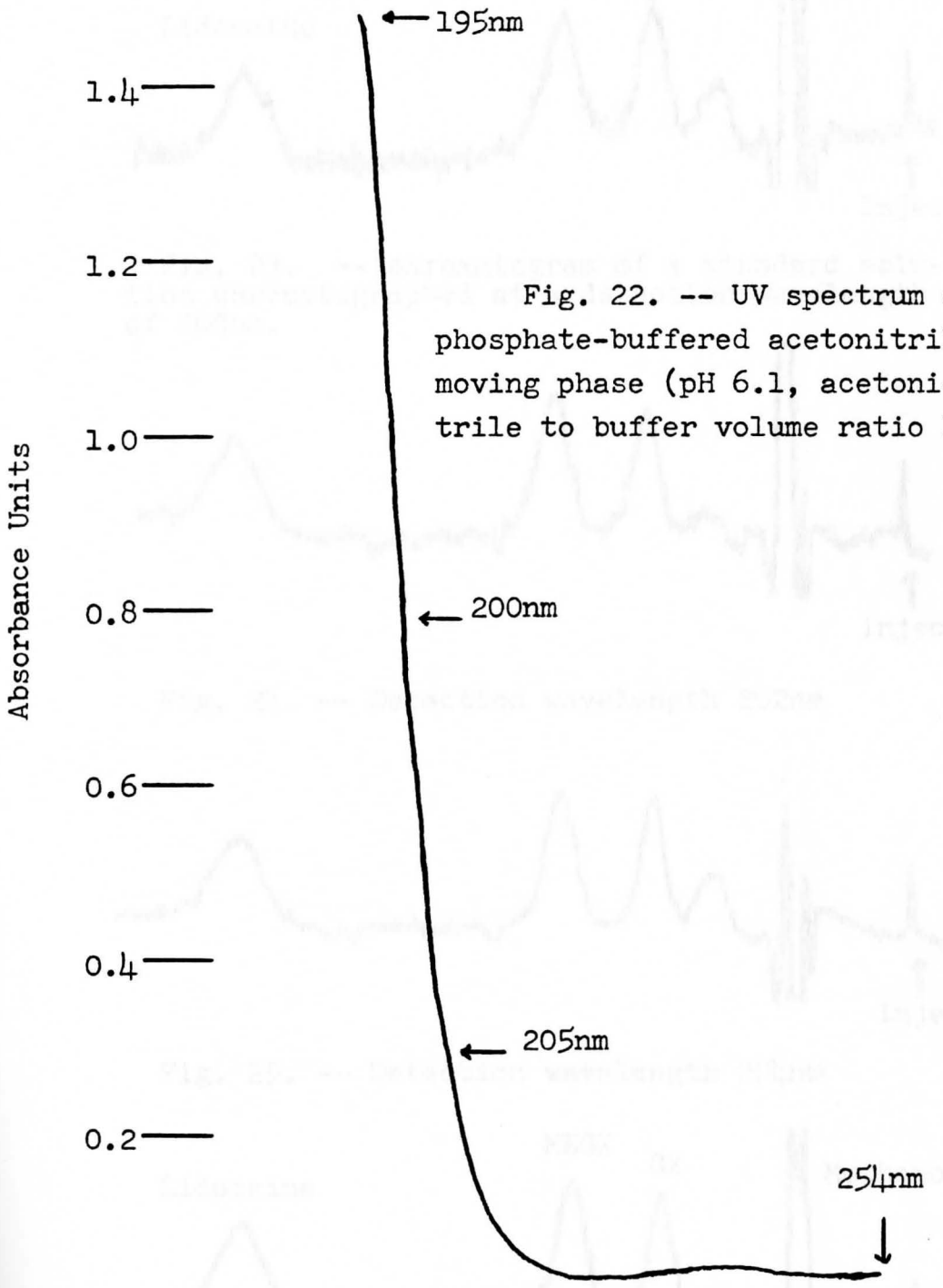


Fig. 22. -- UV spectrum of phosphate-buffered acetonitrile moving phase (pH 6.1, acetonitrile to buffer volume ratio 1:9).

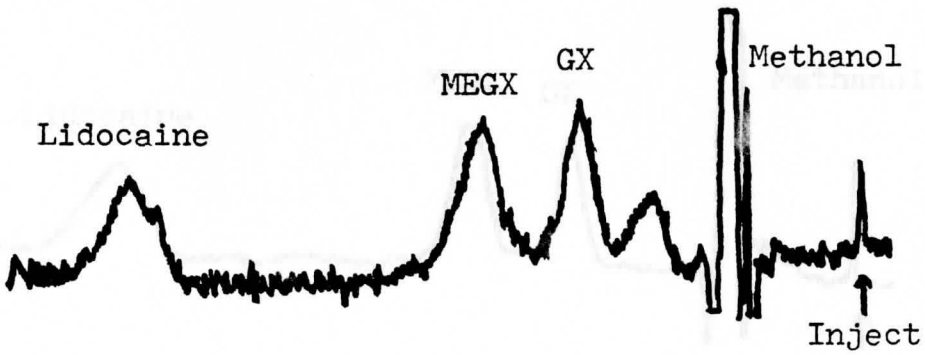


Fig. 23. -- chromatogram of a standard solution chromatographed at a detection wavelength of 200nm.

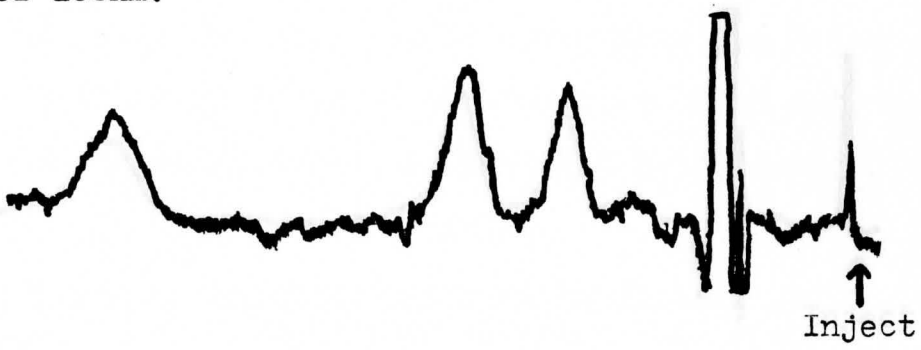


Fig. 24. -- Detection wavelength 202nm

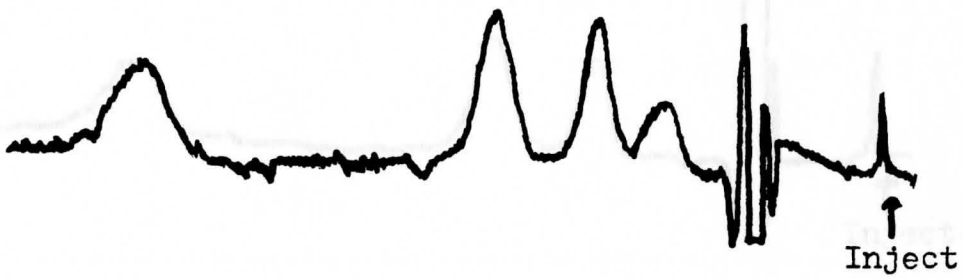


Fig. 25. -- Detection wavelength 204nm

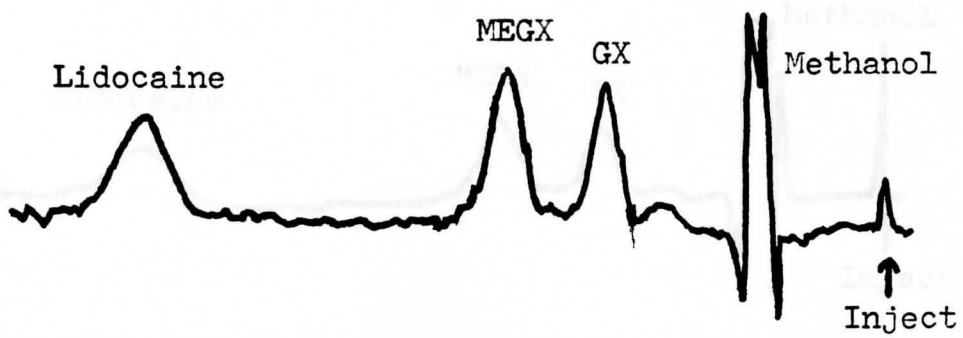


Fig. 26. -- Detection wavelength 205nm

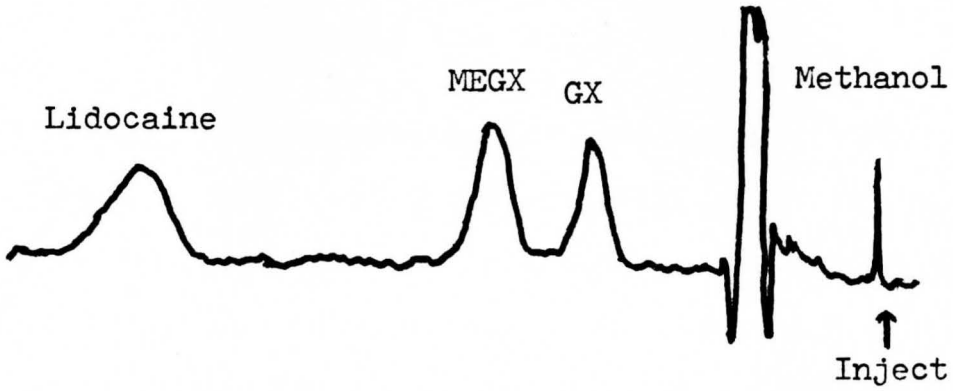


Fig. 27 -- Detection wavelength 206nm

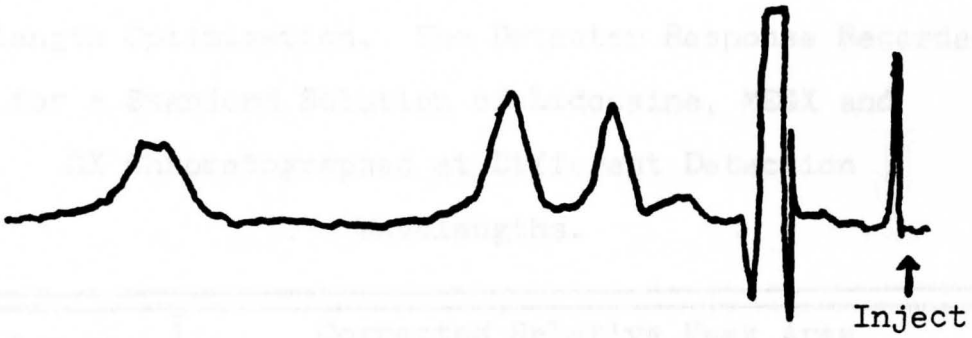


Fig. 28. -- Detection wavelength 208nm

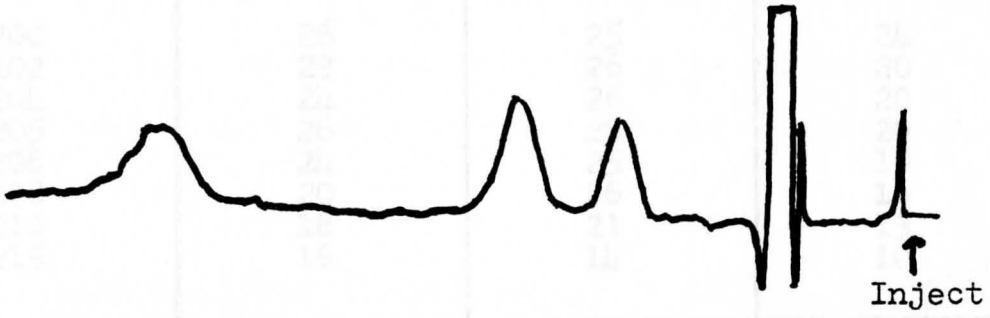


Fig. 29. -- Detection wavelength 210nm

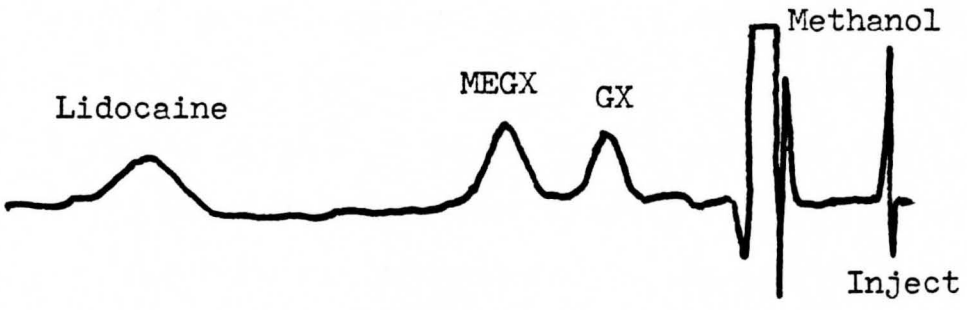


Fig. 30. -- Detection wavelength 215nm

TABLE 3

Wavelength Optimization. The Detector Response Recorded for a Standard Solution of Lidocaine, MEGX and GX Chromatographed at Different Detection Wavelengths.

Wavelength (nm)	Corrected Relative Peak Area		
	Lidocaine	MEGX	GX
200	26	25	24
202	29	26	20
204	24	26	20
205	26	26	20
206	24	25	18
208	20	26	16
210	18	21	13
215	16	14	10

Detector Slit Width

The Beckman 26 spectrophotometer used as the detector in this study is capable of being manually set at any slit width of from 0.0 to 2.0mm. To determine the effect of the slit width setting on the sensitivity and resolution capabilities of the detection a standard solution of MEGX and the drug procaine was chromatographed under similar conditions except that the slit width was set at 0.8mm in one case(Figure 31) and at 2.0mm in the other(Figure 32). As can be seen from these chromatograms, at the narrower slit width setting the baseline is noisier, presumably because there is less light reaching the photodetector thus requiring a higher gain setting. Also apparent from these chromatograms is that the resolution is not noticably better than with the slit width wider setting. For these reasons, therefore, the maximum slit width setting of 2.0mm was used during most of this study.

Shifts in the Elution Characteristics of the System

Three times during this study the absolute elution times for all compounds chromatographed showed an abrupt decrease. The relative elution pattern, it should be noted, was unaffected. The first and largest of these shifts occured, early in the study, after the system had been flushed with a 0.2% solution of sodium azide as was recommended in the operation manual supplied by Waters Associates. The elution characteristics after this first shift remained constant until the system was again flushed with 0.2% sodium azide which resulted in a sec-

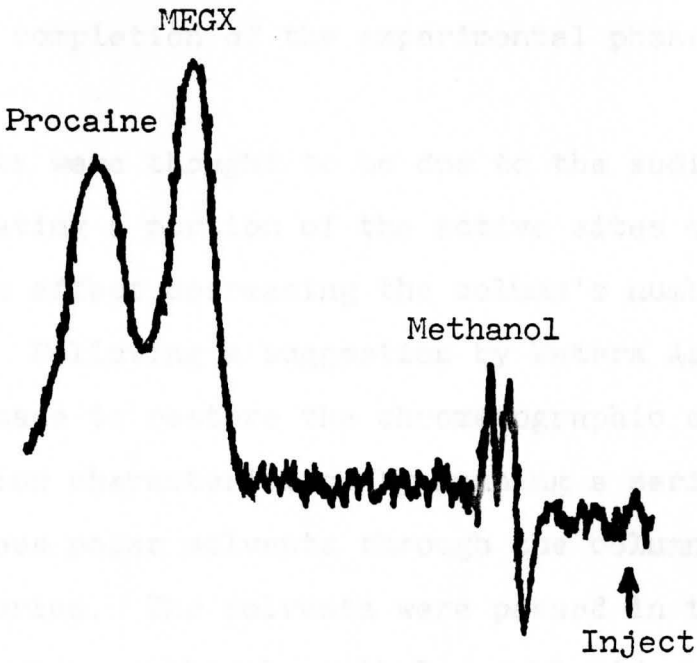


Fig. 31. -- Standard solution of procaine and MEGX chromatographed at a detector slit width of 0.8mm.

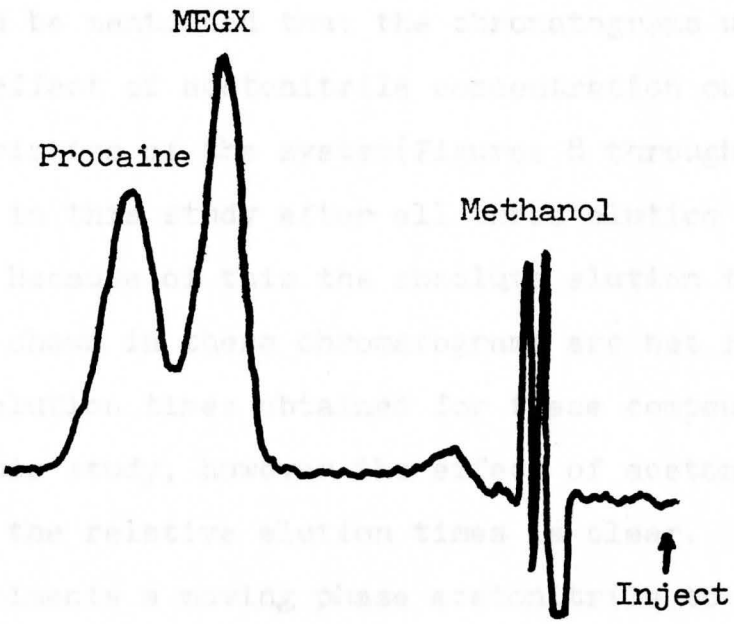


Fig. 32. -- Standard solution of procaine and MEGX chromatographed at a detector slit width of 2.0mm. Moving phase composition and flow rate the same as in Figure 31.

ond smaller shift in the elution times. A third shift was observed near the completion of the experimental phase of this study.

These shifts were thought to be due to the sodium azide somehow deactivating a portion of the active sites of the C-18 column, thus, in effect decreasing the column's number of theoretical plate. Following a suggestion by Waters Associates an attempt was made to restore the chromatographic column to its former elution characteristics by running a series of progressively less polar solvents through the column and then reversing the series. The solvents were passed in the following sequence: water, methanol, methylene chloride and hexane. Approximately 20 ml of each solvent was passed at a flow rate of 3.0 ml/min. This treatment unfortunately had no effect on the column elution characteristics.

It should be mentioned that the chromatograms used to illustrate the effect of acetonitrile concentration on the elution characteristics of the system(Figures 8 through 12) were obtained late in this study after all three elution time shifts had occured. Because of this the absolute elution times for the compounds shown in these chromatograms are not representative of the elution times obtained for these compounds throughout most of this study, however the effect of acetonitrile concentration on the relative elution times is clear. From preliminary experiments a moving phase acetonitrile to buffer volume ratio of 1:9 was selected for routine use in this study. Figure 33 shows a chromatogram with elution times for lidocaine,

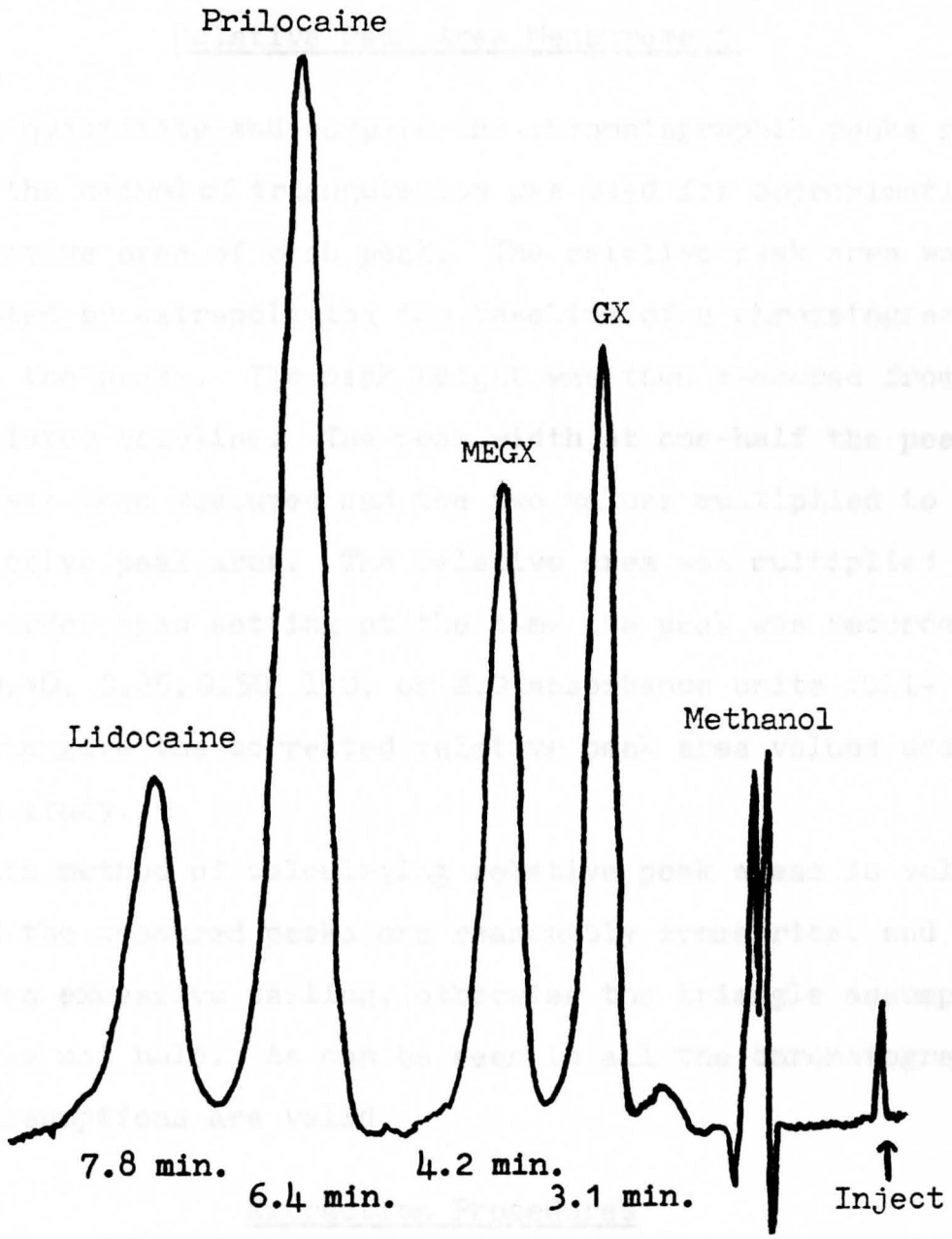


Fig. 33. -- Chromatogram with elution times for lidocaine, MEGX, GX and prilocaine as obtained throughout most of this study. Moving phase pH 6.1, acetonitrile to buffer volume ratio 1:9 and flow rate of 3.0 ml/min.

MEGX, GX and prilocaine which are typical of those obtained throughout most of this study.

Relative Peak Area Measurement

To quantitate and compare the chromatographic peaks recorded the method of triangulation was used for approximating the relative area of each peak. The relative peak area was calculated by extrapolating the baseline of a chromatogram through the peaks. The peak height was then measured from the extrapolated baseline. The peak width at one-half the peak height was then measured and the two values multiplied to give the relative peak area. The relative area was multiplied by the recorder span setting at the time the peak was recorded (i.e., 0.10, 0.25, 0.50, 1.0, or 2.0 absorbance units full-scale) to give the corrected relative peak area values used in this study.

This method of calculating relative peak areas is valid only if the measured peaks are reasonably symmetrical and free from excessive tailing, otherwise the triangle assumption does not hold. As can be seen in all the chromatograms, these assumptions are valid.

Extraction Procedures

As blood contains many formed elements (cells, platelets, etc.) and proteins, which would rapidly clog and destroy a HPLC column, a method to extract the components of analytical interest from the blood is required. The formed elements were most easily removed by allowing the blood to clot in a tube

which was then centrifuged followed by removing the liquid portion of the blood (i.e., serum or plasma) while leaving the formed elements behind as a gelatinous clot. The serum or plasma collected in this study was stored in a freezer at -12°C until use.

Liquid - Liquid Partition

The most commonly used method for removing lidocaine and its metabolites from serum or plasma, as was discussed in Chapter I, involved their extraction from alkalinized serum or plasma into an organic solvent. After mixing and centrifuging, the organic phase may be separated from the aqueous phase and processed for injection into the HPLC unit for analysis. This single extraction technique however has the problem of carry over of denatured protein, which forms a gel at the interface of the serum and organic phases. Some of this gel tends to be removed with the organic phase and may, therefore, find its way into the HPLC system and clog the column. In order to clean up the sample actually injected into the HPLC system a double or back extraction technique similar to those discussed in Chapter I was tested for use in this study.

The exact double-extraction protocol used in this study was as follows. To a 15 ml glass-stoppered centrifuge tube was added 1.0 ml of serum, 50 µl of internal standard solution (prilocaine in methanol), 0.5 ml of 10N sodium hydroxide and 5.0 ml of methylene chloride. The contents were mixed for 1 minute either by inversion or with a mechanical vortexer (see

Chapter IV, page 99) and centrifuged for 5 minutes at 2,900 rpm. The aqueous (top) layer was aspirated to waste and as much as possible of the organic layer was transferred to another tube containing 1.0 ml of 1N hydrochloric acid. This tube's contents were mixed as before and the layers were allowed to separate, centrifuging being unnecessary as no denatured protein was formed at this stage. The organic(bottom) phase was then aspirated to waste by pinching-shut the aspirator tube while inserting the aspirator tip through the aqueous phase into the organic solvent. After the organic phase was removed the tube was pinched again and the aspirator removed. The aqueous layer was alkalinized with 0.5 ml of 10N sodium hydroxide and was extracted as before with 5 ml of fresh methylene chloride. The aqueous layer was aspirated to waste and the organic layer was transferred to a test tube and evaporated to dryness at 60°C in a sand bath. The test tube was removed from the sand bath as soon as possible after evaporation to avoid decomposition of the residue. The residue was dissolved in 100 µl of methanol and as much as possible of the methanol was removed from the test tube using a graduated 100 ul syringe. The volume of methanol collected was noted and then the contents of the syringe were injected into the HPLC system for analysis.

Charcoal Adsorption

Preliminary experiments demonstrated that the charcoal adsorption technique used by Adam et al. (27) for recovering lidocaine from serum also worked satisfactorily for MEGX and

GX. Adam's paper and charcoal adsorption technique were described in Chapter I, page 21. Only slight changes were made in this technique to adapt it for use in this study, the exact protocol followed being described below.

To a centrifuge tube 1.0 ml of serum, 50 µl of an internal standard solution(prilocaine in methanol), 2.0 ml of 2% ammonium hydroxide and 8 mg of charcoal(Norit A) were added. The contents of the tube were mixed on a mechanical vortexor for 15 seconds followed by centrifuging for 5 minutes at 2,900 rpm. The liquid phase was aspirated to waste leaving behind a charcoal button to the surface of which the lidocaine, MEGX and GX were adsorbed. To desorb these substances from the charcoal 1.0 ml of methylene chloride was added to the tube which was mixed and centrifuged as before. As much as possible of the organic solvent was removed from the centrifuge tube and transferred to a test tube and processed for injection into the HPLC system as described for the liquid - liquid partition extraction procedure.

Internal Standard

The extraction and processing procedures just described involve several manipulations which were difficult to duplicate exactly from one sample to another, and for this reason an internal standard was considered necessary. The use of an internal standard involved the addition of the same amount of a reference compound to each sample of a run, thus establishing a ratio of amount of analytical substance (i.e., lidocaine, MEGX and GX) to the amount of internal

standard present in each sample. When the samples were extracted and chromatographed any variation in the handling of the samples, such as the volume of extract recovered or the volume of redissolved residue injected into the HPLC system, would affect the absolute amount of material detected but not the ratio of analytical to internal standard substances. This ratio was directly proportional to the ratio of the corrected relative peak areas of the analytical and internal standard peaks, which since the amount of internal standard initially added to each sample was the same, was directly proportional to the amount of analytical substance originally present in each sample.

Standard curves were constructed by plotting the ratio of analytical to internal standard peak areas for each standard solution as a function of the standard solution's known concentration. By handling any unknown serum samples in the same manner as the standard solutions then the ratio of analytical to internal standard peak areas could be measured and the concentration of the analytical substance determined from the appropiate standard curve.

The HPLC method for lidocaine analysis published by Adams et al.(27), used procaine as the internal standard. It was found in the present study however that the chromatographic peaks for procaine and MEGX were not completely resolved from one another, as shown in Figure 32. During the course of this investigation it was found that the substance prilocaine elutes between MEGX and lidocaine without interfering with either and was therefore used as the internal standard.

Figure 33 shows the elution time of prilocaine in relation to lidocaine, MEGX and GX. A 100 µg/ml solution of prilocaine in methanol was used, 50 µl of which was added to each sample of an analysis run.

Standard Solutions

Serum

A stock serum standard solution was made by weighing pure samples of lidocaine, MEGX and GX reagents into a volumetric flask. The weight of the flask was recorded both before and after each substance was added to it, thus allowing the calculation of the exact amount of each substance present in the flask. The mixture was then dissolved in a small volume of distilled water to which had been added a drop of dilute hydrochloric acid. Once the mixture was completely dissolved, the solution was diluted to the mark of the volumetric flask with bovine serum and mixed by inversion.

The working serum standard solution series used in this study was prepared by pipetting selected volumes of the stock standard solution into volumetric flasks of various capacities, followed by dilution to the mark with bovine serum. The concentrations of the working serum standard solution series was selected to cover the therapeutic and toxic concentration ranges of lidocaine, MEGX and GX. The serum standard solution concentrations used in this study are shown in Table 4. The serum standard solutions were stored frozen at -12°C.

Methanol

A standard solution series of lidocaine, MEGX and GX in methanol was prepared and injected directly into the HPLC system in order to determine extraction efficiencies as will be described in Chapter IV. A stock standard solution and working standard solution series were prepared in a similar manner to that just described for the serum standard solutions except methanol was used to dissolve the chemicals instead of serum.

The concentration range covered by the methanol standard solution series was selected to correspond to the amount of lidocaine, MEGX and GX expected to be injected into the HPLC system after extracting and processing 1.0 ml of each of the working serum standard solutions. The methanol standard solution concentrations are summarized in Table 5. The methanol standard solutions were stored at room temperature, approximately 22°C.

Paired-Ion Chromatography

Paired-Ion Chromatography (PIC) is a new form of reverse phase liquid chromatography. The technique of PIC provides an alternative to the ionic suppression technique for separating compounds by forcing them into their non-ionic, lipophilic forms as was used in this study. The PIC technique involves adding a large organic counter-ion to the moving phase to form a reversable ion-pair complex with the compound of interest in its ionic form. This complex behaves as a elec-

TABLE 4

Concentration of Serum Standard Solutions

Solution	Lidocaine µg/ml	MEGX µg/ml	GX µg/ml
Stock	406	396	396
Working A	0.41	0.40	0.40
" B	0.81	0.79	0.79
" C	1.62	1.58	1.58
" D	4.06	3.96	3.96
" E	8.12	7.92	7.92
" F	16.24	-	-

TABLE 5

Concentration of Methanol Standard Solutions

Solution	Lidocaine µg/ml	MEGX µg/ml	GX µg/ml
Stock	500	504	500
Working A	2.5	2.5	2.5
" B	5.0	5.0	5.0
" C	10.0	10.1	10.0
" D	20.0	20.2	20.0
" E	50.0	50.4	50.0
" F	100	101	100

trically neutral and non-polar, lipophilic compound. Thus, by using the appropriate PIC reagent one can analyze strong and weak acids simultaneously with weak bases(33).

Waters B-7 PIC reagent was tested for possible use in this study. This reagent contains heptane sufonic acid as the counter-ion buffered at a pH of about 3.5 with glacial acetic acid and is designed to separate bases such as the quaternary amine forms of lidocaine, MEGX and GX.

A moving phase composed of equal volumes of methanol and distilled water to which had been added a specified amount of B-7 PIC reagent was prepared and run through the reverse phase HPLC unit. The recorder baseline resulting from the use of this moving phase was very noisy, therefore, a UV spectrum of this moving phase was obtained. Figure 34 shows the UV spectrum of a 50/50 methanol-water solution with B-7 PIC reagent referenced against a 50/50 methanol-water solution without the PIC reagent. As can be seen from this spectrum the absorbance of the PIC moving phase over the wavelength range of interest to this study is very high. The use of this PIC reagent was, therefore, not compatible with the UV absorbance detector used in this study.

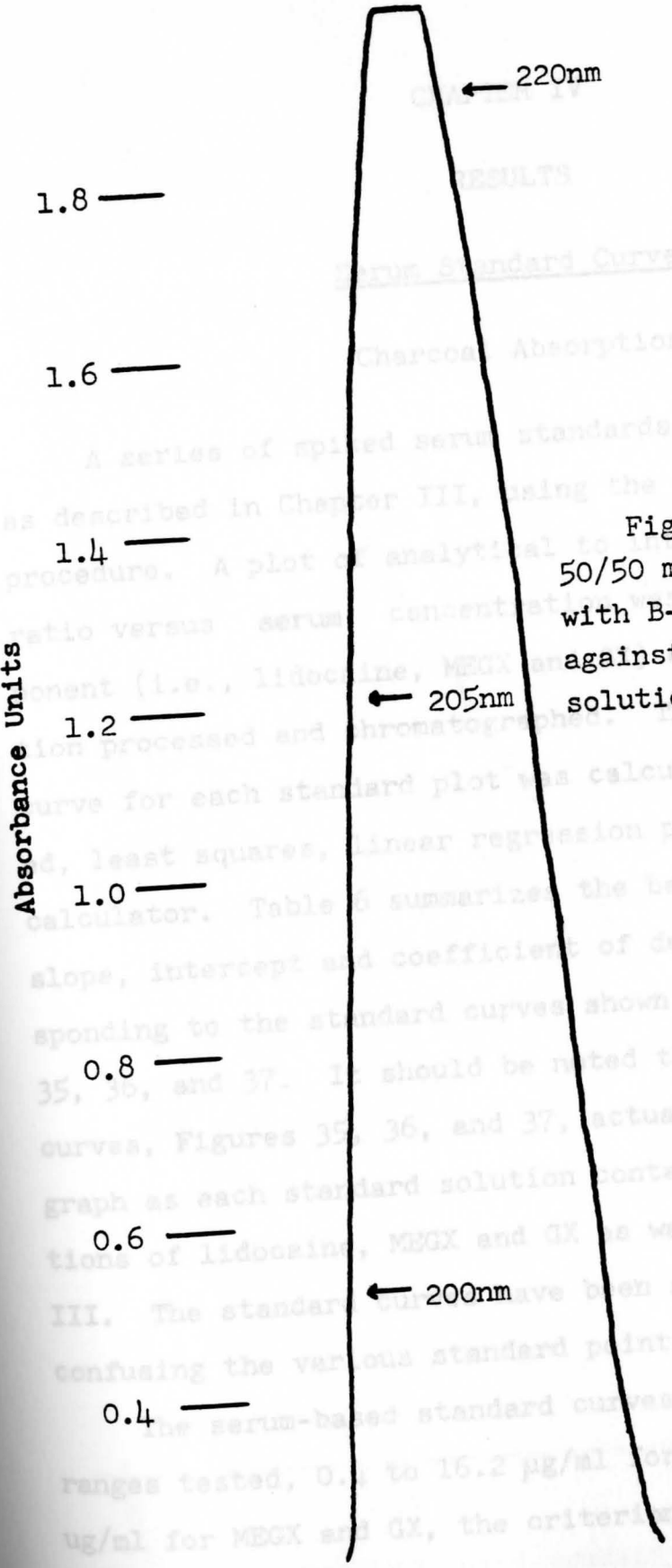


Fig. 34. -- UV spectrum of a 50/50 methanol-water solution with B-7 PIC reagent referenced against a 50/50 methanol-water solution without PIC reagent.

CHAPTER IV

RESULTS

Serum Standard Curves

Charcoal Absorption

A series of spiked serum standards was chromatographed, as described in Chapter III, using the charcoal adsorption procedure. A plot of analytical to internal standard peak area ratio versus serum concentration was graphed for each component (i.e., lidocaine, MEGX and GX) of each standard solution processed and chromatographed. The best-fit linear curve for each standard plot was calculated using an unweighted, least squares, linear regression program on a programmable calculator. Table 6 summarizes the best-fit curve data, (i.e. slope, intercept and coefficient of determination) corresponding to the standard curves shown graphically in Figures 35, 36, and 37. It should be noted that all three standard curves, Figures 35, 36, and 37, actually are part of one graph as each standard solution contained standard concentrations of lidocaine, MEGX and GX as was described in Chapter III. The standard curves have been shown separately to avoid confusing the various standard points.

The serum-based standard curves are linear over the ranges tested, 0.4 to 16.2 µg/ml for lidocaine and 0.4 to 7.9 ug/ml for MEGX and GX, the criterion of linearity being how

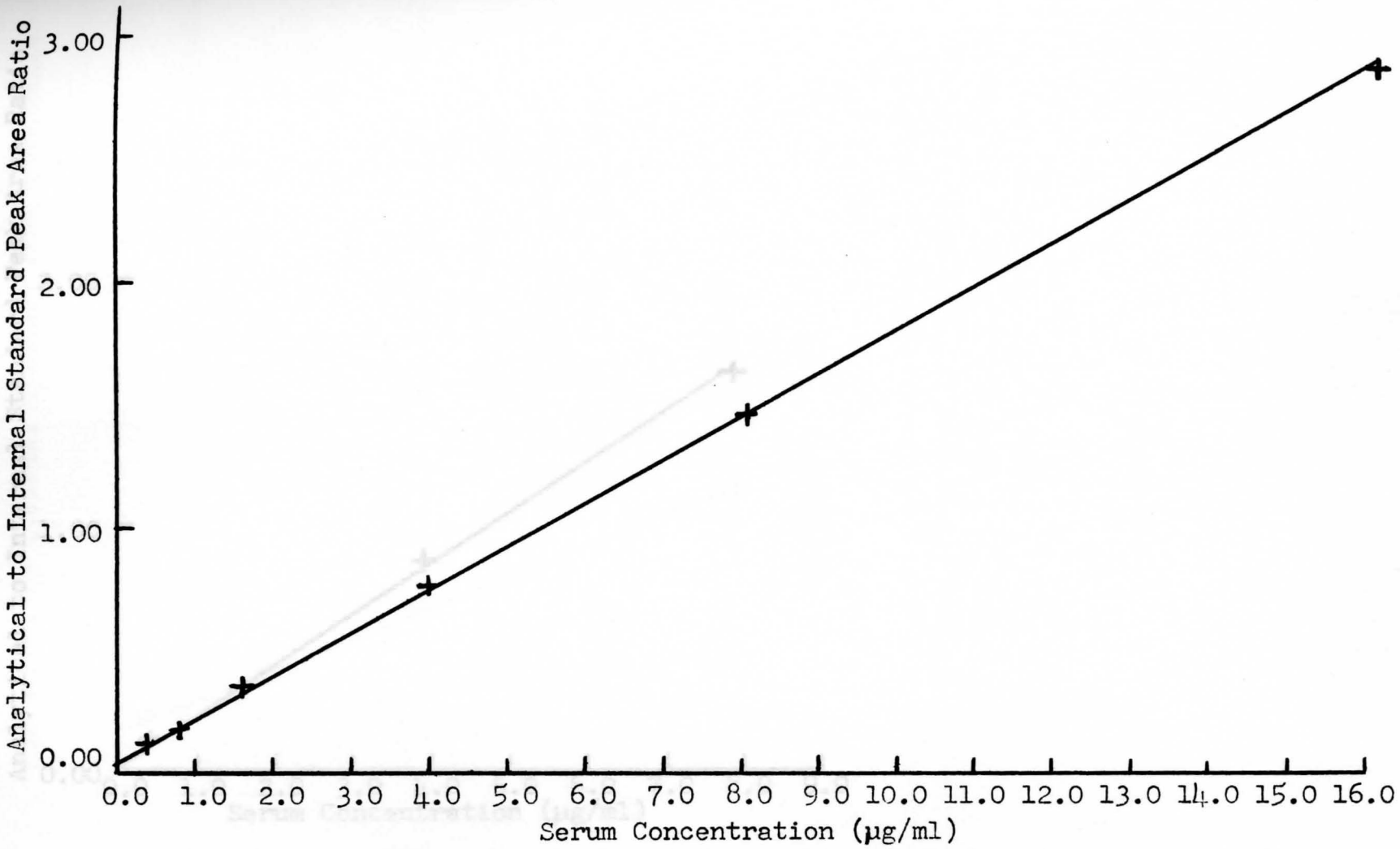


Fig. 35. -- Lidocaine Serum Standard Curve, Charcoal Adsorption. Moving phase: pH 6.1 and acetonitrile buffer volume ratio of 1:9.

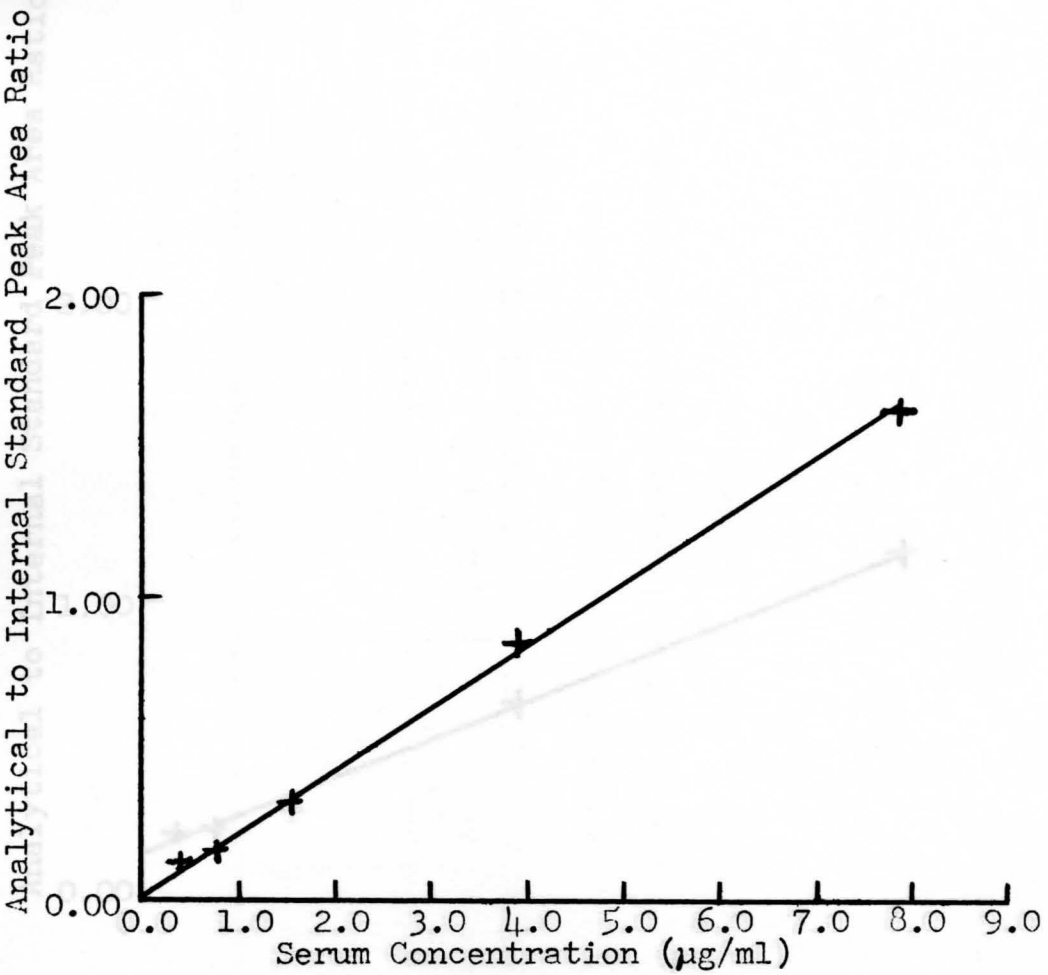


Fig. 36. -- MEGX Serum Standard Curve, Charcoal Adsorption.
Moving phase: pH 6.1 and acetonitrile to buffer volume ratio of 1:9.

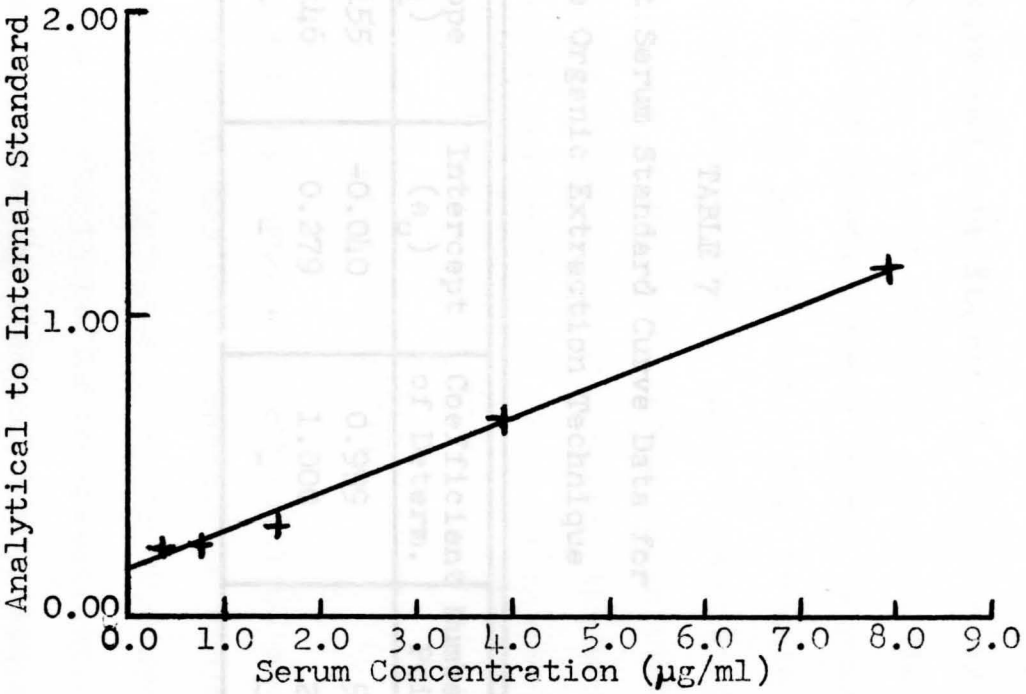


Fig. 37. -- GX Serum Standard Curve, Charcoal Adsorption.
Moving phase: pH 6.1 and acetonitrile to buffer volume ratio of 1:9.

TABLE 6

Best-fit Serum Standard Curve Data for

Charcoal Adsorption Technique

	Slope (a_1)	Intercept (a_o)	Coefficient of Determ.	Number of Points
Lidocaine	0.1733	0.0455	0.999	7
MEGX	0.2035	0.0196	0.999	6
GX	0.1244	0.1624	0.990	6

TABLE 7

Best-fit Serum Standard Curve Data for

Double Organic Extraction Technique

	Slope (a_1)	Intercept (a_o)	Coefficient of Determ.	Number of Points
Lidocaine	0.155	-0.040	0.999	5
MEGX	0.146	0.279	1.000	2
GX	-	-	-	-

close the best-fit curve's coefficient of determination was to the ideal value of 1.000. The coefficients in Table 6 all show a good linear fit. The detection limit was about 0.4 μg/ml lidocaine, MEGX and GX.

Figure 38 shows the chromatogram of the bovine serum, with internal standard added, used to make the serum standard solutions as described in Chapter III. This chromatogram shows that the bovine serum has a peak with an elution time about equal to that of GX as shown in Figure 33. In order to make the GX standard curve, Figure 37, the presence of this interfering peak had to be taken into account. This was done by subtracting the relative peak area of the interfering peak, as determined from Figure 38, from the relative peak area of the GX peaks of the standard solution chromatograms used to make the GX standard curve before calculating the GX to internal standard peak ratios. This technique assumes that the relative area added to each GX peak of the standard solutions by the interfering bovine serum peak was the same as that of the interfering peak alone. Since the bovine serum blank sample was handled in the same manner as the standard solutions, this assumption is true.

Double Organic Extraction

Samples of the working serum standard solution series were also processed through the double extraction procedure described in Chapter III and injected into the HPLC system. The HPLC system in use at the time of the double extraction run was the same as was used when the charcoal adsorption samples

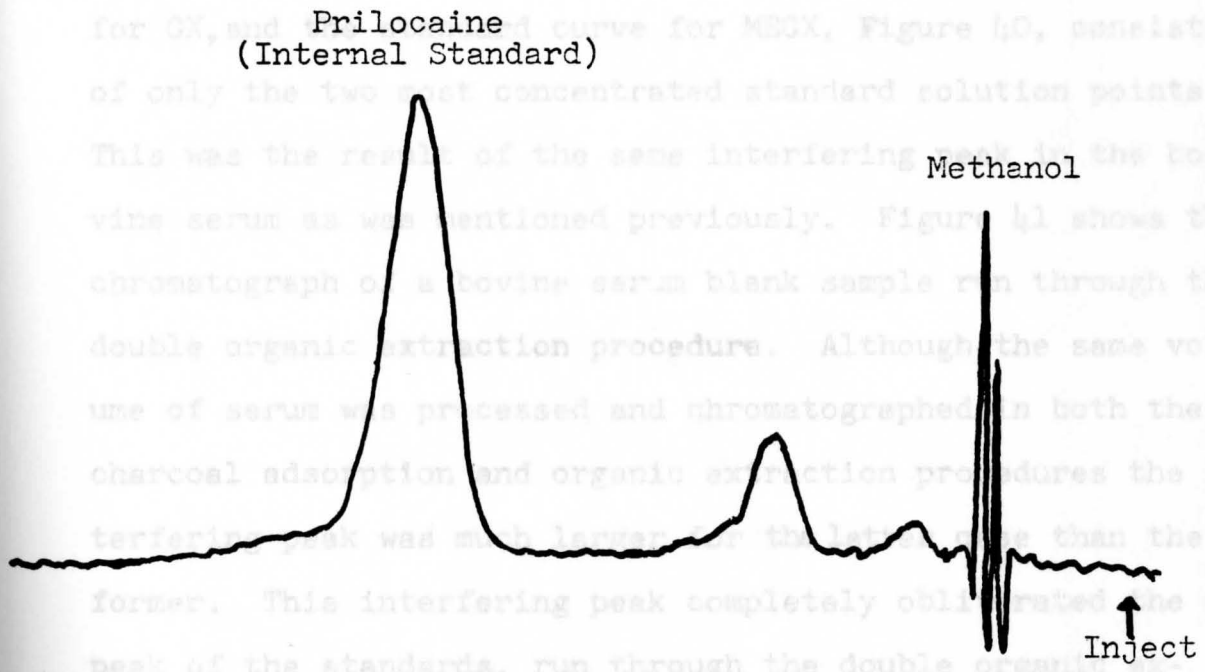


Fig. 38. -- Chromatogram for bovine serum blank (with internal standard), Charcoal Adsorption. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio of 1:11. Recorder span 0.25 absorbance units full-scale.

were run with the exception that the acetonitrile to buffer volume ratio was 1:11 in the former case and 1:9 in the latter case.

Serum standard curves were constructed as described in the preceeding paragraphs. The best-fit linear regression data are summarized in Table 7 and the curves are shown graphically in Figures 39 and 40. There is no standard curve for GX, and the standard curve for MEGX, Figure 40, consists of only the two most concentrated standard solution points. This was the result of the same interfering peak in the bovine serum as was mentioned previously. Figure 41 shows the chromatograph of a bovine serum blank sample run through the double organic extraction procedure. Although the same volume of serum was processed and chromatographed in both the charcoal adsorption and organic extraction procedures the interfering peak was much larger for the latter case than the former. This interfering peak completely obliterated the GX peak of the standards, run through the double organic extractor procedure, and also partially obscured the standard's MEGX peaks.

Figure 41 shows that the bovine serum blank gave rise to another peak in addition to that mentioned above. This other peak had a relatively long elution time, much longer than that for lidocaine, MEGX or GX, and has a relatively large peak area. This peak was not apparent in the charcoal adsorption processed bovine serum blank sample shown in Figures 38. Incidentally the retention times of the peaks obtained using the two extraction procedures were due to the different mov-

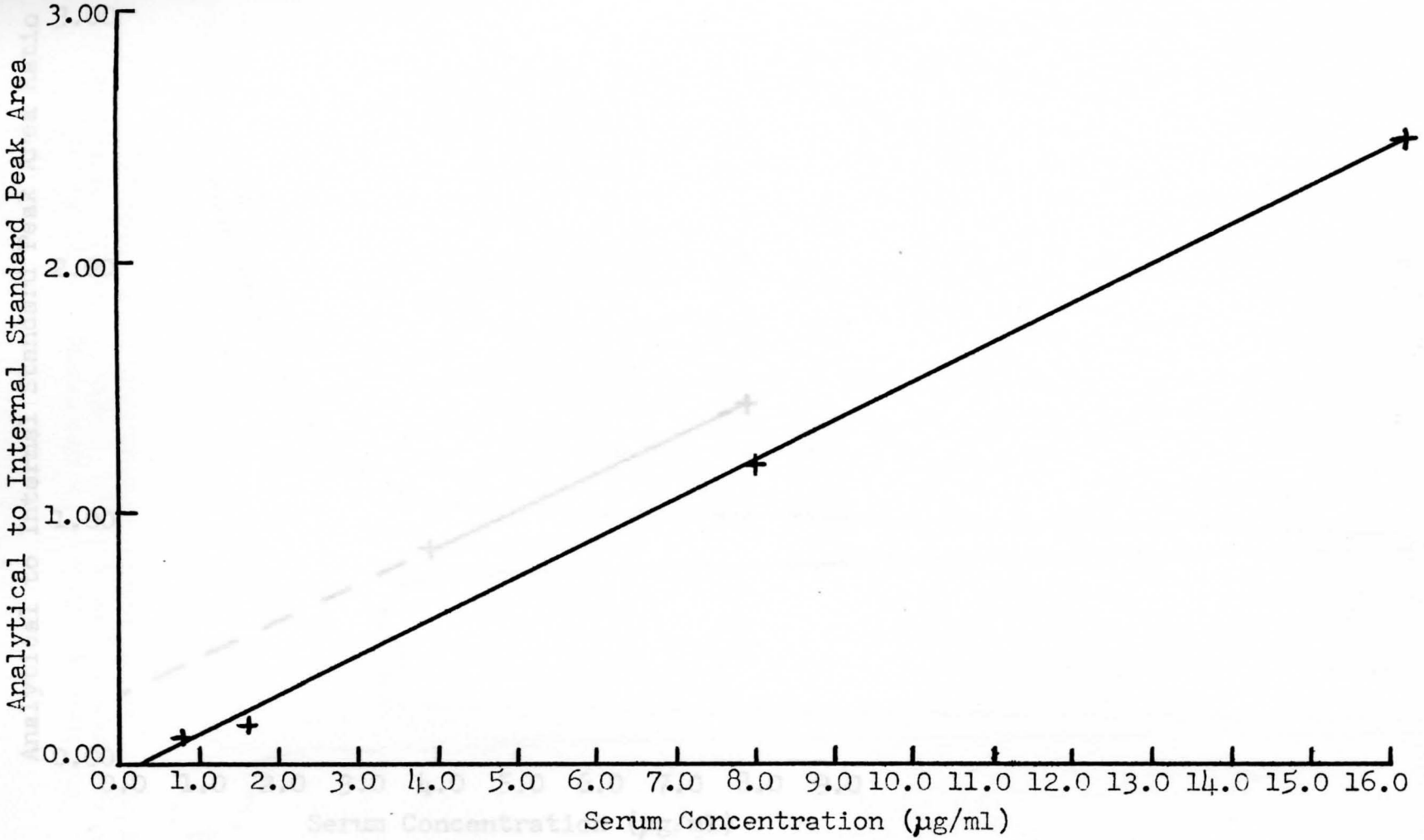


Fig. 39. -- Lidocaine serum standard curve, double organic extration. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio of 1:11.

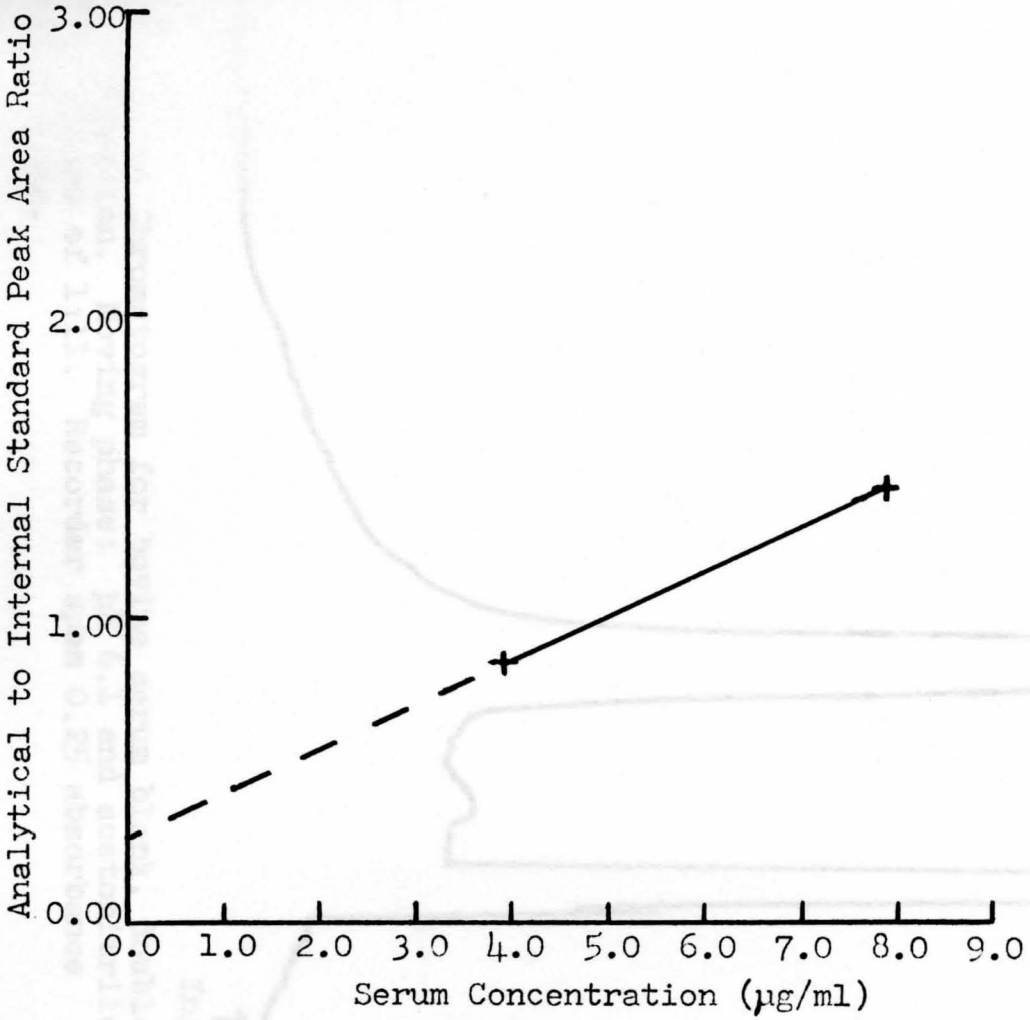


Fig. 40. -- MEGX serum standard curve, double organic extraction. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio of 1:11.

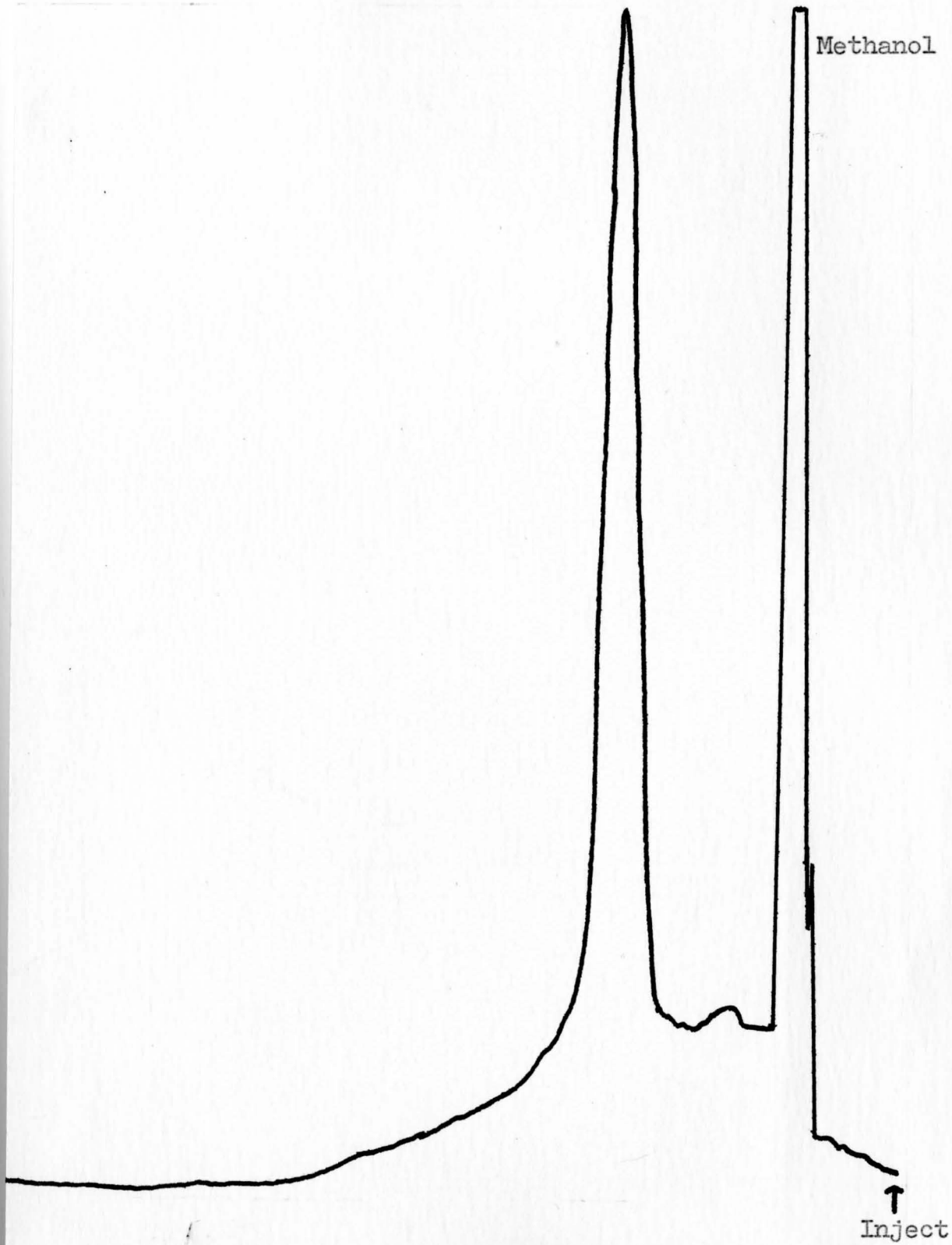


Fig. 41. -- Chromatogram for bovine serum blank, double organic extraction. Moving phase: pH 6.1 and acetonitrile to buffer volume of 1:11. Recorder span 0.25 absorbance units full-scale.

ing phase compositions used for the two runs.

Extraction Efficiency

Methanol Standard Curves

In order to determine the extraction efficiencies of the extraction procedures used in this study standard curves were prepared using solutions of lidocaine, MEGX and GX in methanol, which were injected directly into the HPLC system. The concentration range of the methanol standard solution series was selected, as described in Chapter II, so that the amount of lidocaine, MEGX and GX injected directly into the HPLC would correspond to the amount of lidocaine, MEGX and GX injected for each of the serum working standard solutions after being extracted and processed.

Standard curves for lidocaine, MEGX and GX were constructed by plotting the corrected relative chromatographic peak area for each component of each standard chromatographed, no internal standard involved, versus the amount of each component injected. Figures 42 through 47 show the methanol-based standard curves obtained during this study. One set of curves (Figures 42, 43 and 44) was chromatographed with a moving phase acetonitrile to buffer volume ratio of 1:9 while the other set (Figures 45, 46 and 47) was run with a ratio of 1:11.

The best-fit linear curve for each standard curve was calculated in the same way as was described for the best-fit serum standard curves. The best-fit methanol standard curve

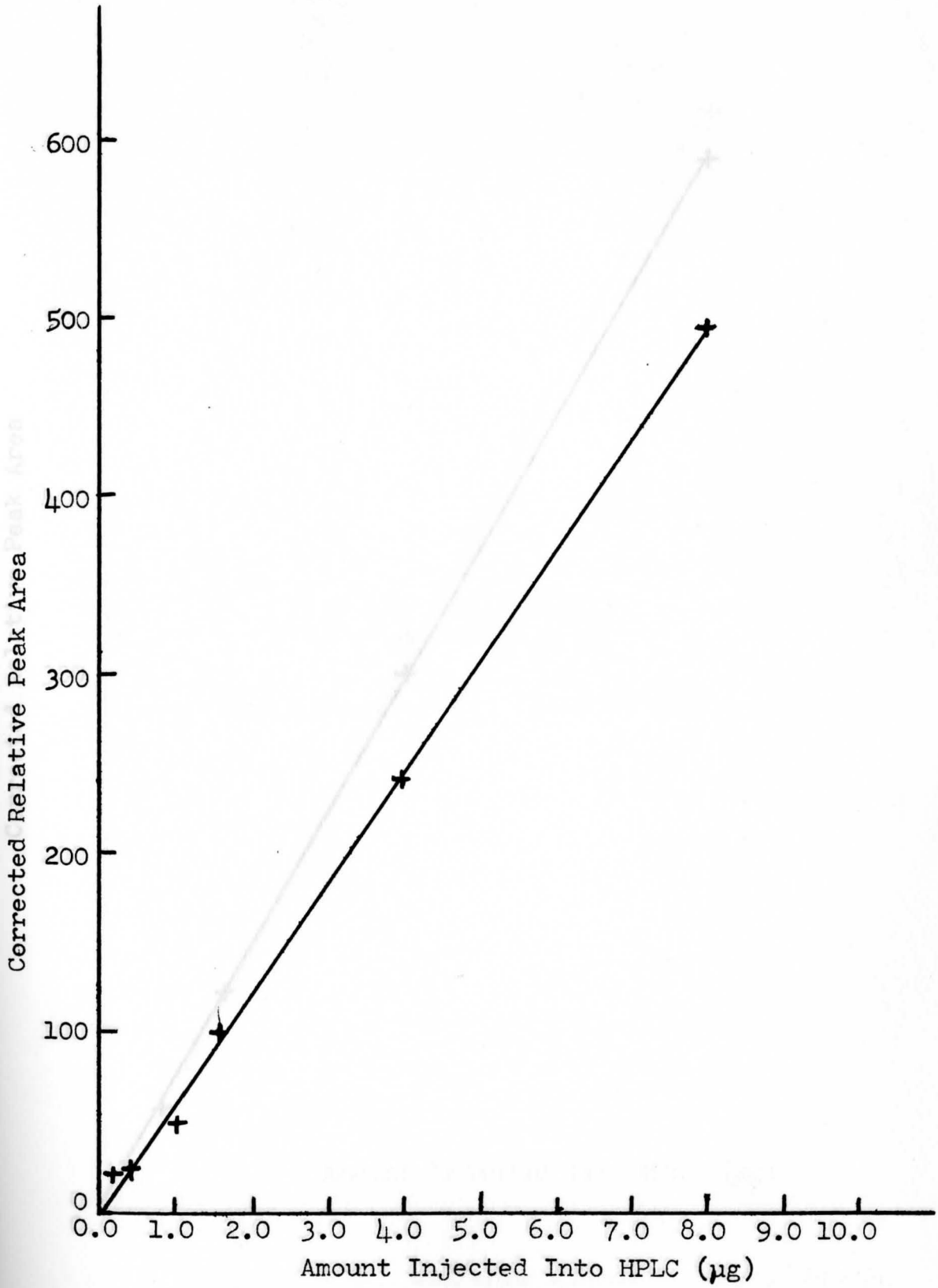


Fig. 42. -- Lidocaine methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:9.

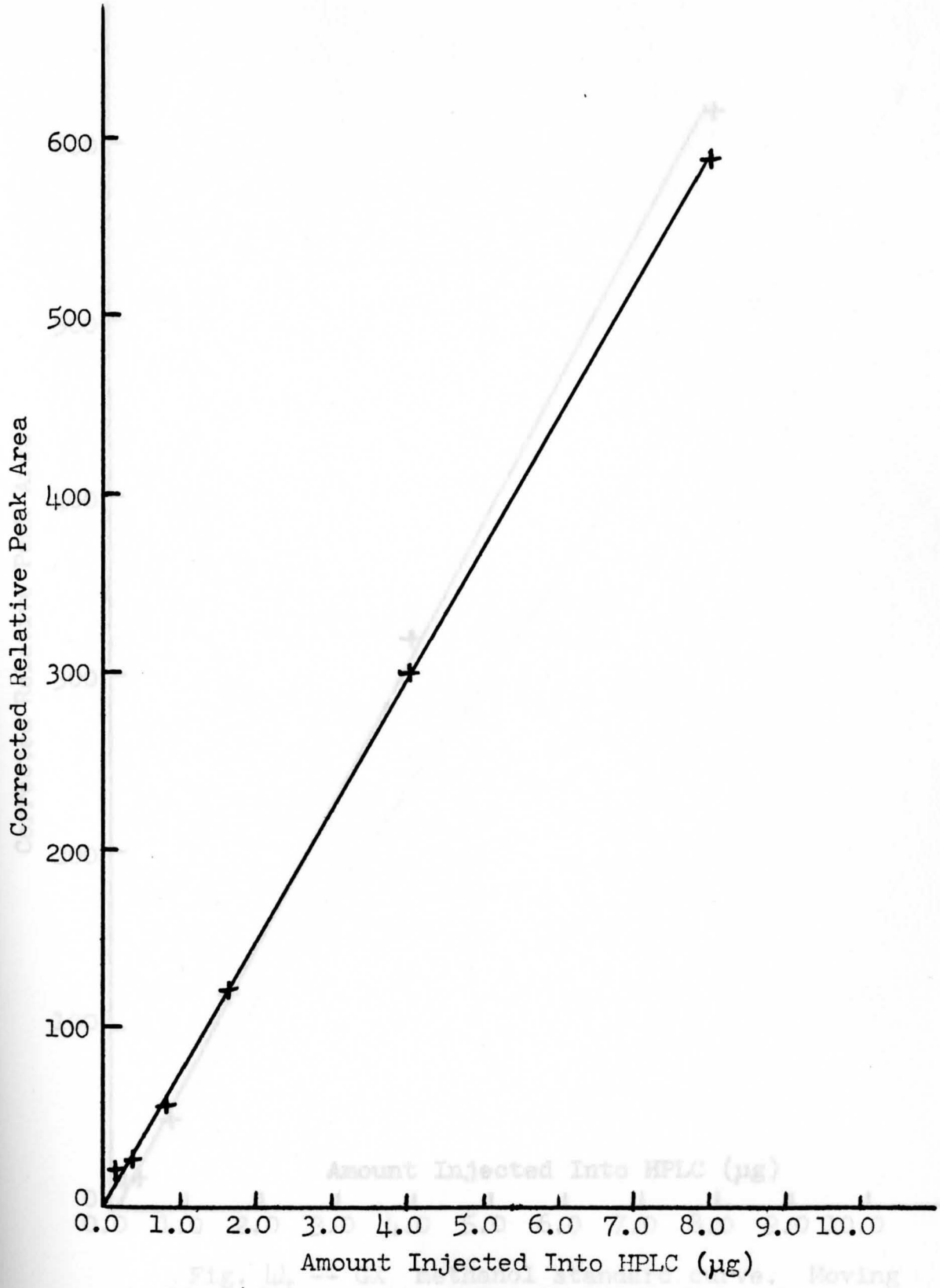


Fig. 43. -- MEGX methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:9.

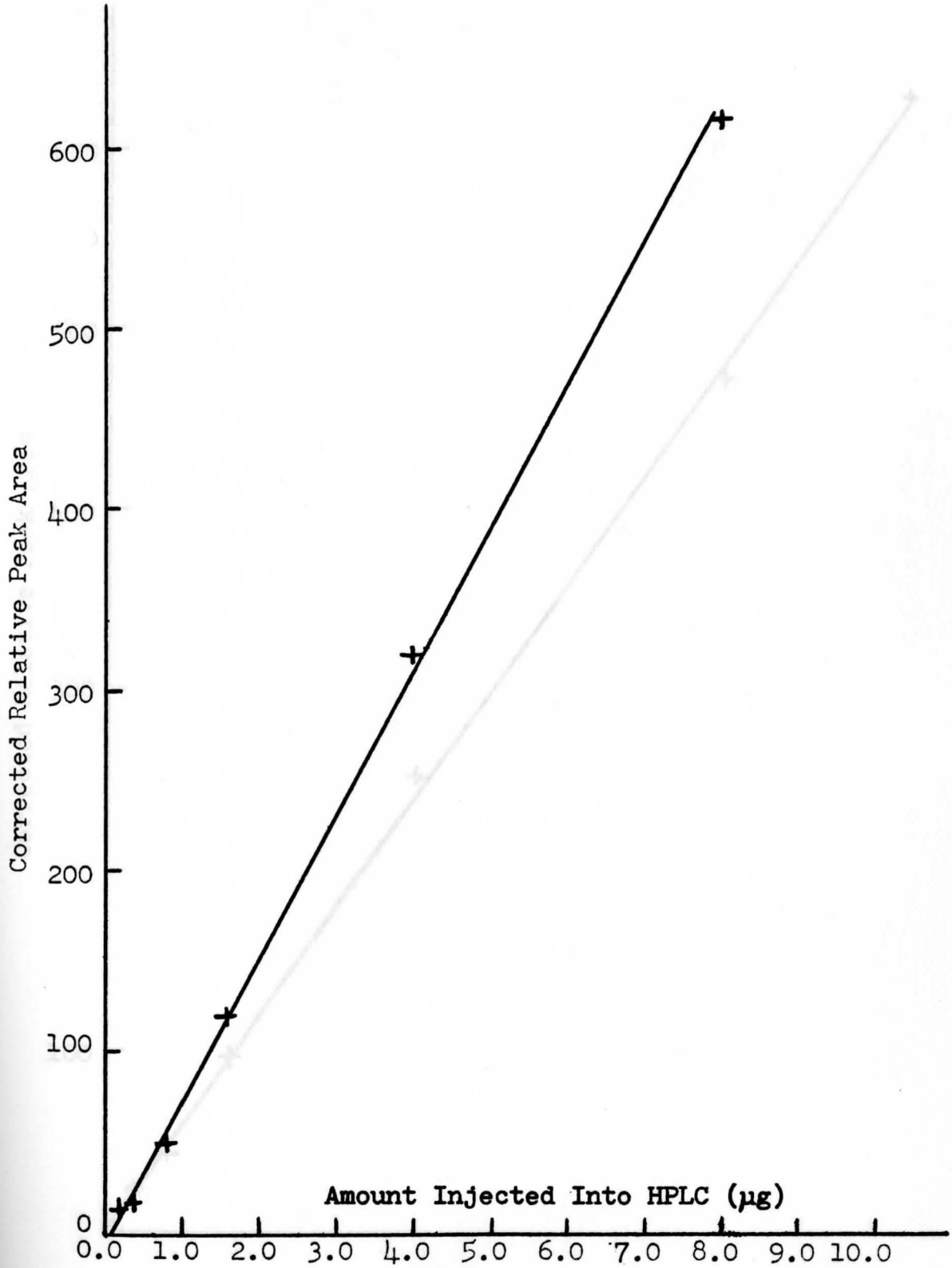


Fig. 44 -- GX methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:9.

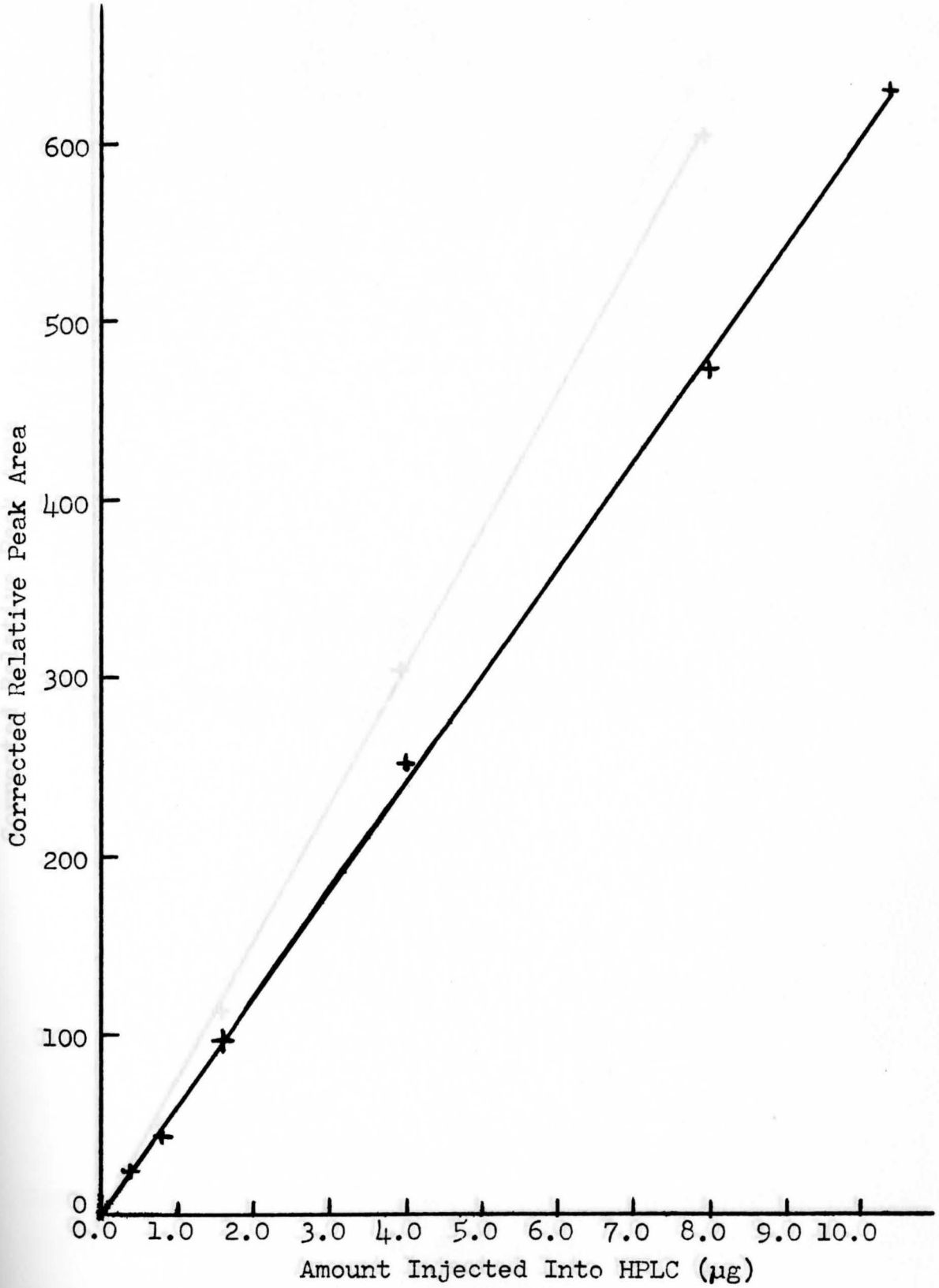


Fig. 45. -- Lidocaine methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:11.

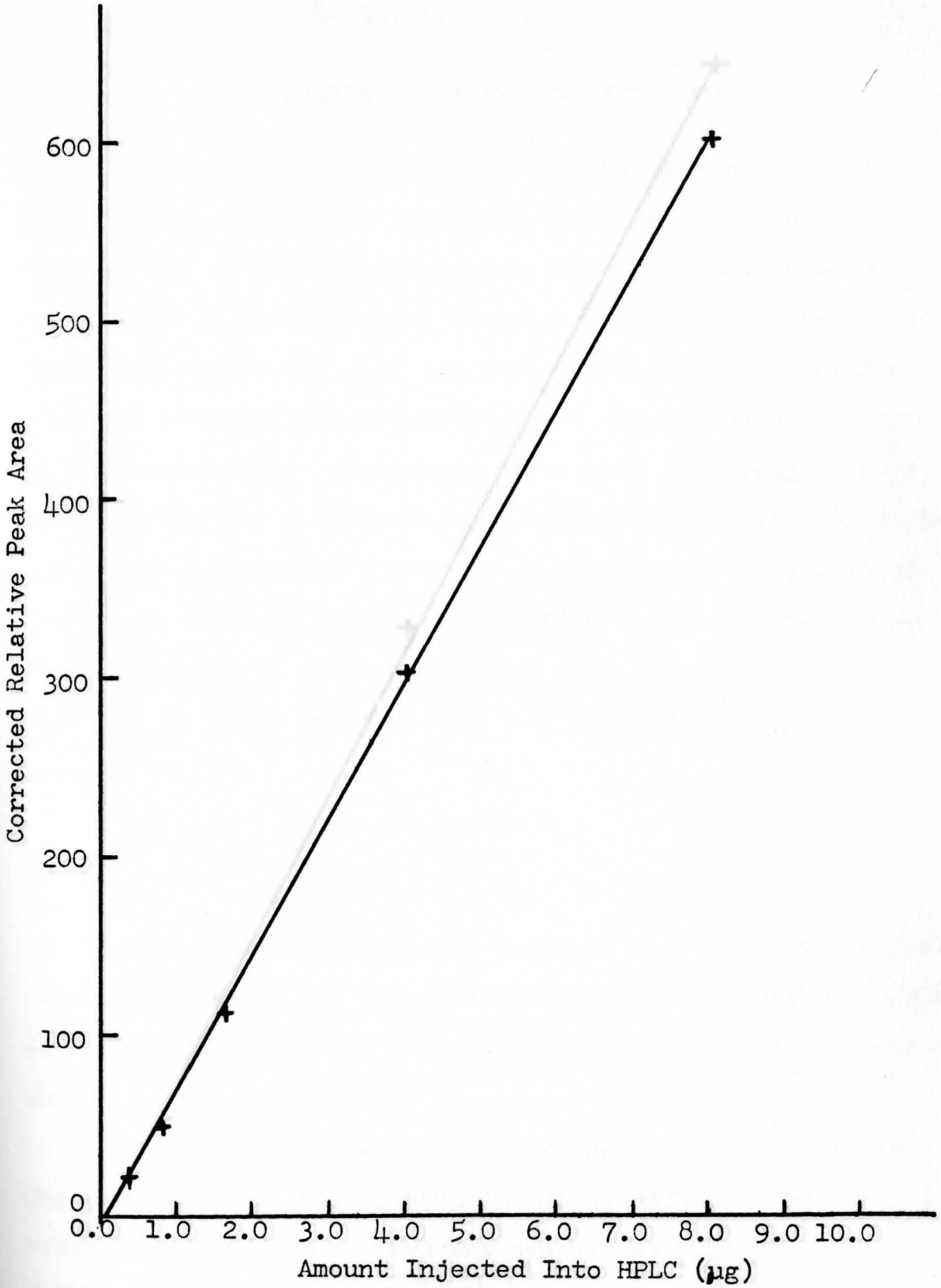


Fig. 46. -- MEGX methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:11.

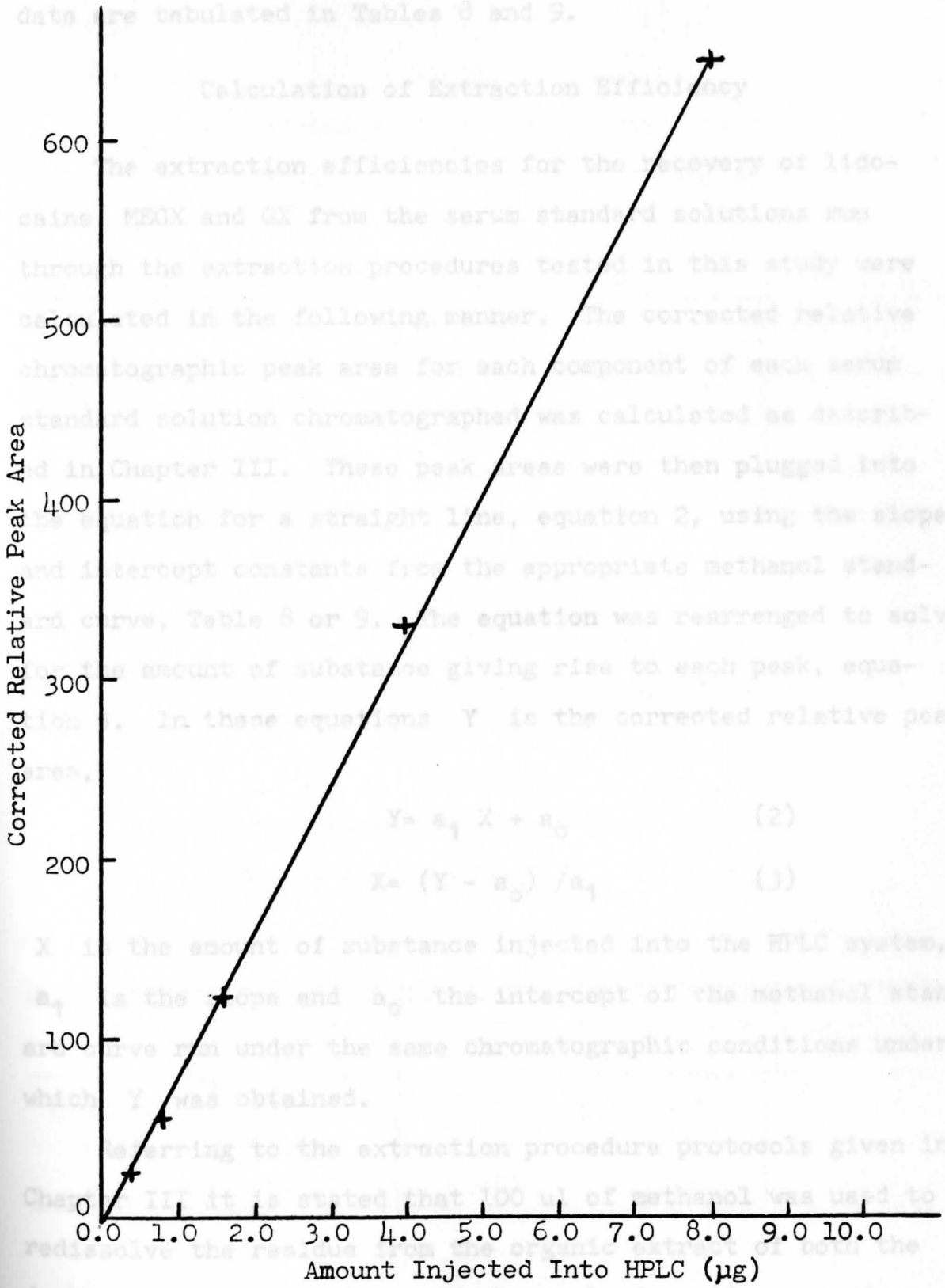


Fig. 47. -- GX methanol standard curve. Moving phase: pH 6.1 and acetonitrile to buffer volume ratio 1:11.

data are tabulated in Tables 8 and 9.

Calculation of Extraction Efficiency

The extraction efficiencies for the recovery of lidocaine, MEGX and GX from the serum standard solutions run through the extraction procedures tested in this study were calculated in the following manner. The corrected relative chromatographic peak area for each component of each serum standard solution chromatographed was calculated as described in Chapter III. These peak areas were then plugged into the equation for a straight line, equation 2, using the slope and intercept constants from the appropriate methanol standard curve, Table 8 or 9. The equation was rearranged to solve for the amount of substance giving rise to each peak, equation 3. In these equations Y is the corrected relative peak area,

$$Y = a_1 X + a_o \qquad (2)$$

$$X = (Y - a_o) / a_1 \qquad (3)$$

X is the amount of substance injected into the HPLC system, a_1 is the slope and a_o the intercept of the methanol standard curve run under the same chromatographic conditions under which Y was obtained.

Referring to the extraction procedure protocols given in Chapter III it is stated that 100 ul of methanol was used to redissolve the residue from the organic extract of both the double organic extraction and charcoal adsorption procedures. Not all of this methanol could be recovered by syringe for in-

TABLE 8

Best-fit Methanol Standard Curve Data for

A Moving Phase Acetonitrile to Buffer

Volume Ratio of 1:9

	Slope (a_1)	Intercept (a_o)	Coefficient of Determ.	Number of Points
Lidocaine	61.499	1.930	1.000	7
MEGX	73.182	1.761	1.000	7
GX	78.24	-4.95	0.999	7

TABLE 9

Best-fit Methanol Standard Curve Data for

A Moving Phase Acetonitrile to Buffer

Volume Ratio of 1:11

	Slope (a_1)	Intercept (a_o)	Coefficient of Determ.	Numbers of Points
Lidocaine	60.020	0.929	0.999	7
MEGX	75.692	-6.133	1.000	6
GX	81.522	-4.089	1.000	6

jection into the HPLC system, therefore, the amount of lidocaine, MEGX and GX dissolved in the methanol and not injected into the HPLC must be taken into account when calculating the extraction efficiencies. This was done by multiplying the known concentration of a serum standard by the volume of methanol injected into the HPLC system for that standard expressed as a fraction of the total amount of methanol used to redissolve the residue(i.e., 100 µl). This value (Z) in equation 4 represents the theoretical amount of substance that

$$Z = C(V_I/V_T) \qquad (4)$$

would have been injected for each peak if the extraction procedure had been 100% efficient. In equation 4: C is the known amount(concentration) of analytical substance(i.e., lidocaine, MEGX and GX) in the volume of serum extracted(i.e., 1 ml), V_T is the volume of methanol used to redissolve the organic extract(i.e., 100 µl) and V_I is the volume of methanol injected into the HPLC system.

The actual percent extraction efficiency for each component of each serum standard solution processed and chromatographed was calculated by comparing the actual amount injected to the amount for a 100% efficient procedure, Z from equation 4. Equation 5 shows the final extraction efficiency equation used in this study.

$$\text{Percent Extraction Efficiency} = (X/Z)100\% \qquad (5)$$

Extraction Efficiency Results

Table 10 summarizes the percent extraction efficiencies

TABLE 10

Percent Extraction Efficiencies for Serum Solutions Extracted by Various Techniques

Serum Standard Solution	Percent Extraction Efficiency		
	Lidocaine	MEGX	GX
Charcoal Adsorption			
A	91.9	80.3	82.3
B	75.1	55.7	49.4
C	72.4	58.1	37.3
D	60.1	58.9	37.2
E	71.2	68.4	43.0
F	62.8	-	-
Average	72.3	64.3	49.8
Double Organic Extraction, with Vortexing			
B	26.5	-	-
C	16.3	-	-
D	-	42.5	-
E	38.5	39.4	-
F	24.8	-	-
Average	25.3	40.9	-
Double Organic Extraction, with Inversion			
B	20.2	-	-
E	27.8	39.7	-
Single Organic Extraction			
A	51.3	-	-

obtained for each component of each serum standard solution processed through the various extraction procedures tested in this study. The percent extraction efficiencies for the recovery of lidocaine from the serum standard solutions run through the charcoal adsorption technique were approximately the same as those values published by Adams et al.(27). The extraction efficiencies for MEGX and GX by this technique tended to be somewhat lower than the efficiencies for lidocaine, with the extraction efficiencies for MEGX being consistently higher than those for GX.

The extraction efficiencies for the double extraction procedure were considerably less than for the same solutions run through the charcoal adsorption technique. Most of the published procedures for extracting lidocaine from serum or plasma, as described in Chapter I, involved only a single organic extraction from alkalized serum and were claimed to have extraction efficiencies for lidocaine of well over 90%. For reasons discussed in Chapter III a single extraction procedure was not considered advisable for routine use in this study. However, to determine if the low extraction efficiencies of the double extraction procedure were due to a large loss of material with the addition of the second or back extraction cleanup step a single extraction technique was tested. The single extraction procedure of Keenahan(19) was followed as it closely corresponds to the first extraction step of the double organic extraction procedure used in this study. The percent extraction efficiency for the recovery of lidocaine from the only serum standard solution processed through the

single extraction procedure was 51.3%. This is about twice the observed extraction efficiency for the same standard solution run through the double organic extraction procedure, but only half that of the claimed 98% extraction efficiency for the method.

In order to test if the low extraction efficiencies of the organic extraction procedures might be due to the denatured protein gel formed at the interface of the aqueous and organic phases upon mixing by vortexer, a run was made wherein all mixing was done by inversion for 60 seconds. The amount of gel formed by this mixing technique was, in fact, noticeably smaller than with the vortexing technique, however the extraction efficiencies for lidocaine in the standard solution run through both procedures were significantly lower for the mixing by inversion technique than for the vortexing technique.

Results for Serum from Patients Receiving Lidocaine

As has been discussed in the previous sections the charcoal adsorption technique: (1) appeared to be freer from interferences, (2) was the only technique for which complete standard curves for lidocaine, MEGX and GX were obtained and (3) had the highest extraction efficiencies for these substances of any of the extraction procedures tested in this study. For these reasons the charcoal adsorption technique was used exclusively for the assay of actual patient serum samples.

Four serum samples from three patients supposedly receiving lidocaine were obtained from two different sources.

Unfortunately very little information was supplied with these samples. Therefore, it is not known what medications, other than lidocaine, these patients may have been receiving nor why or in what form the lidocaine was administered to these patients.

Calculating Patient Serum Lidocaine, MEGX and GX Concentration

The lidocaine, MEGX and GX concentrations in a serum sample of unknown concentration were calculated in the following manner. The corrected relative peak area of any peak identified by retention time as lidocaine, MEGX and GX and the internal standard peak were calculated. The analytical to internal standard relative peak area ratios were then determined. These ratios were used in conjunction with the appropriate serum standard curve slope and intercept constants from table 6 and inserted into equation 3, page 94, to give the serum concentration of lidocaine, MEGX or GX in micrograms per milliliter.

Patient Serum Results

Table 11 summarizes the results of analysis for lidocaine, MEGX and GX in serum from patients known to be receiving lidocaine at the time the samples were collected. The serum from the patient designated T. M. showed the clearest example of MEGX and GX being present with lidocaine. This sample showed the first pattern of lidocaine metabolism, discussed in Chapter I page 9, where the lidocaine concentration (2.9 ug/ml) was within the therapeutic range with MEGX

TABLE 11

Results for Serum from Patients Receiving Lidocaine

Patient	Lidocaine µg/ml	MEGX µg/ml	GX µg/ml
T M	2.9	0.5	0.5
N K "1	0.0	0.0	Interference
N K "2	3.5	Present	"
N J	9.4	-	"
CAP-T_4 Control	4.0	-	"

(0.5 µg/ml) and GX (0.5 µg/ml) each about one-sixth of the lidocaine concentration.

Figure 48 shows the chromatogram for the T.M. sample. It should be noted that the recorder span was changed at the points indicated to allow the internal standard peak to read on-scale while also providing sufficient scale expansion for the lidocaine, MEGX and GX peaks to appear. The corrected relative peak area values are unaffected by this variation as each peak's relative area was multiplied by the recorder span at which it was recorded(i.e., full-scale absorbance setting of 0.10, 0.25, 0.50, 1.0 or 2.0 absorbance units). This technique of changing the recorder span between peaks was unnecessary for all the other patient and standard serum samples as the amount of internal standard used was one-tenth as much as was added to the T.M. sample. The corrected realtive peak area of the internal standard peak of the T.M. chromatogram was divided by ten before the analytical to internal standard peak area ratios were calculated, to allow the concentrations of lidocaine, MEGX and GX to be determined from the serum standard curve data of Table 6.

Samples N.K. #1 and N.K. #2, whose chromatograms are shown in Figures 49 and 50, were marked as having been collected from the same patient on the same date, however one sample was marked "2 A.M."(N.K. #1) and the other as "second specimen"(N.K. #2). N.K. #1 and N.K. #2 appear to be samples collected before and after lidocaine administration as sample N.K. #2 shows a definite peak at the retention time for lidocaine while the other sample does not.

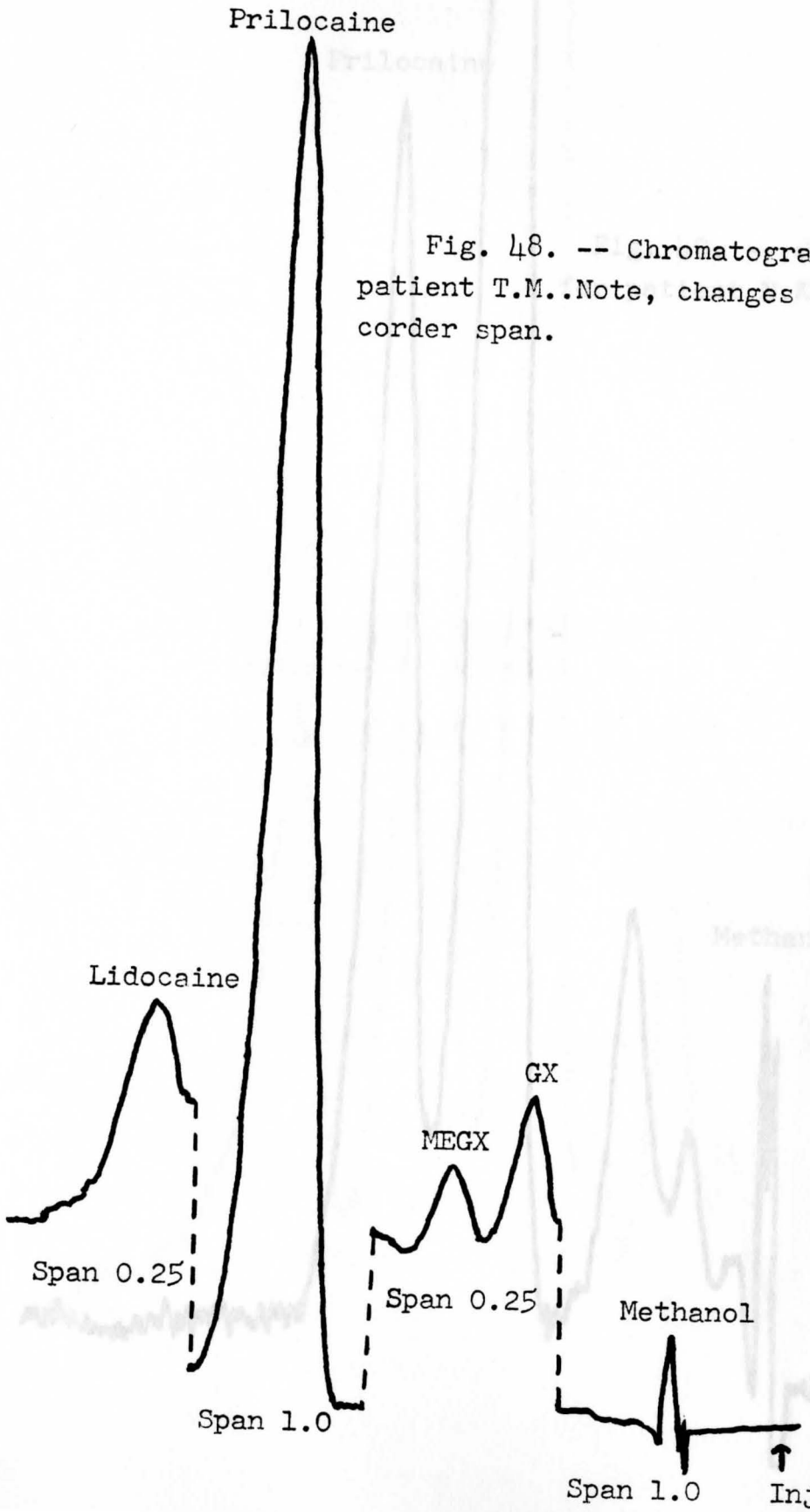


Fig. 48. -- Chromatogram for patient T.M..Note, changes in recorder span.

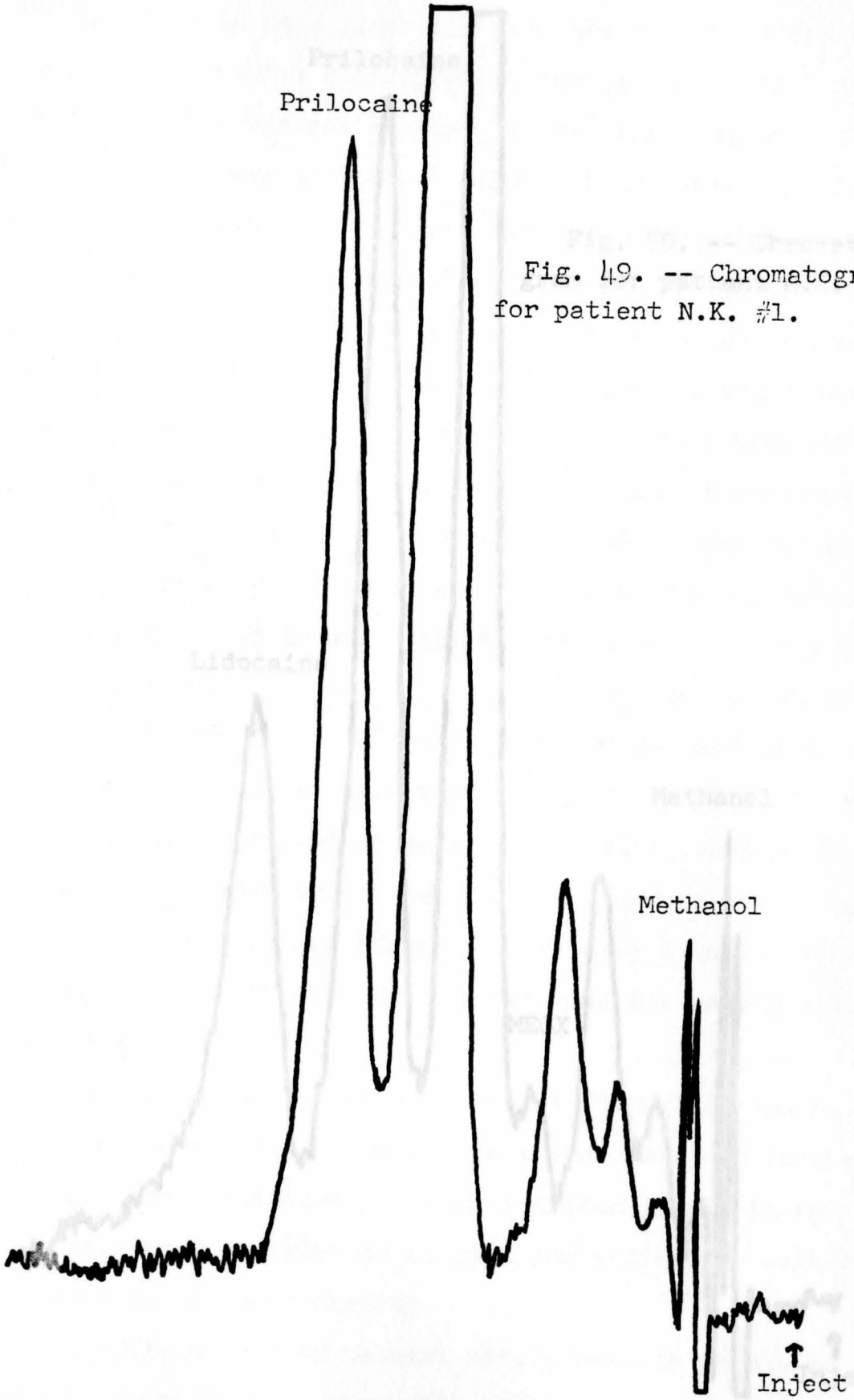


Fig. 49. -- Chromatogram for patient N.K. #1.

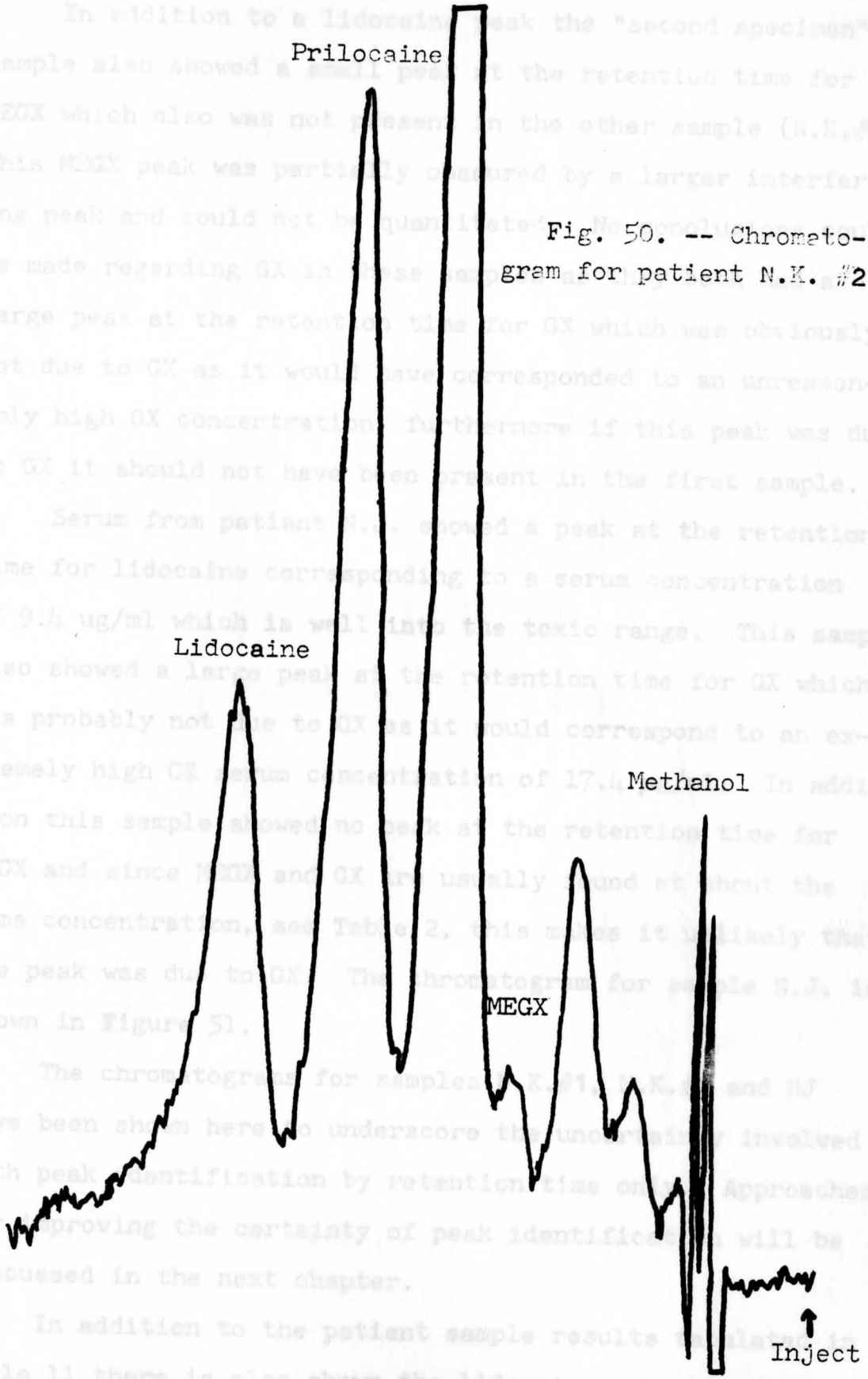


Fig. 50. -- Chromatogram for patient N.K. #2.

In addition to a lidocaine peak the "second specimen" sample also showed a small peak at the retention time for MEGX which also was not present in the other sample (N.K.#1). This MEGX peak was partially obscured by a larger interfering peak and could not be quantitated. No conclusions could be made regarding GX in these samples as they both had a large peak at the retention time for GX which was obviously not due to GX as it would have corresponded to an unreasonably high GX concentration, furthermore if this peak was due to GX it should not have been present in the first sample.

Serum from patient N.J. showed a peak at the retention time for lidocaine corresponding to a serum concentration of 9.4 ug/ml which is well into the toxic range. This sample also showed a large peak at the retention time for GX which was probably not due to GX as it would correspond to an extremely high GX serum concentration of 17.4 µg/ml. In addition this sample showed no peak at the retention time for MEGX and since MEGX and GX are usually found at about the same concentration, see Table 2, this makes it unlikely that the peak was due to GX. The chromatogram for sample N.J. is shown in Figure 51.

The chromatograms for samples N.K.#1, N.K.#2 and NJ have been shown here to underscore the uncertainty involved with peak identification by retention time only. Approaches for improving the certainty of peak identification will be discussed in the next chapter.

In addition to the patient sample results tabulated in Table 11 there is also shown the lidocaine concentration

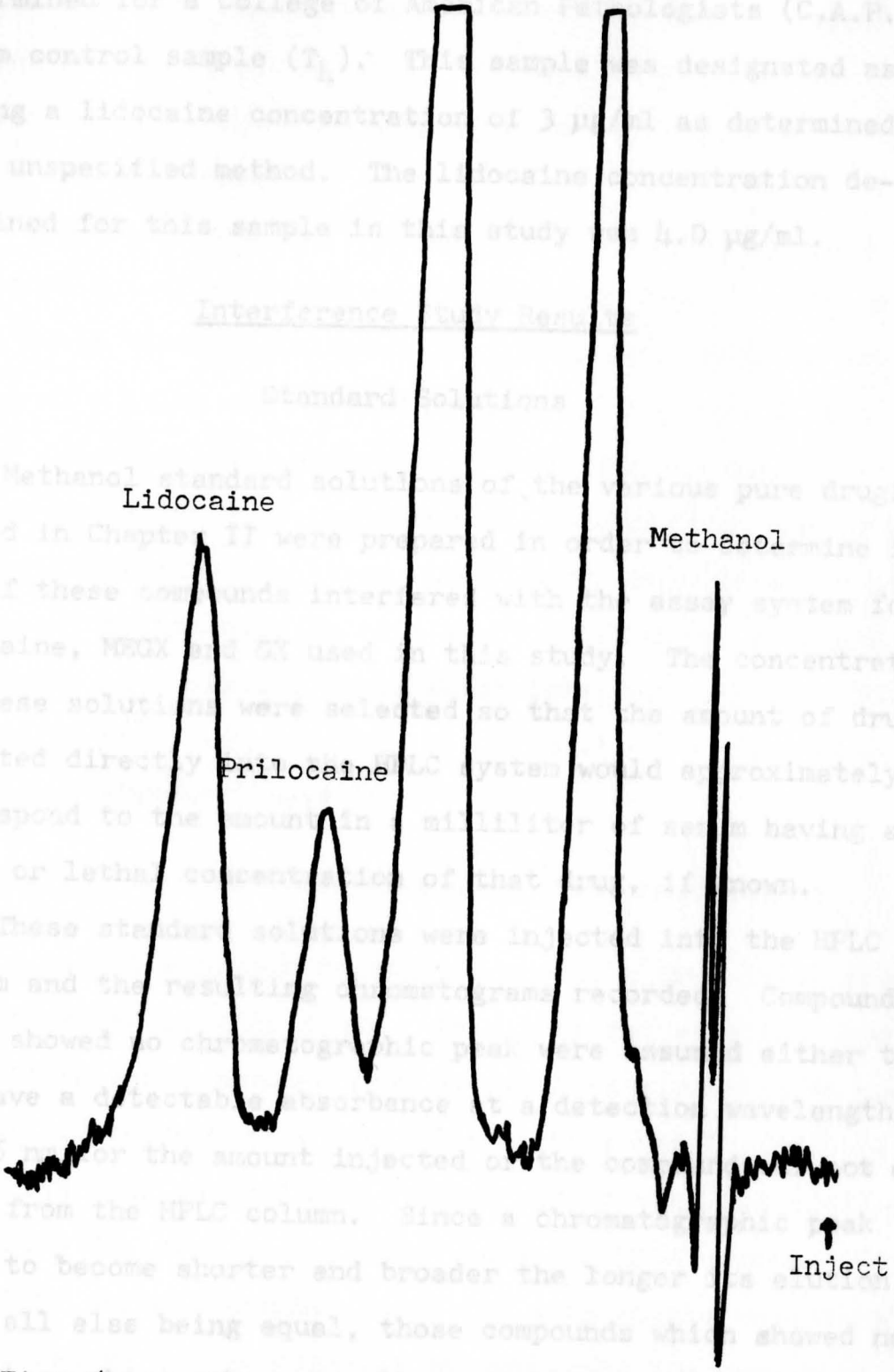


Fig. 51. -- Chromatogram for patient N.J.

determined for a College of American Pathologists (C.A.P.) serum control sample (T_4). This sample was designated as having a lidocaine concentration of 3 µg/ml as determined by a unspecified method. The lidocaine concentration determined for this sample in this study was 4.0 µg/ml.

Interference Study Results

Standard Solutions

Methanol standard solutions of the various pure drugs listed in Chapter II were prepared in order to determine if any of these compounds interfered with the assay system for lidocaine, MEGX and GX used in this study. The concentrations of these solutions were selected so that the amount of drug injected directly into the HPLC system would approximately correspond to the amount in a milliliter of serum having a toxic or lethal concentration of that drug, if known.

These standard solutions were injected into the HPLC system and the resulting chromatograms recorded. Compounds which showed no chromatographic peak were assumed either to not have a detectable absorbance at a detection wavelength of 205 nm for the amount injected or the compound was not eluted from the HPLC column. Since a chromatographic peak tends to become shorter and broader the longer its elution time, all else being equal, those compounds which showed no chromatographic peaks were allowed to run for at least 30 minutes before another sample was injected. In this way if a peak for one compound eluted from the column after another

sample had been injected the shape of the peak in relation to the injection points would indicate an overlap had occurred in which case the samples were reinjected in reverse order for confirmation.

Table 12 shows the elution times for the various drug standard solutions prepared and chromatographed. The table also shows the elution times for lidocaine, MEGX, GX and prilocaine solutions chromatographed at the same time as the other solutions for comparison. This table reveals that of the drugs tested only procineamide, ethosuximide and hydrochlorothiazide have elution times which would interfer with the assay system used in this study. All three of these compounds have elution times which interfer with the MEGX and/or GX peaks but not with the lidocaine or prilocaine (internal standard) peaks.

Serums from Patients Receiving Known Medications

A second approach to studing possible drug interferences was attempted in this study. This approach entailed processing either serum samples from patients receiving known medications or commercially available serum control samples of specified drug composition through the charcoal adsorption procedure followed by injection into the HPLC system. The resulting chromatographic peaks for each sample were then identified as far as possible by comparing the retention times of the sample peaks to the retention time of a standard solution, if available, of the specified drug(s).

Table 13 lists: the serum samples run, what medication(s)

TABLE 12

Elution Times for Some Medications Commonly Given in Conjunction With Lidocaine

Medication	Elution Time (min.)
Isoproterenol	1.6
Hydrochlorothiazide	2.6
GX	2.7
Procainamide	2.8
Ethosuximide	3.0
MEGX	3.7
Prilocaine	5.5
Lidocaine	6.9
Atropine	9.0
Glutethimide	32.0
Phenytoin	38.8
Propranolol	46.8
Digoxin	No Peak Observed
Diphenhydramine	" " "
Diazepam	" " "

TABLE 13

Results for Serum from Patients

Receiving Known Medications

Serum Number	Known Medications	Identified Peaks	Number of Unidentified Peaks
1	Primidone	Primidone	2
2	Primidone and Phenytoin	Primidone and Phenytoin	3
3	Phenytoin	-	1
4	Quinidine	-	1
5	Quinidine	-	1
6	Phenobarbitol	-	2
7	Phenytoin Glutethimide Barbituates Others	Phenytoin Glutethimide	4

the patient was supposedly receiving, the chromatographic peaks which could be identified and the number of peaks for each sample which could not be identified. The most significant result of this segment of the study was that the drug primidone was found to have about the same elution time as the internal standard prilocaine. Both of the samples listed in table 31 as containing primidone showed a peak with an elution time of about 5.2 minutes. This in itself would not be sufficient evidence to identify these peaks as being due to primidone. A standard solution of this drug was unavailable, however the study by Adams et al.(27) established the relative elution time of primidone on a comparable HPLC system. The relative elution pattern of several drugs chromatographed in common to both studies(i.e., lidocaine, procaine, procaineamide and ethosuximide) was the same. Assuming primidone would also have the same relative position in both studies the peak at 5.2 minutes would fit this pattern.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Sensitivity

The HPLC system used in this study had adequate sensitivity for lidocaine, but only marginal sensitivity for MEGX and GX due to the fact that these metabolites are usually present in serum at concentrations of from one-tenth to one-sixth the serum's lidocaine concentration. This could be significantly improved with the proper detection equipment. Commercially available UV detectors capable of detecting at a wavelength at or near 205 nm and designed specifically for the HPLC unit would, no doubt, provide greater sensitivity than the homemade arrangement actually employed in this study.

Selectivity

The results of sera from patients receiving lidocaine therapy demonstrate that extreme caution must be used in interpreting the chromatograms obtained with a single wavelength detector, where peak identification is by retention time only. In order to increase the specificity of the system a dual-wavelength detection system is highly recommeded. Using such a detector two detection wavelengths could be selected for the simultaneous monitoring of the column effluent. The ratio of the two responses could then be determined for individual standard solutions of lidocaine, MEGX and

GX. Each ratio would be characteristic of the compound for which it was determined and could be used to verify the identity of a chromatographic peak. The detection wavelengths would have to be choosen such that lidocaine, MEGX and GX would all have an appreciable absorbance at both wavelengths.

Another approach to increasing the reliablity of the results would be to use two different analysis methods. Since lidocaine, MEGX and GX exist mostly in an ionic form as a quaternary amine at a pH of 6 or less, these substances should be analyzable by the ion exchange (cation) mode of HPLC. An ion exchange HPLC method might prove to be less susceptible to interferences than the adsorption HPLC method used in this study. In any case, as the resolution is determined by completely different parameters in the two modes the peak patterns for any given sample analyzed by both methods would certainly be different. Thus, if both assay modes gave the same lidocaine, MEGX and GX results for a sample of blood from a particular patient the analyst could then be sure of his interpretations. Subsequent monitoring of that particular patient's blood could then be done by a single method with reasonable confidence.

APPENDIX

UV spectrums for lidocaine in various reverse phase solvents and aqueous buffer systems were obtained in order to evaluate their possible use as moving phases in this study. Some of these spectrums are shown on the following pages.

In addition to the solvents and buffers for which spectrums are shown other solvents were found to have high UV absorbances and for this reason were not useable as moving phases in this study. The reverse phase solvents found to have high UV absorbances were: acetone, furfural, acetate buffer (pH 4.6) and phthalate buffer (pH 3.5 and 5.2).

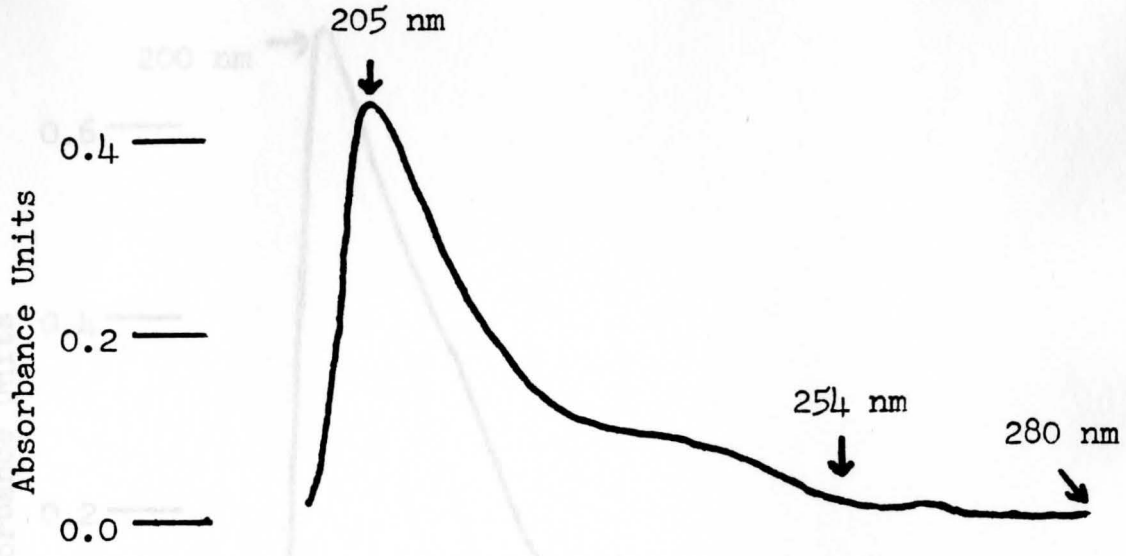


Fig. 52. -- UV Spectrum of lidocaine (5.0 µg/ml) in 1-propanol referenced against 1-propanol.

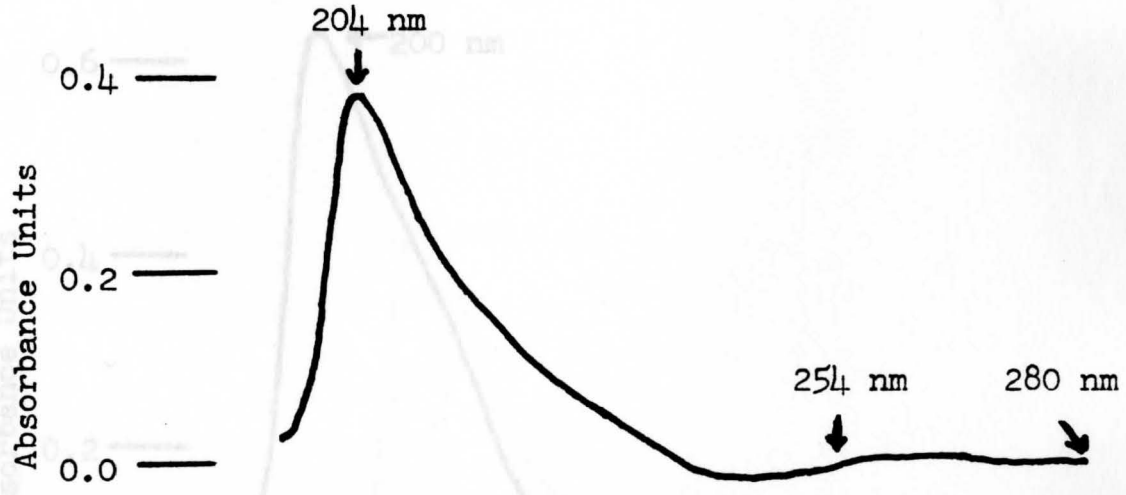


Fig. 53. -- UV Spectrum of lidocaine (5.0 µg/ml) in ethanol referenced against ethanol.

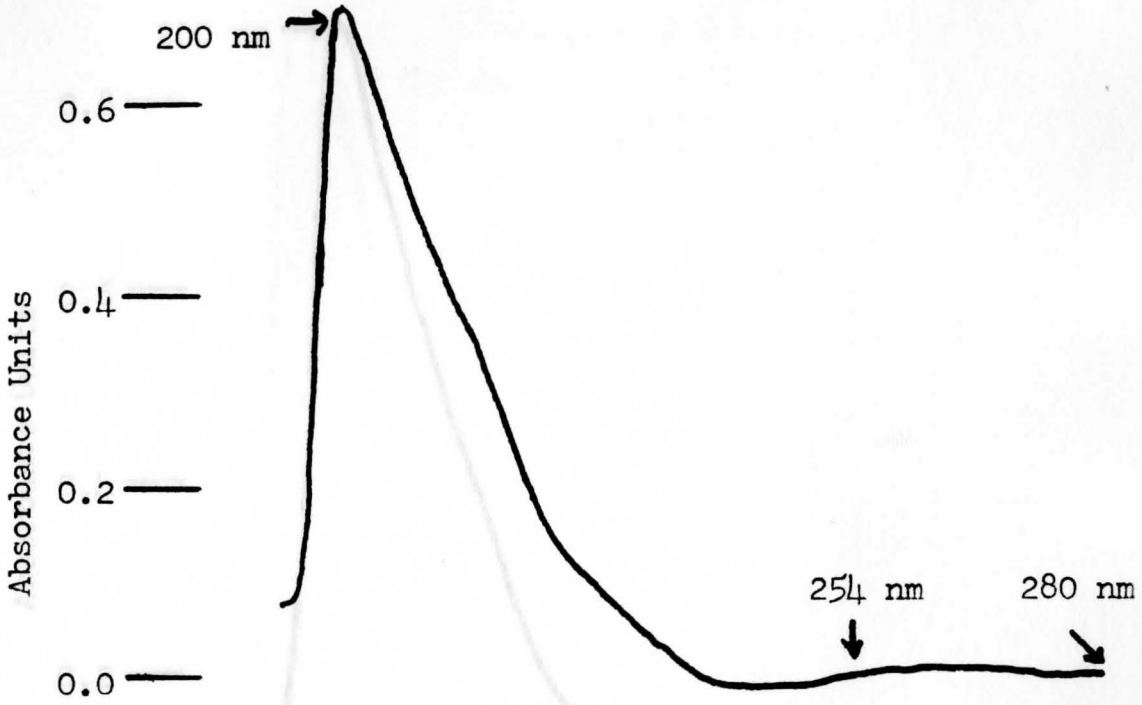


Fig. 54. -- UV spectrum of lidocaine (10 µg/ml) in a potassium chloride-hydrochloric acid buffer of pH 2.0.

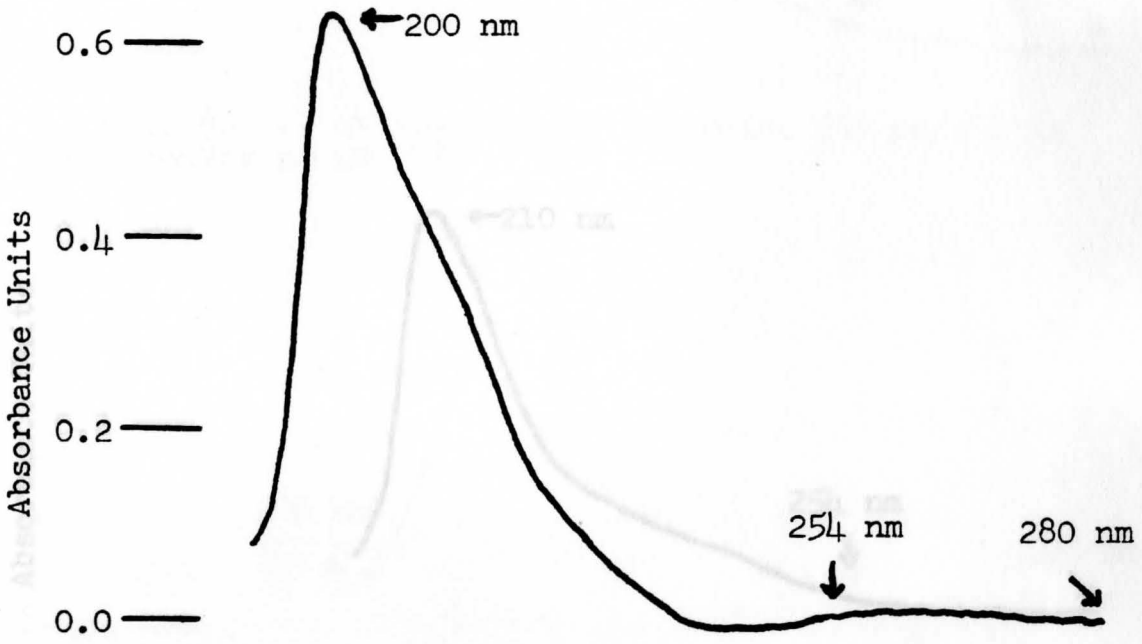


Fig. 55 -- UV spectrum of lidocaine (10 µg/ml) in a phosphate buffer of pH 7.1.

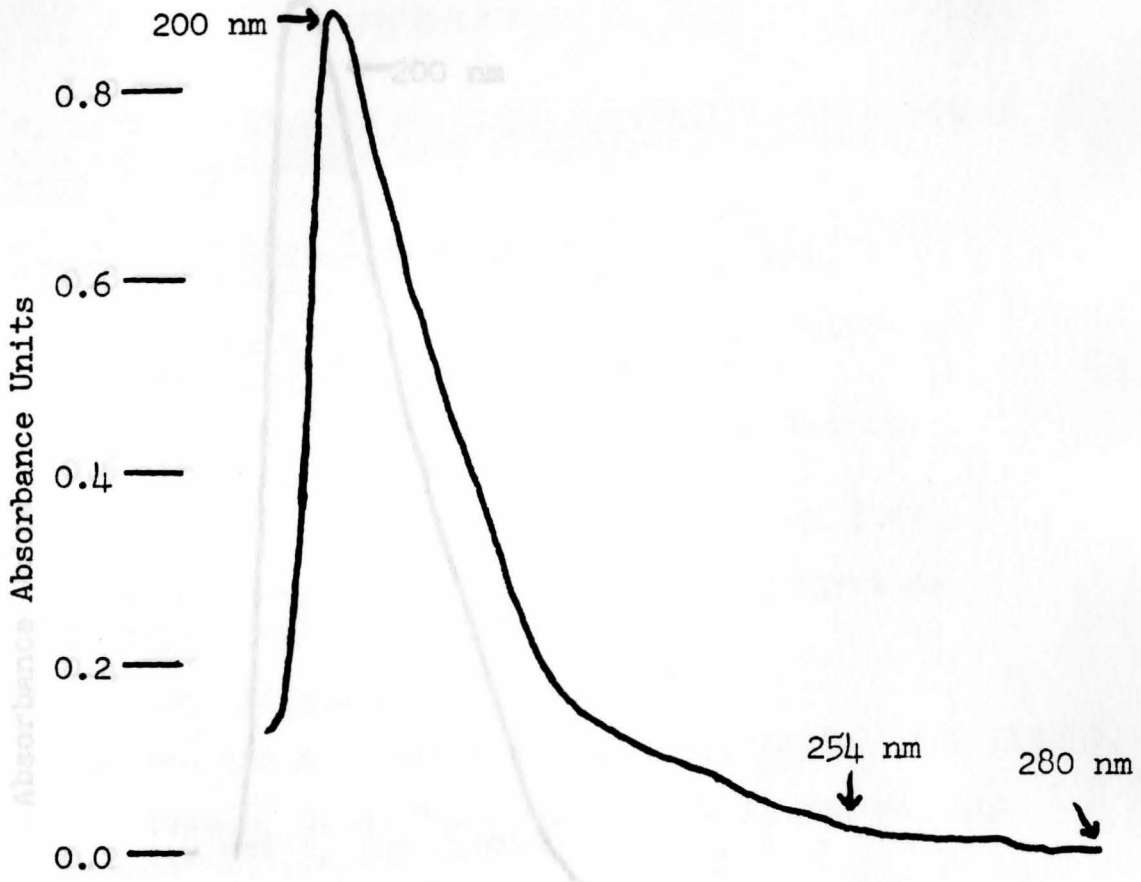


Fig. 56. -- UV spectrum of lidocaine (10 µg/ml) in a boric acid buffer of pH 8.9.

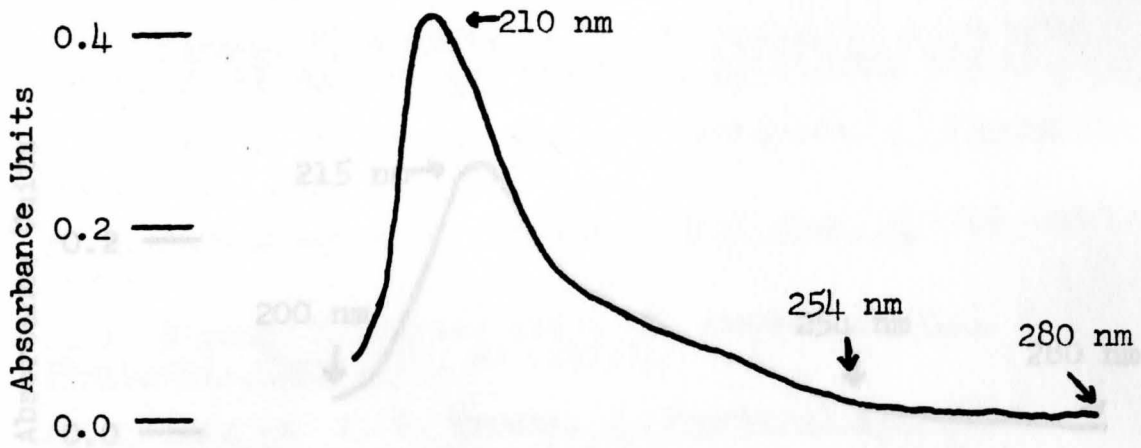


Fig. 57. -- UV spectrum of lidocaine (10 µg/ml) in a tris buffer of pH 8.1.

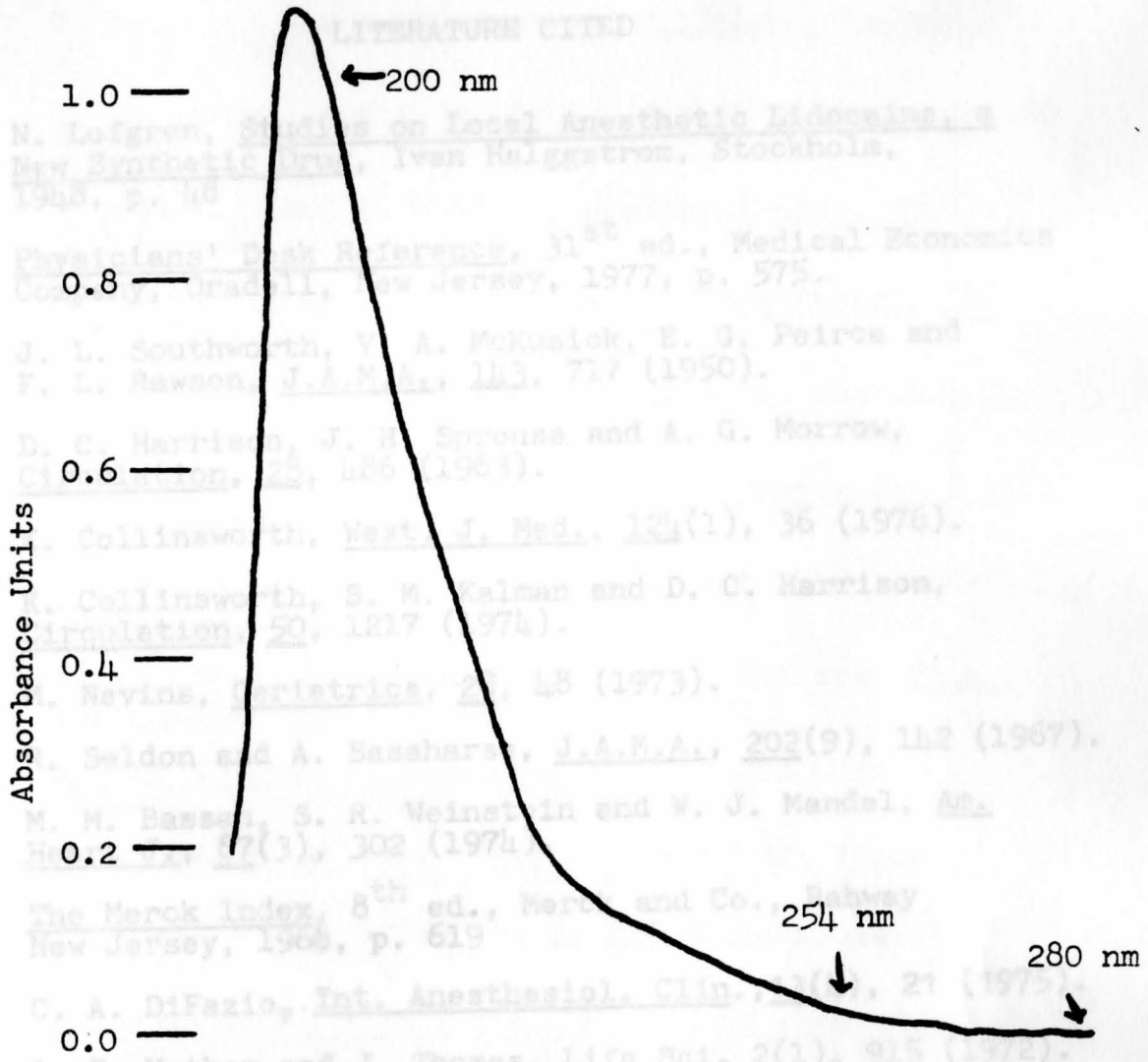


Fig. 58. -- UV spectrum of lidocaine (10 µg/ml) in a Borax buffer of pH 9.8.

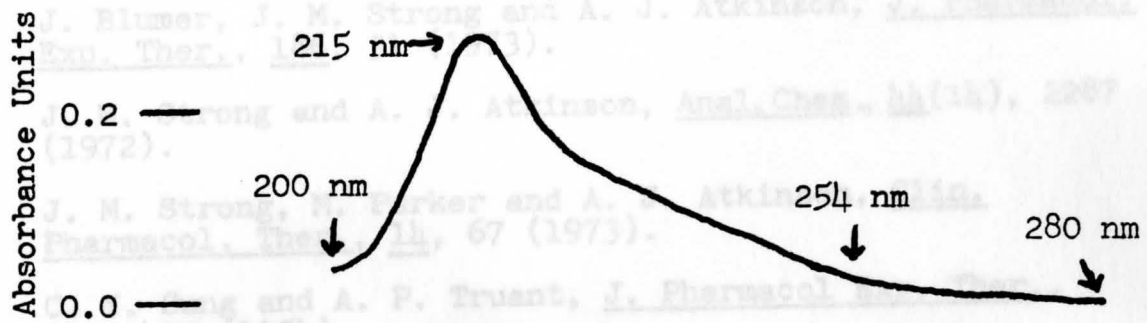


Fig. 59. -- UV spectrum of lidcaine (10 µg/ml) in a Carboxylate buffer of pH 10.7.

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