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Liquid chromatography of methylxanthines with selective amperometric detection

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Liquid chromatography of methylxanthines with selective amperometric detection

by

Eugene Carlton Lewis

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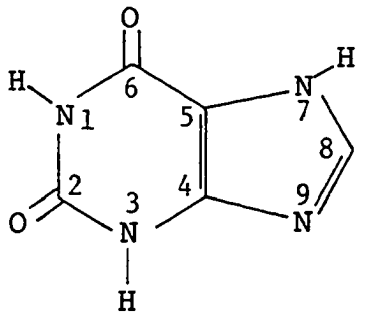
I. INTRODUCTION

> "Electroanalytical chemists who cut their teeth on the reduction of cadmium ion encounter many new problems in dealing with biochemicals." -- A. L. Underwood

Liquid chromatographic analysis with electrochemical detection was conceived over a quarter of a century ago because convenient, continuous, sensitive and selective methods were required for the determination of trace quantities of organic and inorganic substances in complex samples. Revolutionary developments in the theory and application of liquid chromatography during the past decade have been accompanied by renewed interest in the union of electroanalysis with liquid chromatography. Such a combination of techniques remains attractive today because analytical methods with great specificity, high sensitivity and low detection limits are in even greater demand. Such demand can be met when amperometric electroanalysis is coupled to the sophisticated separation technique of liquid chromatography.

This dissertation is concerned with the elucidation of the role of various experimental variables which influence the response of amperometric detectors. Application of a flow-through, tubular detector for amperometric detection of liquid chromatographic separations of methylxanthines in samples of physiological fluids is described.

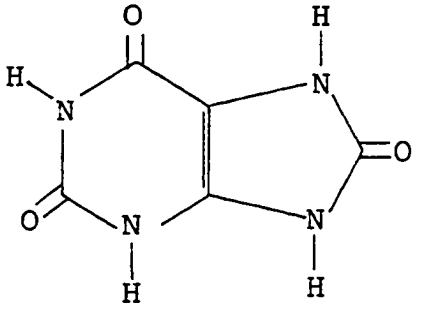
Xanthine (I), or 2,6(1,3)-purinedione, may be substituted with methyl groups at positions 1, 3 and 7 or any combination



(I)

of these positions. Several of the methylxanthines have common names that will be used here. Caffeine is 1,3,7-trimethylxanthine; theophylline is 1,3-dimethylxanthine and theobromine is 3,7-dimethylxanthine. These three methylxanthines are also known as purine alkaloids.

Oxidation of xanthine at the 8 position yields uric acid (II), and the corresponding oxidation of any methyl-



(II)

xanthine yields the corresponding methyluric acid.

If selectivity of an electrochemical method is unsatisfactory because the half-wave potentials of constituents are similar, then liquid chromatography often provides the

additional selectivity desired. This dissertation has as a focal point an alternative approach. The methylxanthines serve as a model system of compounds for which the liquid chromatographic separation may lack the requisite resolution. This model system serves to illustrate that selective response to chromatographically unresolved isomers can be achieved by appropriate choice of detection potential.

Tunable selectivity is a figure of merit for a chromatographic detector. Tabulations of electrochemical properties reveal that even isomers can have different half-wave potentials. In contrast, absorption spectra of organic molecules are often characterized by absorption of a broad range of wavelengths, and isomers generally have similar spectra. Therefore, one must contend with less selectivity if a photometric detector is used. On the other hand, various modes of potential control allow one to "fine tune" amperometric detectors so that even isomers may be distinguished from one another. Thus, the tunable selectivity of an electrochemical detector may be superior to that of the spectrophotometric detector for some applications.

Liquid chromatography with amperometric detection is a promising technique for monitoring concentrations of drugs in physiological fluids. Several of the methylxanthines have therapeutic value and are widely used as drugs (1). For example, theophylline is often prescribed for individuals

suffering from asthma (2, 3). Analytical methods for theophylline are in demand because the narrow therapeutic range dictates that dosage be individualized in management of patient therapy (4-6). Selective methods are required because the physiological sample frequently contains metabolites with similar structures and properties to the unmetabolized drug. These metabolites interfere with the determination if non-selective methods such as ultraviolet spectrophotometry on sample extracts are used (7). A fast and simple chromatographic procedure for the selective determination of theophylline in small volumes of blood serum is described in this dissertation using an ultraviolet detector and an amperometric detector as partners.

Certainly, there are many drugs in addition to the methylxanthines which are probable candidates to illustrate the utility of amperometric detection in the liquid chromatographic analysis of physiological fluids. The methylxanthine group is a good choice for a model system because consumption of beverages containing the methylxanthines is ubiquitous, insuring that these methylxanthines will be found in nearly all samples of physiological fluids. Methylxanthines are familiar because caffeine occurs in coffee, tea, cocoa and cola drinks; theophylline is contained in tea and theobromine is found in cocoa and chocolate products. Thus, the characterization of detector response for methylxanthines is

also of value because of the possible interference of methylxanthines with chromatographic determinations of other substances in biological fluids.

Additional remarks concerning the rationale for this research are appropriate at the outset. Present work in liquid chromatography often requires a number of detectors with various modes of response. Even the most popular liquid chromatographic detectors have severe limitations. Clearly, electrochemical detectors are of great value if the substances to be separated are electroactive but otherwise, can not be detected. Increased qualitative and quantitative characterization of complex samples are realized with minimal investment of additional time when two or more detectors are used in series. As members of such a partnership, amperometric detectors show significant promise.

Perturbations of physiological regulatory mechanisms as a result of disease generally are accompanied by characteristic changes in the chemical composition of the body fluids. In clinical medicine, qualitative identification and quantitative determination of the numerous components of serum and urine are of established diagnostic utility. Robinson and Pauling have advocated chromatographic methods as powerful, yet inexpensive, aids in clinical diagnosis (8, 9). Jellum and co-workers have studied inborn errors in metabolism and have shown that diagnosis of more than

forty such disorders is possible by combination of gas chromatography and mass spectrometry (10-12). Zlatkis and co-workers have used gas chromatography with selective detectors for head-space analysis of urine volatiles in order to diagnose diabetes (13-15). Numerous, exciting, chromatographic successes of a similar nature have been reported (16-21).

Physiological fluids are sufficiently complex in composition to tax the resolving power of liquid chromatography. A single broad spectrum detector may be unsatisfactory for the purpose of multicomponent diagnosis. This dissertation concludes with an exploratory investigation of the utility of amperometric detectors for multicomponent analysis of urine. The presence of the methylxanthines in urine samples is inevitable. Therefore, the effect of coffee consumption on a metabolic profile of urine is reported for the amperometric mode of detection.

II. SURVEY OF PERTINENT LITERATURE

> "The analytical chemist must study ... (all aspects) ... of the problem before developing an analytical solution to a problem that doesn't exist or to a problem that is insignificant."
> -- Merle A. Evenson

A. Electrochemical Detection for Liquid Chromatography

Müller made a fascinating contribution to continuous-flow electroanalysis (22) in 1947. Müller reported the conditions for which reproducible analytical data could be obtained at a stationary platinum electrode placed in a flowing solution for determinations of electroactive substances in that solution. Flow rate, temperature, identity and concentration of supporting electrolyte, scan rate, the electrode history and pretreatment of the electrode were the conditions reported to be critical. Although Müller suggested that the platinum electrode would be of value in the positive potential region beyond the potential limit for mercury, early attention focused on mercury as the electrode material for construction of chromatographic detectors.

The first application of polarography with the dropping mercury electrode for automatic monitoring of chromatographic effluents was not published until 1950 when Drake reported a liquid chromatographic separation of proteins with electrochemical detection (23). Kemula is often given credit

for making the earliest contribution to this area; however, his first papers on this subject appeared in the literature more than a year after the Drake publication (24-27). More than one hundred reports have appeared in the literature describing the application of electrochemical detection in liquid chromatography; nearly half of these papers were contributions of Kemula and co-workers. This tireless effort in the technique Kemula called "chromato-polarography" has been reviewed elsewhere (28, 29).

Additional chromatographic contributions using mercury as the detector electrode have appeared (30-48). The favorable potential range of mercury electrodes for the study of cathodic reactions has been a frequent justification for using mercury. The renewable, reproducible surface of the dropping mercury electrode also contributed to the early acceptance of mercury as the electrode material of choice.

Improved understanding of the surface processes occurring at solid electrodes has led to recent acceptance of solid electrodes for reliable analytical work. Reports of advances in theory and use of solid electrodes in flowing solutions continued to appear after the contribution by Müller (22). The excellent monograph by Adams is recommended for a detailed treatment of electroanalysis with solid electrodes (49). Much interest in solid electrodes originated as a

consequence of curiosity concerning anodic reactions of organic substances. Mercury is of very limited utility for anodic work.

Apparently, solid electrodes were not applied as detectors for liquid chromatography until 1965 (50). Since then copper, various forms of carbon, platinum, platinum coated with mercury, platinum coated with iodide, gold, silver and cadmium have been used as electrode materials for electrochemical detectors for liquid chromatography. A concise, but informative survey of the literature of electrochemical detection in liquid chromatography using these solid electrode materials is presented in Table II.1. Reports of the use of mercury as the electrode material were omitted for the sake of brevity and because mercury electrodes were not used in the research described in this thesis.

B. Pharmacology of Methylxanthines

Methylxanthines have numerous pharmacological actions and are used therapeutically for their effects on cardiac muscle and the cardiovascular system, the central nervous system, the kidney and smooth muscle (1). Caffeine is generally used as a stimulant of the central nervous system. Theophylline and theobromine find application for diverse cardiovascular actions. Of the methylxanthines, theophylline produces the greatest diuretic action. Theophylline is used commonly for symptomatic relief of asthma (2, 3) and in

Table II.1. Solid electrodes as detectors for liquid chromatography

Substances Detected	Electrode Material[a]	Mode of Detection[b]	Mode of Separation[c]	Reference Numbers
INORGANIC SUBSTANCES				
Metals				
Alkaline earth				
Mg(II), Ca(II), Sr(II), Ba(II)	C	COUL	IE(A)	51
Transition				
Fe(III), Au(III), Cu(II)	Cu	COUL	IE(C)	50, 52, 53
Cd(II), Cu(II)	C(rubber)	AMP(dc)	ADS	45
Cu(II), Fe(III)	Pt	COUL	IE(A)	54
Hg(II), Cu(II), Zn(II), Ni(II), Pb(II), Cd(II), Co(II)	C	COUL	IE(A)	51
Sb(III), Fe(III)	Pt(ads. I)	COUL	IE(A)	55
Nonmetals				
Halides	Ag	COUL	IE(A)	51
Nitrate, Nitrite	Cd	AMP(dc)	IE(A,C)	56
Se(IV)	Au	ASV	IE(C)	57
ORGANIC SUBSTANCES				
Acids				
Amino	Cu	COUL	IE(C)	52
	C	COUL	IE(C)	51
Ascorbic	CP	AMP(dc)	IE(A)	58-62
Biogenic aromatic	GC	COUL	LLC(R)	63
Carboxylic	C	COUL	IE(C)	51
Phenolic	CP	AMP(dc)	IE(A)	60, 62, 64
	CP	AMP(dc)	LLC(R)	65
Uric	CP	AMP(dc)	IE(A)	59, 61, 66-68

Alkaloids				
Tetrahydroisoquinoline	CP	AMP(dc)	IE(C)	69
Amines				
Amino acids	See Acids			
Biogenic (Catecholamines L-DOPA)	CP	AMP(dc)	IE(A,C)	58, 59, 61, 62, 70-76
Carcinogenic (benzidine, o-Dianisidine, naphthylamines)	CP	AMP(dc)	IE(C)	77
Ketones				
Benzil	Hg-Pt	AMP(dc)	LLC(R)	78
Nitro Compounds				
Nitroanaline	Hg-Pt	AMP(dc)	LLC(R)	78
Nitrophenol	C(rubber)	AMP(dc)	ADS	45
	Pt, C, Au	AMP(dc,p,dp)	LLC(R)	79
	GC	AMP(dc,p)	IE(C)	46
Nitropyridine derivatives	Hg-Pt	AMP(dc)	LLC(R)	78
Phenols				
Acetaminophen	CP	AMP(dc)	IE(C)	61, 62, 80
Catecholamines	See Amines			
Nitrophenol	See Nitro compounds			
Other phenols	C, Pt, Au	AMP(dc,p,dp)	LLC(R)	79
	C	COUL	IE(C)	51
	CP	AMP(dc)	IE(C)	80

[a]Atomic symbols are used with these exceptions: Pt(ads. I) = Platinum coated with iodide, CP = carbon paste, GC = glassy carbon and Hg-Pt = Platinum coated with mercury.

[b]COUL = coulometric, AMP(dc) = amperometric, ASV = anodic stripping voltammetry, AMP(p) = pulse amperometric, AMP(dp) = differential pulse amperometric.

[c]Type of chromatography IE(A,C) = ion-exchange(anion,cation), ADS = adsorption, LLC(R) = reverse-phase, GPC = gel permeation.

Table II.1. (continued)

Substances Detected	Electrode Material	Mode of Detection	Mode of Separation	Reference Numbers
Other phenols (continued)	CP	AMP(dc,dp)	IE(C)	81
	Pt	COUL	IE(A)	82
Phenolic acids See Acids				
Phenothiazines	GC	COUL	LLC(R)	63, 83
Purines	C(rubber)	AMP(dc)	GPC	84
Uric acid See Acids				
Quinones	CP	AMP(dc)	IE(C)	58
	C	COUL	IE(C)	51
	Hg-Pt	AMP(dc)	LLC(R)	78
Steroids				
Estrogens	GC	AMP(dc,p)	IE(C)	46
Sulfanilamide	CP	AMP(dc,dp)	IE(C)	81

apnea (85, 86), a transient cessation of the breathing impulse which is often observed in premature infants.

Toxic side effects can accompany administration of methylxanthines (1, 4, 5). Considerable attention has been focused on theophylline. Marked differences in the rate of biotransformation of theophylline have been reported (85, 87, 88). The pharmacokinetics of theophylline in children can not be assumed to be identical to adults. Even within a group of children on theophylline therapy, the biologic half-life of theophylline ranged from 1.4 to 7.8 hours in one study (85). Such findings make measurement of the concentration of theophylline in serum or plasma obligatory because this concentration must be maintained within a narrow therapeutic range in the management of asthma and apnea (5, 6). Relief is rarely obtained when serum levels fall below 10 mg/l. For serum levels exceeding 20 mg/l, a greater incidence of untoward side reactions is observed. Toxic effects include nausea, vomiting, headache, decrease in blood pressure, palpitation, or in extreme cases, convulsions and even death (1).

Dosage regimens based on milligrams of theophylline per kilogram of body weight are inadequate. Understandably, sensitive and selective methods for monitoring theophylline concentration in serum are in demand by cautious physicians for guidance in the prescription of individualized dose

regimens. The alternative is the increase of dosage progressively until a toxic reaction develops and then the reduction of the dose until toxic reactions are no longer observed (3).

C. Metabolism of Dietary Methylxanthines

Metabolic transformation of theophylline and the other dietary methylxanthines challenges the selectivity of many analytical methods. Awareness of the metabolic products of methylxanthines gives direction to method development. These potential interferences must be examined in any method involving the selective determination of theophylline in physiological samples.

The fate of the dietary methylxanthines in the body has been the subject of several investigations (89-92), but the report of Cornish and Christman is most comprehensive (91). Their results are summarized in Table II.2. In a more recent report concerning theophylline metabolism (92), Thompson, Nagasawa and Jenne made use of sophisticated liquid chromatographic techniques. They have confirmed the earlier reports and, once again, noted the conspicuous absence of 1-methylxanthine and 3-methyluric acid as urinary metabolites of theophylline. From Table II.2 one notes that all of the possible methylxanthines may be present in the body fluids of individuals who consume beverages containing these dietary methylxanthines.

Table II.2. Urinary metabolites of dietary methylxanthines (91)

Compound Ingested	Major Urinary Metabolites Identified
Theophylline (1,3-dimethylxanthine)	1,3-dimethyluric acid 1-methyluric acid 3-methylxanthine
Caffeine (1,3,7-trimethylxanthine)	1,3-dimethyluric acid 1-methyluric acid 1,7-dimethylxanthine 1-methylxanthine 7-methylxanthine
Theobromine (3,7-dimethylxanthine)	7-methyluric acid 3-methylxanthine 7-methylxanthine

D. Electrochemical Investigations of Purines

Anodic reactions of purines have been studied using cyclic voltammetry at pyrolytic graphite electrodes (93-101). Hansen and Dryhurst studied the oxidation of theobromine and caffeine (97), theophylline (98) and other xanthines (99). Dryhurst has reviewed this work (100, 101), and his reviews and original papers should be consulted for detailed information regarding mechanisms. Among the valuable aspects of these studies of the anodic behavior of purines was the study of the pH dependence of the voltammetric peak potential. Dryhurst summarized this study in reference 100; a portion of his table is reproduced here as Table II.3.

The knowledge that these compounds are electroactive contributed to the choice of the methylxanthines as the

Table II.3. Dependence of anodic E_p on pH for selected purines (100)

Compound	Peak	pH Range	E_p (V vs. SCE)
7-methylxanthine	I	7-12.4	1.19-0.049 pH[a]
	II	0-12.5	1.22-0.042 pH
3-methylxanthine	I	5.5-12.5	1.20-0.056 pH[a]
	II	0-11.9	1.27-0.050 pH
1-methylxanthine	I	0-12.5	1.05-0.049 pH
3,7-dimethylxanthine (theobromine)	I	2.3-5.5	1.67-0.064 pH
1,3-dimethylxanthine (theophylline)	I	4-9	1.35-0.069 pH[a]
	II	2.3-8.5	1.45-0.056 pH
1,7-dimethylxanthine	I	0-12.5	1.31-0.059 pH
1,3,7-trimethylxanthine (caffeine)	I	2.3-5.5	1.59-0.042 pH
uric acid	I	2.3-5.7	0.76-0.069 pH
xanthine	I	0-12.5	1.07-0.060 pH
hypoxanthine	I	0-5.7	1.27-0.067 pH

[a]Adsorption peak.

subject of the research described in this dissertation. The peak potentials are quite similar for isomeric methylxanthines so it is doubtful that electrochemical techniques could be used without prior separation to determine the methylxanthines. Other metabolites are electroactive as well, e.g., uric acid, xanthine and hypoxanthine. A separation would undoubtedly be required for such samples.

This choice of model compounds is more real than ideal. As one observes at the outset, adsorption to the electrode material may be troublesome for chromatographic detection of some compounds at certain pH values with an electrochemical detector.

E. Determination of Theophylline

Although official methods are available for determination of methylxanthines in coffee, tea, cola and chocolate products (102) and in pharmaceuticals (102, 103), these methods are generally unsuitable for measuring the small amounts of theophylline and other methylxanthines in physiological fluids. Schack and Waxler published the first procedure for determination of plasma theophylline (7). Their ultraviolet spectrophotometric method involved two tedious extractions. Nevertheless, the method lacked specificity. A 3-ml sample of plasma (5 ml of whole blood) was required for this assay. After a single attempt to collect a blood sample from an infant, only Dracula would need encouragement to search for a better method.

Fortunately, there is no longer a shortage of methods for determination of theophylline in biological fluids. Chromatographic methods for theophylline in serum, plasma and saliva samples are being published at an explosive rate. The survey given here of this literature is not exhaustive. Gas chromatographic methods for theophylline in small samples

have been published, but require prior extraction of the sample or derivatization (104, 105). One realizes, from reviewing this extensive literature, that the use of liquid chromatography for the purpose of monitoring theophylline concentration is an idea whose time has arrived. Adsorption (106-108), ion-exchange (92, 109), normal phase (110) and reverse phase liquid chromatography (6, 111-113) have been used successfully to monitor theophylline in physiological fluids without significant interference from dietary methylxanthines. In general the claim is made that these liquid chromatographic procedures for theophylline are free of interference from other methylxanthines and metabolites of methylxanthines. Most studies have neglected to examine 1,7-dimethylxanthine, a caffeine metabolite. Theophylline and 1,7-dimethylxanthine were only partially resolved by the ion-exchange procedure of Thompson, et al. (92). No unequivocal reports of complete separation of these two isomers by reverse-phase chromatography have been published. Orcutt, et al. (112) suggested that adjustment of their mobile phase composition to 5% acetonitrile from 7% acetonitrile would obviate interference from a substance in randomly selected serum samples that they presumed to be 1,7-dimethylxanthine. However, they did not report any attempt to accomplish this separation of theophylline from 1,7-dimethylxanthine of known purity. Orcutt, et al.

reported a positive interference in nearly one quarter of the blank serum samples equivalent to 0.25 to 3.5 mg/l if reported as theophylline.

III. INSTRUMENTATION

"... with thimble and thread and wax and hammer and buckles and screws, all such things as geniuses use; two bats for patterns, curious fellows! A charcoal pot and a pair of bellows; some wire and several old umbrellas; a carriage cover, for tail and wings; a piece of harness; and straps and strings; and a big strong box in which he locks these and a hundred other things. ... The greatest invention under the sun! 'An' now,' says Darius, 'hooray fer some fun!'"

-- John Townsend Trowbridge

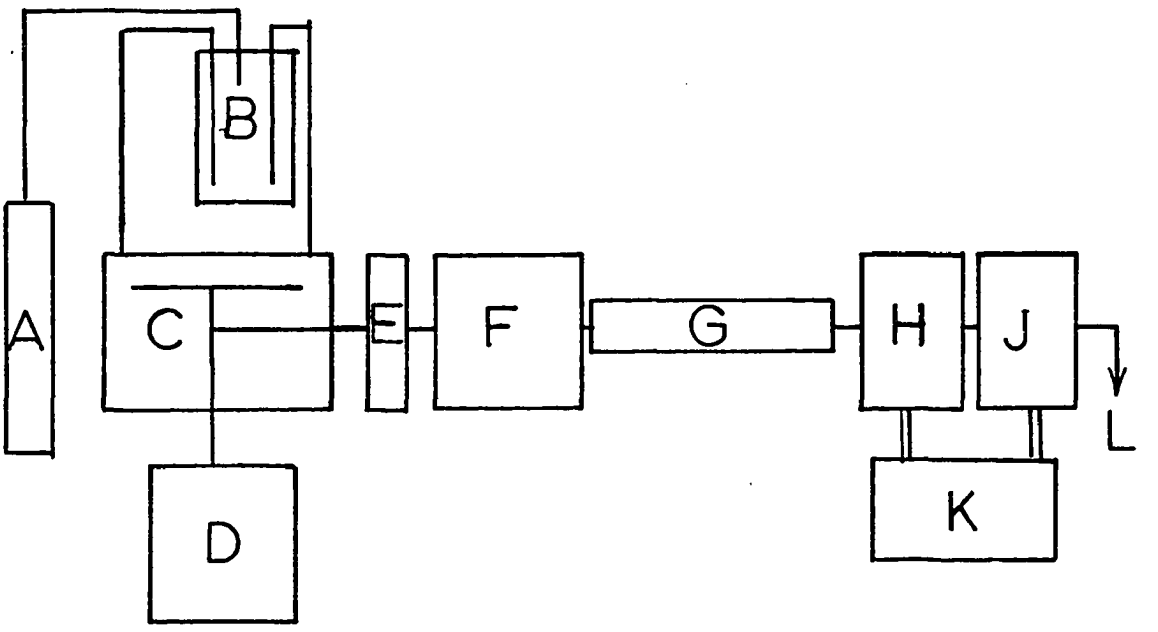
"And now I see with eye serene the very pulse of the machine."

-- William Wordsworth

A. Liquid Chromatograph

The liquid chromatograph was assembled from components that are available commercially. The apparatus was constructed according to published designs (114), and a block diagram of the apparatus is shown in Figure III.1. Low pressure connections were made with Altex tube-end fittings purchased from Larry Bell Associates of Hopkins, Minn.; high pressure connections were made with 316 stainless steel Swagelok fittings available from Hawkeye Fitting and Valve Co. of Des Moines, Ia.

The mobile phase delivery system consisted of a Milton Roy Instrument miniPump® and a Model 709 pulseDampener® obtained from Laboratory Data Control of Riviera Beach, Fla. The adjustable bellows of this dampening device is rated



A Helium cylinder
B Eluent reservoir
C Pump
D Pulse dampener and pressure gauge
E 2-μm filter
F Sample injection valve
G Chromatographic column
H Ultraviolet detector
J Electrochemical detector
K Recorder
L Aspirator

Figure III.1. Block diagram of the liquid chromatograph

for use at pressures between 200 psi and 1200 psi. For discharge pressures exceeding 1200 psi, the bellows is protected by an isolation valve; the bourdon pressure gauge serves as the only pulse dampener when the isolation valve is closed. The mobile phase was sparged of other gases in the reservoir by helium pressure in order to prevent bubble formation in the pulse dampener and bubble formation at the outlet of the chromatographic column.

A Nupro, 2-μm filter made of sintered stainless steel available from Hawkeye Fitting and Valve Co. was placed between the pulse dampener and the injector to protect the chromatographic column from particulate material in the mobile phase.

A Rheodyne Model 7120 loop injector available from Larry Bell Associates was used. The fixed-volume sample loop could be loaded partially or completely as desired at full operating pressure. The volumes of the sample loops available were 10 μl, 20 μl, 50 μl, 100 μl, 151 μl and 220 μl. The latter two loops were made and calibrated locally. A portion of the sample, approximately 0.5 μl, remained within the injector after injection but this consideration was of no consequence for this research because the sample loop was always completely filled; the portion of the previous sample was flushed out completely when loading the loop with the next sample.

A μ Bondapak® C_{18} chromatographic column (Column A) from Waters Associates of Milford, Mass., was used for most of the separations described. A Bondapak® C_{18}/Corasil column (Column B) also from Waters Associates was used for selected experiments.

The effluent from the column passed through one or more detectors. The Model 200 ultraviolet photometric detector from Chromatronix, Inc. (now Spectra-Physics of Santa Clara, Calif.), operating at a wavelength of 254 nm was used for some experiments. The electrochemical detectors and associated circuitry are described in subsequent sections.

Liquid flowing through the chromatographic system contacted only inert materials such as Kel-F, Teflon, quartz, glass and 316 stainless steel.

Chromatograms were recorded with a Model SR-255B Strip chart recorder from the Heath Co. of Benton Harbor, Mich.

B. Tubular Electrochemical Detectors

A cross-sectional diagram of the platinum tubular detector is shown as Figure III.2. A second detector of similar design was constructed with a glassy carbon electrode. These detectors were constructed by Pine Instrument Co., Grove City, Penn. The platinum tubular electrode had an internal diameter of 1.0 mm and a length of 5.0 mm with an internal volume of 15.7 μl. The glassy carbon tubular electrode had an internal diameter of 1.09 mm and a length

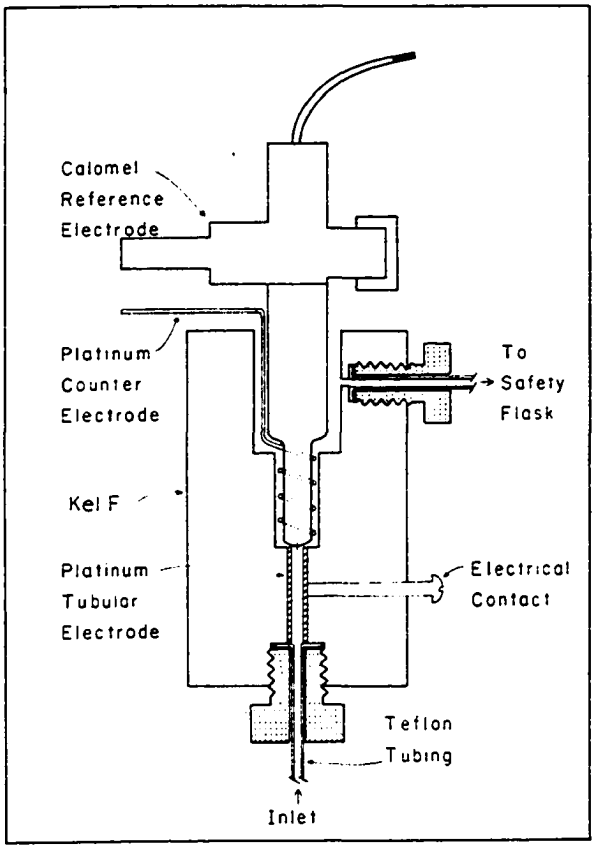


Figure III.2. Cross-sectional diagram of the platinum tubular detector

of 3.17 mm with an internal volume of 11.8 µl. Electrical contact to each of these electrodes was made through the wall of the detector body. The reference electrode used for most experiments was a Model 39270 fiber-junction calomel electrode from Beckman Instruments, Inc., Fullerton, Calif. All potentials are given with respect to this reference electrode unless specifically stated otherwise. A nonaqueous reference electrode consisting of a silver wire in contact with silver nitrate (0.01 M) dissolved in acetonitrile available from Koslow Scientific Co., North Bergen, N. J., was used for selected experiments. The counter electrode was a coil of 21-gauge platinum wire wrapped around the reference electrode probe. Waste liquid was removed by aspiration.

C. Potentiostatic Instrumentation

A PAR Model 174A Polarographic Analyzer from Princeton Applied Research Corp. of Princeton, N. J., was used to control the potential of the electrochemical detector for many experiments. The PAR 174A was designed for voltammetry with various modes of potential control. These modes include d.c., sampled d.c., pulse and differential pulse. When used as a chromatographic detector in the d.c. mode, the potential is held constant at the desired detection potential and the amperometric response is observed. Potential pulses of constant amplitude are applied by the potentiostat in the pulse mode and current is sampled only at the final potential.

The amplitude of the potential pulse may be set at any desired value. A voltage proportional to the absolute value of the current is presented to the recorder. This mode of chromatographic detection is called pulse amperometric detection. Differential pulse operation is similar to pulse operation; however, current is sampled at both the initial and final potential. A voltage proportional to the difference of the absolute values of current at these two potentials is presented to the recorder. Only a limited number of options of pulse amplitudes are available; these are 5 mV, 10 mV, 25 mV, 50 mV and 100 mV. This mode of chromatographic detection is called differential-pulse amperometric detection. The sampled d.c. mode was not used.

A second potentiostat was constructed locally, and the design has been published (115, 116). This potentiostat (ADAM 5) is more versatile than the PAR 174A for amperometric operation because background currents may be subtracted readily by balancing the background signal with an equal signal of opposite sign using a summing amplifier. Attenuation of chromatographic peaks is easily accomplished by changing the feedback resistor of the summing amplifier. Attenuation of chromatographic peaks is inconvenient or impossible with the PAR 174A if any background offset is needed.

IV. EVALUATION OF PARAMETERS WHICH INFLUENCE DETECTOR RESPONSE

> "... with a twitch of his little finger the electrochemist can alter rate constants through many order of magnitude. In view of this almost divine power it is not surprising that devotees of the cult have surrounded it with enough mumbo-jumbo to keep its arcane mysteries concealed from the eyes and understanding of lesser mortals.
> -- J. Albery

A. Introduction

The electrolytic efficiency of a tubular electrode is low, typically less than 1%. The efficiency, and hence, the detector sensitivity, is influenced by a multitude of parameters including flow rate of the solution containing the electroactive material. Small variations of flow rate produce significant changes in the amperometric response, and frequent calibration is required when flow rate cannot be adequately controlled.

Electrochemical detectors have been designed which electrolyze completely (100%) the electroactive constituents of the flowing stream, and these detectors have been applied to liquid chromatography (50-52, 54, 63, 82, 83). Such detectors have been named coulometric detectors and the response is predicted by Faraday's law:

$$Q = nF(\text{moles}) \qquad \text{(IV.1)}$$

where Q is the time integral of the electrode current

(coulombs) and n and F have their conventional electrochemical meaning. Only occasional, precautionary calibration of a coulometric detector is necessary once the efficiency is verified to be 100%. Coulometric electrodes were not used in the research described here.

Amperometric detectors may be used most effectively if the influence of each parameter is elucidated. Which variables are influential? Amperometric response is profoundly influenced by the electrode material and geometry as well as the history of the electrode surface. Solvent-electrolyte combinations which are suitable for electrochemical studies are not always satisfactory for use as chromatographic eluents; effluents must have sufficient conductivity for amperometric response. Temperature, flow rate and pH of the eluent may affect the amperometric response as well as the chromatographic separation. The half-wave potential varies with pH for many organic compounds and the chromatographic retention behavior may be affected by pH. Detection potential and the mode of potential control are among the salient parameters which govern detector response. The half-wave potential depends on concentration if unequal stoichiometric coefficients are involved in the electrode reaction; calibration curves are non-linear if the detection potential is chosen in the vicinity of the variable half-wave potential.

Furthermore, the mechanism of the reaction may also depend on concentration of the substance to be detected.

Amperometric detection with flow-through electrodes can be made in potential regions generally considered inaccessible for conventional voltammetric electroanalysis. The signal-to-noise ratio is diminished, however, by high background currents in this expanded range of potentials. Nevertheless, analytical data can be obtained for substances that are electroactive in the potential region of solvent-electrolyte decomposition or where high background currents completely obscure conventional voltammetric response. Electroactivity of a substance does not disappear just because the solvent or electrolyte is more easily oxidized.

Frequent calibration for amperometric detectors is required because precise control of the many variables is difficult. This chapter focuses on the influence of salient electrochemical parameters on the performance of amperometric detectors.

B. Experimental

1. Chemicals and reagents

Solutions used as eluents were prepared with "distilled-in-glass" acetonitrile available from Burdick and Jackson Laboratories of Muskegon, Mich., and distilled water that had been deionized by passage through a column packed with

Amberlite MB-3, a mixed-bed ion-exchange resin, supplied by Mallinckrodt, Inc. Although absolute purity was uncertain, individual solutions of the organic compounds used in this work appeared to be chromatographically pure, - that is, only a single chromatographic peak was observed - and further purification was unnecessary. Theophylline, theobromine and uric acid were obtained from Matheson, Coleman and Bell. Caffeine was supplied by Eastman Chemical Co. Aldrich Chemical provided the 3-methylxanthine, and Adams Chemical Co. of Round Lake, Ill., furnished the 1-methylxanthine. The supporting electrolyte solutions were prepared from the best grade of material available locally; each chemical was analytical reagent grade (AR) except the phosphoric acid which was equivalent in purity to U.S.P. specifications. The sodium acetate trihydrate, glacial acetic acid and potassium hexacyanoferrate(II)trihydrate were obtained from J. T. Baker, Inc.; anhydrous sodium monohydrogen phosphate, sodium dihydrogen phosphate monohydrate, phosphoric acid and ammonium acetate were supplied by Fisher Scientific Co. The ammonium carbonate originated from West Germany but was obtained through Mallinckrodt, Inc.

2. Preparation of solutions

The solvent-electrolyte solutions used as sample diluents and as chromatographic eluents were prepared as described on the following pages.

Eluent A: Aqueous 0.1 M ammonium carbonate was prepared by dissolution of 19.2 g of ammonium carbonate in two liters of deionized water. This solution was mixed thoroughly with 222 ml of acetonitrile to give a solution consisting of 10% (v/v) acetonitrile.

Eluent B: This acetate-buffered eluent was prepared by mixing 27.2 g of sodium acetate trihydrate and 25.0 ml of glacial acetic acid with sufficient deionized water to make the total volume equal to two liters. This solution had a pH of 4.5. To this solution was added 105 ml of acetonitrile to give an eluent consisting of 5% (v/v) acetonitrile.

Eluent C: This eluent was prepared by addition of 20 g of ammonium acetate to 2 l of deionized water; this entire volume of solution with a pH of 7.0 was mixed with 105 ml of acetonitrile to give an eluent of 5% (v/v) acetonitrile.

Eluent D: To a 2-l volumetric flask were added 4.1 g of sodium acetate trihydrate, 10.8 ml of glacial acetic acid and sufficient deionized water to make the total volume equal to two liters. The analytical concentration of acetate in this solution was 0.1 M and the pH was 3.75. Two liters of this aqueous acetate buffer was mixed with 105 ml of acetonitrile to give a solution of 5% (v/v) acetonitrile.

Eluent E: Sufficient deionized water, 41 g of sodium acetate trihydrate and 108 ml of glacial acetic acid were mixed in a 2-l flask to give an aqueous acetate buffer with an

analytical concentration of 1.0 M and a pH of 3.95. To obtain an eluent consisting of 5% (v/v) acetonitrile, 105 ml acetonitrile was added to two liters of this solution.

Eluent F: Four liters of deionized water was added to a large conical flask containing 56.8 g of sodium monohydrogen phosphate and 55.2 g of sodium dihydrogen phosphate monohydrate. This solution was an equimolar mixture of monobasic and dibasic sodium phosphate with a pH equal to 6.9 before addition of acetonitrile. After dissolution was complete, 210 ml of acetonitrile was added to give an eluent of 5% (v/v) acetonitrile.

Eluent G: This solution was prepared by mixing four liters of deionized water with 22.6 ml of concentrated phosphoric acid and 55.2 g of sodium dihydrogen phosphate monohydrate in a large conical flask. This phosphate buffer had a pH of 2.3. To this solution was added 210 ml of acetonitrile to give an eluent of 5% (v/v) acetonitrile.

Eluent H: To four liters of deionized water was added 55.2 g of sodium dihydrogen phosphate monohydrate and 210 ml of acetonitrile to give a solution of 5% (v/v) acetonitrile.

Stock solutions of theophylline, theobromine, caffeine, uric acid, 3-methylxanthine and 1-methylxanthine were prepared utilizing as diluent the eluent used for that particular experiment. The dissolution of these materials was straightforward except that the solubility of uric acid and

1-methylxanthine was limited. In Eluent F, dissolution of the uric acid and 1-methylxanthine was accomplished by stirring overnight with a magnetic stirrer. Uric acid solutions in Eluent G and Eluent H could not be prepared with a concentration as great as 10^{-4} M, so the solutions prepared with these eluents used as diluents were saturated in uric acid.

3. Procedure for collecting current-potential data

Each tubular electrode was preconditioned by application of a potential more positive than the most positive detection potential to be used in the subsequent experimentation. Eluent was flowing during this anodic preconditioning period. Surface functional groups on glassy carbon that were oxidizable at the desired detection potential were more readily oxidized at more positive potentials. Hence, baseline stability at the detection potential was achieved more quickly than if one held the potential at the desired detection potential without preconditioning. Desired detection potentials were set after preconditioning the electrode in this manner.

The sample containing the compound or compounds to be detected was injected into the flowing stream of eluent. Plots of the peak detector current as a function of detection potential were constructed. The chromatographic column was required if additional peaks were observed as a result of a

mismatch of eluent and diluent. The column could be eliminated if the eluent and diluent used were from an identical batch, i.e. well-matched, provided no change in diluent composition (such as pH variation) accompanied the dissolution of the compound of interest. A flow restrictor was placed in-line between the sample injection valve and the 2-μm filter when the column was removed in order to provide adequate back pressure for operation of the pulse dampening device. Collection of data was tedious, but removal of the column accelerated data aquisition time.

Another expedient was devised to avoid long delays between each injection that resulted if the compound had a long retention time. Repetitive injections at regular intervals were made without waiting for each peak to appear. In this manner, solute peaks appeared at shorter intervals than if one waited for complete elution before the next injection of sample. The plots of peak current as a function of detection potential obtained with the column in-line were presumed to be more representative of the chromatographic response than current-potential curves plotted from data obtained without a chromatographic column. The current-potential curves were determined to be quite similar regardless of which of these procedures was used.

C. Results and Discussion

1. Electrode geometry

Many of the publications reviewed in Chapter II reflect the fact that numerous designs for amperometric detectors are suitable for liquid chromatography. Only the tubular flow-through electrodes described in the previous chapter were used in this research. The tubular electrode has the virtues of simplicity of construction and defined hydrodynamics. A derivation of the equation which characterizes the amperometric response of a tubular electrode was given in a monograph by Levich (117) and repeated by Blaedel et al. (118, 119). The result for the mass transport-limited current is given by Equation IV.2.

$$I_\ell = 5.43nFD^{2/3}L^{2/3}V_f^{1/3}C_o = K_{IV.2}C_o \qquad \text{(IV.2)}$$

In IV.2, L is the length of the tube in the direction of flow (cm); V_f is the flow rate (cm^3/sec), I_ℓ is the electrical current (mA) and C_o is the bulk concentration of electroactive species (mols/l). The other terms have their usual definitions. Equation IV.2 applies for laminar, steady-state flow of a homogeneous solution containing the electroactive substance and an excess of an inert supporting electrolyte. Thus, $\delta C/\delta t = 0$ in all regions of the stream and of the detector, and an insignificant depletion of the electroactive substance in the bulk of the solution stream is assumed.

Ross and Wragg (120) focused on the hydrodynamic flow through a tubular electrode. They found I_ℓ to be proportional to $V_f^{0.33}$ for laminar flow, in agreement with Equation IV.2, and proportional to $V_f^{0.58}$ for turbulent flow. The result of the experimental verification of Equation IV.2 by Blaedel, Olson and Sharma (118) was a $V_f^{0.335}$ dependence of I_ℓ for flow rates less than 10 ml/min. Blaedel, et al. reported a $V_f^{0.47}$ dependence for flow rates between 10 ml/min and 40 ml/min and concluded this result was evidence of turbulent flow. Tubular electrodes with radii ranging from 0.25-0.50 mm and lengths ranging from 2.5-25 mm were used in the studies of Blaedel, et al. The internal volume of tubular electrodes with these dimensions is within the range tolerable for the interstitial volume of a liquid chromatographic detector.

Equation IV.2 satisfactorily represents the current resulting from electrolysis of a homogeneous solution at a constant rate of flow. However, reason suggests that the maximum current measured for a chromatographic peak can not be used directly to calculate from Equation IV.2 the concentration of the electroactive analyte in the sample injected. A sample may be concentrated on a chromatographic column and as a result the volume of effluent containing the analyte is smaller than the volume of sample injected. The solute may also elute in a much larger volume than the volume injected so the peak concentration in the effluent is less than the

concentration of the analyte in the sample. Any quantitative relationship between the peak height (or area) and the concentration of the analyte in the sample must account for the chromatographic process.

Linear calibration curves are obtained for peak height or peak area plotted as a function of analyte concentration, provided the chromatographic conditions are constant. Since the mass transport-limited current $I_{\ell,IV.2}$, is proportional to the steady-state concentration, one may write

$$\frac{I_{\ell,IV.2}}{I_p} = \frac{K_{IV.2}C_o}{K_p C_{max}} \qquad (IV.3)$$

where I_p is the chromatographic peak current and C_{max} is the maximum concentration of the solute in the effluent. Provided the internal volume of the detector electrode is small compared to the volume of effluent containing the analyte, $\delta C/\delta t = 0$ at C_{max} so K_p is expected to be equal to $K_{IV.2}$.

Chromatographic peaks are often approximately gaussian in shape. An ideal, gaussian, chromatographic peak is shown in Figure IV.1. The concentration at any point in a gaussian chromatographic peak, C_t, is given by Equation IV.4 (121).

$$C_t = \frac{M \exp[(-1/2)(\frac{t_r - t}{\sigma_t})^2]}{V_f \sigma_t \sqrt{2\pi}} \qquad (IV.4)$$

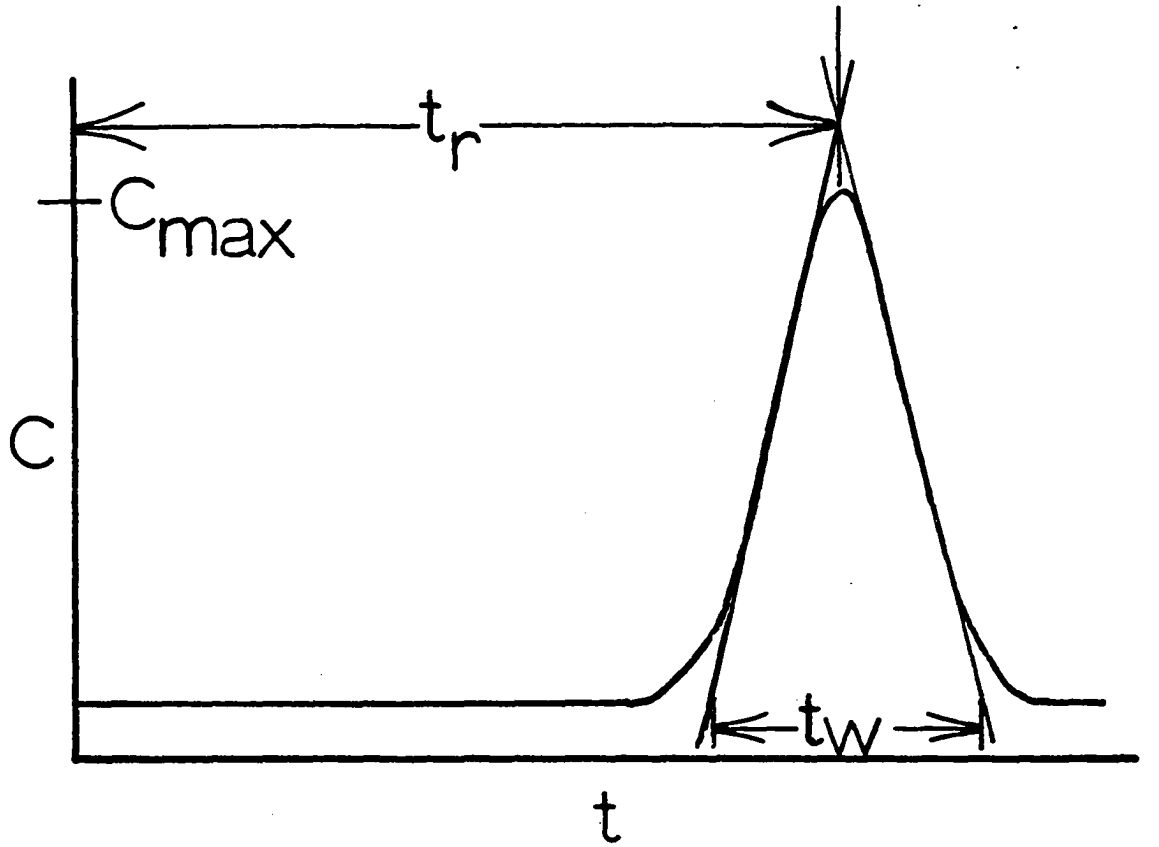


Figure IV.1. An ideal gaussian chromatographic peak

In Equation IV.4, M is the mass of solute given by IV.5,

$$M = C_o V_{inj} \tag{IV.5}$$

where V_{inj} is the volume of sample injected and C_o is the concentration of the analyte in the sample. The standard deviation in time units, σ_t, is one-fourth of the band width at the baseline, t_w, as approximated by a triangulation procedure illustrated in Figure IV.1. Tangents are drawn to the inflection points on each side of the chromatographic peak, and the intersection of these tangents with the baseline gives t_w.

$$\sigma_t = \frac{t_w}{4} \tag{IV.6}$$

In Equation IV.4, t_r is the retention time, and for C_{max}, $t = t_r$. Hence, Equation IV.4 may be simplified to give an expression for C_{max}.

$$C_{max} = \frac{M}{V_f \sigma_t \sqrt{2\pi}} = \frac{4V_{inj} C_o}{V_f t_w \sqrt{2\pi}} \qquad \text{(IV.7)}$$

The product of the flow rate, V_f, and t_w converts the band width at the baseline to volume units in Equation IV.8.

$$V_f t_w = V_w \qquad \text{(IV.8)}$$

Substitution of Equation IV.8 into IV.7 gives a simple relationship between the initial concentration injected and the concentration at the peak maximum.

$$C_{max} = \frac{4V_{inj}}{\sqrt{2\pi} V_w} C_o = 1.596 \frac{V_{inj}}{V_w} C_o \qquad \text{(IV.9)}$$

This relationship is predicted regardless of the mode of detection. Substitution of C_{max} for C_o in Equation IV.3 leads to the conclusion expressed as Equation IV.10.

$$I_p = 1.596 \frac{V_{inj}}{V_w} I_{\ell, IV.2} \qquad \text{(IV.10)}$$

The preceding discussion is not intended as a rigorous derivation. This section was included to bridge the gap between the steady-state prediction of current and the chromatographic peak current for a tubular electrode. The implicit assumption that the dependencies on flow rate and length of the tube were the same regardless of the presence of a chromatographic column was not tested experimentally.

The volume, V_w, was assumed to be much greater than the internal volume of the tubular electrode. The peak current is expected to be less than predicted by Equation IV.10 if the internal volume of the detector becomes significant with respect to V_w.

2. Electrode material

The rate and mechanism of a reaction at an electrode surface is influenced by the physical and chemical properties of the electrode material. Although theoretical progress has been made in the study of interfacial phenomena at electrodes, predictions based only on theory may not adequately account for some of the electrochemical behavior of various materials used as electrodes. The choice of electrode material is often based on empirical information. Hydrogen or oxygen overvoltage, adsorption of substrate or product on the surface, catalytic effects or even the history of the electrode can influence the course of the reaction.

The analytical chromatographer desires that a chromatographic detector be a reliable monitor of the concentration of solutes in the effluent which does not participate in mysterious phenomena which result in a change of detector sensitivity. Yet, no electrode material is truly inert; even electrodes constructed from noble metals such as platinum are subject to complicated surface reactions which can affect sensitivity. The popularity of the dropping mercury

electrode for cathodic electroanalysis is due, in part, to the greater freedom from surface complications as a result of continual renewal of the electrode. Mercury is of very restricted utility for anodic reactions. Moreover, the dropping mercury electrode is an inconvenient monitor of chromatographic effluents so suitable solid electrode materials have been sought.

The exact nature of the surface of solid electrodes at a given potential depends on the potentiostatic history of the electrode. This characteristic of solid electrodes is responsible for the ritualistic preconditioning procedures practiced by electroanalytical chemists working with solid electrodes. A constant potential is chosen for detection of chromatographic peaks, but the amperometric response can depend on the direction from which the detection potential was approached. A typical procedure for preconditioning involves rigorous anodization and cathodization of the electrode followed by several scans of the electrode potential between the potential limits characteristic of the solvent-electrolyte mixture in use. This empirical procedure is performed prior to each analytical measurement in an attempt to insure that the electrode surface is in exactly the same condition for each measurement.

Examination of all electrode materials for suitability as chromatographic detectors was beyond the scope of this

research. The platinum and glassy carbon detectors were chosen because they were available and because the methylxanthines are electroactive at both materials. As a result of the comparisons, the glassy carbon electrode was chosen over the platinum electrode for monitoring the liquid chromatographic separations of methylxanthines in physiological fluids. Exploratory work is described in this section, and the justification of the choice of the glassy carbon electrode is presented.

a. Amperometric response at the platinum electrode

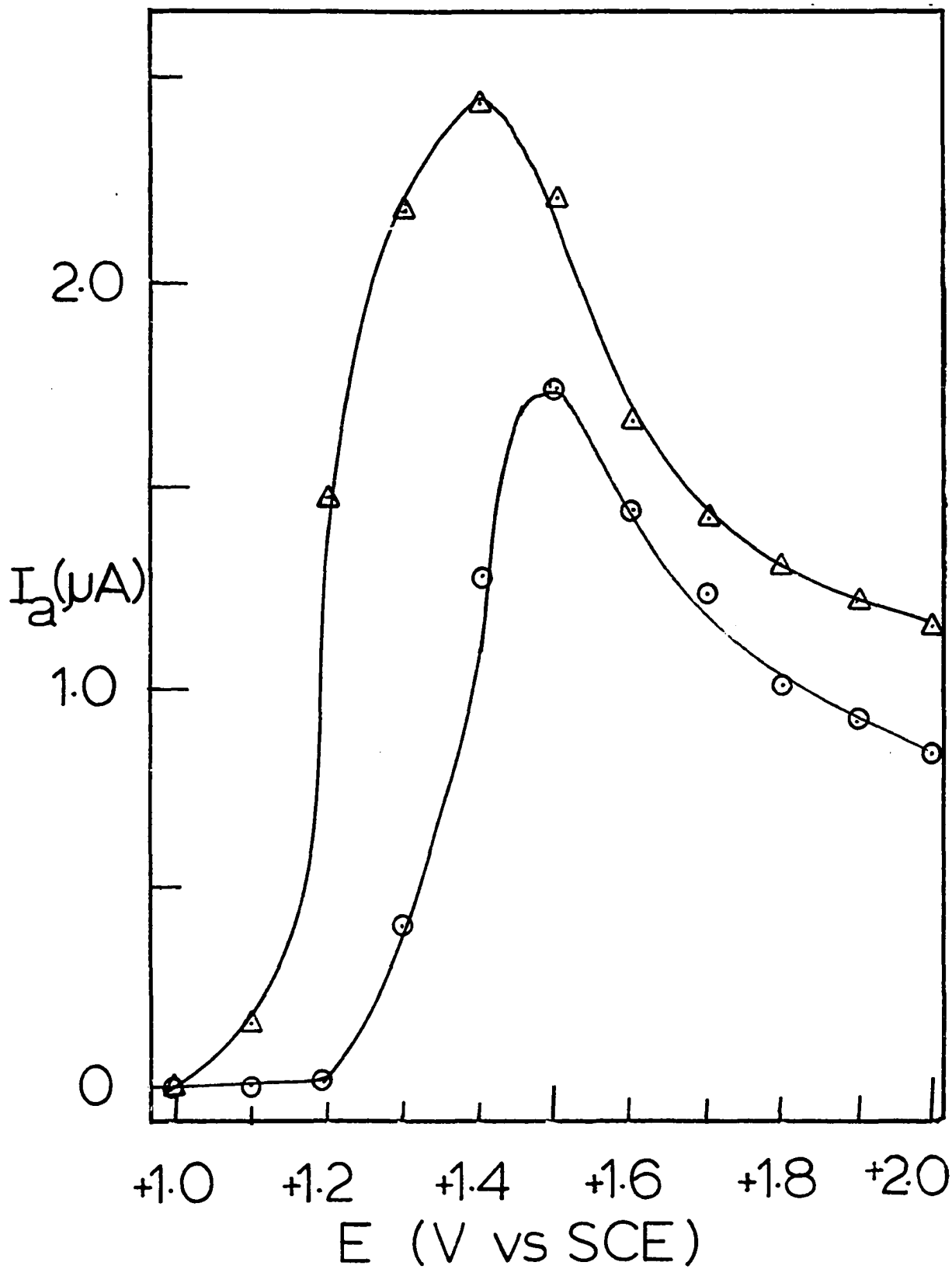
Methylxanthines are oxidized at platinum electrodes at potentials sufficiently positive to assure that the electrode surface is oxidized. The platinum electrode was pre-oxidized at +2.0 V in one series of experiments using Eluent B. A mixture of 1.0×10^{-3} M theophylline and 1.0×10^{-3} M theobromine prepared with Eluent B was separated on Column A. Repetitive injections of a constant amount of sample were made. The detection potential was changed by increments of 0.1 V from +2.0 to +1.0 V. At +1.0 V the direction of the potential change was reversed and data collected for potentials from +1.0 to +2.0 V. A plot of peak current for potentials where detection occurs defines the detection domain for theophylline and theobromine. Only the data collected for the potential change from +1.0 to +2.0 V are shown in Figure IV.2. Current values obtained for the

Figure IV.2. Current-potential curves for amperometric detection of theophylline and theobromine

 1.0 mM theophylline

 1.0 mM theobromine

PAR 174A (dc mode)
Pre-oxidized platinum detector
Column A
Eluent B
10 µl injected


2.0
1.0
0
$I_a(\mu A)$
+1.0
+1.2
+1.4
+1.6
+1.8
+2.0
E (V vs SCE)

potential change from +2.0 to +1.0 V were slightly higher in value, but the shape of the detection domains was the same.

Evolution of oxygen at platinum in acetate buffer limits the useful potential range for conventional anodic voltammetry to potential values less positive than +0.9 V (49). The current-potential curves of the dimethylxanthines in these experiments (Figure IV.2) do not have the appearance of conventional voltammograms. This observation is hardly surprising in view of the fact that the dimethylxanthines are oxidized at ultrapositive potentials; that is, potentials beyond the voltammetric cutoff potential. It is startling that amperometric response follows such a regular pattern in a potential region where background electrolysis proceeds at a significant rate.

Similar experiments were performed for caffeine, theophylline, theobromine and uric acid, but the chromatographic column was replaced by a long length of connecting tubing. Detection domains for the methylxanthines collected in this manner at oxidized platinum share two characteristics. 1) The amperometric response plotted as a function of increasing potential rises to a maximum and diminishes at more positive potentials regardless of the direction of approach to the maximum just as the detection domains determined for theophylline and theobromine. 2) The

magnitude of the current at the maximum in the detection domain was always greater for methylxanthines if the direction of approach to the maximum was from more positive potentials. Apparently, an oxidized platinum electrode surface enhances, by some catalytic mechanisms, the oxidation of methylxanthines. The magnitude of this catalytic enhancement depends on the extent of electrode surface oxidation. Sensitivity for theophylline oxidation at +1.5 V was doubled in one experiment in which the electrode was anodized at +2.4 V prior to detection at +1.5 V.

Uric acid is a constituent of physiological fluid, and the methyluric acids are metabolites of the methylxanthines. If one wishes to determine uric acid as well as the methylxanthines at constant sensitivity, platinum is unsuitable as an electrode material for the chromatographic detector. A drastic loss of sensitivity for uric acid is associated with detection potentials more positive than +1.0 V where oxygen evolution occurs at a vigorous rate in Eluent B. The data obtained are very similar to the data to be shown for Eluent C. Thus, detection potentials that are satisfactory for detection of methylxanthines are not suitable for detection of uric acid even though uric acid is oxidized at less positive potentials.

The catalytic enhancement of methylxanthine oxidations at platinum electrodes by surface oxide was investigated further

in Eluent C, and the results are shown in Figure IV.3. Detection domains for methylxanthines are very dependent on the extent of surface pre-oxidation of the platinum detector. Catalytic enhancement of theophylline oxidation is illustrated in Figure IV.3.A. The platinum electrode was preconditioned by cathodization at -1.6 V and the corresponding anodic response, shown by Curve A1, was then limited to a narrow span of detection potentials. Beyond +1.2 V the amperometric response dropped below the baseline. Anodization of the platinum electrode at an ultrapositive potential of +3.5 V for five minutes had a remarkable effect on the amperometric response. Anodic amperometric response for theophylline was then observed in the potential range +1.0 to +1.7 V as shown by Curve A2 of Figure IV.3.

Theobromine was oxidized readily on a pre-oxidized platinum electrode as shown in Figure IV.3.B (Curve B1). When cathodization of the electrode preceded the collection of data in the same potential range, amperometric response for theobromine was significantly smaller as shown by Curve B2.

When cathodization of the electrode preceded attempts to detect caffeine, no amperometric response was observed between +1.0 and +1.4 V as illustrated by Curve C1. Yet, pre-anodization of the surface elicits amperometric response as shown by Curve C2. The duration of pre-anodization of

Figure IV.3. Effect of platinum electrode history on current-potential response for purines

A 1.0×10^{-3} M theophylline

▲ A1 Precondition at -1.6 V

△ A2 Precondition at +3.5 V (5 min)

B 1.0×10^{-3} M theobromine

⊙ B1 Precondition at +3.0 V (2.5 min)

⬢ B2 Precondition at -1.15 V

C 1.0×10^{-3} M caffeine

● C1 Precondition at -1.0 V

⊙ C2 Precondition at +3.6 V (6 min)

◑ C3 Increasing sensitivity at a fixed detection potential with additional preconditioning at +3.6 V

D 1.2×10^{-4} M uric acid

⊡ D1 Precondition at +0.20 V

■ D2 Precondition at +2.0 V (5 min)

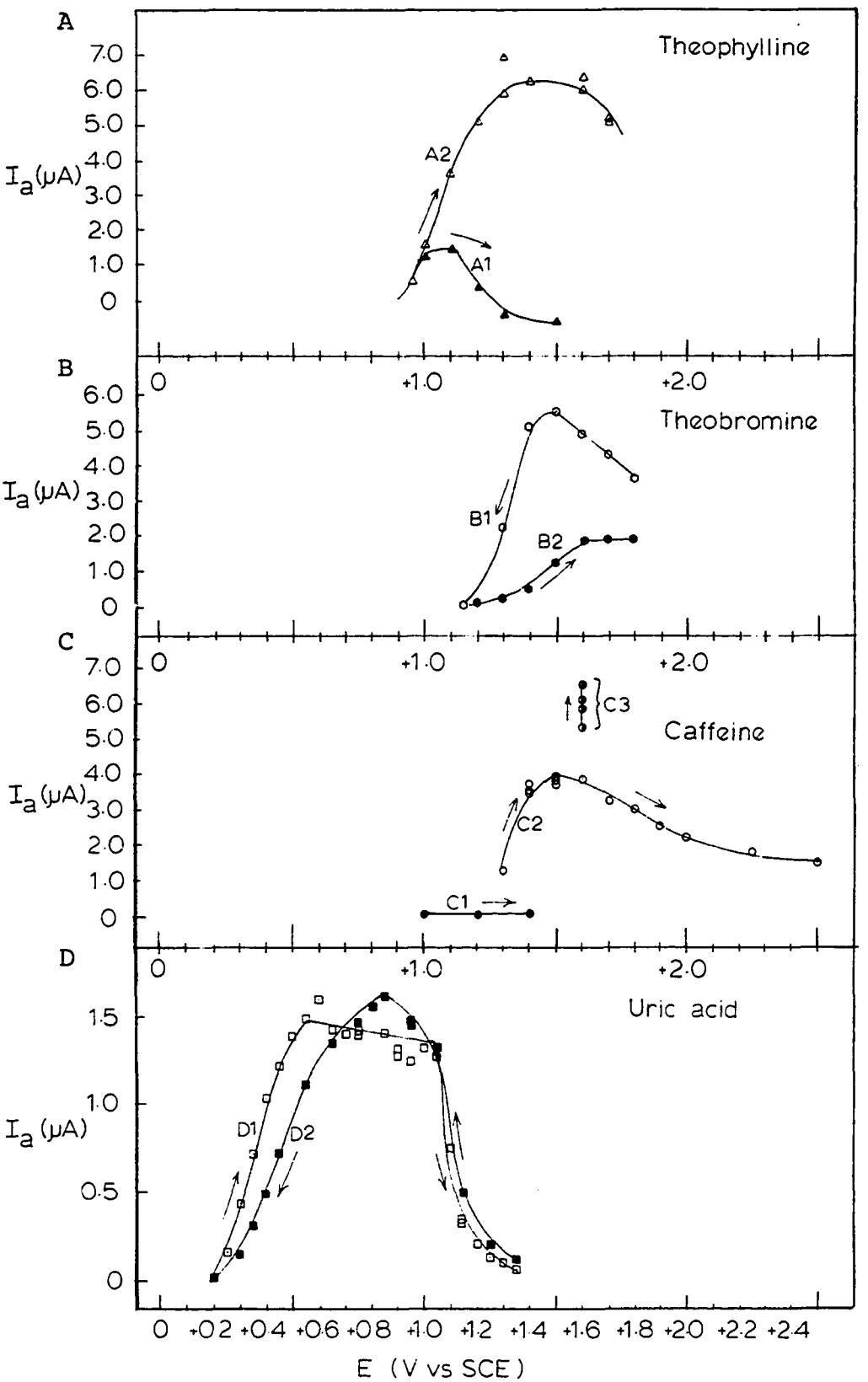
PAR 174A (dc mode)

Platinum tubular detector

No chromatographic column

Eluent C

10 µl injected


A
Theophylline
A2
A1
I_a (μA)
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
B
Theobromine
B1
B2
0
+1·0
+2·0
C
Caffeine
C3
C2
C1
D
Uric acid
D1
D2
1·5
0·5
0 +0·2 +0·4 +0·6 +0·8 +1·0 +1·2 +1·4 +1·6 +1·8 +2·0 +2·2 +2·4
E (V vs SCE)

the platinum electrode is important. Sensitivity is increased at a detection potential of +1.6 V for caffeine when the extent of surface pre-oxidation is increased. This effect is shown in Figure IV.3.C by Curve C3.

Uric acid oxidation is not enhanced by pre-anodization of the platinum electrode. Inhibition of uric acid oxidation in the rising portion of the detection domain is evident for an oxidized electrode by comparison of Curve D1 with Curve D2 in Figure IV.3.D. A dramatic loss of sensitivity for uric acid oxidation accompanies application of detection potentials exceeding +1.0 V in Eluent C as well as Eluent B.

The influence of platinum as the electrode material for organic oxidations is of considerable fundamental interest. However, as a practical material for an amperometric chromatographic detector of organic substances, platinum is inconvenient, and fortunately, a more suitable material is available.

b. Amperometric response at the glassy carbon electrode

Oxidation of the surface of a glassy carbon electrode is a complex process involving the formation of hydroxyl, carbonyl, carboxy and quinoid functional groups (122-124). As the potential is varied, the proportions of these surface functional groups change. Blaedel and Jenkins (124) reported that an amperometric, point-by-point construction of a voltammogram could be advantageous, when otherwise,

high background currents on glassy carbon would obscure the faradaic current. Blaedel and Jenkins allowed the current to decay to an equilibrium value at a fixed potential, before recording that current on the assumption that the measured steady-state was the faradaic current at that potential. They called the technique "steady-state voltammetry". The similarity of this steady-state approach and the method used here to collect current-potential curves is unmistakable.

While the oxidation of the glassy carbon surface is complex, the glassy carbon electrode was determined to be more suitable than platinum as a material for anodic chromatographic detection. The condition of the surface can be adjusted readily by anodization at a potential more positive than the desired detection potential. This empirical procedure minimizes but does not eliminate hysteresis effects such as the noncoincidence of peak current measured at a particular detection potential. Experiments with the glassy carbon electrode are described in subsequent sections, and detection domains on glassy carbon are shown. A limiting current plateau is observed in the current-potential curve of compounds oxidized at potentials less positive than the voltammetric cutoff potential on glassy carbon. On platinum a limiting current plateau is not well defined.

Glassy carbon is certainly not ideal as a material for an electrochemical chromatographic detector because the sensitivity was found to decrease for repetitive injections of an identical amount of each methylxanthine being studied. This effect was also observed with the platinum electrode. Adsorption of the reaction substrate or product may be responsible for loss of sensitivity by partial blockage of surface sites or by some other mechanism of surface deactivation. This loss of sensitivity was most serious for the first two injections, but the sensitivity was generally found to stabilize with subsequent injections. This loss of sensitivity amounted to as much as 5% for methylxanthines. Such a loss of sensitivity may be tolerable for some applications such as analysis of physiological fluids. An automatic method for reconditioning the electrode might be useful if the loss of sensitivity becomes too serious.

3. Solvent-electrolyte composition

Supporting electrolytes are required in electroanalytical measurements to minimize solution resistance and, quite importantly, to minimize contribution to the amperometric response by electrical migration. It is desirable to buffer the medium to minimize pH variation when hydrogen ions are involved in the reaction at the electrode, so buffers are often chosen as supporting electrolytes. Eluents for

liquid chromatography are often similar in composition to solvent-electrolyte mixtures required for electroanalysis. The choice of solvent-electrolyte composition for liquid chromatography with electrochemical detection is dictated by a factor not considered in other electroanalytical investigations; the solution flowing through the detector should be a satisfactory eluent. Admittedly, one may alter the composition of effluent prior to detection but at the cost of increased chromatographic band broadening. Eluents A through H were determined to be satisfactory for the separation of the dietary methylxanthines. Only these eluents were studied because the focus of the research was on separations of methylxanthines.

a. Ammonium carbonate in the eluent Eluent A was used in an early series of experiments with the pre-anodized platinum detector. The pH of this eluent was observed to increase gradually when the reservoir was sparged of dissolved gases by dispersed helium. With continued use of Eluent A, the sensitivity of the detector decreased, eventually becoming zero, and could only be restored by dissolution of additional ammonium carbonate in the eluent. Ammonium carbonate is slightly volatile, and extended dispersion of helium resulted in complete elimination of the electrolyte from the eluent.

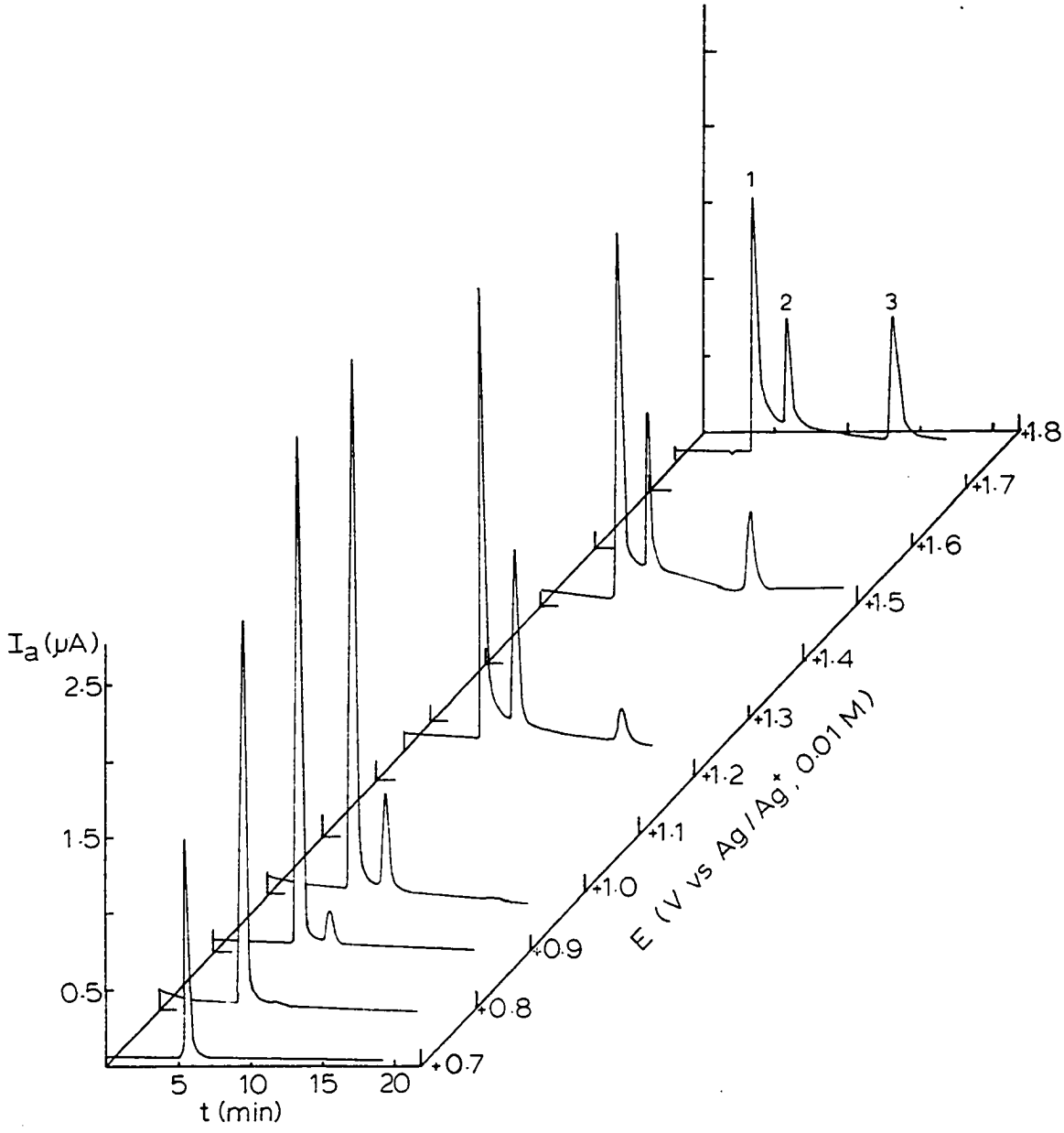
Figure IV.4. Chromato-voltammogram of the dietary methyl-xanthines

1 1.8 µg theophylline

2 1.8 µg theobromine

3 2.0 µg caffeine

PAR 174A (dc mode)
Pre-oxidized platinum detector
Column A
Eluent A 2.0 ml/min


1
2
3
I_a (μA)
2·5
1·5
0·5
5
10
15
20
t (min)
+0·7
+0·8
+0·9
+1·0
+1·1
+1·2
+1·3
+1·4
+1·5
+1·6
+1·7
+1·8
E (V vs Ag/Ag⁺, 0·01 M)

The separation of the dietary methylxanthines is shown in Figure IV.4 using the pre-oxidized platinum detector at a variety of detection potentials. This series of experiments was very promising. The pH of eluent became greater than 8 with extended dispersion of helium and eventually, the chromatographic column was destroyed because the silica support dissolved, and the column became clogged. Further use of ammonium carbonate was abandoned.

b. Acetate buffer in the eluent Detection domains for theophylline and 3-methylxanthines were determined chromatographically for 1.0 mM solutions using Eluent D and the glassy carbon detector; these detection domains are shown in Figure IV.5. The appearance of the detection domains is intriguing because one expects to observe a plateau in the region of amperometric response. The observed response has a maximum at approximately the potential where the background increases markedly due to the electrolysis of water.

$$2H_2O = O_2 + 4H^+ + 4e^- \qquad \text{(IV.11)}$$

To discover if variation of the pH at the solution-electrode interface is responsible for loss in sensitivity at ultrapositive potentials, the buffer capacity of the eluent was increased. This was accomplished by increasing the analytical concentration of acetate to 1.0 M in Eluent E. The detection domain for theophylline in Eluent E was

Figure IV.5. Current-potential curves for amperometric detection of 3-methylxanthine and theophylline at glassy carbon

⊡ 1.7 µg 3-methylxanthine

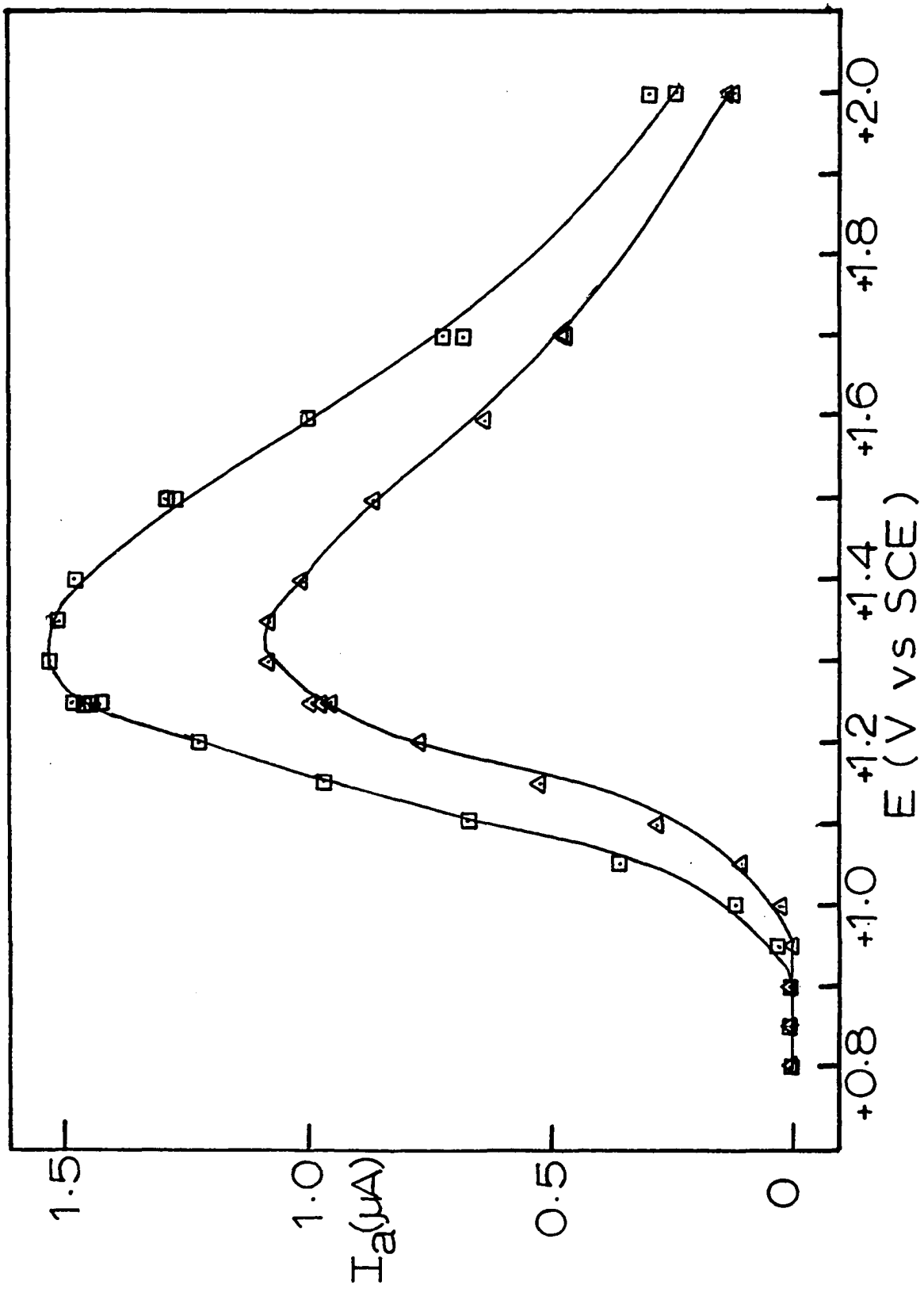
△ 1.8 µg theophylline

PAR 174A (dc mode)

Glassy carbon detector

Column A

Eluent D


1.5
1.0
0.5
0
Ia(μA)
+0.8
+1.0
+1.2
+1.4
+1.6
+1.8
+2.0
E (V vs SCE)

similar in appearance to that shown for Eluent D; however, the relative loss in sensitivity with increasing potential was greater in Eluent E in spite of the increased buffer capacity. This suggests that acetic acid or acetate anions contribute to the loss of sensitivity at ultrapositive potentials and not the interfacial pH variation.

Uric acid is oxidized at less positive potentials than theophylline in Eluent D. The detection domain of uric acid was determined at glassy carbon in Eluent D. The detection domain of uric acid had the appearance of a conventional voltammogram with a plateau in the response in the range +0.7 to +1.2 V. Sensitivity diminished at potentials greater than +1.2 V at which evolution of oxygen is vigorous. Reproducibility was poor at a constant detection potential even for potential values in the plateau region but this experiment was valuable because it demonstrated that the peaked appearance of the detection domain in acetate was a result of oxygen evolution rather than the specific buffer or buffer capacity. A plateau in response is not observed unless the background process at ultrapositive potentials is separated from the rising portion of the current potential curve by a wider span of potentials.

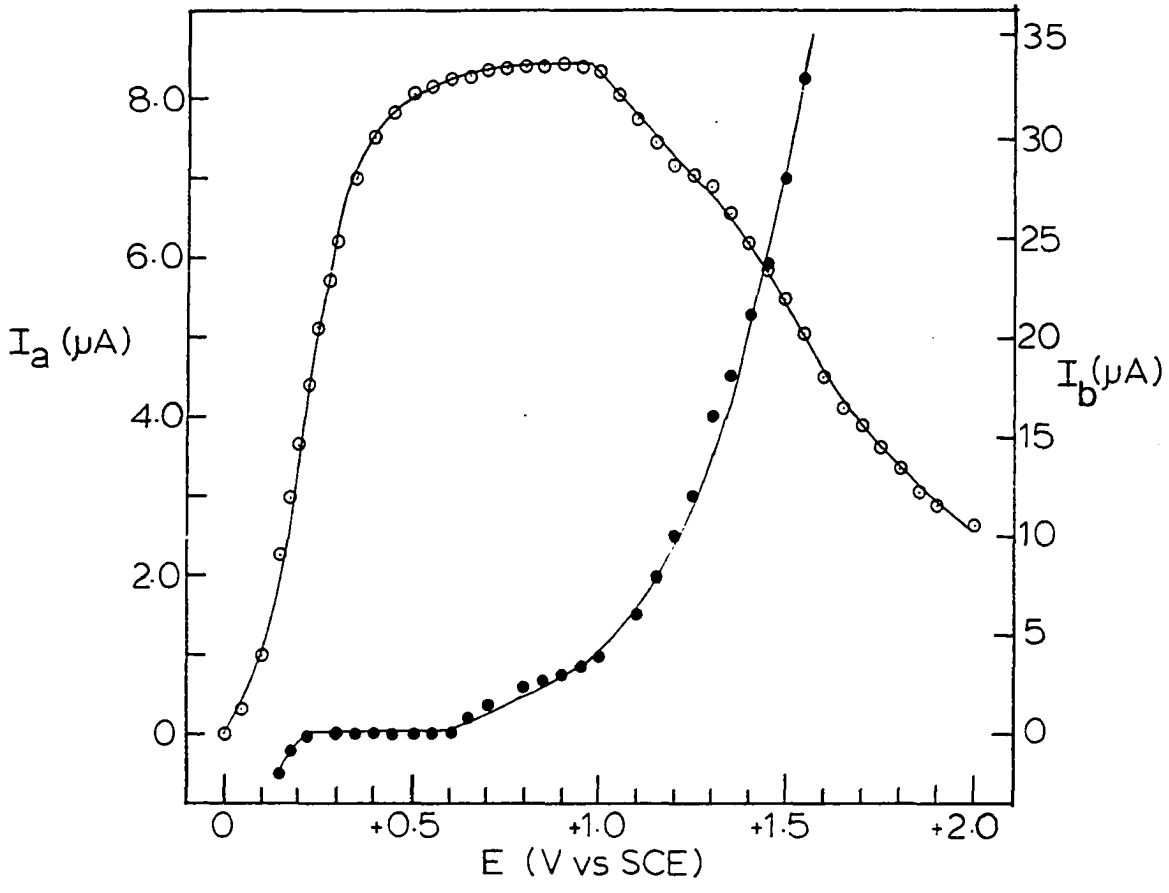
c. Phosphate buffer in the eluent The electrochemical oxidation of ferrocyanide ion is independent of pH. Thus, ferrocyanide was selected as a model to test further the

Figure IV.6. Current-potential curve for ferrocyanide

⊙ 1.0 mM ferrocyanide in Eluent F

● background Eluent F

PAR 174A (dc mode)
Glassy carbon detector
No chromatographic column
Eluent F 1.2 ml/min


8.0
6.0
4.0
2.0
0
I_a (μA)
35
30
25
20
15
10
5
0
I_b(μA)
0
+0.5
+1.0
+1.5
+2.0
E (V vs SCE)

conclusion that loss of detector sensitivity is not related to change of pH in the interfacial region at ultrapositive potentials. A phosphate-buffered eluent was chosen for this study. A solution of 1.0 m$\underline{M}$ K_4 $Fe(CN)_6$ was prepared in Eluent F. Injections of 100 µl of this solution into Eluent F flowing directly through the detector resulted in the response shown in Figure IV.6. The background current at each potential is also shown in this figure. A plateau in the response is obvious in the detection domain of ferrocyanide ion in the potential range +0.6 to +1.0 V. Once again, loss in sensitivity occurs with the onset of oxygen evolution at ultrapositive potentials. Interfacial change of pH may be significant at ultrapositive potentials, but blockage of reaction sites at the electrode surface by sorbed oxygen is concluded to be a more likely explanation for the loss of sensitivity in this potential region.

Observation of a plateau in amperometric response for purines is more probable if a compound is examined that is oxidized at less positive potentials than for onset of oxygen evolution. The rising portion of the current-potential curve for a purine that is oxidized more readily would be further removed from the onset of oxygen evolution just as the rising portion of the current-potential curve for ferrocyanide ion is separated from the influence of the background process by a plateau. The data of Table II.3

Figure IV.7. Current-potential curve for amperometric detection of 1-methylxanthine

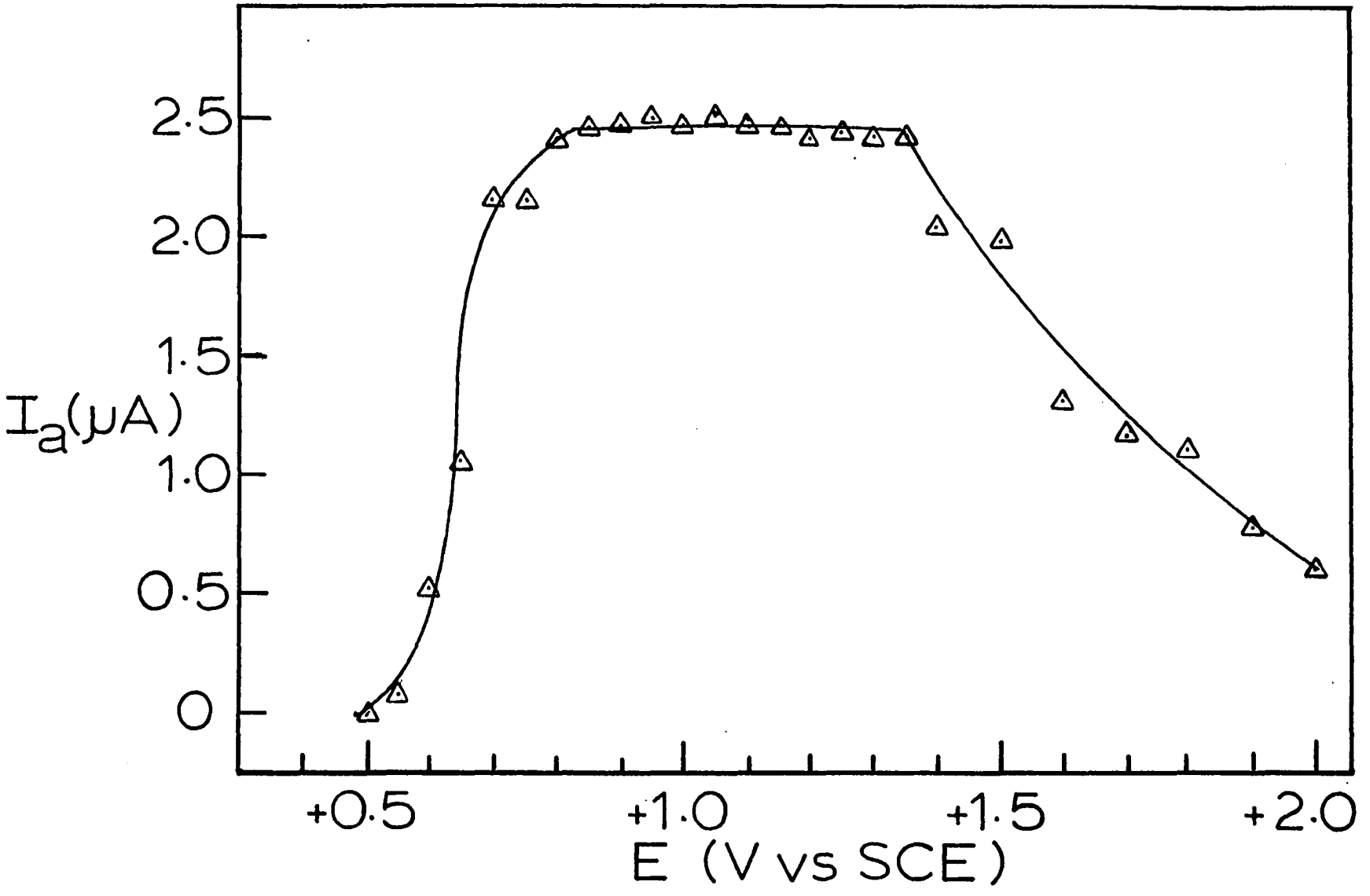
PAR 174A (dc mode)

Glassy carbon detector

Column A

Eluent F 0.70 ml/min

100 µl of 1.0×10^{-4} M 1-methylxanthine injected (1.66 µg)


2.5
2.0
1.5
1.0
0.5
0
$I_a(\mu A)$
+0.5
+1.0
+1.5
+2.0
E (V vs SCE)

suggest that 1-methylxanthine would serve well as a representative methylxanthine for this investigation because 1-methylxanthine is oxidized more readily than the other methylxanthines. The half-wave potential of uric acid on glassy carbon and platinum is sufficiently less than the potential for onset of oxygen evolution and uric acid would also be a good choice for demonstration of a plateau in detector response in the phosphate-buffered eluent. Eluent F has a high buffer capacity and was chosen for the initial experimentation.

The detection domain for 1-methylxanthine is shown as Figure IV.7. A plateau in response is observed on glassy carbon for 1-methylxanthine in Eluent F. The background process is further implicated in the loss of sensitivity because amperometric response was observed to decrease at potentials between +1.3 and +2.0 V.

d. Influence of pH of the eluent on amperometric response The pH of the effluent can influence the detection domain if hydrogen ions are involved in the electrochemical reaction. Uric acid was chosen as the substance to demonstrate this effect. The detection domains for uric acid on glassy carbon are shown in Figure IV.8 for Eluent F, Eluent G and Eluent H. The background current at each potential beyond +0.7 V is also shown for each eluent. A plateau is observed at each value of pH. The difference in the values

Figure IV.8. Current-potential curves of uric acid: the pH dependence of the detection domain

⊙ 1.0×10^{-4} M uric acid in Eluent F (pH 6.9)

△ saturated uric acid in Eluent H (pH 4.3)

⊡ saturated uric acid in Eluent G (pH 2.3)

● background for Eluent F

▲ background for Eluent H

■ background for Eluent G

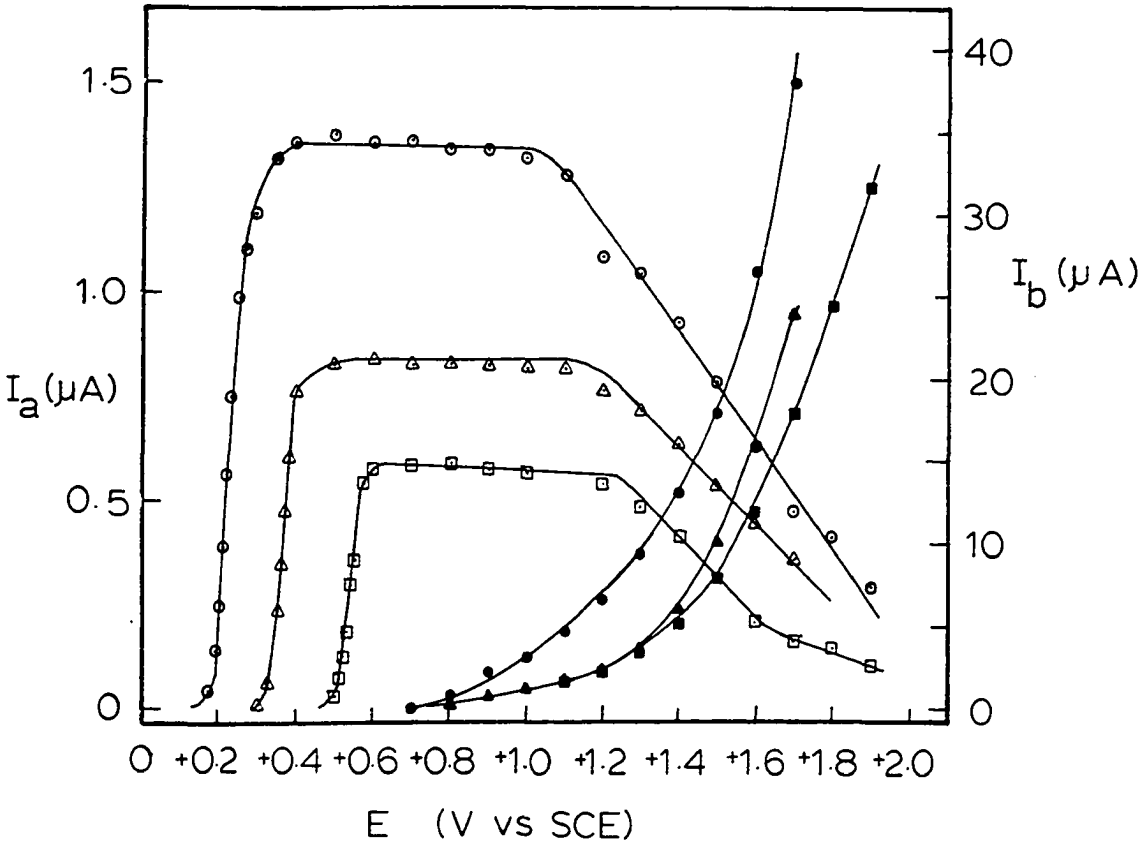
ADAM 5 (dc mode)

Glassy carbon detector

Column A

Eluents as indicated

Flow rate: Eluent F (0.58 ml/min)
Eluent H (0.80 ml/min)
Eluent G (0.65 ml/min)


1·5
1·0
0·5
0
I_a(μA)
40
30
20
10
0
I_b (μA)
0 +0·2 +0·4 +0·6 +0·8 +1·0 +1·2 +1·4 +1·6 +1·8 +2·0
E (V vs SCE)

of plateau current between experiments is due, mainly, to the difference in uric acid concentration between experiments. Solubility of uric acid is quite limited in these eluents. The concentration of uric acid in Eluent F was 1.0×10^{-4} M, but saturated solutions of uric acid were used in Eluent G and Eluent H with uric acid concentration less than 1.0×10^{-4} M. Solubility of uric acid is enhanced by formation of the urate under basic conditions. The first acid dissociation constant of uric acid has a value of $pK_a = 3.89$ at 12 °C according to the Handbook of Chemistry and Physics (125).

The data obtained in the rising portion of the detection domain can be used to estimate the number of electrons involved in the reaction and the half-wave potential by plotting the wave equation. The dependence of the half-wave potential on pH can also be determined from the data shown in Figure IV.8. A summary of the wave analysis for uric acid is given as Table IV.1. Non-integer values of n are indicative of the irreversibility of the electrode reaction on glassy carbon. The pH dependence of the half-wave potential determined using only the data from the two well buffered solutions (Eluent F and Eluent G) is in remarkable agreement with the data of Dryhurst (100). He reported a shift in potential of 69 mV with each unit change in pH and a change of 68 mV/pH is noted between pH 6.9 and pH 2.3 in

Table IV.1. pH dependence of uric acid oxidation

pH	n	$E_{1/2}$ (V vs. SCE)
6.9	1.5	+0.230
4.3	1.6	+0.365
2.3	1.5	+0.542

Table IV.1. However, the data at pH 4.3 is not in agreement. Eluent H is not well-buffered and the pH in the interfacial region will change with electrolysis.

Further comment can be made with regard to the loss of sensitivity at ultrapositive potentials as a result of observations of the detection domains for uric acid in eluents of various pH values. That interfacial pH variation might be responsible for the loss in sensitivity seems to be an improbable explanation. The response in Eluent H would be expected to be influenced to a greater degree than the response in Eluent F and Eluent G if interfacial pH variation were responsible for the loss in sensitivity at ultrapositive potentials because Eluent H has a low buffer capacity. No dramatic difference in the relative response is noted for uric acid in Eluent H in this region of ultrapositive potentials. I conclude that the loss of sensitivity is caused by blockage of active surface sites, presumably by molecular oxygen. The potential for onset of oxygen evolution shifts in a negative direction with an increase of pH and the potential for initial loss of

sensitivity parallels the potential for onset of oxygen evolution. Davenport and Johnson (56) observed similar behavior for reduction of nitrate and nitrite in tubular cadimum detectors at very negative potentials. They attributed the loss in sensitivity to formation of H_2 bubbles that adhered to the wall of the detector, preventing nitrate and nitrite from reaching the surface. Blockage of the surface on the molecular level seems probable as well. If adsorption precedes the electron transfer, the competition for surface sites by oxygen or hydrogen could cause a loss of sensitivity. This phenomenon was not investigated further because all of the methylxanthines could be detected without resorting to ultrapositive potentials.

Phosphate-buffered eluent was chosen for further study of the methylxanthines. Ammonium carbonate was rejected because deterioration of the expensive chromatographic column was noted when this eluent was used. The only rationale that may be offered in retrospect for not utilizing acetate-buffered eluent is that reproducibility of the data obtained with phosphate-buffered eluent was better in early experiments. Acetate-buffered eluent may be entirely satisfactory for electrochemical detection of methylxanthines.

No influence on amperometric response by the acetonitrile was observed. A systematic study of various solvents and solvent mixtures was not conducted.

4. Control of electrode potential

Tunable selectivity of an amperometric detector for liquid chromatography is governed mainly by the potential of the electrode. Selectivity and, to the some extent, sensitivity may be adjusted by variation of the detection potential. The relationship between the voltammmogram and the detection domain is apparent, so the vast literature of organic electrochemistry is available for guidance in the search for appropriate conditions for detection in liquid chromatography. Another comforting aspect of this observation is that voltammograms may be obtained readily for previously unstudied systems with considerably less labor than is required for construction of detection domains, provided the substance is electroactive within the range of potentials available for voltammetry.

Analysis of the relationship between the applied potential and the measured electrode current provides insight into the reaction mechanism. The precise manner in which the potential influences the outcome of a reaction at the electrode is generally considered to be more puzzling for most organic reactions than for inorganic reactions. Reaction paths may have several elementary steps prior to and following the electron transfer. Studies of heterogeneous organic electrochemical reactions are oftentimes ambiguous and incomplete. In troublesome applications of

electrochemical detection in liquid chromatography the chromatographer may find insight into the cause and possible solution of the problem by consulting the literature of organic electrochemistry. A number of monographs on this subject have appeared recently (49, 126, 127).

Tunable selectivity is an inherent property of amperometric detectors, but the optional feature of this selectivity implied by the word "tunable" is limited. If a detector is operated in the amperometric mode, then all substances that are oxidized at potentials less positive than the detection potential will be detected. The response and selectivity may be made more versatile by controlling the potential of the detector in another manner. The detector may be focused or "fine-tuned" by operation in the differential pulse mode. The detection potential is alternately applied at two potentials that define the limits of the detection window. The current at one potential is subtracted from the current at the second potential. If both potentials at the limits of the detection window are potentials for which a plateau in amperometric response is observed, then some immunity to that substance is obtained because the current difference is negligible or, at least, very small. One can then focus mainly on the substances that have a rising portion of their current-potential curve in the potential window chosen. I would like to suggest that this more

versatile form of selectivity obtained in the differential pulse amperometric mode of detection be called "discretional selectivity". Demonstration of the benefit of discretional selectivity is reserved for the following chapter.

Alternately varying the potential between two values may also give benefit in addition to discretional selectivity. Adsorption of reactants and products on solid electrodes is often a serious problem in electroanalysis, but the electrode may be reconditioned or cleaned by choice of a potential which causes any adsorbed species to be desorbed. Another mode of detection is possible because the difference in current need not be determined. One merely alternates the electrode potential between the two values of potential and measures the current at only one of the potentials, the detection potential for the pulse amperometric mode. This technique could have advantage for studies of substances that are oxidized at ultrapositive potentials. The product of the oxidation could be reduced at a potential free of interference from high background currents. The pulse amperometric mode of detection may have additional advantages in terms of tunable selectivity because not all substances would be electrolyzed to give a detectable product.

The detection domains for 1-methylxanthine using the pulse and differential pulse amperometric modes of detection are presented here for the purpose of illustrating the

influence of these modes of potential control. A comparison of the linear dynamic range, sensitivity and detection limit for theophylline is also presented. The need for frequent calibration is illustrated. Much work remains to characterize and optimize parameters for pulse amperometry; this limited comparison of the three techniques may be unfair. Hopefully, these data will give impetus to further study and instrument development. The methylxanthines will serve as a challenging model system for this purpose.

A solution containing 1-methylxanthine at a concentration of 1.0×10^{-4} M was injected repeatedly onto Column A in a manner similar to that used to construct amperometric detection domains. Detection potential windows in the vicinity of the half-wave potential were selected. A detection domain for the differential pulse amperometric mode of detection was constructed by plotting the maximum current as a function of the potential midway between the two limits of the detection potential window. This plot is shown in Figure IV.9 for detection potential windows of 25 mV, 50 mV and 100 mV in amplitude. Immunity, or loss of detectability at potentials more positive than the half-wave potential, is demonstrated when the potential pulse is chosen to occur between two values that are on a plateau for the amperometric response. The sensitivity is greatest for the window of largest amplitude. Sensitivity is maximal

Figure IV.9. Differential pulse amperometric detection domains for 1-methylxanthine

Pulse	Amplitude
⊙	100 mV
⊡	50 mV
△	25 mV

Cycle time 0.5 sec

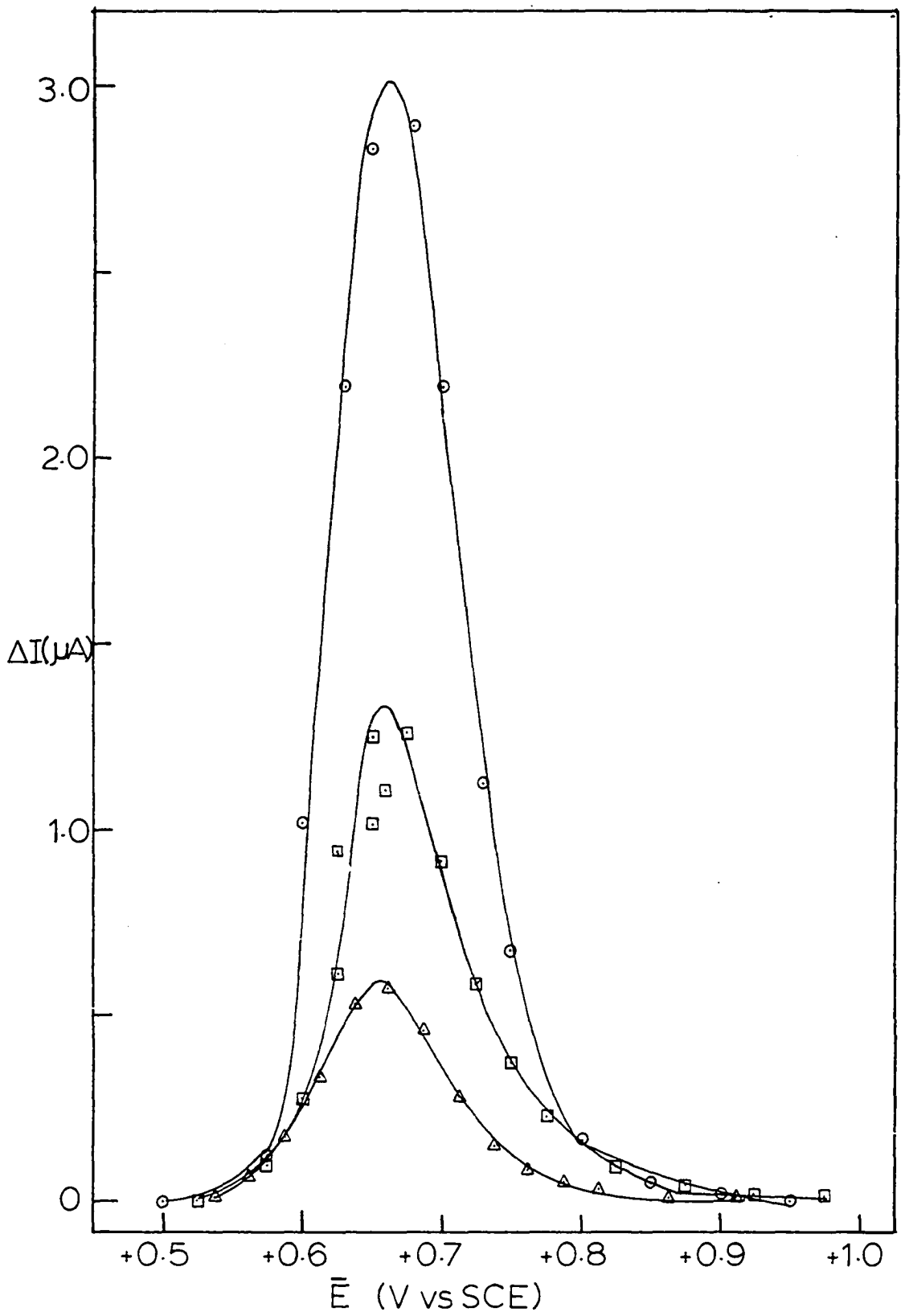
PAR 174A (differential pulse mode)

Glassy carbon detector

Column A

Eluent F 0.70 ml/min

100 µl of 1.0 x 10^{-4} M 1-methylxanthine injected (1.66 µg)


3.0
2.0
1.0
0
ΔI(μA)
+0.5
+0.6
+0.7
+0.8
+0.9
+1.0
Ē (V vs SCE)

for the differential pulse amperometric mode if the mean potential is the half-wave potential. The sensitivities of amperometric and differential pulse amperometric detection appear to be nearly the same when the plateau in the amperometric response of Figure IV.7 is compared to the maximum in the differential pulse amperometric detection domain for the 100-mV window. Since one would expect the maximum response for the differential pulse amperometric mode if $I = I_{\ell}$ at E_2 and $I = 0$ for E_1, greater sensitivity could be obtained if detection windows greater than 100 mV were available. If the sensitivity of the two modes of potential control were only equal, then one would only chose the differential pulse amperometric mode when immunity is desired.

The detection domain for 1-methylxanthine using the pulse amperometric mode of detection was determined using the same conditions as were used for the amperometric and differential pulse amperometric modes of detection. Detection domains of 1-methylxanthine are shown in Figure IV.10 for each of the three modes of detection. The need for frequent calibration is demonstrated because the pulse amperometric response depends on the potentiostatic history of the glassy carbon electrode. An increase in sensitivity of two to three-fold for the pulse amperometric mode is noteworthy when compared with the amperometric mode of detection.

Figure IV.10. Comparison of the pulse amperometric detection domain of 1-methylxanthine with the amperometric and differential pulse amperometric detection domains for 1-methylxanthine

⊡ 1 Pulse amperometric mode

⬡ 2 Pulse amperometric mode

△ 3 Amperometric mode (identical to Figure IV.7)

⊙ 4 Differential pulse amperometric mode (identical to Figure IV.9 for 100 mV pulse amplitude)

Cycle time 0.5 sec

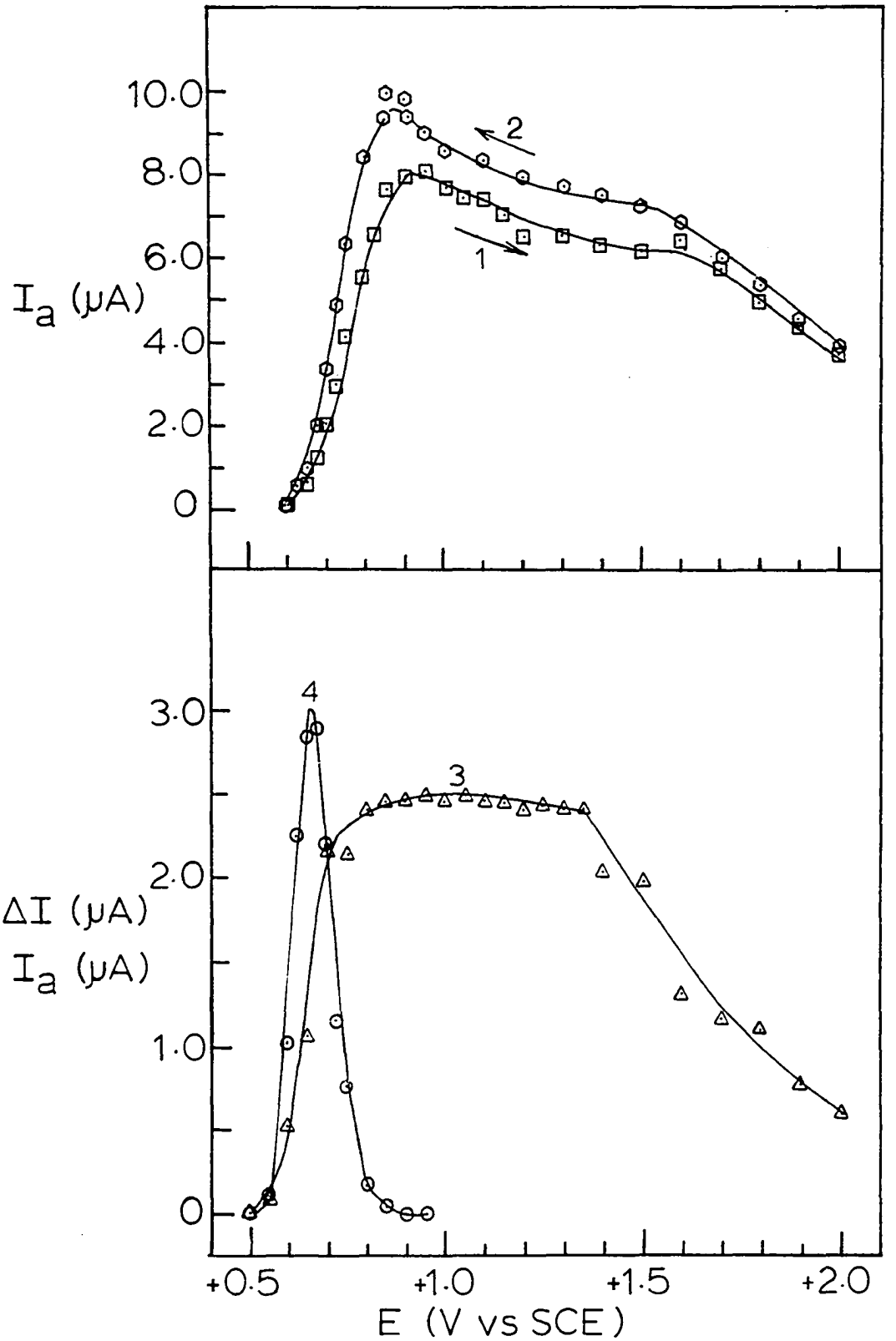
PAR 174A (mode as indicated)

Initial potential for pulse amperometric mode (+0.50 V)

Glassy carbon detector

Column A

Eluent F 0.70 ml/min


10.0
8.0
6.0
4.0
2.0
0
I_a (μA)
2
1
3.0
2.0
1.0
0
4
3
ΔI (μA)
I_a (μA)
+0.5
+1.0
+1.5
+2.0
E (V vs SCE)

There is every reason to believe that the differential pulse amperometric mode of detection would have sensitivity equivalent to the pulse amperometric mode if greater potential amplitudes were available. However, a sacrifice of immunity must be made when larger detection windows are used in the differential pulse amperometric mode.

Standard solutions of theophylline were prepared in Eluent F in the concentration range 10^{-7} M to 10^{-3} M. Injections of 100 µl of each of these solutions were made onto the pellicular chromatographic column, Column B. Calibration curves were constructed for each of the modes of potential control of the detector. These curves are shown in Figure IV.11.

A detection potential of +1.30 V was chosen for the amperometric mode of detection because this potential is on the plateau in a detection domain of theophylline. A linear calibration curve was obtained for injections ranging from 0.09-18 µg. The detection limit is estimated to be about 20 ng of theophylline for the amperometric mode of detection. Baseline noise from the oscillating flow of eluent is responsible for this limitation. A smaller detection limit would be feasible if a better way of suppressing oscillations of the flow rate were used.

The linear dynamic range for theophylline was not nearly as great for the differential pulse amperometric mode of

Figure IV.11. Calibration curves for theophylline for various modes of potential control

—⊙—	Amperometric mode	$E = +1.30$ V	Column B (2.1 ml/min)
--⊡---	Pulse amperometric mode	$E_1 = +0.80$ V $E_2 = +1.10$ V	Column B (2.1 ml/min)
···⊙····	Differential pulse amperometric mode	$E_1 = +0.95$ V $E_2 = +1.05$ V	Column B (2.1 ml/min)
—△—	Pulse amperometric mode	$E_1 = +0.750$ V $E_2 = +1.350$ V	Column A (0.70 ml/min)

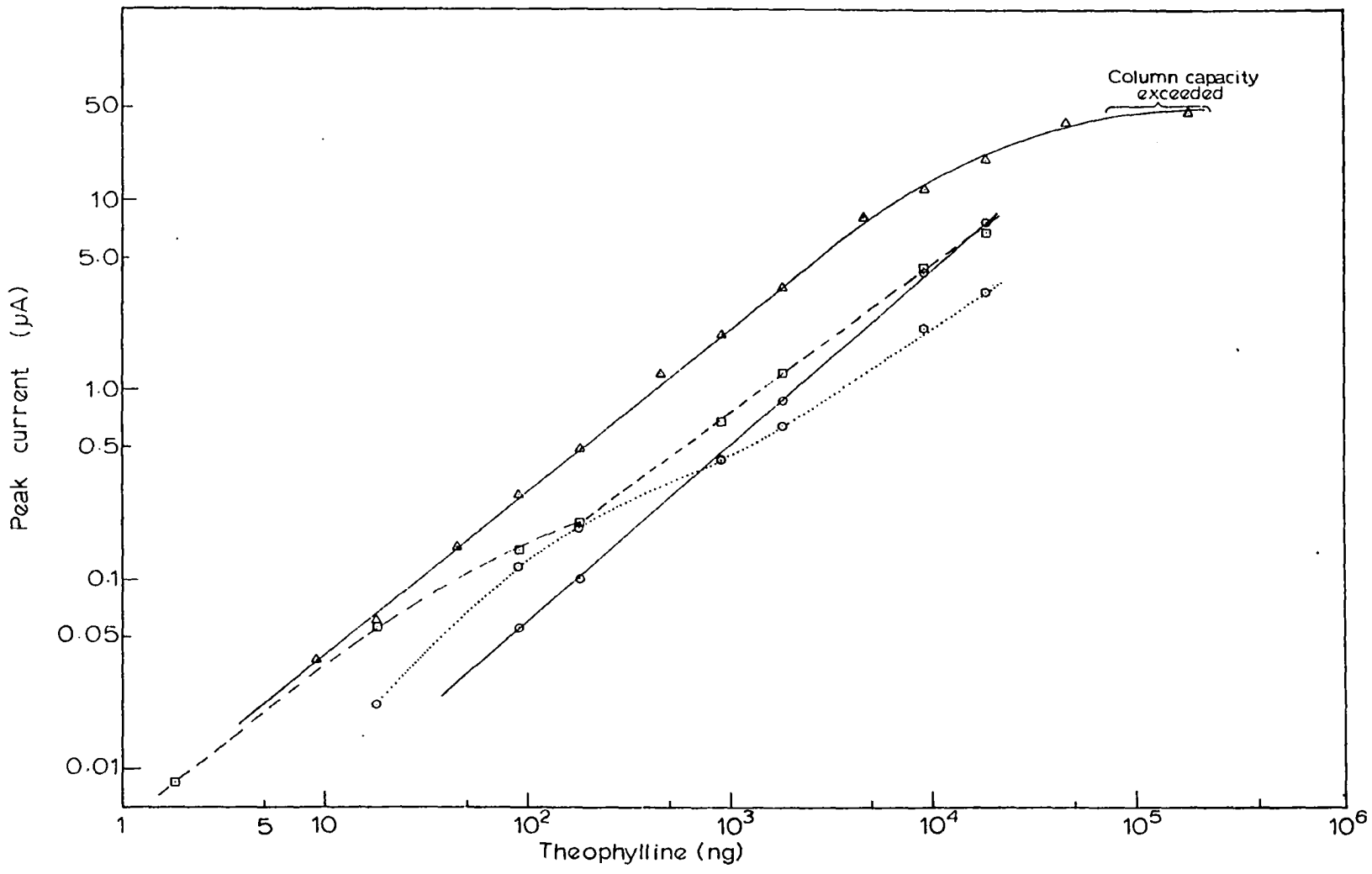
Cycle time 0.5 sec for pulse amperometric and differential pulse amperometric mode

PAR 174A (mode as indicated)

Glassy carbon detector

Column (as indicated)

Eluent F


Column capacity exceeded
Peak current (μA)
50
10
5.0
1.0
0.5
0.1
0.05
0.01
1
5
10
10^2
10^3
10^4
10^5
10^6
Theophylline (ng)

detection although the dynamic range is greater than the dynamic range of the amperometric mode. A lower detection limit may be anticipated from the calibration data shown for this mode of detection. The nonlinear response with this mode of detection is not surprising in view of the complexity of the mechanistic studies of Hansen and Dryhurst (97-99). They reported that one product of theophylline oxidation on pyrolytic graphite is a dimer of theophylline and in addition the number of electrons involved varied. If these are the only mechanistic complications then the detection domain will be dependent on concentration because the half-wave potential will vary with concentration. Any reaction that is irreversible will have a detection domain that is concentration dependent. Thus, the detection domain in differential pulse amperometric mode will be influenced to the greatest extent and the linear dynamic range will be curtailed. A short linear dynamic range is not a serious problem for theophylline determinations because the concentration generally falls within a narrow range. If one changed the sample size to accommodate a pediatric specimen, then a new calibration curve would be required. A wider detection potential window should obviate the problem of a small linear dynamic range. The obvious limit is that of the pulse amperometric mode which will now be considered.

The full benefit of the pulse amperometric mode of detection is not illustrated by the calibration curve shown for the potential window of +0.8-+1.1 V. A more positive final potential is required to reach the plateau in amperometric detection domain. A second calibration curve for the pulse amperometric mode is shown for the potential window of +0.75-+1.35 V. The linear dynamic range of nearly four orders of magnitude is obtained if potentials in the vicinity of the half-wave potential are avoided. The detection limit using the pulse amperometric technique is less than a nanogram for the detection window of +0.80 to +1.1 V.

A separate study was conducted to compare the precision of the three modes of potential control for injections of theophylline on Column A. The data obtained are summarized in Table IV.2. The first peak in a series of experiments was invariably greater than subsequent peaks in the same series of injections. Adsorption of the product of the reaction may saturate the surface and thereafter response levels off. Two series of experiments are shown for differential pulse amperometric mode of detection. The first peak in each series was greater than subsequent peaks. The injections in each series of experiments were made at regular intervals without waiting for each peak to completely elute. The flow of solution must have a cleaning effect on the electrode because the enhanced response of the first

Table IV.2. Comparison of modes of potential control for detection of theophylline[a]

Determination	Amperometric (+1.35 V)	Differential pulse Amperometric (+0.95 → +1.05 V)		Pulse Amperometric (0.85 → 1.35 V)
		Series 1	Series 2	
1	4.23 μA	2.19 μA	2.22 μA	7.82 μA
2	4.19	2.13	2.17	7.60
3	4.19	2.13	2.15	7.54
4	4.19	2.13	2.12	7.48
5	4.17	2.12	2.12	7.54
6			2.13	7.40
7			2.14	7.40
8				7.36
Mean	4.19	2.14		7.52
Std. Dev.	0.0219	0.0318		0.1455
RSD	5.22 ppt	14.9 ppt		19.4 ppt

[a] 9.2 μg of theophylline injected.

peak returned if the electrode was washed with flowing eluent for ten minutes between injections. Such effects will be bothersome in chromatography because an early peak may affect subsequent peaks in the same chromatogram.

The periodic variation of the potential in the pulse amperometric and differential pulse amperometric modes had no beneficial cleaning effect for these detection windows. In fact, the opposite was true; the loss in sensitivity after the first injection was 3 to 4% for pulse and differential pulse and only 1% in the amperometric mode. Initial potentials chosen in the vicinity of the half-wave potential would not be expected to be satisfactory potentials for conditioning or cleaning the electrode but another potential may be very satisfactory for this purpose.

D. Summary

Influential electrochemical parameters were illustrated in this chapter using as examples the oxidation of methylxanthines, uric acid and ferrocyanide ion. The influence of the tubular geometry was discussed. Platinum and glassy carbon were examined as electrode materials; glassy carbon was chosen as the superior material for liquid chromatographic detection of the methylxanthines because greater freedom from troublesome surface phenomena is realized if glassy carbon is utilized. Several solvent-electrolyte combinations that also are satisfactory chromatographic eluents for the

dietary methylxanthines were studied. The influence of pH on detection domains was illustrated using uric acid as an example. Detection domains were shown to extend beyond the voltammetric cutoff potential where high background currents would otherwise obscure the electrochemistry of the substance of interest. Sensitivity diminishes in this domain of ultrapositive potentials, but analytical data can be obtained for compounds that would be considered non-electroactive in voltammetry. This full range of detection potentials will not be accessible with many other solid electrode materials. Modes of potential control were compared. Tunable selectivity is an inherent property of amperometric detectors, but this selectivity may be enhanced by using differential pulse amperometric detection which gives a measure of immunity to compounds that have a plateau in amperometric response in the detection window chosen. Sensitivity, linear dynamic range and detection limit were compared for the three modes of potential control using theophylline as the substance detected.

V. CHROMATOGRAPHY OF METHYLXANTHINES

> "There is no dearth of methods that are entirely satisfactory for the determination of elements when they occur alone. The rub comes in because elements never occur alone, for nature and man both frown on celibacy. Methods of determination must therefore be judged by their 'selectivities'." -- G. E. F. Lundell

A. Introduction

The previous chapter focused on the parameters which influence the response of amperometric detectors for liquid chromatography, but no chromatograms were shown for the glassy carbon detector. With the exception of chromatograms shown for the pre-oxidized platinum detector, presentation of chromatographic separations was reserved for this chapter.

Isomers often present a challenging separation problem, so the methylxanthines are particularly appropriate for the choice as a model system to illustrate the merits of tunable selectivity and discretional selectivity when the separation is difficult. The conditions for chromatographic separation of a large variety of mixtures are described in this chapter. The three modes of potential control are assessed for chromatographic detection. Finally, a new injection technique is proposed that is useful for monitoring the response of electrochemical detectors for liquid chromatography. This technique should also be useful for many chromatographic procedures with other detectors.

B. Experimental

1. Chemicals and reagents

Adams Chemical Co. of Round Lake, Ill., supplied the following methylxanthines and metabolites: 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid and 1,3-dimethyluric acid. Xanthine and 8-chlorotheophylline were obtained from Aldrich Chemical Co. The 8-chloro-theophylline was contaminated by a substance having the retention time of theophylline. Matheson, Coleman and Bell supplied the uric acid, theophylline and theobromine. Caffeine was provided by Eastman Organic Chemicals. Creatinine was furnished by Sigma Chemical Co. The 7-(2,3-dihydroxypropyl) theophylline, called dyphylline generically, was generously provided by Dr. J. D. McCallister of Mallinckrodt, Inc.

2. Preparation of solutions

The eluents were prepared as indicated in the previous chapter. Solutions of the compounds to be studied were prepared by dissolution of the compound of interest in a diluent carefully matched to the eluent. Artifactual peaks were sometimes observed in chromatograms at the retention time of an unretained peak. These artifactual peaks could generally be eliminated if portions of the same batch of

eluent were used as diluent. Some compounds were sparingly soluble in these diluents, but dissolution could be effected by stirring overnight with a magnetic stirrer.

3. Procedure

The glassy carbon tubular electrode was preconditioned by pre-oxidation at +2.0 V for two to five minutes. Desired detection potentials were set after conditioning the electrode in this manner. Only one such pre-oxidation was performed for each set of experiments in which detection domains were examined.

The sample containing the compound or compounds of interest was injected onto Column A. Current-potential curves for these compounds were plotted from chromatograms obtained at differing detection potentials.

For selected experiments, the flow rate was calibrated by collecting effluent in a graduated cyclinder for a known period of time.

C. Results and Discussion

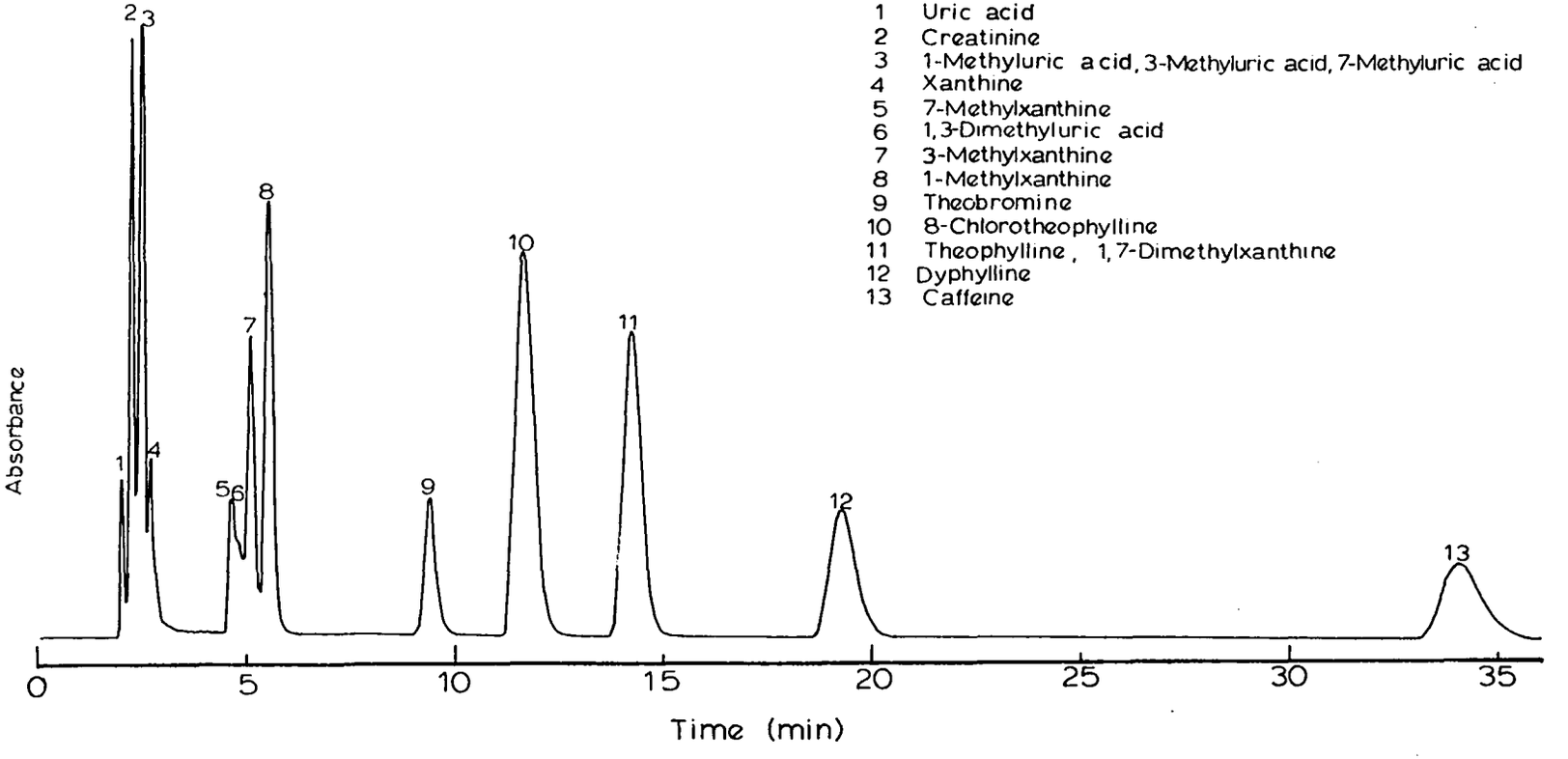
The conditions for the separation of methylxanthines presented in this chapter differ from the conditions described by previous workers who used acetate-buffered eluents (6, 111-113). A chromatogram for a solution containing the methylxanthines, as well as urinary metabolites of the methylxanthines, was obtained using the ultraviolet detector for

Figure V.1. Liquid chromatographic separation of methylxanthines and selected urinary metabolites with ultraviolet detection

UV detector (254 nm)

Column A

Eluent F


1 Uric acid
2 Creatinine
3 1-Methyluric acid, 3-Methyluric acid, 7-Methyluric acid
4 Xanthine
5 7-Methylxanthine
6 1,3-Dimethyluric acid
7 3-Methylxanthine
8 1-Methylxanthine
9 Theobromine
10 8-Chlorotheophylline
11 Theophylline, 1,7-Dimethylxanthine
12 Dyphylline
13 Caffeine
Absorbance
Time (min)
0
5
10
15
20
25
30
35

the purpose of comparison of the separation developed for this research with published separations. This chromatogram is shown in Figure V.1. Several other compounds were included in the mixture used to obtain this chromatogram. Dyphylline is used in the treatment of asthma and will be found in some patients' serum (128). As a common internal standard for chromatographic separations of methylxanthines 8-chlorotheophylline should be resolved from the other methylxanthines. Motion sickness drugs such as Dramamine® also contain 8-chlorotheophylline, and thus it may be found in some specimens. Uric acid, creatinine and xanthine were included in the mixture only because they are common constituents of physiological fluids and serve as reference points for similar separations at other values of pH. The amounts of each constituent are unknown.

Perfect resolution of the methylxanthines and metabolites of the methylxanthines has not been obtained for these chromatographic conditions. Note particularly that theophylline is not resolved from a caffeine metabolite, 1,7-dimethylxanthine. Optimization of chromatographic conditions might produce further separation, but such measures may be unnecessary if the tunable selectivity or discretional selectivity of an electrochemical detector is adequate.

Several chromatographic approaches to improve the separation were investigated. It was known from the abortive

work with Eluent A that the order of elution of theobromine and theophylline could be reversed by variation of pH. Attempts to separate theophylline and 1,7-dimethylxanthine were not successful by use of Eluent H with a previously unused Column A. Only partial resolution of these two isomers was obtained by this eluent, and resolution did not substantially improve when two of these columns were used in series.

In several papers reviewed in Chapter II, an acetate-buffered eluent was used for the reverse-phase separation of methylxanthines (6, 111-113) on Column A. The separation of theophylline and 1,7-dimethylxanthine was attempted by using the conditions described in those papers, but no resolution was obtained in contradiction to the suggestion of Orcutt, Kozak, Gillman and Cummins (112) that this separation could be performed if the acetonitrile concentration was reduced to 5% for acetate-buffered eluent. This suggests that there exists large variation in the manufacture of these columns or, as has been observed during the course of this entire investigation, that these columns have a limited lifespan with a gradual loss of efficiency.

Eluent F was chosen over Eluent H for work with the electrochemical detector. The choice was not based on clear-cut reasoning. Dryhurst indicated that adsorption on the electrode surface was only observed in methylxanthine

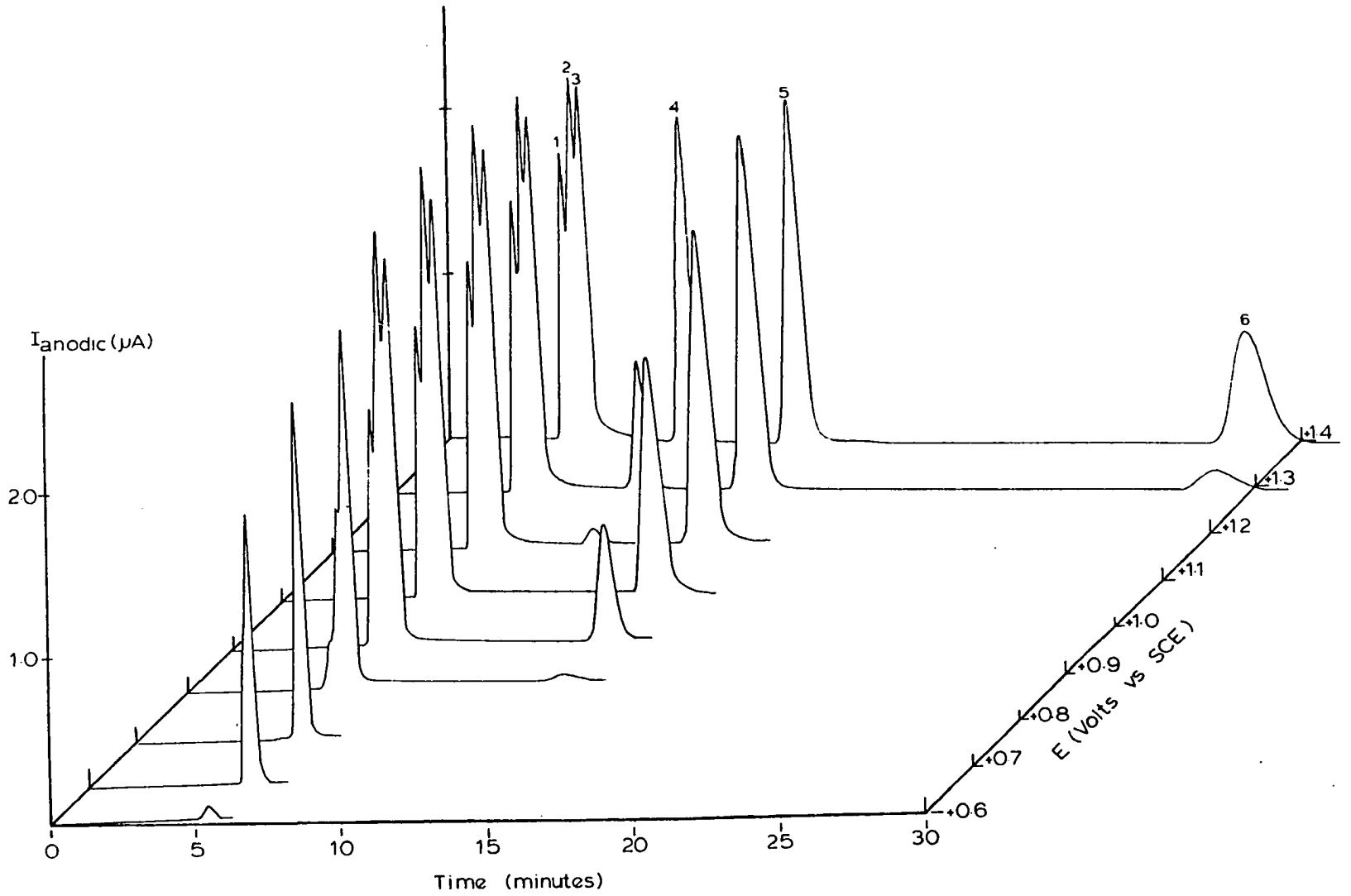
oxidations at pH values greater than the pH of Eluent H. On the other hand, the retention times of theophylline and other methylxanthines were greater for Eluent H than for Eluent F. Eventually Eluent F was chosen primarily because solubility of many physiological substances was expected to be greater at the pH of Eluent F than at the extreme value of Eluent H. Eluent F has a pH between the physiological pH values of urine and serum and is a compromise. The experience with uric acid related in the previous chapter may be regarded as a representative illustration.

A mixture containing each of the methylxanthines except 1,7-dimethylxanthine was prepared. Injections of this mixture onto Column A were made at a variety of detection potentials. A plot showing the chromatograms as a function of detection potential is called a "chromato-voltammogram". A chromato-voltammogram for the methylxanthines is shown in Figure V.2. Tunable selectivity is dramatically demonstrated by this chromato-voltammogram. At +1.40 V each of the methylxanthines is detected. Theobromine and caffeine are tuned out at potentials less positive than +1.15 V. Response for theophylline is still quite adequate at +1.15 V. The number of samples analyzed in a given time period may be increased if one wants to determine only theophylline by injection of a subsequent sample before all undetected components of the previous sample are eluted. The isomeric

Figure V.2. Chromato-voltammogram of methylxanthines at glassy carbon

Peak	Identity	
1	1.77 µg	7-methylxanthine
2	1.61 µg	3-methylxanthine
3	1.59 µg	1-methylxanthine
4	3.67 µg	theobromine
5	4.09 µg	theophylline
6	7.27 µg	caffeine

ADAM 5 (dc mode)
Glassy carbon detector
Column A
Eluent F 2.65 ml/min


I anodic (μA)
2·0
1·0
0
5
10
15
20
25
30
Time (minutes)
+0·6
+0·7
+0·8
+0·9
+1·0
+1·1
+1·2
+1·3
+1·4
E (Volts vs SCE)
1
2
3
4
5
6

monomethylxanthines have greatly different half-wave potentials and also illustrate the tunable selectivity of amperometric detectors.

A plot of peak height for each methylxanthine as a function of detection potential is shown in Figure V.3. Such a family of current-potential curves showing the detection domains of the methylxanthines is useful for optimization of sensitivity and selectivity. A plateau in amperometric response is observed for each compound. The distortions in the plateau of the detection domain for 1-methylxanthine result because of the incomplete resolution of 1-methylxanthine from 3-methylxanthine and 7-methylxanthine. These distortions parallel the loss in response for the unresolved monomethylxanthines as these compounds are "tuned out". The loss in response that accompanies ultrapositive detection potentials is not shown in this figure. In separate studies, response was observed to diminish at ultrapositive potentials; the response for 1-methylxanthine shown in Figure IV.7 may be regarded as typical of the other methylxanthines.

As mentioned, 1,7-dimethylxanthine was omitted from the previous experiments because it is unresolved from theophylline. A separate study was conducted for these two unresolved isomers, and the detection domains are superimposed in Figure V.4. The detection domain of each of the methylxanthines is unique and the differences may be utilized to illustrate the selectivity of amperometric detectors.

Figure V.3. Family of current-potential curves: partial detection domains for methylxanthines

Conditions identical to Figure V.2

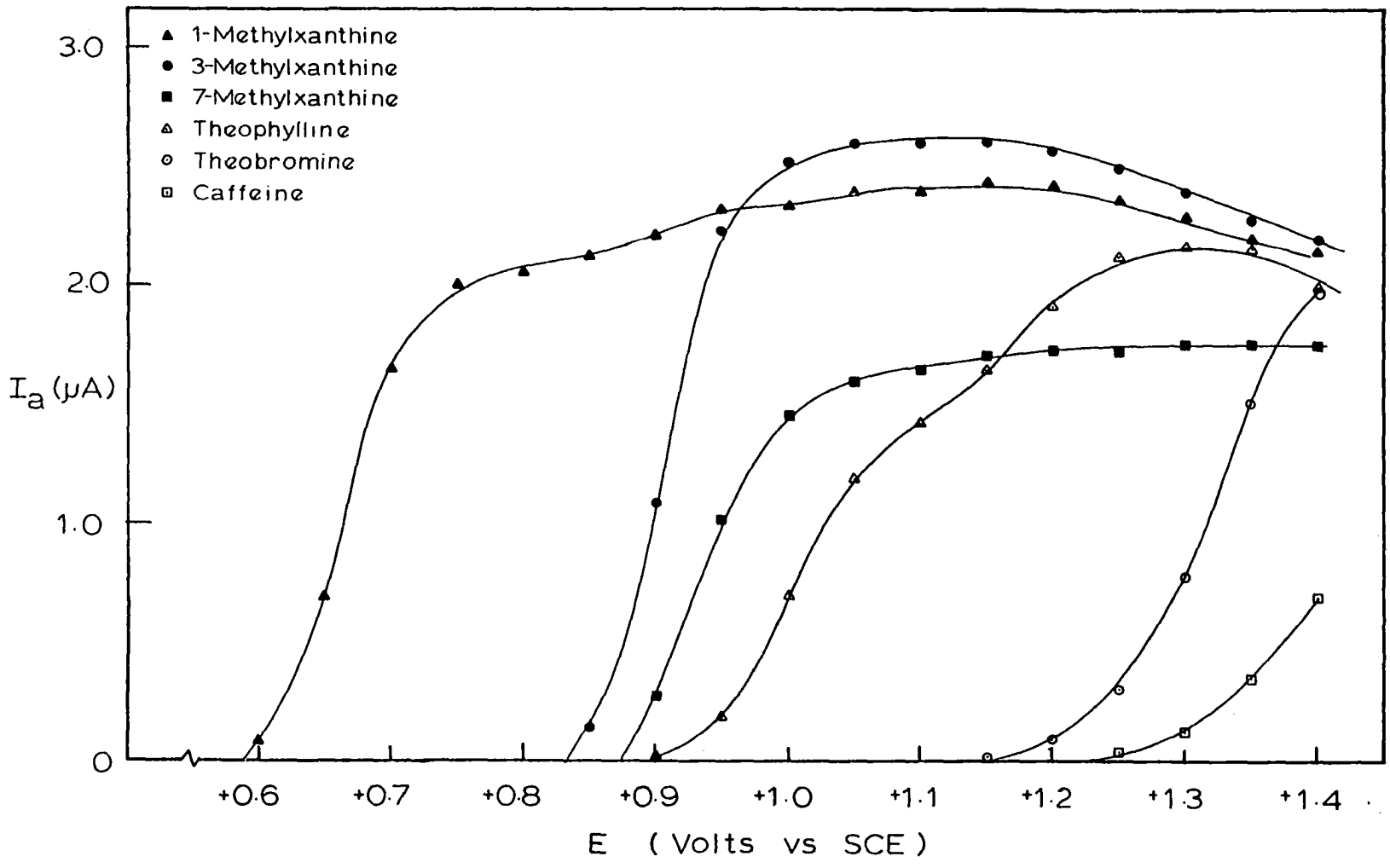

1-Methylxanthine
3-Methylxanthine
7-Methylxanthine
Theophylline
Theobromine
Caffeine
3.0
2.0
1.0
0
I_a (μA)
+0.6
+0.7
+0.8
+0.9
+1.0
+1.1
+1.2
+1.3
+1.4
E (Volts vs SCE)

Figure V.4. Detection domains for two unresolved dimethylxanthines

⊙ 18 µg of 1,7-dimethylxanthine

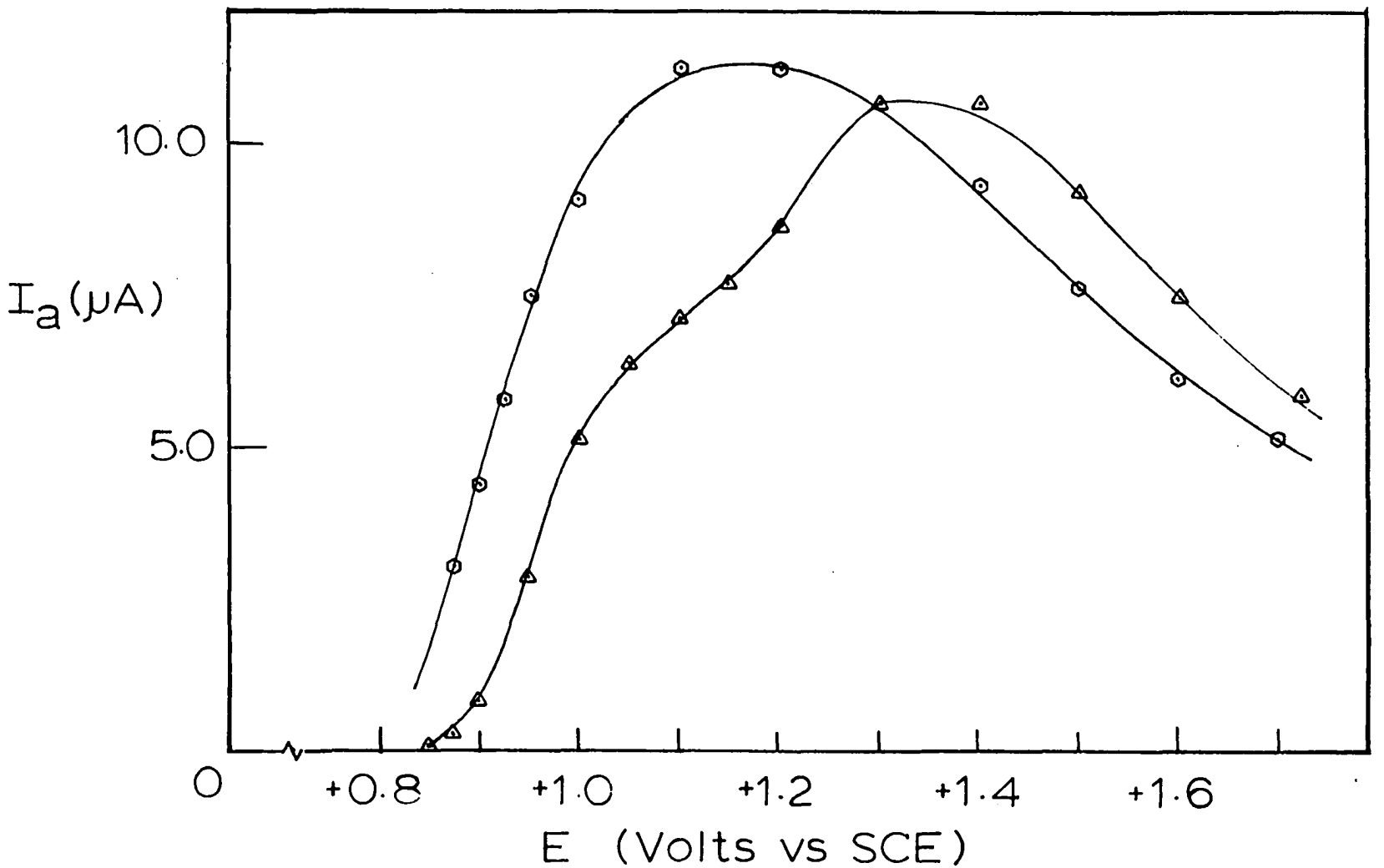
△ 18 µg of theophylline

ADAM 5 (dc mode)

Glassy carbon detector

Column A

Eluent F 1.50 ml/min


10.0
5.0
I_a (μA)
0
+0.8
+1.0
+1.2
+1.4
+1.6
E (Volts vs SCE)

Now that the detection domains of the methylxanthines are established, various modes of potential control may be discussed. The real merit of differential pulse amperometric detection is discretional selectivity. The instrument (PAR 174A) available to demonstrate the immunity of differential pulse amperometric detection was not designed for this purpose, but sufficiently promising data were obtained to encourage development of an improved instrument. Much work will have to be performed to optimize such an instrument as a part of a detection system for liquid chromatography. The methylxanthines should provide a useful model system for optimization of pulse parameters in that development.

The degree of immunity obtained with the PAR 174A operated in the differential pulse mode is illustrated in Figure V.5. This is the same mixture of methylxanthines used to obtain the data shown in Figure V.2, and Figure V.3. The detection window for differential pulse amperometric detection of the upper chromatogram was chosen to focus on theophylline while ignoring the methylxanthines oxidized at less positive and more positive potentials. Note particularly that the most easily oxidized compound, 1-methylxanthine, is hardly detected in this potential window. Response has been enhanced for the remaining dietary methylxanthines in the chromatogram shown in lower part of Figure V.5. Flow

Figure V.5. Differential pulse amperometric detection of methylxanthines with discretional selectivity

A Focusing on theophylline

E_1 = +1.025 V E_2 = +1.125 V

B Focusing on theobromine and caffeine

E_1 = +1.300 V E_2 = +1.400 V

Same mixture of methylxanthines as shown in Figure V.2

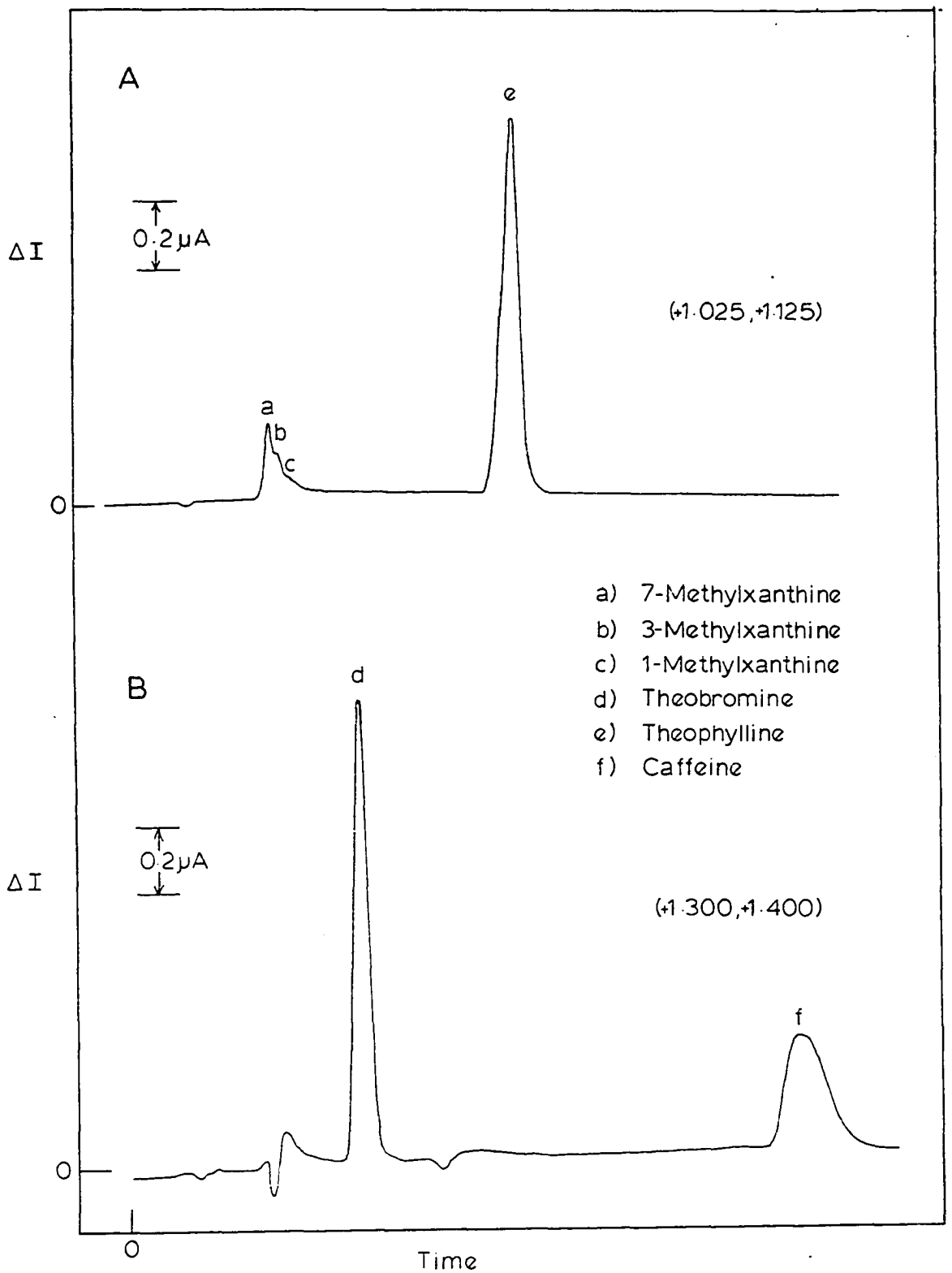
Cycle time 0.5 sec

PAR 174A (differential pulse mode)

Glassy carbon detector

Column A

Eluent F


A
e
0·2μA
ΔI
(+1·025,+1·125)
a
b
c
0
a) 7-Methylxanthine
b) 3-Methylxanthine
c) 1-Methylxanthine
d) Theobromine
e) Theophylline
f) Caffeine
B
d
0·2μA
ΔI
(+1·300,+1·400)
f
0
0
Time

rates were not identical for the two chromatograms shown in Figure V.5 because these experiments were performed at different times; retention times are not shown because the flow rates were not calibrated exactly.

An exploratory experiment was conducted using various pulse amperometric detection windows. Pulse detection windows were examined that had an initial potential sufficiently positive to oxidize all of the methylxanthines yet had a final potential less positive than the onset of the detection domain for some of the methylxanthines. Only the current at the final potential was sampled. All of the methylxanthines were detected for such windows even though the current at the final potential could not have been anodic in many cases. The absolute value circuitry of the PAR 174A in the pulse mode causes all peaks to be displayed in the same direction. I conclude that the products of the methylxanthine oxidation are being detected at the less positive final potential. This prospect was not investigated further because there was no advantage for detection since all of the methylxanthines could be detected without resorting to the ultrapositive potentials. Sensitivity for the pulse amperometric mode of detection of the oxidation product was not as great as sensitivity for pulse amperometric formation of the oxidation product.

Theophylline and 1,7-dimethylxanthine are difficult to separate chromatographically and the detection domains shown in Figure V.4 are quite similar. Since the detection domains are not identical, a simultaneous determination might be possible. An ultraviolet detector and an electrochemical detector could be used in series. Only an electrochemical detector would be required if the detection potential could be varied in a programmed manner with the storage of the frequently sampled current at a variety of detection potentials. Such multiplexing is not possible with the PAR 174A, but by multiplexing the detection potentials in this manner one could obtain the full sensitivity of pulse amperometric detection and also by selective subtraction, the immunity of differential pulse amperometric detection. Realistically, there is very little interest in the determination of 1,7-dimethylxanthine. Perhaps, differential pulse amperometric detection could be used to determine theophylline with only trivial interference from 1,7-dimethylxanthine even though the two compounds are unresolved. The response for equal concentrations of these dimethylxanthines was compared using several modes of detection.

Each of the possibilities suggested is represented in Table V.1. The simultaneous analysis using an ultraviolet detector and an amperometric detector in series certainly seems feasible. The various pulse amperometric detection

Table V.1. Response ratios for equal amounts of unresolved dimethylxanthines[a]

Mode of Detection	Peak Response		Ratio
	Theophylline	1,7-dimethylxanthine	
Ultraviolet			
254 nm	0.557 AU	0.526 AU	1.06
Amperometric			
+1.275 V vs. SCE	6.26 μA	4.39 μA	1.43
Pulse Amperometric			
+0.800, +1.100 V	2.68 μA	5.79 μA	0.463
+0.800, +1.200 V	7.98 μA	10.57 μA	0.754
+0.800, +1.300 V	12.01 μA	12.13 μA	0.990
Differential Pulse Amperometric			
+1.175, +1.275 V	0.622 μA	0.094 μA	6.62

[a]18 μg of theophylline or 1,7-dimethylxanthine injected.

windows should encourage the multiplexing approach once such instrumentation is available. The immunity to 1,7-dimethylxanthine obtained with differential pulse amperometric detection is encouraging because the amount of the caffeine metabolite (1,7-dimethylxanthine) that may be found in serum samples would contribute only a trivial amount of the signal resulting mainly from a therapeutic concentration of theophylline. Further improvements in immunity can be anticipated with improvements in instrumentation.

One important aspect of the detection domain of theophylline has been ignored in the interpretation of the data given in Table V.1. The detection domain of theophylline is dependent on concentration. The detection domain of 1.0×10^{-3} M theophylline in Figure V.3 and 2.27×10^{-4} M theophylline in Figure V.4 show small differences in the half-wave potential that may be partly due to the difference in concentration of theophylline. The calibration curve of theophylline shown in the previous chapter for differential pulse amperometric detection of theophylline is more suggestive of variation of the half-wave potential as a function of concentration. The oxidation of theophylline in Eluent F is mechanistically complex. Response ratios do vary for different concentrations. This variation may be tolerable for the determination of theophylline because the concentration falls within a narrow range in the samples to

be analyzed. However, calibration and determinations of response ratios must be made frequently and in the concentration range of interest.

D. The Sentry Standard Technique

Such problems as just mentioned as well as variation of the condition of the electrode surface lead to the desire for a frequent monitor of detector response. If the product of the electrode reaction fouls the detector, a control or standard injected with each chromatogram would give a prompt warning. A new injection technique, to be christened the "Sentry Standard Technique", was devised to monitor detector response on a continuing basis.

Every chromatogram has spaces where no peak appears. This space may be at the end of the chromatogram but oftentimes the spaces are located elsewhere in the chromatogram. As detectors become more selective, the gaps between peaks become wider and more numerous. The gaps between peaks may be utilized by making one or more injections containing known amounts of a substance that elutes in the blank space. These injections are made without waiting for the chromatogram to fully develop so little or no additional time is required. Any compound may be chosen as a sentry standard, even the substance one wishes to determine. This versatility of choice of the sentry standard is illustrated in Figure V.6.

Figure V.6. The sentry standard technique

A. Separation of methylxanthines

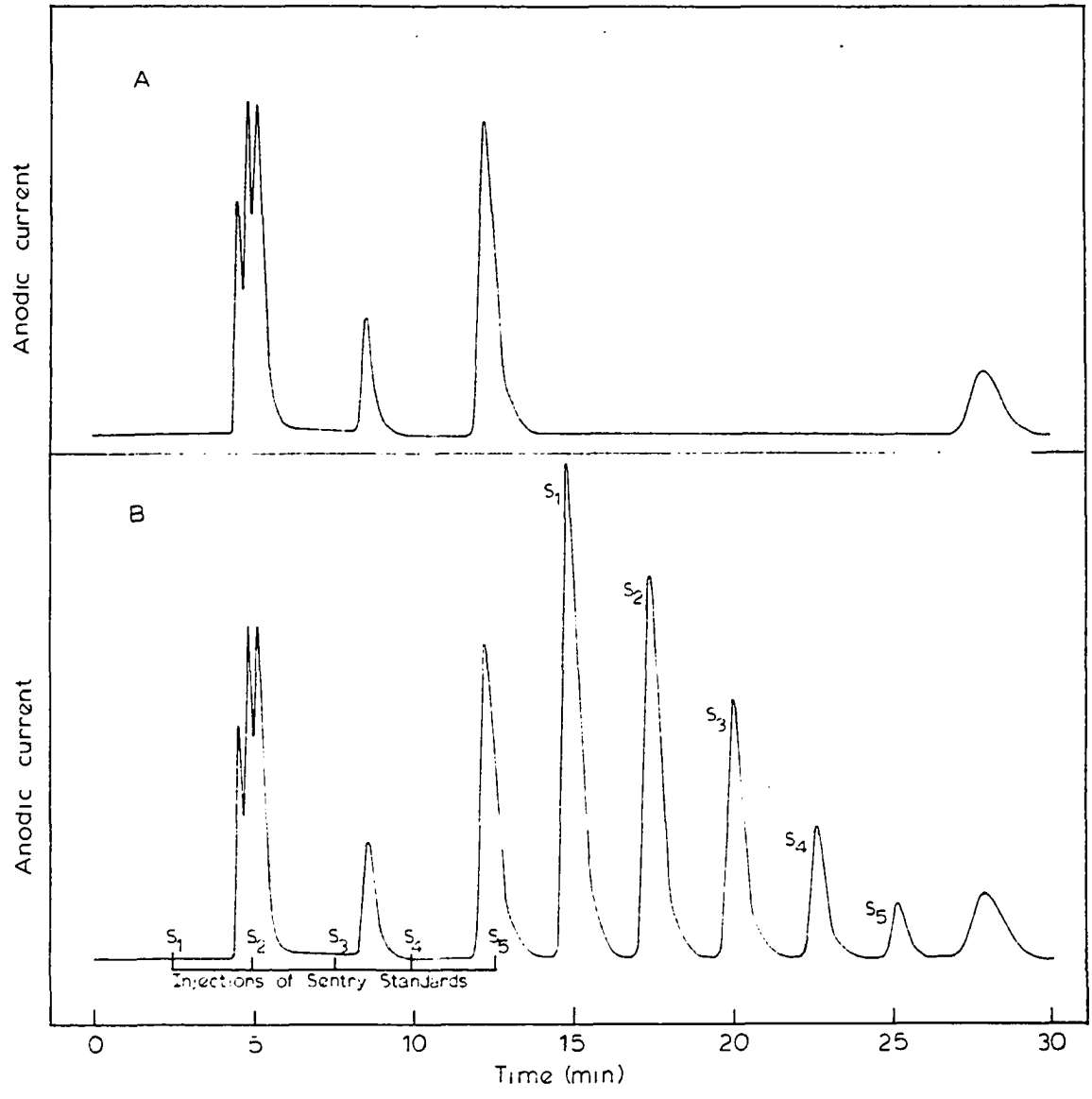
B. Separation of methylxanthines with theophylline sentry standards

ADAM 5 (dc mode)

Glassy carbon detector

Column A

Eluent F


A
B
Anodic current
Anodic current
S1
S2
S3
S4
S5
S1
S2
S3
S4
S5
Injections of Sentry Standards
0
5
10
15
20
25
30
Time (min)

Shown in this figure is a chromatogram for the separation of the methylxanthines with amperometric detection. A gap of nearly thirteen minutes is observed between the theophylline peak and the caffeine peak, but by making multiple injections of standard solutions of theophylline a standard curve may be prepared. No increase in band spreading for the caffeine peak was observed even though several sentry standards were injected. However, additional band spreading may be noted if the loading capacity of a column is approached. For quality control applications, one may wish to inject two sentry standards at each limit of the tolerance for that particular constituent. In therapeutic monitoring of a drug, the low therapeutic and high therapeutic levels would be desirable sentry standards. The sentry standard technique should be very helpful if detector response is nonlinear. Of course, standard curves may be prepared more quickly using this technique; compounds with long retention times are excellent candidates for this time saving technique. Once the band width of the peak is known, one may easily choose the time interval between injections.

In addition to being a desirable monitor of detector response, the sentry standard technique may be a possible alternative to the internal standard technique. Of course, the technique does not have all the advantages of the internal standard technique. If one has a limited sample

with the constituent concentration near the detection limit, he may wish to make direct injections of the sample without prior dilution with an internal standard. The sentry standard may also be considered when an appropriate internal standard can not be found readily.

The sentry standard technique should find application both in liquid chromatography and gas chromatography. The technique should be most useful for the monitoring and calibration of selective detectors and nonlinear detectors. Detectors for liquid chromatography with tunable selectivity include electrochemical, fluorescence and variable wavelength spectrophotometric detectors. In gas chromatography one may find the sentry standard technique useful for electron capture detectors and other selective detectors. Gas chromatography interfaced with mass spectrometry often is performed in the selected ion mode. The sentry standard could also be useful for calibration of response in such applications.

One caution must be exercised. Injection artifacts could cause multiple injections to obscure important parts of the chromatogram. Sometimes injection artifacts may be eliminated such as in the case when abstractors are used in gas chromatography (129, 130). In liquid chromatography with anodic electrochemical detection, matching of eluent and the sentry standard diluent generally eliminates most artifacts.

The sentry standard technique requires a knowledge of the general appearance of the chromatogram, but this is equally true of the internal standard technique. Both techniques are best suited to routine analyses where the retention times are known. In routine determinations one generally has a knowledge of the appearance of a chromatogram, and one would notice if the response of a sentry standard varied significantly because of co-elution of another substance.

The advantages of the sentry standard multiple injection technique are:

1. timesaving and simple,
2. versatility in placement of sentry standards,
3. versatility in choice of sentry standards,
4. standardization is performed more frequently and therefore accuracy improves.

The full benefit of the sentry standard technique will be realized as the number of applications increase. Most assuredly, the sentry standard injection technique will be useful for monitoring electrochemical liquid chromatographic detector response.

VI. LIQUID CHROMATOGRAPHIC DETERMINATION OF THEOPHYLLINE IN BLOOD SERUM

> "... there is an increasing tendency to devote more and more time to determinations which deal with the final act of a chemical analysis, and less and less time to the chemical analysis itself - in other words, to consider chemical analysis as dealing with one or two variables instead of the dozen or more that are often involved. The gradual loss of the analytical viewpoint is evident in contemporary articles that purport to deal with chemical analysis. ... Such methods ... are about as helpful to the analyst as the method for catching a bird which the old folks used to recommend to children - namely to sprinkle salt on its tail. To do that, one must obviously have the bird in hand, and in that case there is no need for the salt."
>
> -- G. E. F. Lundell

A. Introduction

An analytical method developed for a model system is subjected to the acid test when applied to genuine samples. Objective comparison of electroanalytical techniques with other analytical techniques may be made only if genuine samples are analyzed in a realistic manner. A suitable technique for the determination of theophylline in blood serum must be sufficiently responsive to accommodate small volumes of sample.

A fast and simple procedure for the selective determination of the concentration of theophylline in small volumes

of serum is described in this chapter. Recovery of theophylline is virtually quantitative. Theophylline is resolved from several other drugs that are administered in patient therapy for asthma. A truly realistic assessment of the potential interference from other drugs that are administered in combination with theophylline would involve extensive trials with human patients. Such trials are beyond the scope of this dissertation.

The use of two detectors as partners is advantageous to pinpoint interferences that can remain hidden if only a single detector is used. Accordingly, the pulse amperometric mode of detection is compared with the ultraviolet spectrophotometric mode of detection for the determination of theophylline. A pharmacokinetic study of theophylline is also reported using these two modes of detection. The interference from the unresolved caffeine metabolite, 1,7-dimethylxanthine, illustrates the utility of using these two modes of detection in a partnership.

B. Experimental

1. Chemicals and reagents

Ammonium hydroxide, nitric acid, ammonium thiocyanate, ferric ammonium sulfate and silver nitrate were supplied by J. T. Baker, Inc. Quinbron® capsules, manufactured by Mead Johnson Laboratories, were obtained from the Student

Health Service of Iowa State University. Phenobarbital was supplied by Merck and Company, Inc. Lyophilized Q-PAK®-Chemistry Control serum (unassayed) was generously provided by Hyland Division Travenol Laboratories, Inc.

2. Preparation of solutions

Ammonia: One hundred milliliters of concentrated ammonium hydroxide was added to 150 ml of deionized water and thoroughly mixed to give 6 M ammonia.

Ferric Alum indicator: Eight grams of ferric ammonium sulfate was dissolved in 100 ml of deionized water. A few drops of concentrated nitric acid were then added and the solution was thoroughly mixed.

Silver nitrate (0.1000 M): Primary standard grade silver nitrate was dried for two hours at 105 °C. After cooling to room temperature in a desiccator, 16.988 g of silver nitrate was added to a one-liter volumetric flask. Deionized water was added to the mark and the solution was mixed thoroughly. This solution was protected from light by wrapping the flask with aluminum foil.

Ammonium thiocyanate (0.1 M): To a one-liter volumetric flask, 9.032 g of ammonium thiocyanate was added. Deionized water was added to the mark, and the solution was mixed thoroughly. This solution was standardized by a Volhard titration.

Eluent F: Four liters of deionized water was added to a large conical flask containing 56.8 g of sodium monohydrogen phosphate and 55.2 g of sodium dihydrogen phosphate monohydrate. After dissolution was complete, 200 ml of this phosphate buffer was withdrawn for use as the sample diluent. To the remaining solution in the conical flask, 200 ml of acetonitrile was added to give an eluent of 5% (v/v) acetonitrile. Matched eluent was used as diluent for the sentry standards.

Sentry Standards: Stock sentry standard was prepared by dissolution of 200.0 mg of dried theophylline in matched Eluent F. A ten-fold dilution was made by addition of 10.00 ml of this solution to a 100-ml volumetric flask with addition of matched Eluent F to the mark. The concentration of this solution of theophylline was 20.0 mg/l. To four 100-ml volumetric flasks were added, respectively, 2.50 ml, 5.00 ml, 10.00 ml and 15.00 ml of this 20.0 mg/l standard. Matched Eluent F was added to the mark of each flask and the four solutions were thoroughly mixed. These sentry standards account for the dilution of the sample when prepared for analysis. The signals obtained for these standards were equivalent to serum samples with theophylline concentrations of 5.0 mg/l, 10.0 mg/l, 20 mg/l and 30 mg/l when prepared for analysis by the procedure presented later.

Additional sentry standards were prepared by appropriate dilutions with matched Eluent F.

Reconstituted lyophilized serum: To reconstitute the lyophilized serum, exactly 10.00 ml of deionized water was added to the vial containing the serum constituents. The stopper was replaced and the solution was mixed gently until dissolution was complete.

An aqueous solution of theophylline with a concentration of 20.0 mg/l was used as diluent for selected experiments. The reconstituted serum was stored at 4 °C when not in use.

3. Assay of theophylline by Volhard titration

The United States Pharmacopeial Convention, Incorporated, has a program to make available reference standards for use in official assays and tests. These reference standards are collaboratively tested and approved for such use by appropriate U.S.P. Committees and Panels. Theophylline is listed as a reference standard but is currently out of stock. In order to standardize the method for the determination of theophylline in blood serum, the purity of the material used as the standard in this research was determined.

The theophylline used for this work was "chromatographically pure". It may be said with assurance based on many chromatographic experiments that this theophylline was not contaminated by significant amounts of other methylxanthines

or other compounds of similar structure. Accordingly, the official method (103) for assay of theophylline in pharmaceutical preparations was used to determine the purity of the theophylline used in this research. The result of seven titrations using the procedure described below was 99.92% theophylline, and the standard deviation was 0.66%.

4. Assay procedure (103)

Dry the theophylline for four hours at 105 °C and allow the anhydrous theophylline to cool in a desiccator. Place about 250 mg of anhydrous theophylline, accurately weighed, in a 500-ml Erlenmeyer flask, add 50 ml of deionized water and 8 ml of 6 M ammonia, and gently warm the mixture on a steam bath until complete solution is effected. Add exactly 25.00 ml of 0.1000 M silver nitrate, mix, add several glass beads and boil on a hot plate for 15 minutes. Cool to between 5 °C and 10 °C for 20 minutes, then filter through a filtering crucible under reduced pressure and wash the precipitate with three successive 10-ml portions of deionized water. Acidify the combined filtrate and washings with 6 ml of concentrated nitric acid. Cool, add 2 ml of ferric alum indicator, and titrate the excess silver nitrate with standardized 0.1 M ammonium thiocyanate.

C. Determination of Theophylline in Serum

1. Procedure

To a 15-ml conical centrifuge tube, add 50 µl of acetonitrile with a capillary pipet. Add 100 µl of serum to the acetonitrile and aspirate the liquid into both pipets several times to insure complete precipitation of protein. Allow the capillary pipets to remain in the centrifuge tube, add 0.85 ml of the phosphate-buffered diluent reserved in the preparation of Eluent F, mix well and rinse each of the pipets several times with the solution in the tube. Mixing is accomplished by gently blowing through the pipets using a conventional mouthpiece and tubing provided with the pipets. Discard the pipets and place the centrifuge tubes in the centrifuge. Centrifuge long enough to settle the precipitated protein. Withdraw the supernatant liquid into a 1.0 ml syringe being careful not to aspirate any precipitate. There should be sufficient supernatant liquid for three injections. Inject the supernatant liquid onto the chromatograph using a 220-µl sample loop. The use of a pre-column is strongly recommended as the best insurance for an expensive column from blockage by any particulate matter that may be accidently injected. Wait approximately three minutes and begin the injections of the sentry standards at appropriate intervals. Calculate

the result by division of the peak height (or area) of theophylline in the sample by the peak height (or area) of the sentry standard and multiplication by the product of ten times the actual concentration of the sentry standard. The result is the original concentration of theophylline in serum.

The sample preparation may be scaled down for pediatric specimens by addition of 20 µl of serum to 20 µl of acetonitrile and subsequent addition of 0.36 ml of aqueous phosphate-buffered diluent after complete precipitation of protein. This procedure gives sufficient supernatant liquid for a single injection. The same sentry standards may be used; however, the dilution is 1:20 and not 1:10. The result is calculated by division of the peak height (or area) of theophylline in the sample by the peak height (or area) of the sentry standard and multiplication by the product of 20 times the actual concentration of the sentry standard.

When using the pulse amperometric mode of detection for this determination, set the initial potential at +0.75 V and adjust the detection potential to +1.35 V by scanning +0.600 volts more positive and then activating the "hold" switch of the PAR 174A.

Table VI.1. Synopsis of recovery data for theophylline in serum

Mode of Detection	Added mg/l	Found mg/l	Recovery %
Ultraviolet (254 nm)	20.0	20.2	101.0
	20.0	19.3	96.5
	20.0	20.7	103.2
	20.0	19.6	97.8
	20.0	19.6	97.8
Mean		19.9	99.3
Std. Dev.		0.56	2.76
Pulse Amperometric (+0.75V, +1.35 V)	20.0	19.3	96.5
	20.0	18.6	93.0
	20.0	20.4	102.0
	20.0	18.4	92.0
	20.0	19.6	98.0
	20.0	20.3	101.5
	20.0	20.9	104.5
	20.0	20.3	101.5
	20.0	20.5	102.5
Mean		19.8	99.1
Std. Dev.		0.88	4.42

2. Recovery study

To test the validity of the sample preparation, a recovery study was conducted. Lyophilized serum samples from the same lot number were reconstituted and used for this study. Data are summarized in Table VI.1. Small peaks were

observed at the retention times of 1,7-dimethylxanthine and caffeine when the ultraviolet mode of detection was used. A small peak was observed for the blank serum sample at the common retention time of theophylline and 1,7-dimethylxanthine using the pulse amperometric mode. The blank response at the retention time of theophylline was subtracted from the response of the spiked serum to obtain the recovery data presented in Table VI.1. The recovery was essentially quantitative in both samples analyzed immediately and samples analyzed 24 hours after reconstitution. Any loss due to protein binding was negligible.

3. Interference study

Many drugs are administered in addition to theophylline for treatment of asthma. The potential interference of only a few of these substances was examined. Dyphylline and phenobarbital elute after theophylline under the conditions described here for the chromatographic determination of theophylline. Caffeine also elutes much later. These three substances were detected by the ultraviolet detector; however, no significant response was observed for any of the three compounds using the pulse amperometric mode of detection at the same concentration level. Phenobarbital is apparently not electroactive in the detection window chosen. Although caffeine is electroactive, the detector was not sufficiently sensitive to detect caffeine at existing concentrations with

the pulse amperometric mode of detection. Dyphylline in serum elutes immediately after theophylline. The sensitivity of the detector in the pulse amperometric mode for dyphylline was not determined, but sensitivity appeared comparable to that for caffeine.

Among the methylxanthines only the caffeine metabolite, 1,7-dimethylxanthine caused significant interference with determination of theophylline because of the coincidence of the retention times. To demonstrate that the peak at the retention time of theophylline is associated with consumption of caffeine, I excluded coffee and other beverages containing methylxanthines from my diet for 70 hours. A blood specimen was collected by venipuncture for analysis after fasting overnight. Four cups of coffee were consumed within thirty minutes after collection of the fasting specimen. Additional blood specimens were collected at the times indicated in Table VI.2. Each specimen was allowed to clot and was centrifuged; the serum was analyzed. A theophylline sentry standard was used for comparison. Although the peak at the retention time of theophylline in each sample is attributed to 1,7-dimethylxanthine, the concentration is, nevertheless, reported as theophylline because this interference would add to the calculated value for theophylline.

The pulse amperometric mode for detection of theophylline is less subject to the interference by 1,7-dimethylxanthine

Table VI.2. Interference of coffee consumption with determination of theophylline

Hour	Interference (mg/l) UV	Pulse
0	0.2	0.0
1.5	0.7	---
3.0	1.4	---
4.5	1.5	0.7

than the ultraviolet mode. This finding may be somewhat surprising in light of the response ratio of 0.990 reported in the previous chapter for a slightly different detection window and much larger amounts of theophylline and 1,7-dimethylxanthine. This observation demonstrates the necessity of frequent determinations of the response ratios in the concentration range of interest. The interference from 1,7-dimethylxanthine may actually be expected to be greater than indicated in Table VI.2 because the entire course of the metabolism of caffeine in this dose was not followed. More importantly, this amount of 1,7-dimethylxanthine has not been supplemented by additional metabolite from previous coffee consumption as would be expected for habitual consumption of coffee. Orcutt et al. (112) indicated that the interference may be as great as 3.5 mg/l.

Other methylxanthines are medically contraindicated in asthma (2, 3). Beverages containing caffeine and other methylxanthines should be withheld from patients treated

with theophylline because toxic reactions may occur. This medical contraindication is fortunate in terms of the analytical determination. However, the ultraviolet detector would detect any caffeine and warn the clinician of noncompliance by the patient, and the electrochemical detector would give a better estimate of actual theophylline concentration without the necessity of repeating the determination on a new specimen. If only the electrochemical detector is used, the analysis time is reduced by nearly one half because there is no need to wait for the elution of caffeine or phenobarbital before making the next injection of sample. If the ultraviolet detector is used, one generally must wait until these compounds have eluted before making the next injection of sample.

4. Pharmacokinetic study of theophylline

Many would argue with some justification that the determination of theophylline in reconstituted lyophilized serum is not "The Analysis of Things as They Are"; they would call out for my blood. Accordingly, a pharmacokinetic study was conducted. Beverages containing methylxanthines were excluded from my diet for a period of 18 hr with the consequential mental agony since habitual consumption of coffee and cola is my normal dietary practice. After a fasting period of eight hours, a blood specimen was collected by venipuncture. Two Quinbron® capsules containing a total dose of 300 mg of

Figure VI.1. Liquid chromatographic separation of theophylline in blood serum

Cycle time 0.5 sec

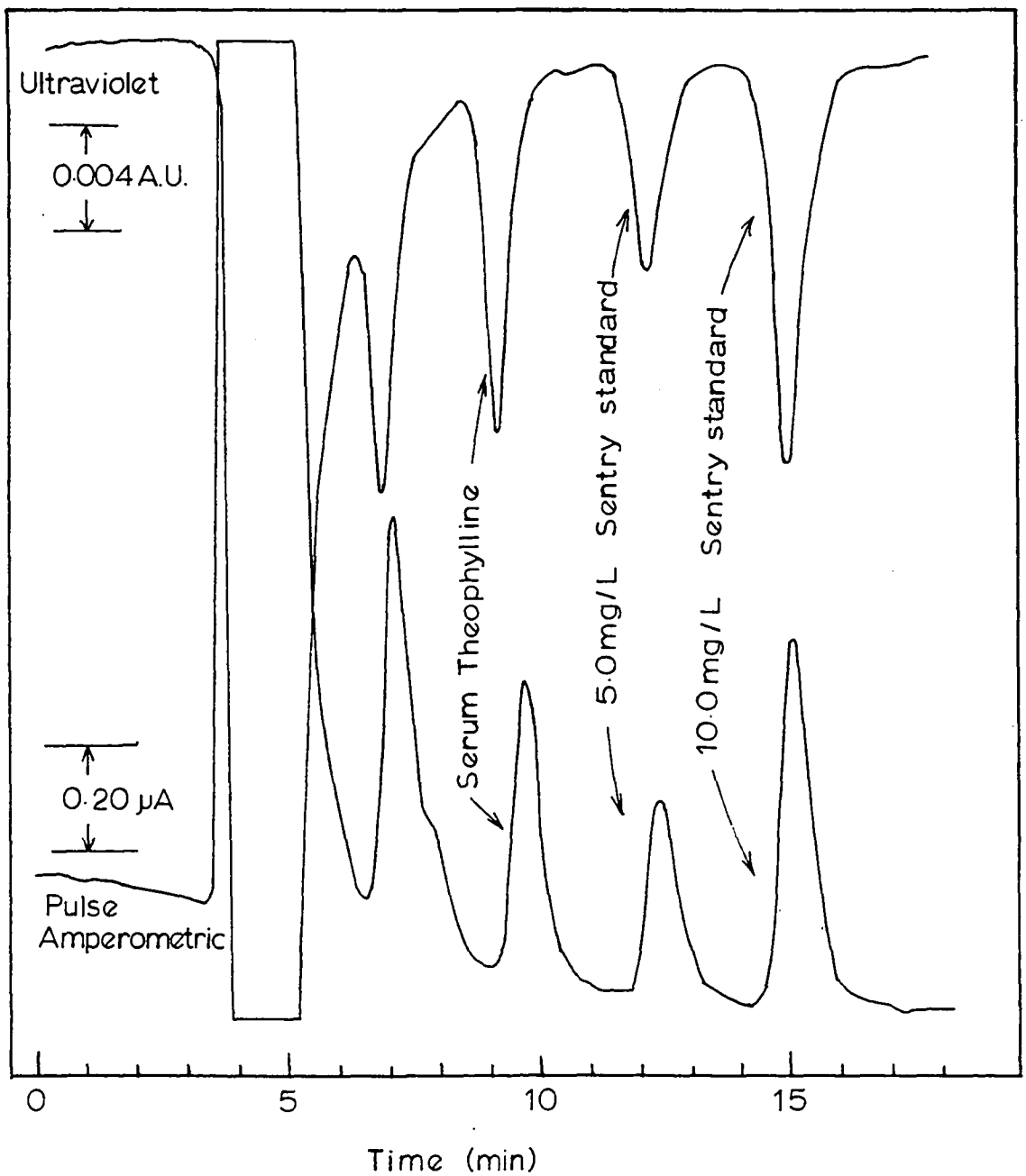
PAR 174A (pulse mode) E_1 = +0.75 V

Glassy carbon detector E_2 = +1.35 V

Ultraviolet photometric detector (254 nm)

Column A

Eluent F


Ultraviolet
0.004 A.U.
0.20 μA
Pulse
Amperometric
Serum Theophylline
5.0 mg/L Sentry standard
10.0 mg/L Sentry standard
0
5
10
15
Time (min)

anhydrous theophylline and 180 mg of glyceryl guaiacolate (an expectorant) were taken orally. Quinbron is an extended-duration form of theophylline. Blood specimens were collected at appropriate intervals to map the pharmacokinetic response. The specimens were allowed to clot and were then centrifuged. Serum was removed and stored at 4 °C until ready for analysis. Theophylline in these serum samples was determined chromatographically using ultraviolet detection as well as pulse amperometric detection. A typical pair of chromatograms with peaks for sentry standards is shown in Figure VI.1. Although these chromatograms are shown on the same figure, they were obtained on separate portions of the same sample.

The pharmacokinetic response for both modes of detection is illustrated in Figure VI.2. Apparently, the restriction of caffeine for 18 hr was not adequate because the first specimen gave a small peak at the retention time of 1,7-dimethylxanthine which was detected by both detectors. It seems doubtful that this peak resulted from a normal constituent of serum. This conclusion is supported by the fact that no peak at this retention time was observed 70 hr later by pulse amperometric detection. The response at 70 hr by ultraviolet detection was a mere 0.2 mg/l reported as theophylline. Furthermore, one observes from Figure VI.2 that the pulse amperometric response was always less than the ultraviolet response during the early hours of the

Figure VI.2. Pharmocokinetic plots for theophylline in serum

▲ ultraviolet detection

● pulse amperometric detection

Cycle time 0.5 sec

PAR 174A (pulse mode)

Glassy carbon detector

E_1 = +0.75 V

E_2 = +1.35 V

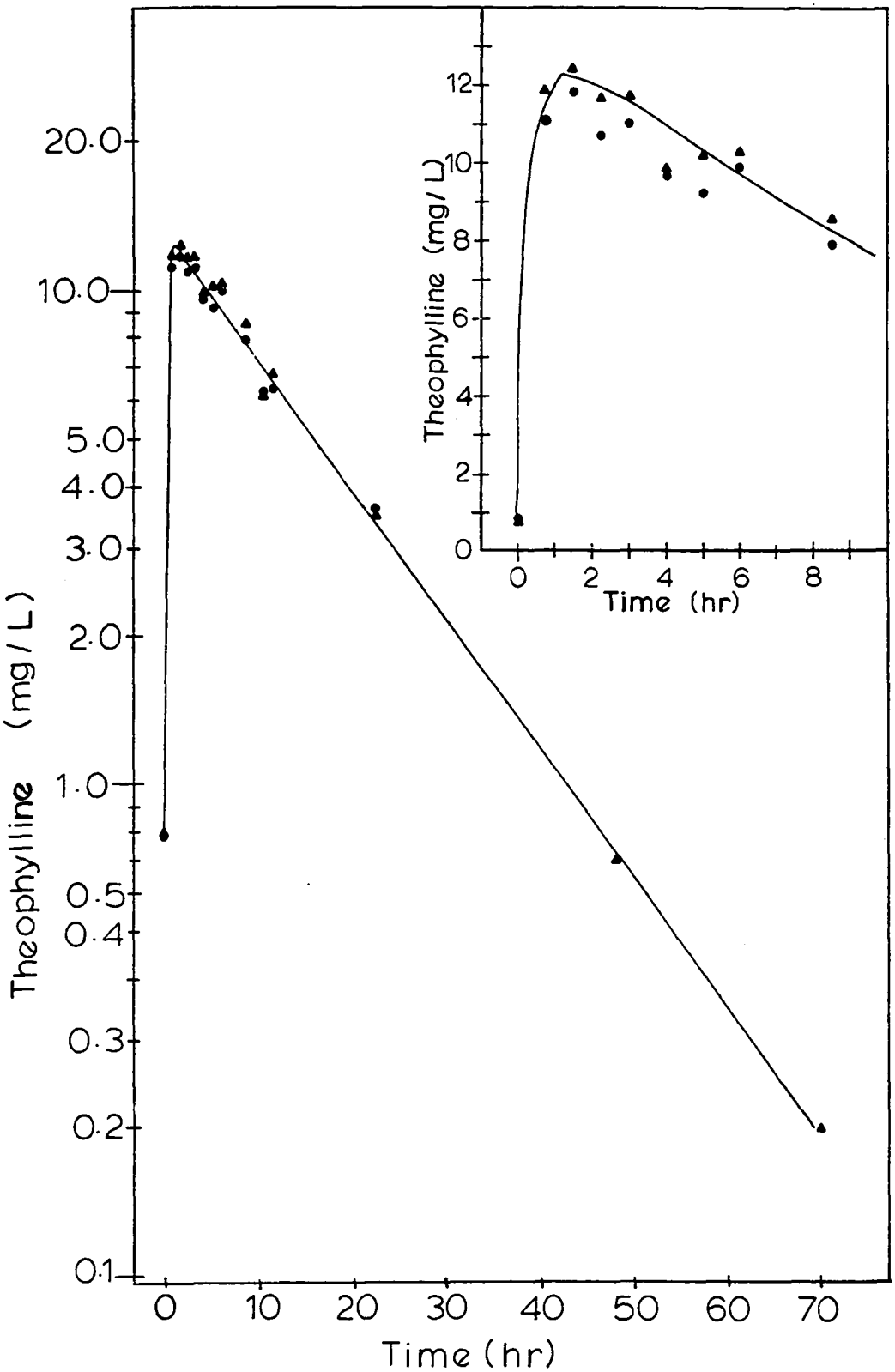
Ultraviolet photometric detector (254 nm)

Column A

Eluent F

Dose 4.4 mg/kg

Half-life 12 hours (extended-duration form of theophylline)


Theophylline (mg/L)
20.0
10.0
5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Time (hr)
Theophylline (mg/L)
12
10
8
6
4
2
0
0
2
4
6
8
Time (hr)

pharmacokinetic study by about 0.6 mg/l on the average. Admittedly, this value is within the error of the methods, but the trend is apparent and probably is not coincidental.

I conclude that only 1,7-dimethylxanthine and theophylline will normally be eluted together. No other serum constituent occurring in normal amounts will elute at this retention time. One may be assured that if the ultraviolet and electrochemical detectors are used in combination, any significant deviation of the two results is a consequence of caffeine consumption provided no other drugs are being taken. If other drugs are being taken and a significant difference in the two results is noted, one should suspect that the drug or a metabolite of that drug elutes at the same retention time.

D. Comparison of Pulse Amperometric Detection with Ultraviolet Detection for the Determination of Theophylline

During the course of the pharmacokinetic investigation, numerous sentry standards were injected so that the sensitivity of the ultraviolet detector and the pulse amperometric detector could be compared objectively. These data are summarized in Table VI.3. The numerals in parentheses indicate the number of determinations used to calculate the mean and the standard deviation. Sensitivity is defined here as the slope of the calibration curve divided by the standard deviation at that point. This definition of sensitivity is rational because both modes of detection then may be compared

Table VI.3. Comparison of sensitivity of an ultraviolet detector and an electrochemical detector for determination of theophylline

Detector	Amount Injected	Peak Response[a]	σ	σ	Sensitivity
UV (254 nm)	(ng)	(AU)	(AU)	(ppt)	(ng^{-1})
	110	0.0072(11)	0.00031	43.2	21.6×10^4
	220	0.0146(11)	0.00071	48.7	9.4×10^4
	440	0.0293(8)	0.00077	26.3	8.7×10^4
		For Ultraviolet Detection			
		slope	6.69×10^{-5} AU/ng		
		intercept	1.75×10^{-4} AU		
Pulse Amp. (+0.75, +1.35)	(ng)	(μA)	(μA)	(ppt)	(ng^{-1})
	44	0.126(1)			
	66	0.197(1)			
	110	0.347(10)	0.0126	36.3	20.2×10^4
	220	0.624(12)	0.0233	37.4	10.9×10^4
	440	1.164(8)	0.0302	25.9	8.4×10^4
	660	1.703(3)			
		For Pulse Amperometric Detection			
		slope	2.53×10^{-3} μA/ng		
		intercept	0.043 μA		

[a]Number of determinations shown in parentheses.

on the same basis. As indicated in Table VI.3, the sensitivity of the two modes of detection are equivalent for the conditions chosen. An ultraviolet detector would be expected to be slightly less than three times as sensitive than indicated if the optimum wavelength of 273 nm were used (7, 112). Nevertheless, the electrochemical detector has sufficient sensitivity to permit this determination of theophylline on serum samples of realistic volume (20-100 µl). Since the electrochemical detector compares favorably with the ultraviolet detector for determinations in "real" samples under realistic conditions, there is every reason to suggest that ultraviolet detectors and electrochemical detectors form a beneficial partnership.

VII. METABOLIC PROFILES OF URINE BY LIQUID CHROMATOGRAPHY

> "It is the fixity of the internal environment which is the condition of free and independent life... All the vital mechanisms, however varied they may be, have only one object, that of preserving constant the condition of life in the internal environment." -- Claude Benard

A. Introduction

Regulatory mechanisms in the body serve to maintain the composition of the body fluids within narrow limits of concentration. Virtually every physiological activity of the body contributes to homeostasis. Poor regulation as a result of a functional or infectious disease is generally accompanied by characteristic changes in the chemical composition of body fluids. The composition of the body fluids is, therefore, an excellent indicator of the malfunctioning of these regulatory mechanisms. In clinical medicine, determination of the concentrations of the numerous components of physiological fluids is of established diagnostic utility.

Current analytical methods lack the necessary resolution to perform a complete multicomponent analysis of physiological fluids. Most of the quantitative clinical tests presently available are for a single substance or only a few substances. A multicomponent analysis is accomplished by performing many individual tests on a single specimen. A

biochemical profile presents the results of a battery of such tests performed for an individual patient. Such biochemical profiles are of special utility for following the course of disease and, in preventive medicine, for early diagnosis of disorders with equivocal or unrecognized symptoms. Biochemical profiles consisting of twenty tests are available at a cost that is not too exorbitant because the automation of these tests has reduced the time required for the complete analysis. Yet, this cost may be prohibitive for widespread preventive medicine and for poor persons without medical insurance. A new approach is required for inexpensive diagnosis and preventive medicine.

The process of intervention to maintain the substances normally present in the body at optimum concentrations has been called "orthomolecular medicine" by Robinson and Pauling (8, 9). By observing changes in the substances that are normally present (the orthomolecules) as well as other changes in the biochemical profile, an orthomolecular diagnosis is made based on extensive statistical data from studies of patient populations. Pattern recognition techniques are used to discover diagnostically significant differences in normal and abnormal populations.

The goal of this new approach is to develop an analytical method such that the state of health could be checked at frequent intervals for all persons regardless of their

economic status. The requirements of the analytical method as outlined by Robinson and Pauling (8) are:

1) The sample should be collected by the patient with minimal restrictions on the sampling procedure. Urine may be the sample of choice, but saliva and breath may be alternative samples.

2) One sample should be sufficient for checking essentially all abnormal and normal conditions. Quantitative analysis of several hundred substances would be required.

3) To keep the cost low, the analysis should be performed by an instrument with minimal intervention by technicians.

4) Objective evaluation and interpretation of the analytical results should be made by comparison with information stored in the memory bank of a computer.

Chromatographic methods have been developed for detection and determination of hundreds of substances in small samples of physiological fluids. These methods may be automated and the data can be analyzed by a computer. Thus, chromatographic methods are attractive for multicomponent analysis of physiological fluids for the purpose of clinical diagnosis.

Many diagnostic successes by methods based on chromatographic separations have already been reported. Dr. Albert Zlatkis and his research group at the University of Houston

have reported characteristic gas chromatographic profiles in diabetes mellitus (13-15). Their work is particularly interesting because several modes of detection were used. Gas chromatography with mass spectrometric detection was the most informative technique; however, the selectivity of the flame photometric detector for sulfur gave enough information to warrant a patent application for the routine method of analysis (131). According to the patent application, a sample trap containing Tenax GC as an adsorbent for organic molecules "was used for analysis or urine samples from normal and diabetic humans, for analysis of human breath, for air analysis, and for analysis of sulfur containing volatiles from cigarette smoke, coffee, and urine of a human with cirrhosis."

Jellum, Stokke and Eldjarn (10-12) have reported the successful diagnosis of more than forty inborn errors in metabolism using gas chromatography with mass spectrometric detection and computer analysis of the data. Robinson and Pauling have reported the use of gas chromatography as well as liquid chromatography for orthomolecular diagnosis (8, 9). They used a conventional ion-exchange separation of amino acids in urine and were able to detect patterns for diurnal variation in healthy individuals, individuality of well persons, dietary control, mental retardation and multiple sclerosis. Additional patterns could be detected by analysis

of urinary vapor. These preliminary successes herald a new era in clinical diagnosis.

Earlier, in related work at Oak Ridge National Laboratory, C. D. Scott and co-workers (17, 132, 133) suggested the use of liquid chromatographic profiles of urine specimens for clinical diagnosis. High-pressure anion-exchange chromatography was used to resolve at least 140 urinary constituents that absorb light in the ultraviolet region. Each analysis required forty hours. Additional publications have appeared using the instrument developed at Oak Ridge (16-21, 134). The effect of a diet of defined chemical composition (16) and the effect of the restriction of purines in the diet (18) were studied. Chromatographic profiles of mentally retarded children were shown to be different from profiles of normal children and adults (19), and the profiles of normal newborns and children differed from an adult composite urine sample (134). More recently, urinary chromatographic profiles of autistic children were studied (20, 21). In these studies (16-21, 132-134) many chromatographic peaks were observed that depend on the diet of the individual. Differences were also noted between day and night urine collections.

Revolutionary developments in liquid chromatography have been made in the decade since the pioneering work of Scott (133). Even so, the time required for a chromatographic

analysis of urine (6-40 hours) remains unfavorable for the routine screening of patients for undiagnosed disorders. A monumental effort involving a variety of approaches, abundant economic investment and the labor of many scientists will be required if the unequivocal diagnostic power is ever to be realized. The inherent selectivity of the chromatographic approach will be of immense value. Nevertheless, I suspect that some dietary control and other restrictions on the sampling procedure will be required. The time required for such a chromatographic analysis must be shortened significantly so that equipment costs may be distributed over a large number of samples. The time required for a chromatographic analysis can be abbreviated by using detectors as partners and by multiplexing the detection parameter of an individual detector. The utility of amperometric detection was studied and compared with ultraviolet detection for chromatographic profiles of urine.

If the sampling procedure is relegated to the patient, I doubt that all patients would cooperate, if asked to restrict intake of dietary methylxanthines for several days. How does the consumption of dietary methylxanthines affect a urinary chromatographic profile? Could electrochemical detection for liquid chromatography of urine be of utility in concert with other modes of detection for metabolic profiling? An exploratory investigation which begins to

answer these questions is reported in this chapter as an overture to the total effort that will be required.

B. Experimental

Column A was used for each separation described in this chapter. The eluent consisted of 5% (v/v) acetonitrile and 95% aqueous 0.2 M phosphate buffer (Eluent F). A Chromatronix Model 200 photometric detector was available for portions of this work. The glassy carbon detector was used for each experiment involving electrochemical detection. Both potentiostats described previously were used. The PAR 174A was used for all experiments involving differential pulse amperometric detection and some amperometric experiments. The ADAM 5 potentiostat was used when an attenuator was required in the amperometric experiments.

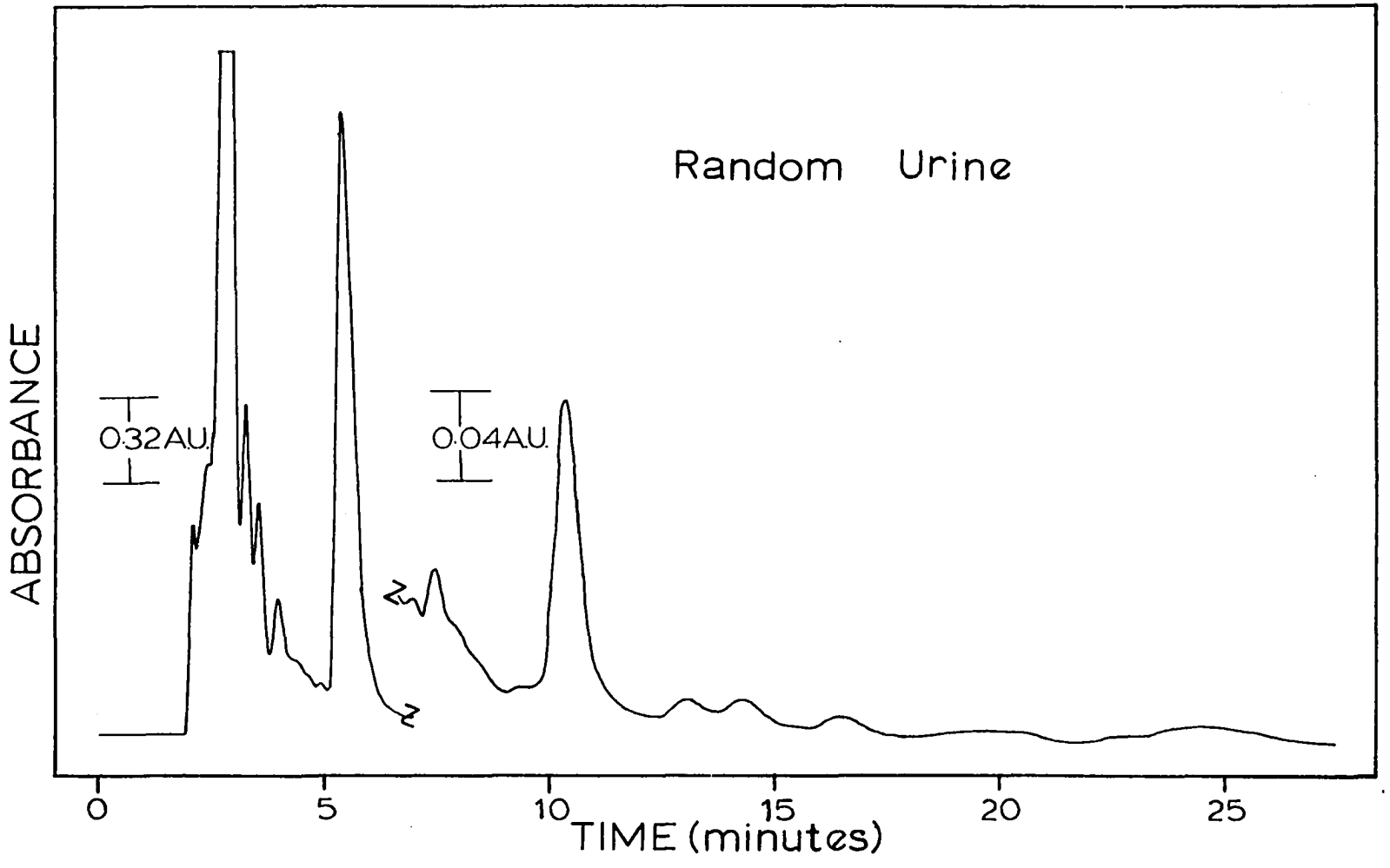
Random urine specimens were collected without regard to an established sampling procedure except where specifically indicated. A sample clarification kit was used for filtration of urine samples prior to injection. The filtered urine was injected without further preparation.

C. Results and Discussion

1. Liquid chromatography of urine with ultraviolet detection

A 100-µl portion of filtered urine was injected; the chromatogram using ultraviolet detection at 254 nm is shown in Figure VII.1. No attempt was made to isolate or identify

Figure VII.1. Liquid chromatogram of a urine sample
Ultraviolet photometric detector (254 nm)
Column A
Eluent F
100 µl of urine injected


Random Urine
ABSORBANCE
0.32A.U.
0.04A.U.
0
5
10
15
20
25
TIME (minutes)

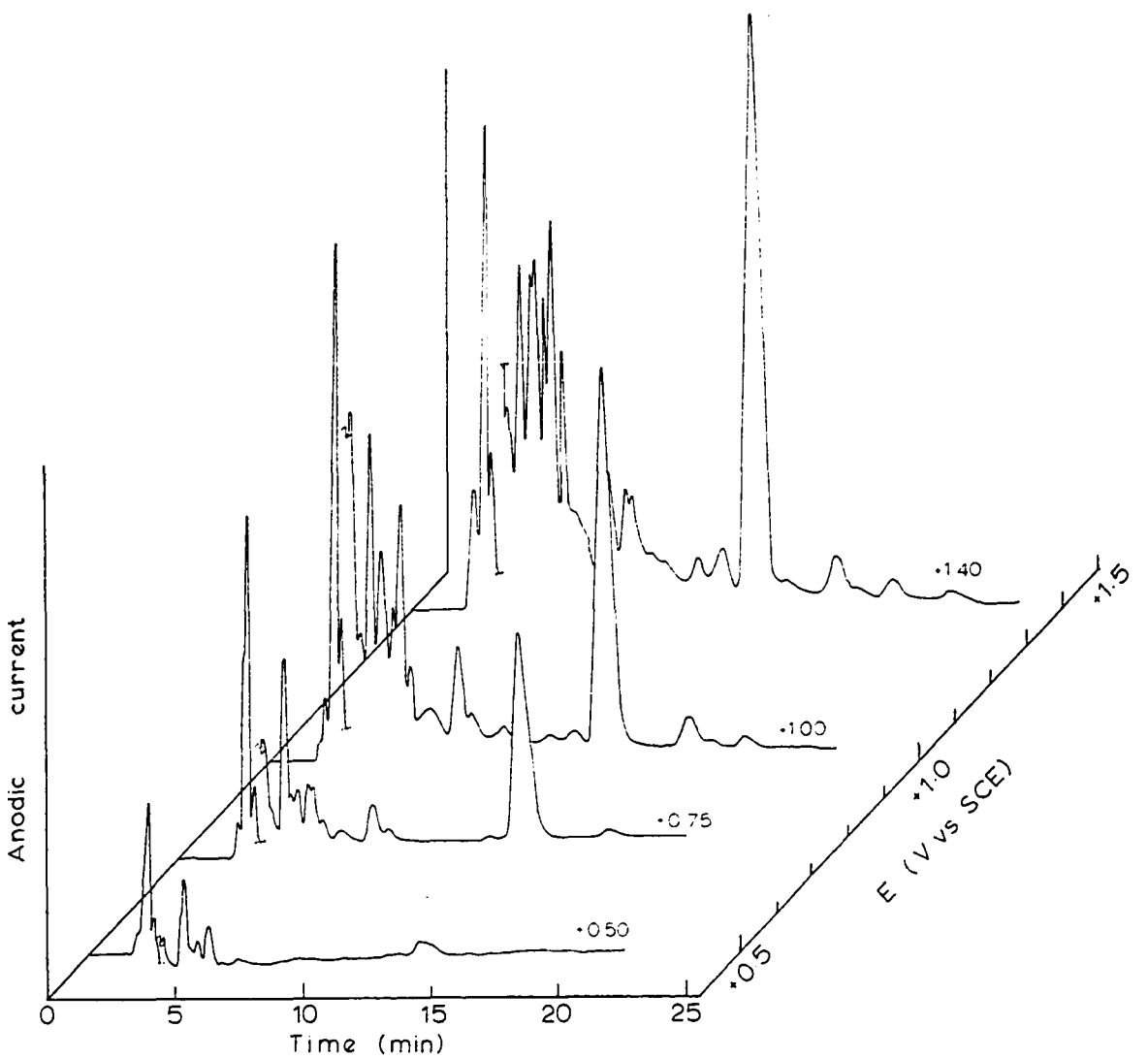
the various constituents. This chromatogram is shown to demonstrate that partial resolution of almost thirty peaks can be obtained in thirty minutes using the reverse-phase column. Scott (133) used anion-exchange chromatography and much larger samples to observe 140 resolved constituents with a separation time of forty hours. I observed no peaks eluting at retention times longer than thirty minutes so I concluded that the entire chromatogram would elute in approximately thirty minutes at this flow rate. If this conclusion is correct, then substantial savings are made in the time required for elution. However, the peaks are not completely resolved. Many of these peaks include more than a single component. This separation is inadequate if a single detector is used, but additional useful information can be obtained by using ultraviolet detection in combination with various modes of electrochemical detection. This chromatogram was assumed to be representative of other chromatograms of urine specimens because the ultraviolet detector was being repaired during the period that the experiments that follow were performed.

2. Liquid chromatography of urine with amperometric detection

A 100-µl portion of filtered urine was injected at each of the detection potentials indicated on the chromatovoltammogram of Figure VII.2. A limitation that is often cited for electrochemical detectors is that only electroactive

Figure VII.2. Chromato-voltammogram of a urine sample

ADAM 5 (dc mode)
Glassy carbon detector
Column A
Eluent F
100 µl of urine injected


Anodic current
0
5
10
15
20
25
Time (min)
+0.5
+1.0
+1.5
E (V vs SCE)
+0.50
+0.75
+1.00
+1.40

substances are detected. This does not appear to be a limitation for urine profiling; at least thirty electroactive constituents are detected at +1.40 V. The tunable selectivity of an amperometric detector may be utilized to simplify a complex chromatogram by choosing less positive detection potentials. Thus, as a partner with another detector, an amperometric detector could be of value because of selected activity toward a limited number of constituents. Obviously, the ability to vary the sensitivity and selectivity of an amperometric detector may prove valuable if the other detector lacks the requisite sensitivity or selectivity.

Fouling of the detector by accumulation of products of the anodic reaction was not a serious problem judging from the reproducibility of peaks for repetitive injections of urine at a constant detection potential. Based on the data obtained, I conclude that the amperometric detector is as useful as the spectrophotometric detector for chromatographic analysis or urine.

3. Liquid chromatography of urine with differential pulse amperometric detection

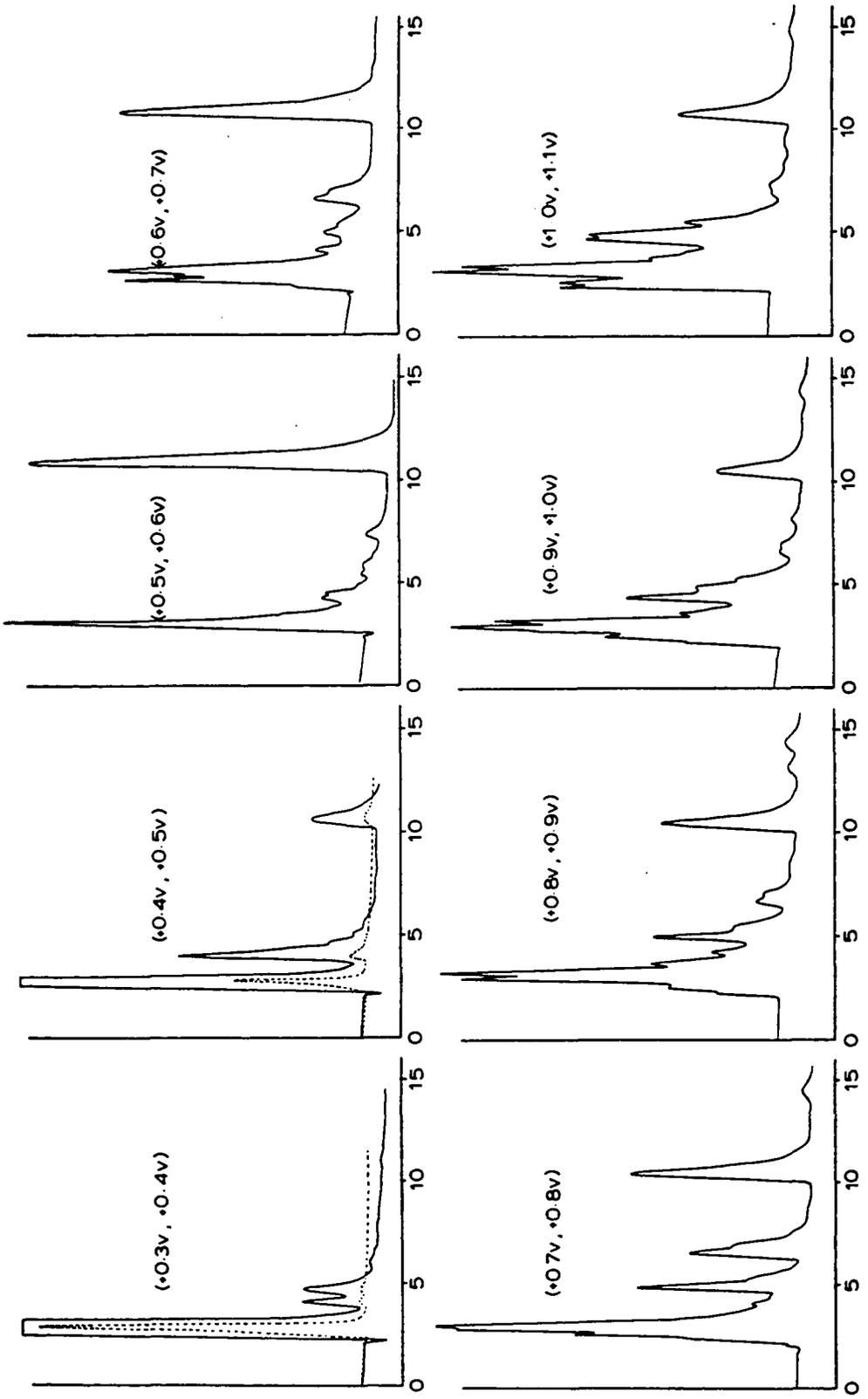
Differential pulse amperometric detection was used for detection of urinary constituents since some immunity from signals for more easily oxidized compounds was obtained. A 100-µl portion of urine was injected for each of the detection potential windows shown in Figure VII.3. Some of

Figure VII.3. Liquid chromatograms of a urine sample with various differential pulse detection windows

——— 1 µA full scale current

····· 10 µA full scale current

Cycle time 0.5 sec
PAR 174A (differential pulse mode)
Initial and final potential indicated on each chromatogram
Glassy carbon detector
Column A
Eluent F 1.33 ml/min
100 µl of urine injected


(+0.3v, +0.4v)
(+0.4v, +0.5v)
(+0.5v, +0.6v)
(+0.6v, +0.7v)
(+0.7v, +0.8v)
(+0.8v, +0.9v)
(+0.9v, +1.0v)
(+1.0v, +1.1v)
0
5
10
15

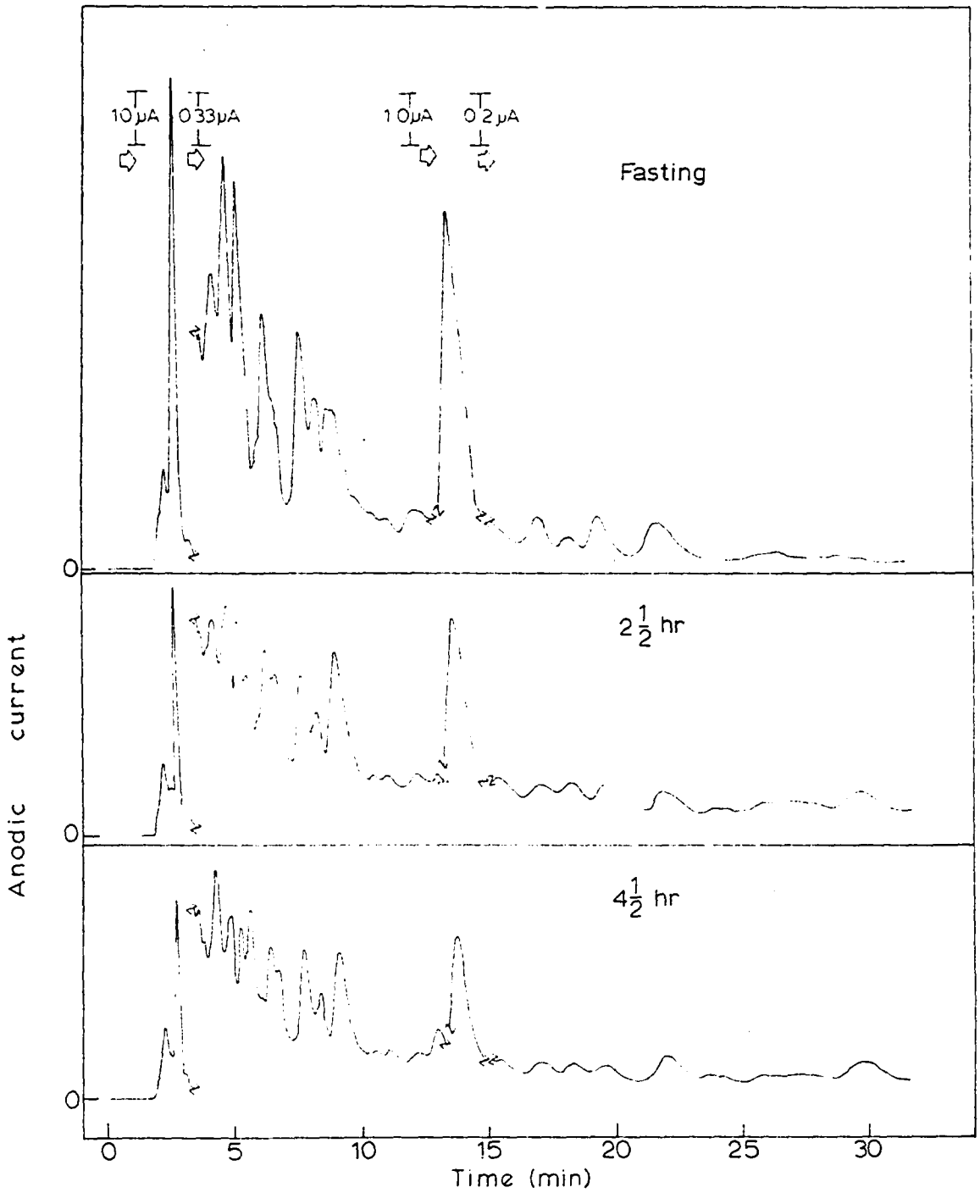
the more easily oxidized compounds are very concentrated so immunity is not complete. Nevertheless, the sensitivity and selectivity are different in the various detection windows. Perhaps such empirical data will serve as an impetus to researchers concerned with the hardware and software of systems for digital acquistion of data. If the detection potential could be multiplexed between, perhaps, ten potential values each second, many chromatograms could be reconstructed by a computer from the ten chromatograms at these ten potentials. Such instrumentation would have a high initial cost which would be justified if a larger number of samples could be analyzed in a shorter period. Obviously, much work remains to be done on optimizing detection parameters and instrument design.

4. Effect of coffee consumption on the chromatographic profile of urine

The effect of coffee consumption was studied as a prelude to the complete investigation of the effects of the dietary methylxanthines on metabolic profiles. The chromatographic profiles for this study are given in Figure VII.4. I chose a detection potential for which all of the methylxanthines are detected amperometrically. Methylxanthines were excluded from my diet for 48 hr, and I fasted for 8 hr before collecting a urine specimen. A direct injection of this urine sample yielded the upper chromatogram in Figure VII.4. In an

Figure VII.4. Effect of coffee consumption on the chromatographic profile of a urine sample

ADAM 5 (dc mode) E = +1.40 V
Glassy carbon detector
Column A
Eluent F 1.5 ml/min
100 µl of urine injected


1.0μA
0.33μA
1.0μA
0.2μA
Fasting
2½ hr
4½ hr
Anodic current
0
0
0
0
5
10
15
20
25
30
Time (min)

experiment not shown in Figure VII.4, I spiked a portion of this fasting urine sample with caffeine. The added caffeine was eluted with a retention time of 30 min. A separate portion of this urine was spiked with theophylline and the peak for theophylline was observed at about 13 min just prior to the attenuation change. During the time that this experiment was in progress, I consumed 5-6 cups of coffee. After 2.5 hr, I collected a second urine specimen. A peak with the retention time of caffeine was observed as illustrated by the middle chromatogram of Figure VII.4. A peak with the retention time of theophylline is not very obvious in this chromatogram, but for the chromatogram of a specimen collected two hours later, this peak is more obvious. This peak probably results from 1,7-dimethylxanthine, a caffeine metabolite, rather than theophylline which has the same retention time because theophylline is not found as a urinary metabolite of caffeine (91). Other changes in the chromatograms may reflect caffeine metabolism or coffee consumption. One point is clear, the amounts of the methylxanthines that are found in urine are easily detected and could interfere with chromatographic profiling.

I conclude on the basis of results reported here that electrochemical detection in liquid chromatography has a promising future for selective analysis of complex physiological samples. Electrochemical detectors may very soon be

regarded as respected partners with the currently popular detectors for liquid chromatography of physiological fluids.

VIII. REFLECTIONS AND SUGGESTIONS FOR FUTURE WORK

> "It is one of our most exciting discoveries that local discovery leads to a complex of further discoveries. Corollary to this we find that we no sooner get a problem solved than we are overwhelmed with a multiplicity of additional problems in a most beautiful payoff of heretofore unknown, previously unrecognized, and as-yet unsolved problems."
> -- R. Buckminster Fuller

As willpower triumphed over endurance, many temptations encountered in this research were resisted. Among these temptations was the enticement to further investigate electrochemical behavior at ultrapositive potentials. The discovery that analytical data can be obtained in the region beyond the potentials accessible to voltammetry was somewhat startling at the outset. Admittedly, there were hints that ultranegative and ultrapositive potentials could be useful analytically (135, 136), and furthermore, the Kolbe electrolysis is a well known example of synthesis at ultrapositive potentials. Yet, much work in organic electrochemistry has focused on a search for solvents, electrolytes and electrode materials with wider potential domains in order to investigate electrode reactions of compounds that are electrolyzed at potentials generally considered inaccessible in aqueous solutions. Much valuable electrochemical data will be revealed to those who are willing to use ultrapositive potentials in various solvents and electrolytes

to investigate electrode reactions of many compounds, especially those compounds that are not generally considered to be electroactive. The possibilities of using ultra-positive potentials are limited only by mental inertia.

There are three obstacles that must be surmounted before electrochemical detectors for liquid chromatography gain wide acceptance. The benefit of using an electrochemical detector in partnership with other detectors for separations of substances in genuine samples must be generally recognized. Secondly, applications need to be developed for electrochemical detection of substances that are difficult to detect with other liquid chromatographic detectors. Gradient compatability is the third hurdle.

Electrochemical techniques are dismissed oftentimes as impractical or useless for complex samples, or at best, only suitable for specialized applications. Yet, synergistic benefits are realized when electrochemical detectors are used in combination with other detectors for liquid chromatography. Such benefits also will be realized for a single electrochemical detector when operated in a multiplexed pulse amperometric mode. Tunable selectivity and discretional selectivity aid in chemical characterization. The methylxanthines should not be regarded as an isolated example. Polynuclear aromatic hydrocarbons can be separated by liquid chromatography and detected amperometrically (137).

Tunable selectivity is certainly a figure of merit for such separations. For example, anthracene and phenanthrene are difficult to separate; yet these geometric isomers have very different half-wave potentials, and one may amperometrically detect anthracene without interference from phenanthrene. A simultaneous determination is possible when these two compounds are completely unresolved by the liquid chromatographic separation if an ultraviolet detector is used in series with an amperometric detector.

A substance detected by both detectors is determined twice from a single injection. Divergent results warn of a probable interference. The advantage in forensic and clinical determinations of having a confirmatory determination for little additional labor and no additional sample should not be underestimated. Instances will be found where only the electrochemical detector gives satisfactory response when used in partnership with other detectors. This will hasten general acceptance of this partnership.

Carboxylic acids are difficult to detect with ultraviolet detectors, but carboxylic acids and carboxylates are electroactive. The Kolbe reaction has been investigated extensively so the literature gives guidance in the search for appropriate conditions for electrochemical detection (138). A detour was taken on this avenue during the course of this research. Carboxylic acids can be separated and

amperometrically detected in the effluent. Analytical methods will be required for an anticonvulsant drug just granted approval by the Food and Drug Administration for limited use in human beings. This anticonvulsant, known generically as sodium valproate is the sodium salt of 2-propylpentanoic acid. Sodium valproate should be an excellent candidate for electrochemical detection.

Amperometric detectors for liquid chromatography will undoubtedly find application for metabolic profiles of constituents in physiological fluids. This exciting area of clinical analysis is only in its infancy. Electrochemical identification and characterization of the many constituents in urine and other physiological samples should supplement other techniques for chemical characterization.

Eventually, pulse amperometric or differential pulse amperometric detection will be applied to in vivo determinations of substances in the bloodstream of animals or even human beings. The pharmacokinetics of a drug could be monitored on a continuous basis. Another possibility comes to mind when considering patients with kidney failure. The dialysis process needs to be monitored in order to determine when dialysis should be halted. The incorporation in the dialysis unit of an amperometric sensor for substances being removed would provide an excellent monitor of the process.

IX. BIBLIOGRAPHY

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