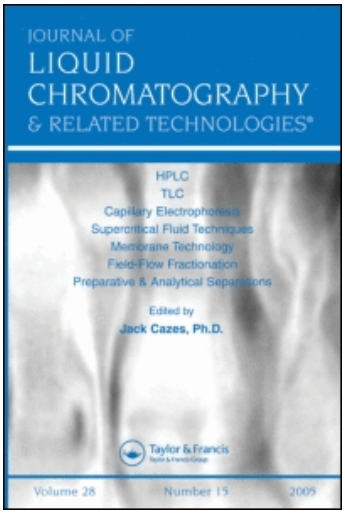

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The Forensic Determination of Drugs of Abuse Using Liquid Chromatography with Electrochemical Detection: A Review

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THE FORENSIC DETERMINATION OF DRUGS OF ABUSE USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION: A REVIEW

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ABSTRACT

The identification and quantitation of drugs of abuse in biological fluids, bulk pharmaceuticals and illicit preparations requires a large commitment of manpower and time within most full service forensic science laboratories. Funding levels within such laboratories rarely allow for large expenditures for very expensive or esoteric equipment, so the majority of the analyses of controlled substances must be performed using techniques which are relatively inexpensive, yet which provide for accurate and sensitive determinations of such compounds in a variety of matrices. Chromatographic methods provide an inexpensive, yet powerful, approach for the identification and quantitation of controlled substances. Liquid chromatography (LC) is particularly well suited for the analysis of a number of important controlled substances which are relatively nonvolatile, or thermally unstable. However, the full potential of LC in the forensic analysis of drugs has not been met to date, due to the lack of suitably sensitive and selective detectors for these compounds. Electrochemical (EC) detection in liquid chromatography (LCEC) offers excellent sensitivity and selectivity for those classes of compounds which possess a suitable electrophore (redox center) in their structures. To date, LCEC has been successfully utilized for morphine and a number of related narcotics, several benzodiazepines, cannabinoids and hallucinogens, fentanyl, and a few tricyclic anti-depressants. Under carefully controlled experimental conditions, or through the use of post-column photolytic derivatization, LCEC can be expanded to allow

for the trace determination of cocaine, barbiturates and many more benzodiazepines. In this manuscript we will examine the use of LCEC for the analysis of controlled substances, evaluate the strengths and weaknesses of the approach, and discuss possible future trends in the use of LCEC in the forensic examination of drugs of abuse.

INTRODUCTION

While there are thousands of compounds which may be prescribed by a medical practitioner during the treatment of patient illness, there are only a few classes of pharmaceuticals which are widely abused. Since these abused substances may pose a substantial risk to the user and to those with whom he may interact, extensive controls have been placed upon the distribution, possession and use of these substances. This rigid regulation necessitates a large commitment of resources, within full service forensic science laboratories, to cases involving the forensic analysis of controlled substances, whether these are submitted in bulk form, in illicit preparations or in biological fluids.

As most forensic science laboratories are publicly-funded institutions, they generally lack sufficient resources to allow for the routine use of very expensive, or overly time-consuming, instrumentation or methods. Rather, the bulk of the analyses in most forensic laboratories are performed using a series of inexpensive and relatively rapid examinations, perhaps followed by the use of an instrumental technique for confirmation of results obtained in the previous tests. However, during the forensic analysis of drug evidence, it is quite important to identify an unknown controlled substance with a high degree of certainty. It may also be important to quantitate the drug, in cases in which there are different penalties for possession of different amounts of the substance. In addition, it may be important to study trace impurities in drug samples confiscated during criminal investigations, in order to establish the origin of the sample, or to determine the synthetic scheme used in a clandestine laboratory. Finally, the results of such drug testing must be of the highest

integrity, in order to withstand any challenges in court. Baker and Phillips have recently enumerated the screening and confirmation techniques commonly used in the forensic analysis of illicit drug evidence [1]; the reader is directed to this excellent review for an in-depth discussion of systematic forensic drug analysis.

Chromatography has long played an important role in the routine identification and quantitation of drugs of abuse in the forensic laboratory. Thin-layer chromatography (TLC) is widely used as a preliminary screening method, primarily because it is inexpensive, and because it does not carry contamination from one sample to the next (each analysis is performed on a fresh plate, thereby insuring the integrity of the analysis). However, TLC suffers from a lack of sensitivity, and may not provide the resolution required for the analysis. Therefore, gas chromatographic (GC) methods are commonly utilized when a sensitive approach is needed for the identification and quantitation of a drug in a complex matrix. For those compounds which are thermally unstable, or relatively non-volatile, high performance liquid chromatography (LC) may be used. Although Doms and Lott [2] have attributed the rather scant use of LC in the forensic laboratory to the lack of research on such applications, Gough and Baker [3,4] point out that GC methods are generally adequate for the trace determination of all but a few classes of drugs of abuse. It is our opinion that the use of LC in the forensic examination of drugs of abuse does more than fill a gap in the availability of GC methods for certain classes of compounds. Rather, LC should provide a powerful confirmatory tool for an identification based on preliminary analyses, and may greatly assist in tracing the origin of confiscated samples or elucidating the synthetic route used in a clandestine laboratory.

One of the major limitations of LC methods in the analysis of drugs of abuse involves the lack of a detection method which is universally sensitive [5]. In the several recent reviews of the use of LC in forensic drug analysis [2-6], it has been shown that ultraviolet absorbance (UV) detection, while providing for fairly universal detection, lacks the necessary sensitivity for many of the major drugs of abuse. Increased sensitivity is available using fluorescence (FL) detection,

but there are relatively few naturally fluorescent compounds. Like FL, electrochemical (EC) detection also offers a sensitive alternative to UV detection. If a compound possesses a redox center (a moiety that may be electrochemically oxidized or reduced), EC can provide limits of detection in the picogram range. In general, it has been found that amines - both aromatic (aniline derivatives) and aliphatic (with relative electroactivity of 3°>2°>1°) - and phenolic compounds are amenable to oxidative EC detection at modest working potentials. Reductive EC is generally applicable to compounds containing nitro, N-oxide, azo, peroxide, thioamide or quinone functionalities.

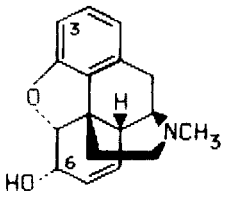
To date, oxidative EC detection has been used much more extensively than reductive approaches, for several reasons. First, oxygen is easily reduced electrochemically, so it must be thoroughly removed from the mobile phase and sample solutions in order to provide for low background currents and avoid the presence of an interfering (oxygen) peak in sample chromatograms. Also, reductive EC at potentials greater (more negative) than about -500-mV are often best performed using mercury electrodes, due to this material's high hydrogen overpotential (its resistance to the reduction of hydrogen in the supporting electrolyte or mobile phase). Solid electrodes generally lack such an overpotential, leading to higher noise levels at the reductive potentials needed for many applications. There are a number of different EC detectors available, including amperometric and coulometric devices, having flow-through, thin-layer (TL), wall-jet (WJ) or mercury drop (SMDE or DME) cell configurations. Also, there are several electrode materials which may be used to enhance the electrochemical response for a particular analyte, including glassy carbon, carbon paste, mercury, gold, silver and platinum. It is not feasible to discuss EC detectors in detail; an excellent discussion and review of LCEC may be found elsewhere [7].

Among the commonly encountered drugs of abuse, as discussed by Gough and Baker [3,4], the most important are morphine and related narcotics, cannabis, and cocaine. Several other classes of compounds, including barbiturates,

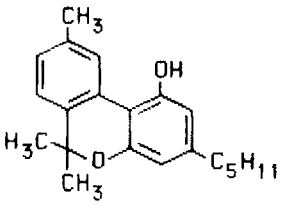
benzodiazepines, amphetamines, hallucinogens and several miscellaneous controlled substances, are also encountered, but to a relatively minor extent. It is the purpose of this manuscript to review the application of LC with electrochemical detection (LCEC) for the trace determination of controlled substances. The use of LCEC for drugs in each of the compound classes noted above will be discussed in turn, and the advantages and deficiencies of LCEC approaches will be described. Discussion of the electrochemistry of several of these componds will necessitate reference to chemical structures; Figure 1 should be consulted for clarification during these discussions. Finally, the projected future of LCEC in the analysis of drugs of abuse will be discussed.

Morphine and Related Narcotics

Morphine is the most prevalent alkaloid found in opium and poppy straw, comprising about 10% by weight [1]. Opium also contains a number of other alkaloids, of which codeine, thebaine, noscapine, papaverine and narceine are the most important. In addition, a number of related narcotics have been synthesized and are subject to abuse, including heroin (3,6-diacetylmorphine), codeine, methadone and pethidine. There are a plethora of reports regarding the use of EC detection in LC assays for these compounds. This fact stems from the ease with which morphine, and a number of its analogues, may be electrochemically oxidized. The oxidative electroactivity of morphine and morphine analogues has been studied [8], and through structure-response relationships it has been determined that the 3-hydroxyl group on these compounds is the electroactive center. This is in agreement with an earlier study [9], in which the similarity between the catecholamine dopamine, and a portion of the morphine structure, was noted. Catecholamines, because of the presence of a phenolic hydroxyl group in their structures, are easily oxidized using relatively low electrochemical potentials. This oxidation may involve dimerization, or formation of a radical cation intermediate which may react with a nucleophile, disproportionate, or undergo bond cleavage [8].



MORPHINE



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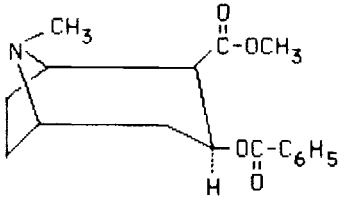
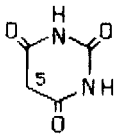
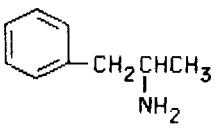


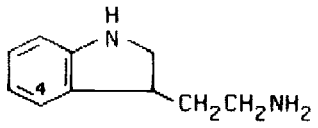
COCAINE



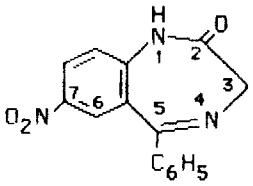
BARBITURIC ACID



AMPHETAMINE



TRYPTAMINE



NITRAZEPAM

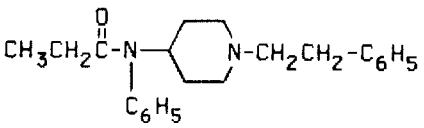


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FIGURE 1. STRUCTURES OF DRUGS OF ABUSE AND PRECURSORS

The range of the assays in which LCEC has been incorporated for the determination of morphine or related narcotics is shown in Table 1. The abbreviations used in this table are: morphine (MOR), oxymophone (OXY), nalorphine (NR), naloxone (NX), naltrexone (NT), morphine-3-glucuronide (MOR-3-GLUC) and apomorphine (APO). In all of these accounts but one [20], glassy carbon working electrodes and Ag/AgCl reference electrodes were used. Westerink and Horn [20], used a carbon paste working electrode and a saturated calomel reference electrode. In general, quantitation in these assays was accomplished using the internal standard method. A number of internal standards were used, including naltrexone [10], dextrorphan [11], nalorphine [12,17], 5-hydroxyquinoline [13], normorphine [14], 3,4-dihydroxyphenylpropionic acid, 3,4-dihydroxybenzylamine [16], and norapomorphine [17]. Several of these reports were also concerned with the possibility of interferences from common drugs or endogenous materials, which could be coextracted from the biological matrix. White [11] studied a large number of phenol-, indole- and phenothiazine-based drugs as possible interferents, and found that only five of the compounds examined had retention times similar to that for morphine. None of these five were commonly prescribed or encountered illicitly, so interference in the morphine assay was considered to be unlikely. In other studies, commonly prescribed analgesics [12] and sedatives [13,17] did not interfere in the determination of morphine in cerebrospinal fluid, blood or plasma.

While morphine is easily detected at relatively low oxidative potentials, most of the opium alkaloids extractable from natural products are not oxidizable except at high potentials. This is due to the fact that these other alkaloids contain a phenol ether in place of the hydroxyl functionality at the 3-position of morphine. Thus, codeine, thebaine, noscapine, papavarine, narceine and narcotine are not readily detectable electrochemically. Not surprisingly, this selectivity for morphine has been used to advantage for the direct determination, without prior separation or clean up, of morphine in poppy straw extracts [21]. It is clear that if one is interested in determining the origin of seized plant material based

Table I. LCEC for Narcotics

SAMPLE		CHROMATOGRAPHY		ELECTROCHEMISTRY		RESULTS		
Analyte	**Matrix**	**Column**	**Mobile Phase**	**Geometry**	**Potential**	**Extr. Eff.**	**LOD**	**Ref.**
MOR OXY NR, NX, NT	PLASMA	C-18 (5um) 300-x4-mm	20 : 80 MeOH : H20 50mM $(CH3)_4NOH$ pH 6.1 (H_3PO_4)	THIN LAYER	+800-mV	NOT GIVEN	1-ng 2-ng 5-ng	9
MOR NX	BRAIN	C-18 (10um) 250-x4.6-mm	25 : 75 MeOH : H20 50mM $(CH3)_4NOH$ pH 6.1 (H_3PO_4)	THIN LAYER	+600-800-mV	>80%	2.5-ppm	10
MOR MOR-3-GLUC	BLOOD	SILICA (7um) 200-x4.6-mm	9:1 MeOH:NH_4NO_3 pH 10.2	WJ/TL HYBRID	+600-mV	81%	0.5-ng	11
MOR	PLASMA CSF	C-18 (10um) 300-x4-mm	5 : 8 : 87 ACN : MeOH : 0.07M KH_2PO_4	THIN LAYER	+790-mV	78.4 ± 2.2%	0.2-ng	12
MOR	SERUM	C-18 (10um) 300-x4-mm	36 : 64 MeOH : 70mM NaH_2PO_4, pH 5.8 .5mM $C_7H_{15}SO_3H$	THIN LAYER	+600-mV	78%	50-pg	13
MOR	PLASMA	C-18 (10um) 250-x4-mm	50 : 50 : 0.1 MeOH : H20 : NH_4OH	THIN LAYER	+650-mV	85%	20-pg	14
MOR	BLOOD	C-18 (5um) 250-x4.6-mm	10 : 2.5 : 6 : 81.5 MeOH : THF : CH_3CO_2H : Citrate pH 3.75	THIN LAYER	+725-mV	NOT GIVEN	0.5-ng	15
MOR	BRAIN	C-18 (5um) 250-x4.6-mm	1 : 99 THF : 50mM Citrate (pH 4.25)	THIN LAYER	+725-mV	72.1 ± 4.8%	1-ng	16
MOR	SERUM PLASMA	C-18 (10um) 300-x4-mm	85 : 15 MeOH : 10mM KH_2PO_4	WJ/TL HYBRID	+1.00-V	79%	20-pg	17
APO	TABLETS	PHENYL (10um) 300-x4-mm	5 : 15 : 80 MeOH : ACN : 50mM KH_2PO_4	THIN LAYER	+500-mV	NOT GIVEN	N/A	18
APO	BRAIN PLASMA	C-18 (5um) 300-x4-mm	34 : 66 MeOH : 20mM Na_2HPO_4 /30mM Citrate pH 3.2	THIN LAYER	+700-mV	92 ± 2.8%	1-ng	19

on the relative proportions of other alkaloids in an extract, this determination cannot be made using straightforward oxidative LCEC. In these cases, sufficient material will be available to allow for the use of LC-UV approaches. However, if one is interested in quantitating morphine and its major glucuronide conjugate, or any of the related narcotics having oxidative electroactivity, in biological fluids, then LCEC is the method of choice.

As a phenol ether, heroin is not easily detected by the oxidation of the moiety at the 3-position. However, most opium alkaloids (including heroin) contain an aliphatic tertiary nitrogen atom in their structure, and it has been well documented that this functionality, when under the proper pH and solvent conditions, may be oxidized electrochemically [22-24]. The mechanism of oxidation of tertiary amines involves the lone pair of electrons on the nitrogen atom. Therefore, the pH of the solvent generally must be above 10, based on pK_b values in the literature, in order to keep the amine in its basic (unprotonated) form, thus allowing for its electrochemical oxidation.

Recently, the oxidative electroactivity of this functional group was used to create an LCEC approach for the determination of heroin and a number of related alkaloids [25]. In this report, separations were performed using reversed phase conditions, including a 250- x 4.6-mm C-18 (10-μm) column and mobile phases composed of between 40% and 55% acetonitrile (ACN) with 0.02M KH_2PO_4 buffer, adjusted to pH values between 6 and 8 using NaOH. Apparently, the presence of ACN in the mobile phase enhanced the basicity of the solution to such an extent that the alkaloids predominantly remained in their basic form. Using this novel approach, heroin, morphine, codeine, acetylcodeine, thebaine, narcotine, and 3- and 6-monoacetylmorphine were detected. Only papaverine, one of the naturally-occurring opium alkaloids, could not be detected using LCEC; this lack of electroactivity was attributed to the fact that this compound contains a secondary nitrogen, instead of the tertiary center found on the other compounds studied.

On its face, this newer oxidative method for alkaloids greatly improves the usefulness of LCEC for the routine analysis of narcotics, especially when attempting to determine the origin of an opium product using the relative proportions of alkaloids in its extract. While not explicitly studied, noscapine and narceine (two other compounds commonly included in such studies of opium alkaloid content) should also be amenable to detection under these experimental conditions. However, the pH needed for detection of the amines places severe constraints upon the type of column that can be used for the separation. Silica-based reversed phase columns are unstable at such high pH conditions, so polymeric columns may be necessary to avoid loss of efficiency and reduced column life. Even this alternative may not be totally acceptable, as the chromatographic efficiency obtained using polymeric based columns may be insufficient for the assay [26].

Even though it has been shown that LCEC is quite selective for alkaloids in a variety of matrices, it is possible to increase the specificity of these determinations through the use of dual electrode EC detection approaches [27]. For example, if two working electrodes are oriented parallel to the flowing stream in the EC detector cell, two different potentials may be monitored simultaneously. In a manner that is entirely analogous to dual wavelength UV detection, the responses at these two working electrode potentials may be compared to provide a "response ratio" for the analyte being detected. The response ratio can then be used to confirm a peak identity initially assigned on the basis of retention time.

In the past two years, we have incorporated on-line, post-column photolytic derivatization in order to increase the number of compound classes amenable to EC detection. In this approach, the eluent from the column is introduced into a knitted, open-tubular Teflon™ irradiation chamber which is wound about a low-pressure mercury lamp. In this chamber analytes undergo continuous irradiation (254-nm), the period of which is determined by the volume of the

irradiation chamber and the flow rate incorporated. During this residence time, analytes are photolytically converted from their non-detectable, parent forms to stable fragments which are electrochemically oxidizable. Following this on-line, continuous photolysis step, the eluent is directed to a dual electrode EC cell, where the photoproducts are detected. This system, termed LC-photolysis-EC (or LC-hv-EC), has been successfully applied to a number of compounds which either have no inherent electroactivity, or which can only be detected using reductive EC approaches. The list of compounds that have been determined to be amenable to this approach includes nitro-compounds, organothiophosphate pesticides, beta-lactam antibiotics, and a number of drugs of abuse (described herein). A review of derivatization approaches for LCEC, including LC-hv-EC, was recently published [28].

Using the LC-hv-EC system, there are three "modes of selectivity" available to the analyst. First, as with any chromatographic method, the retention time for an unknown is compared to that of standards in order to identify the unknown. The response ratio obtained using the dual parallel EC approach may then be used to strengthen this initial identification. Finally, many of the compounds which are amenable to LC-hv-EC have no inherent electroactivity, so if a second injection is made with the irradiation unit switched off, the peak for the analyte of interest will disappear from the chromatogram. Thus, an identification based on these three separate factors (retention time, response ratio and lamp on/off behavior) is much more reliable than that commonly available using retention time alone.

Morphine, nalorphine and pethidine have been studied using LC-hv-EC [29]. Even though these three narcotics exhibit native electrochemical activity, it was determined that the EC response ratios could be altered by subjecting the compounds to post-column irradiation. Thus, even though irradiation was not needed to improve the detectability of the analytes by LCEC, the photolysis step could be used to confirm the identity of the opiates in an extract.

Cannabis Products

The identification of a Cannabis product, whether in the form of plant material, resin or extract, is commonly performed using microscopy, color tests and TLC [1]. With respect to products within this class, LC finds its primary usefulness in the identification and quantitation of specific derivatives of cannabinol in extracts or biological fluids. To a smaller extent, the relative proportions of various cannabinoids may also be determined using LC methods, to assist in the identification of the origin of the material. Masoud and Wingard [30] have reported on the use of oxidative LCEC for the determination of cannabinoids in street-grade Cannabis and hashish, as well as in an alcohol extract of leaves and tops from living plants. Although the authors make no conjecture regarding the mechanism of the electrochemical oxidation of the cannabinoids, the electroactive moiety in the structure of cannabinol, and its derivatives, is presumably the phenolic hydroxyl group. In this study, which incorporated a C-18 (10-µm) column, 75:25 MeOH:50mM H_3PO_4 as mobile phase, and a carbon paste working electrode operated at +1.0V (vs. Ag/AgCl), eleven constituents were separated, and three major cannabinoids were identified. The three cannabinoids, Δ^8- and Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), could be quantitated to the 100-ppb level (2-ng injected). While CBD was found in all of the samples analyzed, only the plant extract contained both the hallucinogenic constituent Δ^9-THC and Δ^8-THC. The fact that there were 8 other (unidentified) constituents apparent in the chromatograms suggests that this method may be useful when attempting to assess the origin of a particular exhibit. However, few cases involving Cannabis products require such analyses, so it is not surprising that there have not been further reports using LCEC for such cannabinoid profiling.

Cocaine

Samples of recovered cocaine rarely consist of the pure substance. Rather, the drug is commonly diluted, or "cut", with other local anesthetics or with

sugars. Since it is often important to quantitate cocaine in seized samples, the chromatographic method employed in such analyses must be capable of resolving the components of the mixture, or a selective detection method must be employed to provide for such discrimination. After ingestion, it may be important to identify cocaine and its principle metabolite, benzoylecgonine, in body fluids. In addition, it may be important to identify synthesis or degradation by-products, such as ecgonine, benzoylecgonine and cinnamoylcocaine. GC procedures, when coupled with preliminary TLC screening, are generally adequate for all of these tasks [3]. However, LC methods have been used to advantage for some analyses, especially where thermal degradation of samples occurred upon injection onto the GC.

Schwartz and David [25] have utilized oxidative LCEC for the determination of cocaine, benzoylecgonine and ecgonine, wherein the authors again took advantage of the tertiary nitrogen atom in the structure of these compounds (as described previously for heroin and other narcotics). Chromatographic resolution and adequate detector sensitivity were obtained using a 250- x 4.6-mm C-18 (10-µm) column with 50:50 ACN:20mM KH_2PO_4 as the mobile phase. The detection of ecgonine and benzoylecgonine exhibited a marked sensitivity to pH, while cocaine did not exhibit such sensitivity. When increasing pH from 7 to 8, the responses for ecgonine and benzoylecgonine increased by factors of 4 and 6, respectively, while that for cocaine decreased by about 10%. Using a thin layer cell with a glassy carbon electrode operated at +1.2V vs. Ag/AgCl, cocaine could be detected down to the 2-ng level. Limits of detection (LODs) for ecgonine and benzoylecgonine, while not explicitly reported, would probably be between 4- and 8-ng, based upon the relative peak heights for these compounds in a mixed standard chromatogram provided in the paper.

LC-hv-EC has been applied to the determination of cocaine in the presence of common adulterants, including local anesthetics, ascorbic acid, nicotinamide and several sugars [31]. Under the pH conditions utilized in this study (pH 3), cocaine exhibited no inherent oxidative electroactivity. However, through the

use of on-line, post-column photolytic derivatization, cocaine could be detected down to the 50-ppb level (11.2-ng injected). The chromatographic conditions employed included a 100- x 5-mm C-18 (10-µm) column, and mobile phase composed of 40:60 MeOH:0.1M NaCl adjusted to pH3, with 7-ml/L butylamine added. Mannitol, lactose and inositol did not respond, either in the lamp-on or -off mode, so their presence in samples did not interfere with the determination of cocaine. The local anesthetics, due to the presence of either an aromatic or tertiary aliphatic amine in their structures, had significant oxidative electroactivity in the lamp-off mode, so they were easily distinguished from cocaine. It is worth noting that the oxidative electroactivity of aliphatic tertiary amines is determined not only by pH, but also by the chemical environment surrounding the moiety in the compound's structure. In addition to the "qualitative discrimination" between cocaine and the local anesthetics, based upon lamp-on and -off behavior, it was also possible to use changes in the electrochemical responses for these compounds, as a function of the applied potential, to advantage. In other words, during the initial stages of the experiment the relationship between the current response (signal) and the applied potential was studied. It was found that, following photolysis, cocaine had significant electroactivity at potentials as low as +650-mV. However, under the same conditions, oxidation of the local anesthetics was not facile until potentials greater than about +900-mV were applied. Thus, by incorporation of a lower potential (+750-mV) during the analysis of simulated illicit cocaine preparations, it was possible to obtain marked selectivity for cocaine, even in the presence of the other anesthetics.

The induced electroactivity of cocaine, following photolytic derivatization, probably involved the dissociation of the molecule to release benzoate anion. This anion had previously been found to demonstrate oxidative electroactivity under photolytic conditions [32]. Based on these findings, it may also be inferred that benzoylecgonine would be amenable to LC-hv-EC. However, ecgonine, which lacks the benzoate functionality in its structure, would presumably not respond

to this approach. Thus, LC-hv-EC would best be used as a rapid screening method for the identification and quantitation of cocaine in illicit preparations. Although it has not been demonstrated, LCEC approaches may also be useful for the determination of contaminants in these preparations, which can aid in the tracing of samples to a particular supplier or clandestine laboratory. At this time, LCEC methods lack the sensitivity needed for the determination of cocaine, and its metabolites, in body fluids.

Barbiturates

It has been reported that barbiturates are the most common drugs involved in suicidal and accidental self-poisonings, and are the subject of widespread abuse because of their popularity with the medical establishment as sedatives, hypnotics and anti-convulsants [33]. Since they are widely prescribed, barbiturates are rarely produced in the clandestine laboratory. Thus, confiscated samples may often be characterized based on their morphology. Traces of barbiturates in containers or biological fluids may be screened by TLC, and GC and LC have been used for identification and quantitation of these substances. Almost all of the reported LC approaches have incorporated UV detection, due to the lack of inherent fluorescent or electrochemical behavior for these compounds. However, the barbituric acid backbone is a poor chromophore at 254-nm, so LC-UV may lack the requisite sensitivity for some analyses unless lower wavelengths, with their accompanying lower selectivity, are used for detection.

There have been no reports of the use of straightforward LCEC for the determination of barbiturates. However, it was recently discovered that post-column photolytic derivatization could be used to create oxidizable species from these non-electroactive parent compounds [34]. Following separation on a 100- x 5-mm C-18 (5µm) column, using 40:60 MeOH:0.2M NaCl as the mobile phase, the barbiturates were photolyzed for about 2 minutes and were then

directed to a dual (parallel) glassy carbon EC detector. When the working electrodes were set at +1.1V and +0.85V (vs. Ag/AgCl), fourteen 5,5'-disubstituted oxybarbiturates could be detected, with limits of detection ranging from 2- to 6-ng (10- to 30-ppb injected). The response ratios for these compounds (+1.1V/+0.85V) fell between 1.4 and 1.6. The identities of the oxidizable species formed from the parent compounds were not elucidated, but it was clear that the barbituric acid backbone was primarily responsible for the photolytic-EC response. Literature reports regarding the UV irradiation of barbiturates in aqueous solution find the major products to be a number of malonuric acid derivatives and amides, and the amide structure has been demonstrated to be electrochemically oxidizable following photolysis [32].

LC-hv-EC was validated for the determination of butalbital [34] and phenobarbital [29] in human serum extracts, by comparison of the analytical results using this newer method with those obtained using accepted methods in a practicing toxicology laboratory. The three modes of selectivity inherent in the LC-hv-EC system were found to be advantageous for proper identification of the analytes of interest, in cases where the serum extracts contained endogenous components which were poorly resolved from the barbiturates. It appears that this method could easily be incorporated in the forensic laboratory for the routine identification and quantitation of barbiturates, following initial TLC screening procedures.

Amphetamines and Related Stimulants

Substituted phenethylamines, and many other stimulants, are not encountered with the regularity of the above-noted classes of drugs. However, they are the subject of abuse, and may be submitted for examination in the forensic laboratory [3]. TLC, GC and LC approaches have all been used for the examination of these materials, but even these methods may be insufficient for the identification of the drug, due to the similarities between many of these

compounds, and the occasional need to identify optical isomers (when the jurisdiction requires such determinations). The majority of these compounds are primary or secondary amines, and are devoid of any appropriate electrochemically active substituent. However, there has been one report in which the principle metabolite of amphetamine, p-hydroxyamphetamine, was quantitated in mouse brain extracts [35]. In this article, a thin-layer EC cell, utilizing a glassy carbon working electrode, was incorporated following separation of the metabolite from endogenous materials using a 250- x 4.6-mm C-18 (5-µm) column with an eluent composed of 50:5:0.5 citrate buffer (pH 4.5):MeOH:THF. With an applied potential of +750-mV (vs. Ag/AgCl), the workers were able to detect p-hydroxyamphetamine to the 0.5-ng level. The extraction efficiency for this metabolite was 85 ± 2%, and 5-hydroxytryptamine and tryptophan (two important biogenic substances) did not interfere with the quantitation.

Methylphenidate, a CNS stimulant, has been analyzed using LC-hv-EC [29]. This compound, which has no inherent oxidative electroactivity, probably undergoes photolytic dissociation to form the benzoate anion, which is then amenable to oxidative EC detection at +1.1V (vs. Ag/AgCl) [32]. Unfortunately, the LOD for this compound using LC-hv-EC was 150-ng (750-ppb injected), so this technique would not be useful for the determination of methylphenidate in biological samples. It may be concluded, therefore, that LCEC is definitely not a method of choice for the determination of amphetamines and related stimulants, as few of these compounds exhibit native electroactivity. Fortunately, the number of cases each year in which such determinations are required are relatively limited, and GC approaches generally offer the necessary sensitivity and selectivity [3].

Hallucinogens

LSD (lysergic acid diethylamide) and several derivatives of the indole tryptamine represent the more important members of this group of compounds

[1]. LC is ideally suited to the trace determination of these compounds, due to their thermal lability and otherwise poor GC behavior. LSD may be detected with excellent sensitivity using FL detection, so this is the method of choice in most reports. From the standpoint of electroactivity, the structure of LSD contains two tertiary amine moieties, thus the compound should be oxidizable under the proper pH conditions. To date, however, there have been no reports of such an approach.

Among the hallucinogenic tryptamine analogues that arise in the forensic analysis of drug evidence, three are derived from the mushroom Psilocybe semilanceata. These components - baeocystin (4-phosphoryloxy-N-methyltryptamine), psilocin (4-hydroxy-N,N-dimethyltryptamine) and psilocybin (o-phosphonyl-4-hydroxy-N,N-dimethyltryptamine) - may be used to characterize the origin of hallucinogenic mushrooms recovered from suspected recreational users. Christiansen and Rasmussen [36] incorporated LC with UV, FL and oxidative EC detection for the determination of psilocybin and psilocin. Psilocin, due to the presence of the 4-hydroxyl functionality on the tryptamine backbone, had excellent oxidative behavior. Using a thin layer cell and a glassy carbon working electrode operated at +650-mV (vs. Ag/AgCl), psilocin could be detected down to 75-pg. This was significantly better than the LODs available using UV (254-nm) or FL (excitation at 267-nm, emission at 320-nm) detection, which were determined to be 7.5-ng and 20-ng, respectively. Psilocybin, on the other hand, lacks the phenolic functionality, so its oxidative electroactivity must be a result of the tertiary amine or phosphonyl moieties. The LODs for this compound were 10-, 5- and 5-ng for UV, FL and EC, respectively. These authors did not study the response of baeocystin; since this compound lacks the tertiary aliphatic amine group, it is not clear that oxidative EC detection would be possible.

Bufotenine (5-hydroxy-N,N-dimethyltryptamine), DMT (N,N-dimethyltryptamine) and DET (N,N-diethyltryptamine) are three other hallucinogens which may be subject to misuse. Although these three have not been explicitly studied

using LCEC, their structures suggest that oxidative LCEC would be useful for their determination. Bufotenine should be readily oxidized at the 5-hydroxy position, while (under the proper pH conditions) it should be possible to take advantage of the tertiary amine functionality within the structures of DET and DMT. It is also possible that psilocybin and baeocystin may be amenable to LC-hv-EC. If the phosphonyl group can be photolytically cleaved to form phosphate products, then detection should be facile, as it has previously been demonstrated that phosphorous-containing species are generally responsive under photolytic-EC conditions [32]. Thus, it appears that LCEC could provide a straightforward, sensitive and selective approach for a number of hallucinogens.

Benzodiazepines

As a class, 1,4-benzodiazepines have been steadily growing in popularity over the past several years, and are generally prescribed as substitutes for barbiturates in their role as anti-depressents or sedatives. These compounds generally have poor GC behavior [37], while LC-UV methods often lack the sensitivity required for their determination in biological fluids [38]. The use of electrochemical methods, in both static systems and in flowing streams (such as LCEC), for the determination of these compounds in pharmaceutical preparations and biological fluids, has recently been reviewed [39]. The most common EC approaches for these compounds involve reduction of the 4,5-azomethine functionality, which is common to all 1,4-benzodiazepines. In addition, the nitro moiety present on the 7-nitro-1,4-benzodiazepines, including clonazepam, nimetazepam and nitrazepam, may be electrochemically reduced at modest potentials. The nitrone functional group of chlordiazepoxide and its metabolite, norchlordiazepoxide, may be reduced as well. The electrochemical potentials employed for these reductions require rigorous purging of the dissolved oxygen content in both mobile phases and samples, as the presence of oxygen in the mobile phase leads to high background currents, and oxygen in samples gives rise

to a large, tailed peak which may obscure significant portions of the chromatogram.

In his review [39], and in an earlier paper with Hackman [40], Brooks discussed the use of a modified dropping mercury electrode (DME) for the reductive amperometric detection of chlordiazepoxide, norchlordiazepoxide and medazepam (as the internal standard) in plasma extracts, following their separation on a 300- x 3.9-mm C-18 (10-μm) reversed phase column. The authors utilized a mobile phase composed of 53:5:42 MeOH:IPA:7.5mM acetate buffer, and evaluated direct current (DC) and differential pulse polarographic (DPP) detection at potentials of -950-mV and -820-mV (pulse width of 100-mV), respectively (both vs. Ag/AgCl). DPP offered an LOD for chlordiazepoxide that was two orders of magnitude lower than that obtained using DC amperometry. Since the modified DME cell had a low (less than 1-μl) effective dead volume, good sensitivity was obtained for the 1,4-benzodiazepines, allowing for detection down to the 50-ppb (5-ng injected) level. However, it was found that the system did not offer similar sensitivity for several 1,4-benzodiazepin-2-ones studied, including demoxepam and nordiazepam. This lack of sensitivity was attributed to a kinetic phenomenon which is peculiar to hydrodynamic voltammetry, but which is not well understood. Since many of the important compounds within this class are 1,4-benzodiazepin-2-ones, this report seems to suggest that there may be severe limitations on the applicability of reductive, DPP detection in the LC analysis of such materials when they are present in dilute concentrations in difficult matrices.

Other reports in the literature regarding reductive LCEC approaches for these compounds are generally in agreement with Hackman and Brooks. For example, Kral and Kainz [41] utilized reductive DPP detection at a static mercury drop electrode (SMDE), following reversed phase separation, for the quantitation of diazepam in pharmaceutical preparations. Their conditions included a 250- x 4-mm C-18 (5-μm) column, a mobile phase containing MeOH:EtOAc:acetate buffer

(pH 6), and detection at -1.15-V (base potential of -0.4-V, pulse amplitude of -0.75-V, vs. Ag/AgCl). Using a 20-µl injection volume, they were able to quantitate diazepam to the 20-ng (1-ppm) level using reductive DPP detection; this sensitivity was sufficient for the determination of diazepam in pharmaceutical preparations.

An early report regarding the use of reductive LCEC for benzodiazepines incorporated a home-made, wall-jet EC cell geometry [42]. The cell was constructed to allow for the easy interchange of glassy carbon, carbon paste or mercury pool working electrodes, and incorporated a silver wire as a "quasi reference electrode". After connection to a 250- x 2.6-mm C-18 (5-µm) column, with 60:40 MeOH:50mM ammonium acetate mobile phase, each of these working electrode materials was tested for the detection of nitrazepam using both DC and DPP amperometry. Under these experimental conditions, the mechanism of detection for this compound involved reduction of the nitro functionality. Glassy carbon, when used in the DC mode at -1.3-V and -0.93-V, gave LODs of 30-ng and 3-ng, respectively, while in the DPP mode at -0.78-V the LOD was 2-ng. Similar LODs were obtained using carbon paste at these potentials. The mercury pool electrode provided LODs of 30-ng and 3-ng when operated in the DC mode at -0.83-V and in the DPP mode at -0.68-V, respectively. Unfortunately, diazepam and chlordiazepoxide do not contain the 7-nitro functionality present in the structure of nitrazepam, and could only be detected at high DC potentials using the glassy carbon electrode. At -1.3-V, these two compounds had LODs of 300-ng (30-ppm injected) each, which did not compare favorably with the UV(254nm) LODs for diazepam and chlordiazepoxide of 6- and 4-ng, respectively. More recent reports, wherein optimized DME cell geometries were utilized, have exploited the increased sensitivity of mercury electrodes, at lower potentials, for these compounds [39-41].

Nitrazepam has been used as a test analyte in two reductive LCEC reports [43,44], in order to characterize novel approaches for improving DME detection. In the first of these [43], a DME was designed which permitted the use of fast

drop rates; the theoretical advantage of faster drop times lies in improved signal to noise. The authors used a 100- x 4.6-mm C-8 (10-µm) column and a mobile phase consisting of 50:50 MeOH:H_2O, 0.1M KNO_3, 1mM HNO_3. In addition, an electrochemical scrubber was used to eradicate the oxygen (and other reducible impurities) from the mobile phase. Amperometric detection at -600-mV (vs. Ag/AgCl) was achieved using both DC and normal pulse polarography (NPP). Through careful synchronization of the drop time and the pulse, nitrazepam could be detected to the 4-ng and 1-ng level, using DC and NPP methods, respectively. In addition, the method was validated through an application to the trace determination of nitrazepam in serum extracts. With an average extraction efficiency of 67 ± 3%, the drug could be quantitated to the 4-ppb level.

Another report by these authors [44] described the application of alternating current (AC) polarographic detection for four benzodiazepines, following chromatographic separation under conditions identical to those described above [43]. The fast dropping mercury electrode was again employed, but NPP and DC amperometry were replaced by the AC approach. In this method, a sinusoidal voltage fluctuation is superimposed on the DC potential applied at the electrode, which leads to an alternating current response. Theoretically, AC polarography should provide for improved sensitivity, because the faradaic (analytically useful) current can be readily differentiated from the capacitive ("background") current. This theoretical advantage was aptly demonstrated, in that an LOD of 0.3-pg was realized for nitrazepam under optimized conditions. The authors also used this method to detect bromazepam, diazepam and clonazepam, incorporating the higher potential necessary for reduction of the azomethine functionality, but no sensitivity data were supplied for these compounds.

Oxidative LC-hv-EC was recently applied to the trace determination of a number of benzodiazepines and metabolites, including clonazepam, diazepam, nordiazepam, nitrazepam, demoxepam, midazolam, flurazepam, chlordiazepoxide, norchlordiazepoxide and benzophenone [29]. Due to the presence of a tertiary amine in its structure, flurazepam could be detected at +1.0-V (vs. Ag/AgCl),

without photolysis, to the 2-ng level. The sensitivity of detection was not significantly improved using photolysis. However, the remainder of the compounds studied had no inherent oxidative electroactivity. Following their separation on a 100- x 5-mm C-18 (5-µm) column, using 65:35 MeOH:0.2M NaCl as mobile phase, the compounds were subjected to photolytic residence times of approximately 2-min. Using dual (parallel) glassy carbon working electrodes at +1.0-V and +0.85-V, LC-hv-EC provided LODs ranging from 100-pg to 500-pg for chlordiazepoxide, norchlordiazepoxide, nitrazepam, clonazepam, diazepam, nordiazepam and demoxepam. Midazolam and benzophenone could be detected, under these conditions, to 1.1-ng and 750-pg, respectively. The method was validated through the determination of chlordiazepoxide and norchlordiazepoxide in human urine extracts. There was good agreement between the quantitative results from this newer method and those previously obtained using GC-MS. While the mechanism of photolytic generation of oxidatively electroactive species from benzodiazepines was not well understood, it was postulated that the method involved detection of a substituted amide functionality. Similar amide-containing species had previously demonstrated oxidative electroactivity following photolysis [32].

From these reports, it appears that LC-hv-EC offers the best LCEC approach for the determination of all benzodiazepines. Reductive EC methods require rigorous pretreatment of the mobile phase and sample to remove interfering species, incorporate specialized electronics for optimum detection, and necessitate the often troublesome use of mercury. In addition, it is not yet clear that reductive LCEC offers an improvement in sensitivity over UV detection for benzodiazepines which do not contain the 7-nitro functionality. On the other hand, LC-hv-EC provides LODs for all of the benzodiazepines which are 1 to 2 orders of magnitude lower than those obtained using LC-UV approaches.

Miscellaneous Drugs of Abuse

While the drugs of abuse discussed above are of primary importance in most forensic work, there are several classes of drugs which are encountered on a

more limited basis for which LCEC approaches have been described. Tricyclic antidepressants, including imipramine, desipramine and mianserin, are amenable to oxidative LCEC. Suckow and Cooper [45] have incorporated a thin layer cell with a glassy carbon working electrode operated at +1.05-V (vs. Ag/AgCl) for the oxidative EC detection of imipramine, desipramine and their 2-hydroxy metabolites in plasma extracts. Following separation on a 300- x 3.9-mm C-18 (10-µm) column, using an ion-pairing mobile phase composed of 40:60 ACN:0.1M acetate buffer and 5mM heptanesulfonate, the LOD for all four compounds was 1-ng (40-ppb injected). The lowest quantifiable level of the drugs and metabolites was about 5-ppb in plasma. Detection was based on the oxidation of the cyclic tertiary amine functionality, and commonly encountered substances which do not have this moiety (benzodiazepines or tricyclic antidepressants having the dibenzcycloheptane nucleus) did not interfere with the assay.

A similar study was performed for mianserin and two of its metabolites, desmethylmianserin and 8-hydroxymianserin [46], in which nearly identical chromatographic and electrochemical conditions as those cited above [45] were utilized. Once again, the drug and metabolites were readily detected down to 1-ppb in standard solutions or 5-ppb in plasma. Detection of the drug and its desmethylated metabolite was based on the three electron oxidation of the tertiary ring nitrogen atom, while oxidation of the 8-hydroxy metabolite could occur at either the nitrogen site or the phenolic hydroxyl group.

A large number of tricyclic neuroleptics, including dibenzo-thiepins, -oxepines and -selenepins, were recently surveyed for oxidative and reductive electroactivity [47]. The authors used a rotating disk, glassy carbon working electrode in a wall-jet cell configuration for oxidative detection, and a modified DME, operated in the DC mode, for reductive EC. The detectors were connected to a chromatographic system composed of a 250- x 3-mm C-18 (10-µm) column, with a mobile phase of 55-70% MeOH:1.8% CH_3CO_2H:28-43% H_2O, 5mM pentanesulfonic acid. Only 2 of the 32 compounds studied could be electrochemically oxidized at +1.4-V (vs. Ag/AgCl), while all but four of the

compounds could be reduced at -1.0-V. LODs for these reducible compounds ranged from 2-ng to 129-ng, with the majority having detection limits between 10- and 20-ng. For the most part, these EC LODs were lower than those obtained using UV (254nm) by a factor of 2 to 5.

As fentanyl is one of the major controlled substances used in the production of "designer drugs", there has been a recent resurgence of interest in the determination of the parent compound and its analogues [48]. Fentanyl has been determined using LC-hv-EC [29], and due to the tertiary amine in its structure it exhibits oxidative electroactivity in both the lamp-on and -off modes. Using a thin layer, dual (parallel) glassy carbon working electrode cell operated at +1.0-V and +0.9-V (vs. Ag/AgCl), and a 2-min irradiation time, lamp-on and lamp-off LODs were determined to be 20-ng (100-ppb) and 70-ng (350-ppb), respectively. While this sensitivity may not be sufficient for the trace determination of fentanyl and its analogues in biological matrices [49], the selectivity offered by LC-hv-EC should allow for strong confirmation of the presence of these compounds in seized exhibits.

In addition to these compounds, there are several drugs which should be amenable to LCEC approaches, but for which such methods have not been reported to date. Propoxyphene, the drug in Darvon®, is encountered with some regularity in the forensic analysis of biological fluids for drug content. Its structure contains a tertiary aliphatic amine moiety, and as such this compound should be oxidizable at reasonable electrochemical potentials. A similar situation exists for phencyclidine (PCP) and its analogues. Methaqualone and its analogues contain cyclic amino carbonyl functionalities which are similar in some respects to the barbiturates and benzodiazepines. As such, these compounds should be amenable to LC-hv-EC; the presence of a tertiary amine may lend oxidative electroactivity to these drugs as well. Finally, pentazocine and tripelennamine (commonly recovered as "T's and Blues", capsules containing both drugs) should both exhibit oxidative EC responses, as pentazocine contains a phenolic hydroxyl group and a tertiary amino moiety, while tripelennamine has three tertiary amine

functionalities in its structure. It should be accentuated that tertiary amines must be in the proper chemical environment, especially with respect to pH, ligands and adjacent functional groups, in order to allow for facile electrochemical oxidation.

CONCLUSIONS

There are a number of classes of drugs of abuse for which LCEC represents the analytical method of choice. Morphine and several other narcotics may be detected with excellent sensitivity and selectivity using oxidative LCEC approaches, and this method has been widely employed for the determination of these drugs in biological fluids and tissues. However, LCEC is not readily amenable to the characterization of the origin of morphine or poppy straw samples. Oxidative LCEC and LC-hv-EC both offer the selectivity needed for the rapid screening of illicit cocaine samples, but lack the requisite sensitivity for the determination of this substance in body fluids. LC-hv-EC may represent the analytical method of choice for barbiturates and benzodiazepines; the method offers sensitive detection of all of the members of these groups, yet provides for increased selectivity through the use of dual electrode response ratios and qualitative lamp-on and -off responses. Cannabinoids and tryptamine-derived hallucinogens may be electrochemically oxidized, but there have been relatively few reports of such methods in the literature. Amphetamines and several other important drugs are not directly amenable to electrochemical detection, but there are other methods available which are more than adequate for such analyses.

It appears that the application of electrochemical detection for the liquid chromatographic characterization of drugs of abuse is still in its infancy. The best potential (sic) use of LCEC in the forensic drug laboratory lies in the identification and quantitation of controlled substances which have been tentatively identified using TLC methods. Oxidative approaches will probably

continue to outweigh reductive methods, because of the greater ease of operation involved. Derivatization methods, including the use of light as a reagent (as in LC-hv-EC), will find increasing use for analytes which are unresponsive in their native form. Finally, the future will probably bring the application of LCEC to a number of compounds which should be electroactive, but for which such methods have not yet been exploited.

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