DERIVATISATION OF AMINES FOR ELECTROCHEMICAL DETECTION IN HPLC

by

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Dedicated to the ones I love,

Ayah and Emak,

Zie, Din, Zah, Halim, Suraya, Zo, Duan, and Lin.

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ABSTRACT

Three derivatising reagents have been studied as possible pre-column derivatisation agents for aliphatic amines for reversed-phase HPLC with electrochemical detection. O-Acetylsalicyloyl chloride, N-succinimidyl-3-(4-hydroxyphenyl)propionate, and methyl p-hydroxybenzimidate.HCl were examined. O-Acetylsalicyloyl chloride was shown to be a suitable derivatising reagent. Firstly, pure amine derivatives were prepared using the Schotten-Baumann reaction conditions and the structure of the compounds were confirmed by melting point, IR and NMR spectroscopy, and TLC analysis. Analyses were carried out on an ODS-Hypersil column using as mobile phase 50:50 methanol-0.025 M phosphate buffer pH 8 and the derivatives were detected at a glassy carbon electrode set at +0.9 V vs Ag/AgCl. Results for all amine derivatives have indicated the suitability of LCEC detection method with good linearity, precision and sensitivity. Then the amine derivatisation was applied to in-situ determination of amines in dilute aqueous solution to give 100% yield, and a linear response down to 10^{-6} M concentration level. Amine derivatives obtained from N-succinimidyl-3-(4-hydroxyphenyl)propionate and methyl p-hydroxybenzimidate.HCl can also be detected by LCEC but problems were observed with these reagents.

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INTRODUCTION

1. ELECTROCHEMICAL DETECTION IN HPLC

Much work in HPLC has recently been focused on the development of improved detection techniques. This is primarily because better and better detection methods are needed for the analyses of more complex mixtures of organic and inorganic compounds. Reproducible, fast, and accurate methods are always a consideration for the development of detection in HPLC. Recently, multi-wavelength UV, fluorescence, and electrochemical detectors have become quite popular for HPLC. Among these, electrochemical detection has been shown to be very sensitive and selective [1].

1.1. Classification of Electrochemical Detectors

Several types of electrochemical detectors have been developed for HPLC systems, including conductometric, polarographic, and amperometric detectors. Rucki [2], Stulik and Pacakova [3,4] have reviewed in detail the application of these detectors. The main discussion focused on the development of more selective and sensitive detectors. The principal electrochemical detection techniques can be summarised as follows;

1.1.1. Conductometric Detectors

A specific detector, monitoring ionic species in solution streams, the conductometric detector contains a cell for conductivity measurement, containing electrodes made of metal or graphite. The current across the cell is monitored as a constant alternating voltage is applied to the electrodes. Successful detection of changes in the eluent conductivity is possible even when the detector has a small dead volume (a few µl) [5]. These detectors have thus a specific application and will not be considered further.

1.1.2. Electrolytic Detectors

Polarographic and amperometric detectors are the most popular electrochemical detectors for HPLC. The technique is based on the recording of current at a particular voltage, resulting from electrolytic oxidation or reduction of electroactive species. The polarographic detector uses a dropping or static mercury electrode as the working electrode. The amperometric detector is similar but uses glassy carbon or metal electrodes. In each case usually only a small proportion of the electroactive species (1% - 10%) is electrolysed in the cell. With some designs in which the analyte is fully or nearly fully reacted, the detector is termed a coulometric detector. These detectors usually have electrodes with a large surface area to achieve a complete reaction. When complete electrolysis is achieved, unlike the amperometric detector response should not be dependent on electrode area, temperature or variation in eluent

flow rate [2]. Generally, all these detectors have detection limits in the picogram level with good dynamic behaviour and linear response [6].

1.2. Advantages of Electrochemical Detection

Most of the electrochemical detectors for HPLC offer advantages in :-

a) Sensitivity

An important criteria in selecting a good detector, the analysis of analytes can be achieved down to nanogram and picogram levels and the detection limit is much lower than ultraviolet detectors.

b) Selectivity

Only compounds containing an electrochemically active group can be detected and the applied potential can be adjusted to oxidation or reduction potentials to give a selective response for different electroactive compounds.

c) Wide linear range

Electrochemical detection easily covers a linearity of up to four or five orders of magnitude of concentration.

Electrochemical detectors can also have low dead volumes and are considered economic to operate [1].

2. ELECTROCHEMICAL DETECTOR OPERATION AND DESIGNS

2.1. Electrochemical Detection Principles

The measuring principle in liquid chromatography with electrochemical detection (LCEC) is based on the measurement of the current which flows upon anodic oxidation or cathodic reduction of ions or molecules at a constant applied potential. The current measured is equivalent to the amount of analyte converted at the working electrode. According to Faraday's law :

$$Q = nFN$$

where Q = the measured number of coulombs

n = the number of electrons involved in the reaction

F = the faraday (F = 9.65×10^4 Coulombs per mole)

N = the number of moles of samples converted into product

Differentiating the above equation, with respect to time,

$$dQ/dt = nF\ dN/dt = I$$

The observed current (I) is proportional to the number of moles electrolysed per second.

2.2. The Cell Designs

The construction of the measuring cells for electrochemical detectors are based on one of three concepts; wall-jet, thin-film, and porous electrode. The cell consists of a three-electrode system; working, auxiliary, and reference electrodes. The potential is applied between working and reference electrodes.

A current passing through the reference electrode would change its potential. The auxiliary electrode plays an important role to keep the potential difference between reference electrode and working electrode at the desired value, so that, the reference electrode can be a stable and reliable reference half-cell which can be used as a reference point for potential measurements. The position for the auxiliary electrode is placed opposite to the working electrode and across the flow stream. The reason is to prevent reduction after the oxidation, which could happen for reversible reactions and this arrangement gives a greater linear range. A variety of electrode materials can be used, including silver, carbon, platinum or gold [6]. Glassy carbon electrodes are the most common and have been used for the present research project in a thin-film detector.

2.2.1. Wall-jet

Several LCEC detectors have employed the so-called wall jet principle in which the stream of eluent flows as a jet perpendicular to the electrode surface (usually glassy carbon) [7], as illustrated in Figure 1. This design gives a major advantage in a small cell volume and mechanical cleaning but a low conversion is produced by this detector.

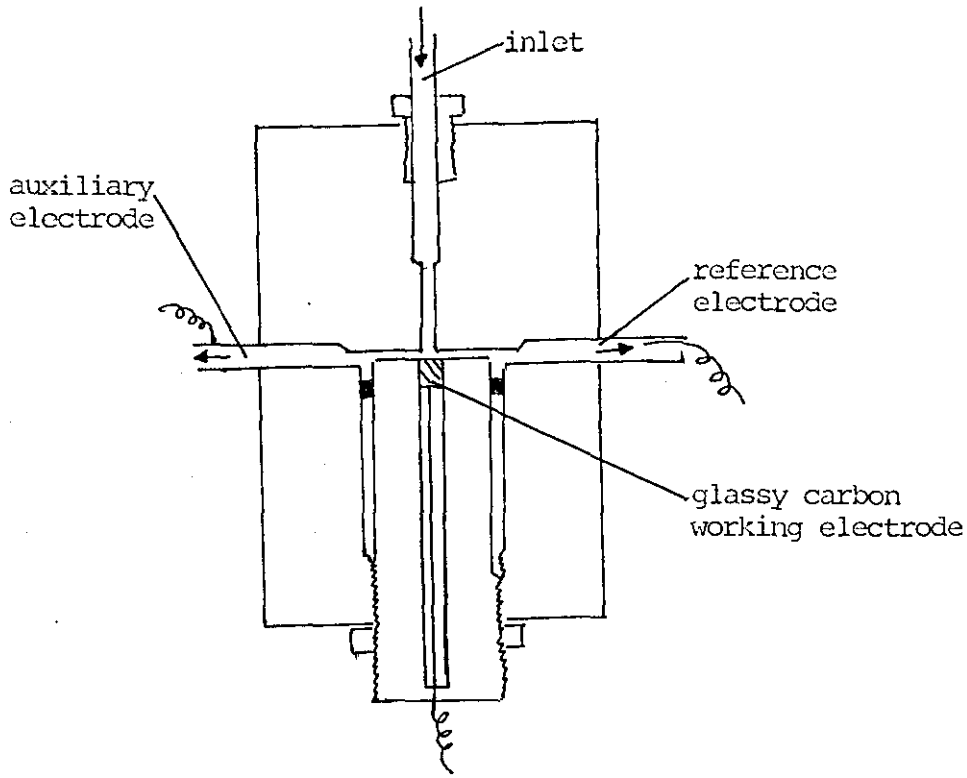


Fig. 1. Schematic diagram of wall-jet detector.

2.2.2. Thin Film

The cell in a thin-film detector is built up from two large surface area of glassy carbon plates as working and auxiliary electrodes. The reference electrode consists of silver tube used as the cell outlet which has been coated internally with silver chloride by applying a conditioning potential. A small amount of NaCl in the mobile phase is important to maintain the reference electrode.

The geometry of a typical thin film cell is shown in Figure 2 [8]. A six angular shape spacer (100 μm) is placed between working and auxiliary electrodes which allows a laminar flow for the eluent as a thin film across the surfaces of the electrodes. The surfaces of the electrodes are highly polished to maintain an efficient reaction of electrolyte.

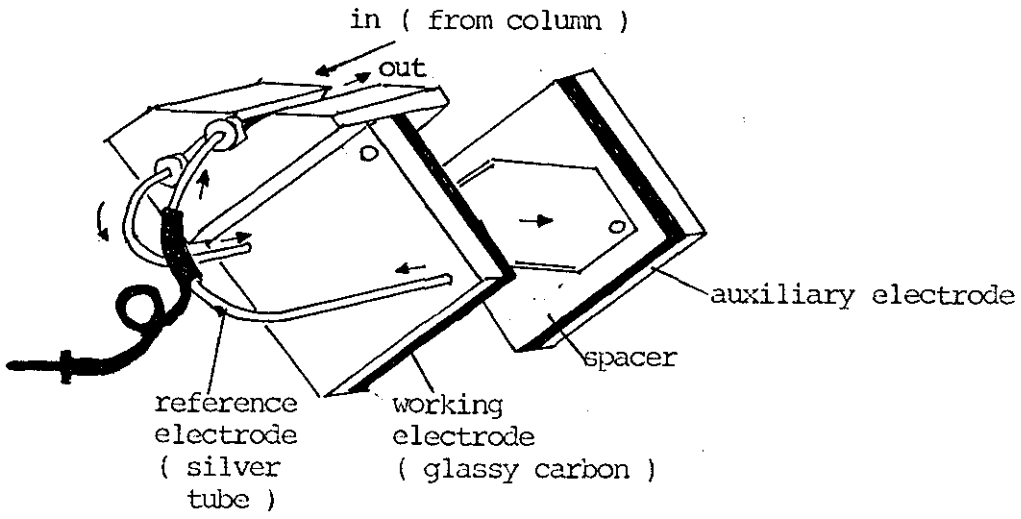


Fig. 2.- Geometry of the thin-layer detector cell

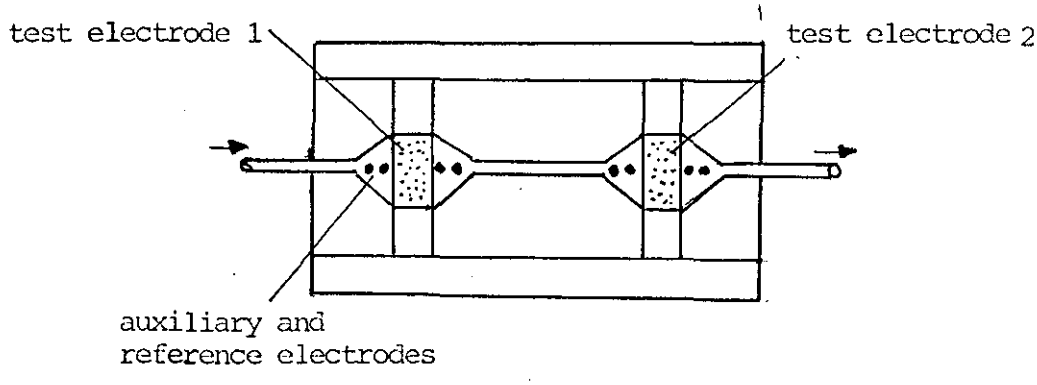


Fig. 3 - Geometry of porous graphite cell

2.2.3. Porous Electrode

The measuring cell contains a high-surface area, porous graphite electrodes through which the eluent passes. The high surface area of these electrodes permits a higher conversion rate and coulometric detection. The cell can also be operated for long periods without a reduction in signal due to contamination. The design of a typical dual detector cell is shown in Figure 3 [9].

2.3. Current-Voltage Relationship

Oxidation or reduction of an analyte containing an electroactive group takes place on the surface of the electrode only if the potential of the working electrode reaches a specific value. The molecules will be oxidised or reduced as rapidly as they reach the electrode, providing the potential is higher than the halfwave potential, $E_{1/2}$. In Figure 4, two voltammograms are presented for the redox reaction in a static system: one of an oxidisable (I) and one of reducible (II) compound. The voltammogram of each compound is characterised by its halfwave potential ($E_{1/2}$) and limiting current. At low potential there is no current and if the voltage is increased as $E_{1/2}$ reached, the current also rises as reaction occurs, until the limiting current reached. At still higher potential, the background reaction becomes exorbitant due to electrochemical oxidation or reduction of aqueous solution. The halfwave potential depends on the species and medium used, while the limiting current is dependent on the maximum mass transport of the analyte towards the electrode [10].

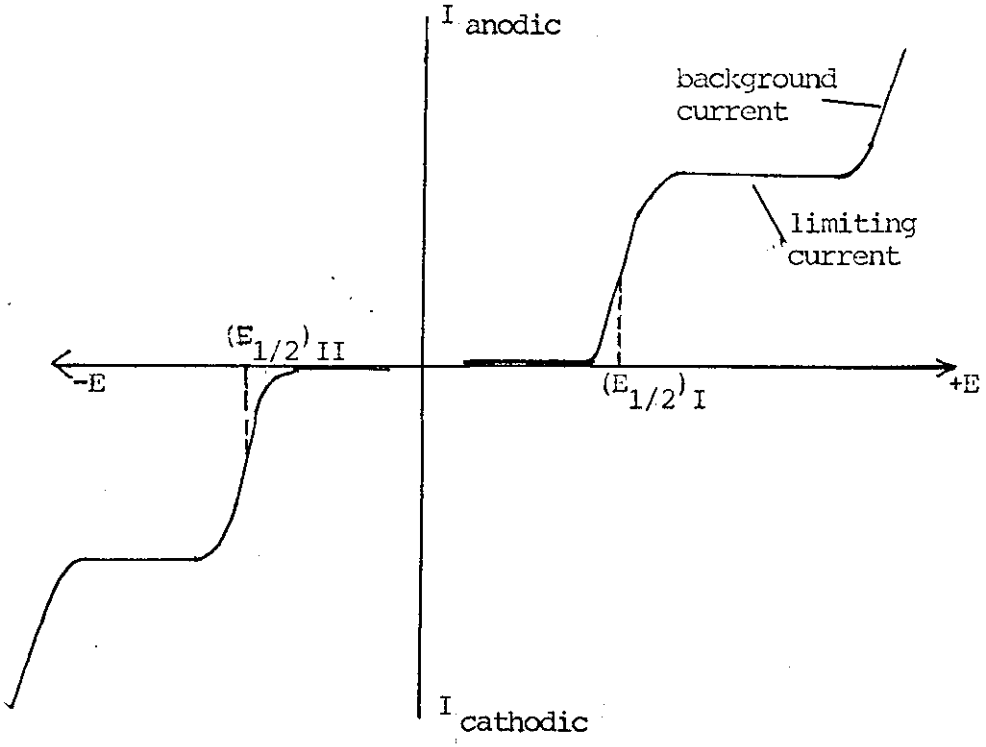
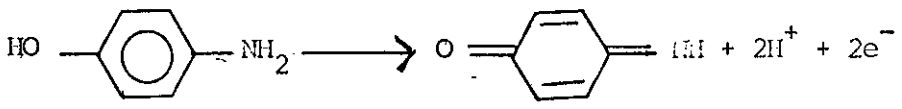


Fig. 4. Voltammograms of an oxidisable (I) and a reducible (II) compound.

2.3.1. Oxidative Electrochemical Detection

In the oxidative mode, the working electrode is the anode and held at positive potential. A typical electrochemical reaction is the detection of phenols [11 - 13].

i.e.



p-aminophenol quinone imine

potential = 1.0 V

Fig. 5. Anodic Oxidation of p-aminophenol

Both inorganic amines [14,15] and biogenic amines and their metabolites have been widely investigated by HPLC with electrochemical detection [15 - 17]. The principal compounds examined are catecholamines (norepinephrine, epinephrine, dopa, dopamine, adrenaline, and noradrenaline), which are found in biological fluids such as the nervous system, urine, and brain fluid. They are easily oxidised at carbon electrodes in the potential ranging from +0.5 V to +1.0 V. The detection limit is the picogram level with good linear behaviour.

Electrochemical detection can also be used to monitor simple phenols at potentials of +0.7 V to +1.1 V. Other compounds which have been studied include vitamins [18,19], alkaloids [20,21], and aromatic amines [22 - 25] (phenylenediamines, benzidines and aminophenols).

2.3.2. Reductive Electrochemical Detection

In contrast to the oxidative mode, the reductive mode uses a cathodic working electrode. However, the major problem with the reductive mode is dissolved oxygen, which is reduced at negative potentials (from -0.5 V to -1.1 V) and this signal can interfere with the signal from the compound of interest. To overcome this problem, oxygen has to be rigorously removed from both the mobile phase and from the sample solution prior to injection into HPLC system. Many aromatic and aliphatic nitro compounds have been examined with reductive electrochemical detection [26 - 28]. Typical reactions are as follows [29]:

$$R - \emptyset - NO_2 + 4e^- + 4H^+ \text{------} > R - \emptyset - NHOH + H_2O$$

$$R - \emptyset - NHOH + 2H^+ + 2e^- \text{------} > R - \emptyset - NH_2 + H_2O$$

Mercury electrodes have been used for determining nitro compounds in the reductive mode by Lloyd [30 - 33], Lyle and Saleh [34]. For example, Lloyd has analysed organic explosive residues using a pendent mercury drop electrode (PMDE) operated at -1.0 V vs Ag/AgCl.

Azo compounds and their derivatives [35 - 36] also give responses to reductive electrochemical detection.

3. PRE-COLUMN DERIVATISATION FOR ELECTROCHEMICAL DETECTION IN REVERSED-PHASE HPLC

Derivatisation is an important technique in HPLC which can be used to increase selectivity and sensitivity of detection where the original compounds do not respond. This approach is mainly used to enhance ultraviolet or fluorescence detection but has also been used to enhance electrochemical detection.

The main reason for derivatisation of trace compounds for electrochemical detection is to be able to detect the analyte at a lower concentration level in a sample [37]. Derivatisation can be accomplished in two ways, i.e. either by changing the original properties of the sample or by addition of an electrochemical active group as a substituent.

The formation of the LCEC active species can be carried out either in a pre- or post-column reaction although the latter is not very common and thus it will not be discussed further. Derivatisation also gives the advantages of selective detection.

3.1. Reagents for Pre-column Derivatisation for LCEC

Several reviews of pre-column derivatisation techniques have been published [38 - 44] and some typical reactions have been summarised in Table 1. Derivatising reagents are chosen depending on analyte of interest. The present study is concerned primarily in the enhancement of the detection of amines and previous studies of their derivatisation will be considered in detail.

Table 1. Summary of some typical reactions for pre-column derivatisation of functional groups

Functional group	Reagent	Applied potential for detection	Reference
isocyanates	1. methoxy-2-phenyl-1-piperazine 2. tryptamine	+0.8 V vs Ag/AgCl +0.8 V vs Ag/AgCl	45 46
hydrazines	salicylaldehyde	+1.0 V vs Ag/AgCl	47
carboxylic acids	p-aminophenol	+0.7 V vs Ag/AgCl	48
cyanide ion	p-benzoquinone	+0.7 V vs Ag/AgCl	49
metallic cations (Ni^{2+}, Cu^{2+}, Hg^{2+}, Co^{3+}, Pb^{2+}, Cd^{2+})	1. diethyl dithiocarbamate 2. pyrrolidine dithiocarbamate	+0.7 V vs Ag/AgCl & +1.2 V vs Ag/AgCl	50 - 52
ethers (as ethylene dibromide)	silver picrate	-0.8 V vs Ag/AgCl	53

3.2. Reagents for Pre-column Derivatisation of Aliphatic Amines for LCEC

Aliphatic amines are widely used industrial chemicals and one of the major raw materials used in the production of other chemicals, pharmaceuticals, pesticides and dyestuffs [54]. Amino compounds are also present as analytes of interest in several biological fluids. Since GLC techniques have difficulty with polar and basic compounds, HPLC offers an attractive approach for the determination of these compounds but as the aliphatic compounds have no chromophore, derivatisation is necessary to enhance detectability.

3.2.1. Previous Studies of Pre-column Derivatisation for Oxidative LCEC

Several derivatising reagents are suitable for the pre-column derivatisation of amines for oxidative LCEC. In most cases the derivatisation reaction has been applied to amino-acids.

Primary alkyl amines and amino-acids can be determined following pre-column derivatisation with o-phthalaldehyde (OPA) in the presence of a suitable thiol. Harsing, Nagashima, Vizi, and Duncalf [55] have derivatised histamine using OPA and 2-mercaptoethanol in sodium tetraborate-methanol. As shown in Figure 6, the derivatisation took 2 min at pH 9.5 before injection into the HPLC system. However, 33% decomposition of the histamine derivative was observed after 60 min. Determinations of thiol were carried out at +0.5 V using a glassy

carbon electrode and the detection limit for histamine was 50 pg at a signal to noise ratio of 3:1. This method has been applied to histamine that was extracted from the different regions of rat brains.

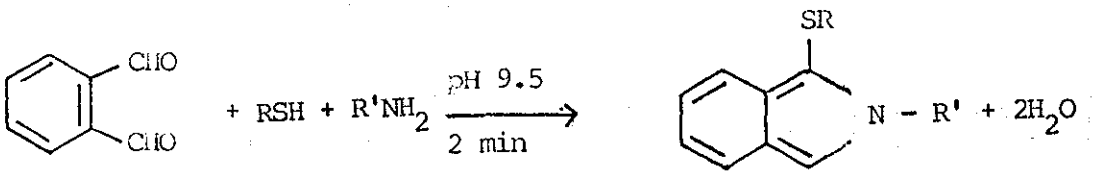


Fig. 6. Reaction between primary alkylamines and OPA in the presence of an alkylthiol

A significant problem with pre-column derivatisation of OPA is the instability of the derivatives, which needs careful timing to maintain reproducibility. Hence, Allison, Mayer, and Shoup [56] have suggested alternative thiols such as tert-butylthiol to produce more stable isoindole derivatives.

Further studies have been done by Jacobs [57] for derivatisation of primary amines using OPA. Because of the instability of the isoindole derivatives, sulfites were substituted for thiol as the co-reagent as shown in Figure 7. These compounds showed good in-situ stability. However, the problem with the derivatives was the tendency to be hydrolysed at low pH, which restricted the chromatographic conditions for analysis.

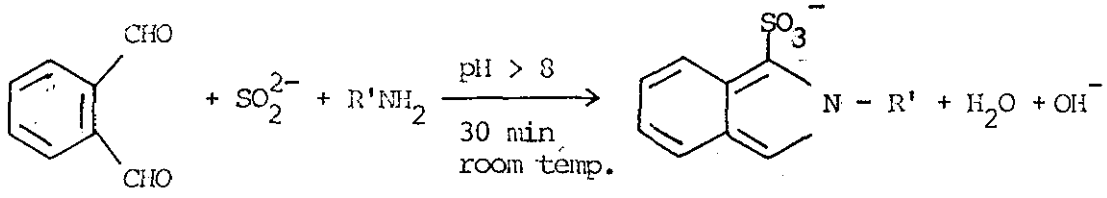


Fig. 7. Reaction between primary alkylamines and OPA in the presence of sulfites

The determination of amines and amino-acids has been studied by Mahachi, Carlson, and Poe [58] using p-N, N-dimethylamino-phenylisothiocyanate. The reaction scheme for derivatisation of an amino-acid is shown in Fig. 8. 21 amino-acids have been derivatised and gave thiohydantoin derivatives which were amenable to detection at a glassy carbon electrode set at 0.85 V vs Ag/AgCl. The separation was complete within 80 min with a C_8 column using a mobile phase containing acetonitrile and phosphate buffer pH 2.0 (75:25). They reported the yield for 10 of 21 amino-acids ranged from 80% - 90% with a detection limit of 0.1 ng to 1 ng. Further studies have been tried to identify the N-terminal amino-acid of a dipeptide and a tripeptide.

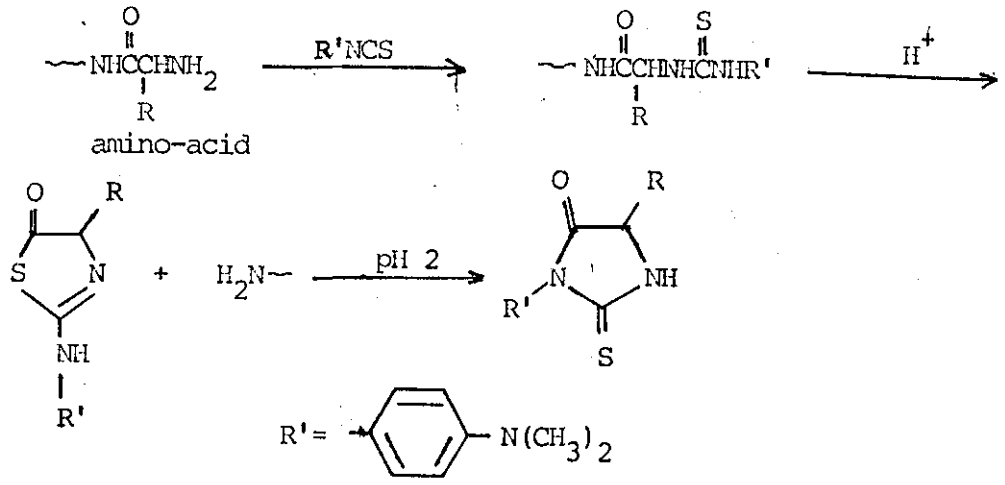


Fig. 8. Reaction scheme for derivatisation of terminal amino-acids with p-N, N-dimethylaminophenylisothiocyanate

Misson and Sternson [59] have also studied p-dimethylamino-phenylisocyanate as a derivatising reagent and applied it to the analysis of extracted arylhydroxylamines in liver homogenates. The reagent is not itself electroactive but reacts with arylhydroxylamines to form electro-oxidisable products (Fig. 9) shown to be suitable for monitoring in HPLC with amperometric detector at 0.5 V vs Ag/AgCl. The derivative detection was linear over the concentration range 1 x 10^{-6} M to 5 x 10^{-8} M.

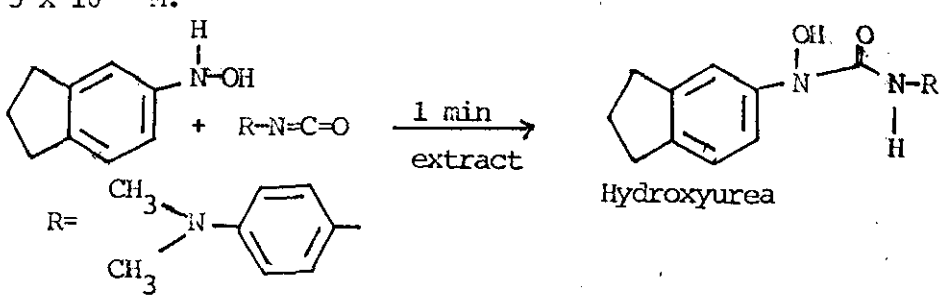


Fig. 9. Derivatisation of arylhydroxylamines with p-dimethylaminophenylisocyanate

Shimada, Tanaka, and Nambara [60] have examined alternative potential derivatising reagents for amines. They prepared N-(4-anilinophenyl)isomaleimide (APIM) and N-(4-anilinophenyl)-isophthalimide (APIP) for pre-column derivatisation of amines in HPLC with oxidative electrochemical detection. Two amines, phenylethylamine and piperidine were selected as the model compounds for primary and secondary amines. The derivatisation reaction took 1 hour in acetonitrile-0.05 M borate buffer (pH 9.0) at room temperature (Fig. 10). N-(4-anilinophenyl)-isomaleimide gave the detection limit of 13 pg at an applied potential +0.4 V vs Ag/AgCl and was claimed to be the most suitable derivatising reagent for pre-column derivatisation in terms of its sensitivity and selectivity.

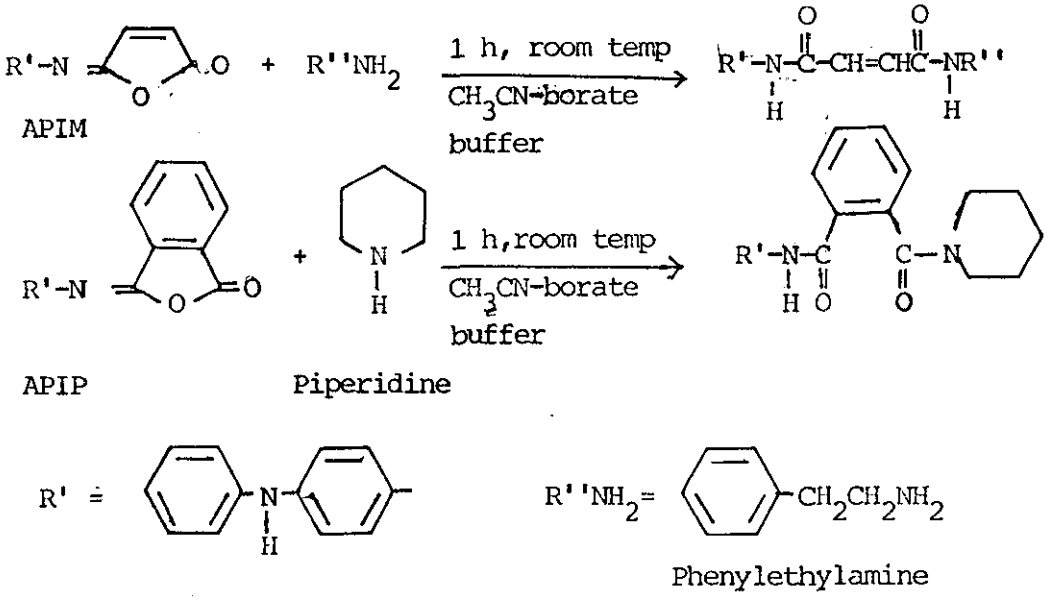


Fig. 10. Derivatisation of amines with N-(4-anilinophenyl)-isomaleimide (APIM) and N-(4-anilinophenyl)-isophthalimide (APIP)

Non-electroactive amines can be derivatised with a derivatising reagent containing a phenolic group. This group can then be utilised for the detection of derivatives by high performance liquid chromatographic technique with electrochemical detection. Shimada, Tanaka, and Nambara [61] have studied two derivatising reagents for the derivatisation of primary and secondary amines. N-succinimidyl vanillate and N-succinimidyl homovanillate were prepared from vanillic acid and homovanillic acid by condensation with N-hydroxysuccinimide. The derivatisation was studied in detail for N-succinimidyl homovanillate employing ethylphenylalaninate as a model compound (Fig. 11). The reaction was carried out in pyridine at 60°C for 30 min, to give the ethyl phenylalaninate derivative in 83% yield. With the potential of the detector at 0.8 V vs Ag/AgCl, the detection limit was 200 pg and a linear response was obtained over the range of 1.0 ng to 25 ng of ethylphenylalaninate. A similar method was applied to piperidine but the results have not been given in detail.

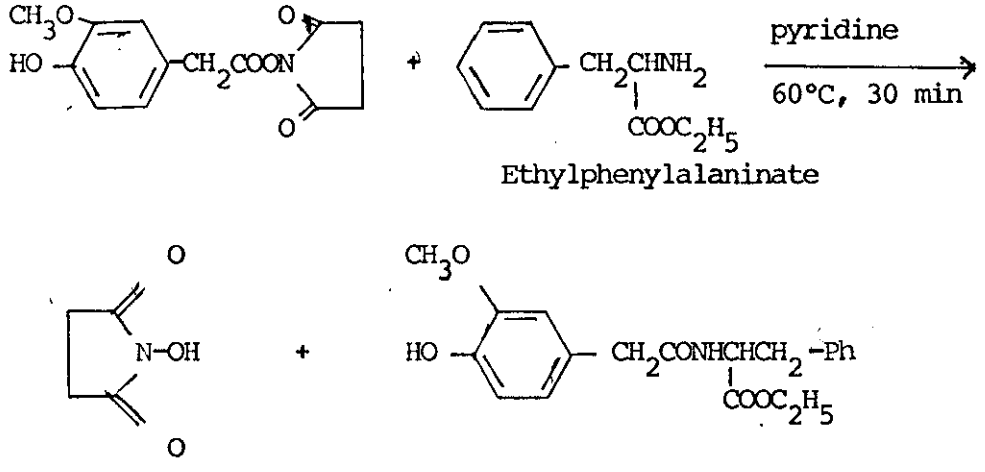


Fig. 11. Derivatisation of ethylphenylalaninate with N-succinimidyl homovanillate

Jacobson, Marshall, Mine, Kirk, and Linnoila [62] have developed a pre-column derivatisation scheme for the analysis of biogenic amines found in very low concentrations in body fluids using the N-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)-propionic acid as a derivatising reagent. They used serotinin and histamine as electroactive and non-electroactive amines for acylation with the derivatising reagent to demonstrate the efficiency of extraction into organic solvents during sample preparation before injection into the HPLC system.(Fig. 12). All the amine derivatives in this studies were separable by HPLC and detectable by electrochemical detection with recoveries of 50% to 90%.

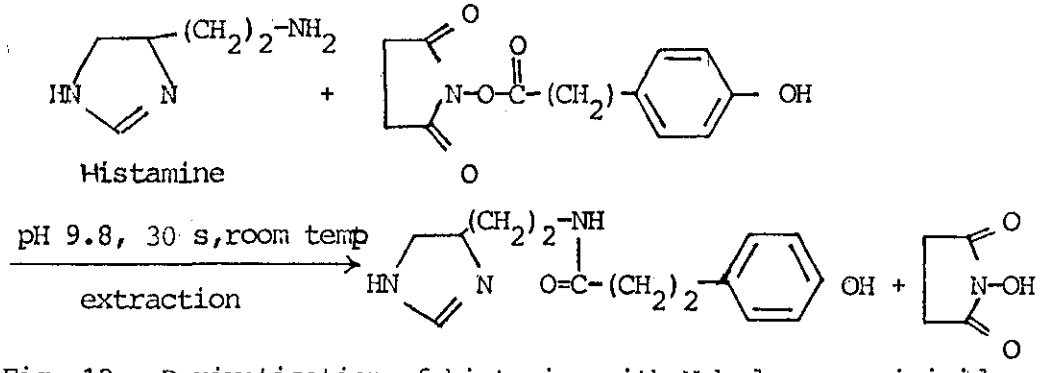
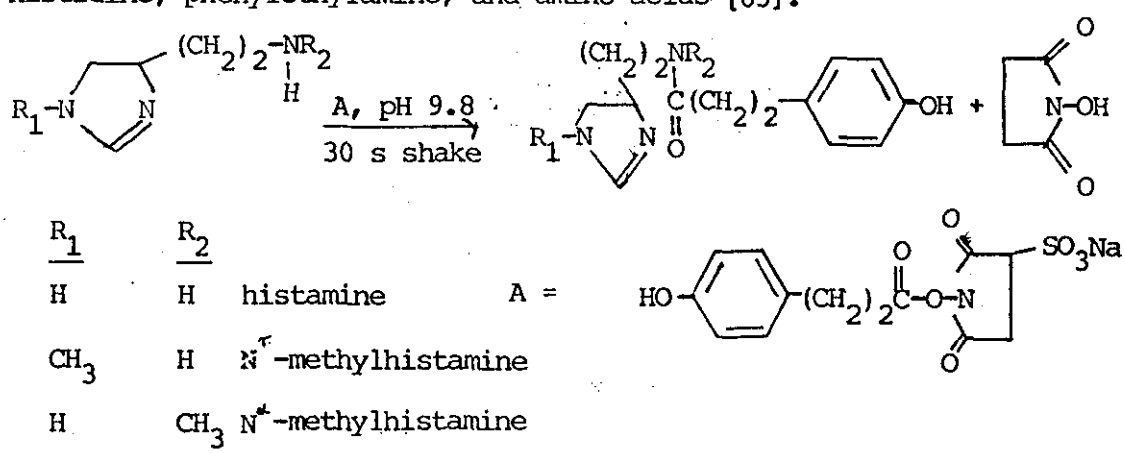


Fig. 12. Derivatisation of histamine with N-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid

In other studies, Mine, Jacobson, Kirk, Kitajima, and Linnoila [63] have further examined derivatisation using succinimide esters. They acylated histamine and N-methylhistamine from rat brain with the soluble Bolton-Hunter reagent, sulpho-succinimidyl-3-(4-hydroxyphenyl)propionate (Fig. 13). Derivatisation was carried out at pH 9.8 to pH 10 at room

temperature and the solution was vortexed for 30 s before extraction. The yield of pure derivative after purification by ion chromatography was 78%. Using a working potential at +0.56 V, the detection limits were 0.1 pmole for histamine and 0.2 pmole of N-methylhistamine. They suggested that the above reagent could possibly also be used for the derivatisation of spermidine, histidine, phenylethylamine, and amino-acids [63].



R_1	R_2	
H	H	histamine
CH_3	H	N^{τ}-methylhistamine
H	CH_3	N^{π}-methylhistamine

Fig. 13. Derivatisation of imidazole amines with sulphosuccinimidyl-3-(4-hydroxyphenyl)propionate

An investigation of reaction of the sulpho Bolton-Hunter reagent, sulphosuccinimidyl-3-(4-hydroxyphenyl)propionate with β-phenylethylamine has been carried out by Gusovsky, Jacobson, Kirk, Marshall, and Linnoila [64]. As shown in Figure 14, derivatisation in 0.1 M Na_2CO_3 - 0.01 M $NaHCO_3$ buffer solution (pH 10) at room temperature was completed in 10 min. A number of endogenous, non-endogenous, aliphatic and aromatic amines were also examined using the sulpho Bolton-Hunter reagent. For the derivative of β-phenylethylamine, the detector response increased with increasing oxidation potential. However, they reported that additional peaks also appeared in the chromatograms as well as the

main peak of interest.

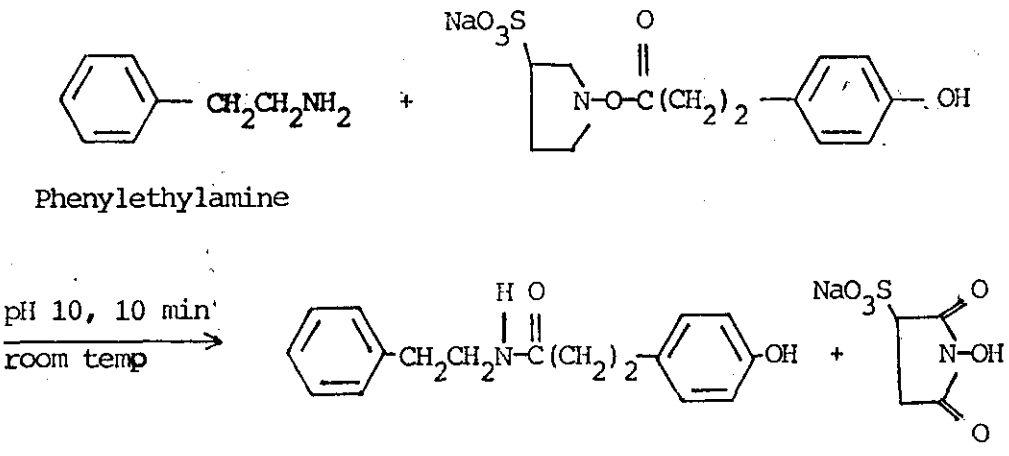


Fig. 14. Derivatisation of phenylethylamine with the sulpho Bolton-Hunter reagent

Tanaka, Shimada, and Nambara [65] have reported a novel ferrocene reagent, N-succinimidyl 3-ferrocenyl propionate for the pre-column derivatisation of amines (Fig. 15). The derivatives contain a ferrocenyl group as an electrophore, which can be oxidised at a low potential and thus detected selectively in the presence of phenols or aromatic amines. Phenylethylamine and tryptamine have been examined with this reagent. The maximum sensitivity could be reached at +0.4 V vs Ag/AgCl with a detection limit of 0.2 pmole.

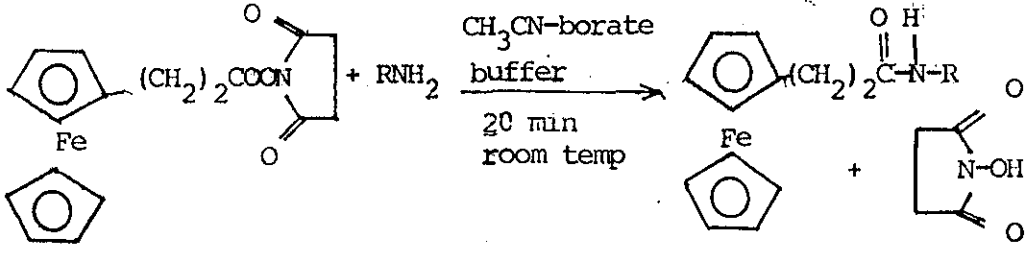


Fig. 15. Derivatisation of amines with N-succinimidyl 3-ferrocenyl propionate

Chou et al. [66] has also applied derivatisation techniques to improve the detection of amines. The derivatising reagent was a polymeric anhydride containing O-acetylsalicyl as a labelling moiety (Fig. 16). Derivatisation has been carried out on primary and secondary amines; such as propylamine, butylamine, nonylamine, diethylamine, and morpholine. The reaction conditions have been optimised and need mild conditions of 60°C for 20 min. The yield of the derivatives ranged from 28% from morpholine up to 96% from butylamine. They used a UV detector but were also able to detect using an electrochemical detector in the oxidative mode with the potential at +1.0 V although the compounds have no apparent electrophore.

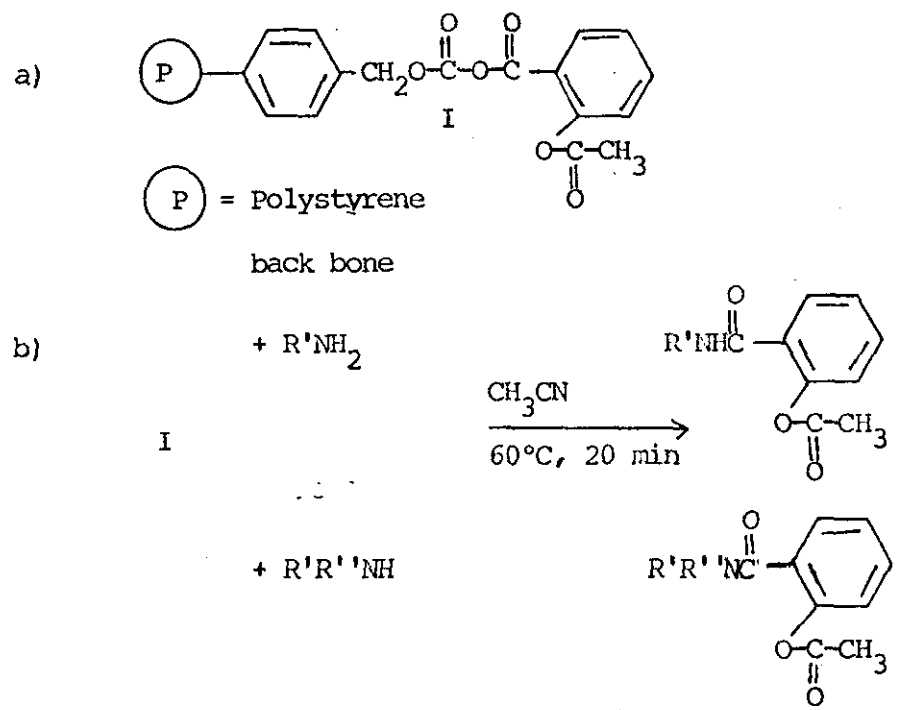


Fig. 16. Derivatisation reaction of primary and secondary amines with polymeric anhydride

3.2.2. Pre-column Derivatisation of Amines for Reductive LCEC

Caudill and co-workers [67] and Jacobs, and Kissinger [68] have proposed the use of 2,4,6-trinitrobenzenesulphonic acid as a derivatising reagent for alkylamines for reductive LCEC. Using 2,4,6-trinitrophenyl derivatives, the low detection limits of LCEC can normally be achieved in picogram levels for easily reduced compounds. Caudill and Wightman [69] have studied the use of 2,4,6-trinitrobenzenesulphonic acid (TNBS) to derivatise γ-aminobutyric acid, β-aminoisobutyric acid, α-aminobutyric acid, β-alanine, L-alanine, glycine, and δ-aminovaleric acid. As shown in Fig. 17, the reaction took 30 min at pH 9.2 with 0.2 M potassium tetraborate. The trinitrophenyl derivatives of amino-acids were detected amperometrically using carbon electrodes and the working electrode potential was -0.6 V vs a saturated calomel electrode (SCE).

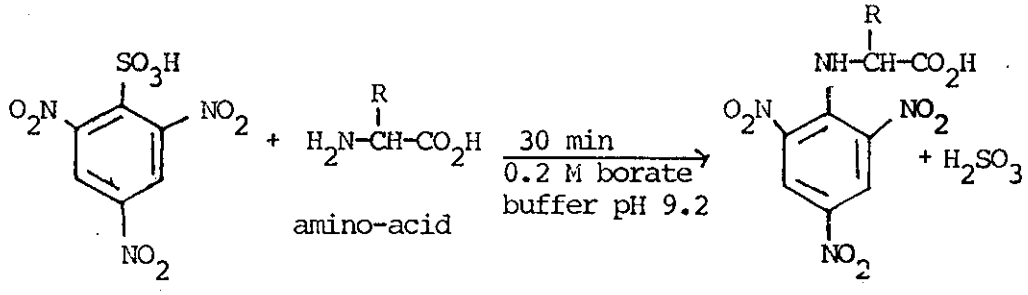


Fig. 17. Derivatisation of amino-acids with 2,4,6-trinitrobenzenesulphonic acid

Jacobs and Kissinger [68,70] studied three reagents: 2,4-dinitrofluorobenzene (DNFB), 2-chloro-3,5-dinitropyridine (DNCP), and 2,4,6-trinitrobenzenesulphonic acid (TNBS) for use as reagents for pre-column derivatisation for LCEC (Fig. 18). Aniline was selected as a model for derivatisation. Both glassy carbon and gold / mercury amalgam electrodes were used for reductive mode electrochemical detection.

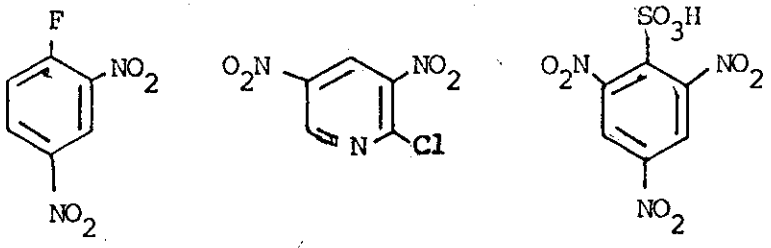


Fig. 18. Derivatising reagents for derivatisation of amines

The derivatisation was carried out in borate buffer at pH 9 (for TNBS) and acetone at pH 8.8 to pH 9.0 (for DNFB and DNCP). The mixture was incubated at room temperature for 1 hour. Each derivative was detected using both LCEC detection at -0.85 V vs Ag/AgCl and UV detection at 254 nm. Only TNBS appeared suited to application as a pre-column derivatisation reagent for LCEC. It gave the most detectable derivatives with minimum production of interfering by-products and was suited to application in aqueous systems.

2,4-Dinitrofluorobenzene, DNFB (Sanger's reagent) is also a potential derivatising reagent for amines using a hybrid technique called " LC-photolysis-EC " (LC-hv-EC) which has been studied by Krull and co-workers [71,72], the mechanism involved

photolytic cleavage of nitrite (NO_2^-) from the parent compound which was detected at an oxidative potential. They have applied this reaction to the determination of amino-alcohols and amino-acids (Fig. 19). They reported the limit of detection for these amines ranging from 20 ppm to 200 pbb.

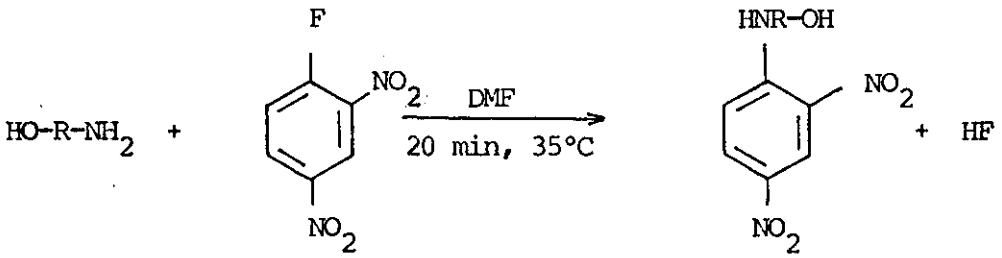


Fig. 19. Derivatisation of amino-alcohols with 2,4-dinitrofluorobenzene (DNFB)

4. PURPOSE OF RESEARCH

Pre-column derivatisation is important in HPLC to enhance detection for compounds which are not readily detected spectrophotometrically. Aliphatic amines with a high polarity are not suitable for GLC but can be separated by HPLC. However, they are difficult to detect, and it is desirable to form a suitable derivative containing an electroactive group.

In comparison with UV and fluorescence detection, so far only a few applications of potential derivatising reagents for pre-column derivatisation of aliphatic amines for HPLC with electrochemical detection have been studied. Most of these have problems related to the effectiveness of derivatisation, separation by HPLC and selectivity and sensitivity of detection.

The present studies have examined two simple and easy derivatising reagents for their suitability for the derivatisation of aliphatic amines. The products can be readily separated by HPLC. Firstly, O-acetylsalicyloyl chloride was studied as a derivatising reagent because it can readily form a derivative containing a free phenolic group which should be extremely electroactive and could be monitored by electrochemical detection in the oxidative mode. Pure amine derivatives have been prepared using O-acetylsalicyloyl chloride. The composition of each product was confirmed by physical and spectroscopic methods. They were then examined by reversed-phase HPLC with electrochemical detection to investigate the suitability of

these products especially the sensitivity and detectability of derivatives under the optimum chromatographic conditions.

Then, the prepared amine derivatives have been used as standards in a study of *in-situ* derivatisation of amines in dilute solution. The yield of derivatives has been examined and the limit of detection and the linearity of analyte have been determined.

A second reagent, N-succinimidyl-3-(4-hydroxyphenyl)-propionate (Bolton-Hunter reagent) has also been studied in some detail as a derivatising reagent for the derivatisation of aliphatic amines. The phenolic group from this reagent is electrochemically active.

In addition in two brief studies, Wood's reagent, methyl p-hydroxybenzimidate.HCl, which should give derivatives with a response to electrochemical detection have been examined and the LCEC properties of the widely used dansyl derivatives were examined. These last compounds are frequently formed for fluorescence detection but are potentially also suitable for electrochemistry.

CHAPTER II

EXPERIMENTAL

1. MATERIALS AND METHODS

1.1. Reagents and Chemicals

O-Acetylsalicyloyl chloride and 3-(4-hydroxyphenyl)propionic acid-N-hydroxysuccinimide ester (Bolton-Hunter reagent) were purchased from Fluka AG, CH 9470, Buchs, Switzerland. N-Hydroxysuccinimide 3-(4-hydroxyphenyl)propionic acid and N,N-dicyclohexylcarbodiimide were purchased from Aldrich Chemical Co. Ltd, England. Methyl p-hydroxybenzimidate.HCl (Wood's reagent) was purchased from Sigma Chemical Co., Poole, Dorset, England. Dansyl-L-aspartic acid, dansyl-L-valine, dansyl-L-proline, and dansyl-L-glycine were purchased from Sigma Chemical Co., Poole, Dorset, England.

Sample amines included benzylamine from Aldrich Chemical Co. Ltd, England, cyclohexylamine from BDH Chemicals Ltd, Poole, England, piperidine from FSA Laboratory Supplies, Loughborough England and N-methylaniline from Hopkin and Williams Ltd, Essex, England. Methanol HPLC grade was purchased from FSA Laboratory Supplies, Loughborough, BDH Ltd, Poole and ROMIL Chemicals Ltd, Shepshed, England. Water, propan-2-ol, tetrahydrofuran, and ethyl acetate HPLC grade were purchased from FSA Laboratory Supplies, Loughborough, England. Sodium chloride was AR grade. Phosphate buffer (pH 8) was prepared by dissolving analytical reagent grade disodium hydrogen orthophosphate (2.1197 g) and

sodium dihydrogen orthophosphate (0.0417 g) in 250 ml water.

1.2. Equipment

HPLC separations were carried out using an Applied Chromatography System (ACS) LC500 (Macclesfield, Cheshire, UK) pump connected to a Rheodyne 7125 injection valve (20 µl sample loop) and Shandon Southern stainless steel column (10 cm x 5 mm i.d.) packed with 5 µm ODS-Hypersil Shandon Southern (Queensferry, UK) (Batch No. 10/1229 and 10/1721). The analytes were detected using Cecil Instruments CE 2012 variable wavelength UV detector (Cambridge, UK) at 254 nm, Perkin Elmer 2000 fluorescence spectrophotometer (Beaconsfield, Bucks, UK) with the excitation and emission wavelengths set at 340 nm and 530 nm, respectively, and Kipp Analytical Model 9205 coulometric detector (Emmen, Netherlands) in series and the peaks were recorded on a Linseis L650 chart recorder.

The spectra of the amine derivatives were measured on Pye Unicam SP3-100 IR spectrophotometer and NMR spectrometer Model EM-360A at 60 MHz. The melting point of the amine derivatives was measured using Gallenkamp melting point apparatus.

1.3. Procedures and Tests HPLC Column

The HPLC column was packed by preparing a slurry of 1.90 g ODS-Hypersil (5 µm) packing material in propan-2-ol and packing upward under pressure (6500 psi) with 100 ml propan-2-ol (upward) and 40 ml propan-2-ol (downward) followed by 50 ml 50:50 methanol: water as eluent using a pressure amplification

pump. The column was tested by using a test column mixture containing benzamide, acetophenone, benzophenone and biphenyl in a mobile phase of 70:30 methanol: water at a flow rate of 0.8 ml/min and UV as a detector set at 254 nm. The efficiency of the peaks was found to be 2524, 2933, 2953, and 3606 respectively.

2. DERIVATISATION REACTIONS

2.1. Derivatisation of Amines using O-Acetylsalicyloyl Chloride.

2.1.1. Preparation of Derivative Standards

The general procedure for derivatisation of amines is based on the Schotten-Baumann reaction conditions [73]. The amine (1.0 g) and O-acetylsalicyloyl chloride (1.0 g) were dissolved in 2 M NaOH (20 ml) and diethyl ether (30 ml), respectively. Then they were mixed and shaken in a flask. The aqueous layer was separated and washed twice with diethyl ether (10 ml). Finally the solution was acidified with 2 M HCl to pH 7.0. The solution was then extracted with chloroform (2 x 5 ml) and after evaporation of the extract to dryness, the product was obtained as a solid which was recrystallised from ethyl acetate and ethyl ether.

The spectra of the amine derivatives were then confirmed by melting point, thin layer chromatography, infra-red and proton nuclear magnetic resonance spectroscopy.

2.1.2. *Chromatographic Analysis of Salicylamide Derivatives*

The general chromatographic conditions for HPLC analysis of amine derivatives were as follows: The eluent, consisting of 50:50 methanol: 0.025 M phosphate buffer (pH 8) containing 0.001 M NaCl was degassed under reduced pressure before use; flow rate was 1.0 ml/min; coulometric detection was monitored at +0.9 V vs Ag/AgCl and chart speed was 1 cm/min.

2.1.3. Measurement of the Optimum Applied Potential of Amine Derivatives

10^{-3} M 2-hydroxybenzamide derivative of piperidine in 50:50 MeOH: water (10 µl) was injected into the HPLC system. The potential of the coulometric detector was increased in steps from +0.5 V to +1.0 V vs Ag/AgCl and current measured as peak height.

2.2. Derivatisation of Amines in Dilute Aqueous Solution

2.2.1. Derivatisation of Piperidine with O-Acetylsalicyloyl Chloride

A series of derivatisations of a solution of 1×10^{-4} M piperidine in 0.1 M NaOH (10 ml) were carried out. Increasing amounts of O-acetylsalicyloyl chloride were added ranging from 10 mg to 200 mg. The derivatisations were allowed to react at room temperature for 1 h. Then, in each case, a sample of the reaction solute (10 µl) was injected into the HPLC system. For detection, the applied potential of electrochemical detector was set at 0.9 V vs Ag/AgCl. The same procedure was used for derivatisation of 10^{-5} M piperidine in 0.1 M NaOH (10 ml).

For general derivatisation of amine solutions, the same procedure as above was used but 200 mg of O-acetylsalicyloyl chloride was used.

2.2.2. Derivatisation of N-Methylaniline with O-Acetylsalicyloyl Chloride using Valdez and Reier [74] Method

Three solutions were prepared for analyses. Solution No. 1 was acetonitrile with molecular sieves added for drying. Solution No. 2 was O-acetylsalicyloyl chloride in dried acetonitrile, 10 mg/ml with molecular sieves added for drying. For solution No. 3, the standard of N-methylaniline solution was made up to 1000 ppm.

In a 2 ml vial, 100 µl of solution No. 1 and 10 µl of solution No. 3 were mixed together with molecular sieves. The sample was then allowed to dry for 10 min, after which time 1000 µl of solution No. 2 was added to the vial. The sample was heated at 75°C for 90 min to 120 min. Finally, the sample (10 µl) was injected into the HPLC system.

3. DERIVATISATION OF AMINES USING 3-(4-HYDROXYPHENYL)PROPIONIC ACID-N-HYDROXYSUCCINIMIDE ESTER (BOLTON-HUNTER REAGENT)

3.1. Preparation of Bolton-Hunter Reagent

The reagent was prepared by modification of the procedure of Rudinger and Ruegg [75]. 3-(4-hydroxyphenyl)propionic acid (1.661 g) and N-hydroxysuccinimide (1.151 g) in tetrahydrofuran (7 ml) were mixed at -5°C for 2 hours with dicyclohexylcarbodiimide (2.475 g) and then kept

at room temperature for 10 hours. The mixture was then treated with acetic acid (0.12 ml) to destroy the excess of carbodiimide. After 1 hour the mixture was diluted with ethyl acetate (10 ml), the dicylohexylurea was filtered off and washed with ethyl acetate and the combined filtrate and washings were evaporated to dryness under reduced pressure. The residue was recrystallised from ethyl acetate (20 ml) by addition of light petroleum ether (40°C - 60°C) to give m.p. 116°C - 120°C (Lit. 120°C - 122°C). The product was homogenous on separation on a TLC plate (silica gel) in eluent ethyl acetate-methanol (95:5, V/V).

3.2. Preparation of Derivative Standards

The amine (200 µl) in 0.1 M $NaHCO_3$ (40 ml), pH 8.5 was reacted with the Bolton-Hunter reagent (300 mg). After 3 hours at room temperature, the solution was extracted and washed twice with chloroform (in the ratio of 2 ml $CHCl_3$: 1 ml $NaHCO_3$). After evaporation to dryness, the product was recrystallised from ethyl acetate and petroleum ether (40°C - 60°C). The first crystals were discarded and the solution was filtered using a sintered filter containing silica gel (60 - 120 mesh). The derivative was eluted from the column with ethyl acetate. The first 1 ml of eluate was discarded and the rest was collected in a series of fractions. After recrystallisation using chloroform and evaporation under N_2, the derivative was collected using filtration under vacuum.

The derivatives were then identified by melting point, thin layer chromatography, infra-red and proton nuclear magnetic resonance spectroscopy.

3.3. High Performance Liquid Chromatographic Analysis of the Bolton-Hunter Derivatives

Derivatised samples (10 µl) in a methanol-water solution were injected directly into the chromatographic system. The separation was carried out on ODS-Hypersil (5 µm) reversed-phase column (10 cm x 5 mm). The eluent of methanol-pH 8 phosphate buffer 50:50 was pumped isocratically at 1.0 ml/min. The peaks were detected electrochemically at an applied potential of +0.9 V vs Ag/AgCl and UV detection at 254 nm.

3.4. Derivatisation of Amines with Bolton-Hunter Reagent in Dilute Aqueous Solution

Solutions of the amines ranging from 10^{-6} M to 10^{-4} M in 0.1 M $NaHCO_3$, pH 8.5 (10 ml) were mixed with the Bolton-Hunter reagent (5 mg). The mixture was allowed to react for 3 hours at room temperature. Then, the solution (10 µl) was injected directly into the HPLC system.

4. DERIVATISATION OF AMINES USING METHYL p-HYDROXYBENZIMIDATE HYDROCHLORIDE

The derivatisation method is based on the procedure of Wood et al. [76]. 2×10^{-4} M amine was prepared in 0.005 M borate buffer at pH 9.2. 4×10^{-2} M Methyl p-hydroxybenzimidate.HCl was also prepared in the same buffer. The reaction was started by adding an amount of methyl p-hydroxybenzimidate.HCl solution (1000 µl) to the amine solution (100 µl) to give a final molar ratio 20:1. After 3 hours at 50°C, the solution (10 µl) was injected onto the reversed-phase HPLC ODS-Hypersil (5 µm) column, (10 cm x 5 mm). The eluent consisting of 70:30 methanol-phosphate buffer (pH 8) containing 0.05 M 1-hexanesulphonic acid was pumped at 1.0 ml/min. The derivatives were detected at the applied potential of +0.9 V vs Ag/AgCl and UV detection at 254 nm.

5. ANALYSIS OF DANSYL DERIVATIVES BY LCEC

10^{-3} M Solutions of commercially prepared dansyl derivatives were dissolved in a methanol-phosphate buffer (pH 8) solution (10 ml). The solution (10 µl) was injected onto the reversed-phase ODS-Hypersil (5 µm) column (10 cm x 5 mm). The eluent, consisting of 50:50 methanol: phosphate buffer (pH 8) was pumped at 1.0 ml/min. The peaks were detected at the applied potential of 0.9 V vs Ag/AgCl and UV detection at 254 nm.

CHAPTER III

RESULTS

3.1. Preparation of Standard Amine Derivatives using O-Acetylsalicyloyl Chloride

Samples of the standard amine derivatives had been prepared by previous student but were not fully characterised [77]. The preparation of derivatives used a Schotten-Baumann reaction conditions.

a. 2-Hydroxybenzamide of Piperidine

The piperidine derivative was pale yellow crystals in 70% yield; m.p. 126°C - 129°C (Lit. [78] 142°C); IR spectrum (in nujol on KBr discs): 3350 cm^{-1} (H-bonded -OH group), 1600 - 1630 cm^{-1} (C = O of amide), and 1580 cm^{-1} (C = C of aromatic ring); ^{1}H-NMR ($CDCl_3$), δ_H = 1.7 (s, 6H for $-\overline{N-CH_2-(\underline{CH_2})_3-CH_2}$), δ_H = 3.6 (s, 4H, for $-\overline{N-\underline{CH_2}-(CH_2)_3-\underline{CH_2}}$) δ_H = 6.7 - 7.3 (m, 4H, for aromatic ring), and δ_H = 9.3 (s, 1H, for phenolic proton). The thin layer chromatography on silica gel in chloroform showed a single spot at R_f = 0.39.

b. 2-Hydroxybenzamide of N-Methylaniline

The N-methylaniline derivative was obtained as pale buff-coloured crystals in 73% yield; m.p. 108°C - 109°C; IR spectrum (in nujol on KBr discs): 3350 cm^{-1} (H-bonded -OH group),

1600 - 1630 cm^{-1} (C = 0 of amide), 1580 cm^{-1} (C = C of aromatic ring), and 2880 cm^{-1} (N-CH_3 group); ^{1}H-NMR ($CDCl_3$); δ_H = 3.5 (s, 3H, for -CH_3 on nitrogen), δ_H = 6.3 - 7.6 (m, 9H, for aromatic proton), and δ_H = 9.7 (s, 1H, for phenolic proton). The thin layer chromatography on silica gel in chloroform showed a single spot at R_f = 0.56.

c. 2-Hydroxybenzamide of Benzylamine

The benzylamine derivative was obtained as colourless crystals in 80% yield; m.p. 135°C - 138°C (Lit. [79] 134°C); IR spectrum (in nujol on KBr discs): 3350 cm^{-1} (H-bonded -OH group), 1600 - 1650 cm^{-1} (C = 0 of amide), 1580 cm^{-1} (C = C of aromatic ring); ^{1}H-NMR ($CDCl_3$): δ_H = 4.5 (d, 2H, for -CH_2), $_H$ = 6.6 - 7.7 (m, 9H, for aromatic proton), and δ_H = 8.3 (s, 1 H, for phenolic proton). The thin layer chromatography on silica gel in chloroform showed a single spot at R_f = 6.9.

d. 2-Hydroxybenzamide of Pyrrolidine

The pyrrolidine derivative was pale yellow crystals in 63% yield; m.p. 124°C - 137°C; IR spectrum (in nujol on KBr discs): 3350 cm^{-1} (H-bonded -OH group), 1600 - 1630 cm^{-1} (C = 0 of amide), and 1590 cm^{-1} (C = C for aromatic ring); ^{1}H-NMR ($CDCl_3$), δ_H = 1.8 (s, 4H, for $-\overline{N-CH_2-(CH_2)_2-CH_2}$), δ_H = 3.6 (s, 4H, for $-\overline{N-CH_2-(CH_2)_2-CH_2}$), δ_H = 6.5 - 7.3 (m, 4H, for aromatic ring) and δ_H = 9.3 (s, 1H, for phenolic proton). The thin layer

chromatography on silica gel in chloroform showed a single spot at R_f = 0.30.

3.2. Separation of Amine Derivatives

Piperidine, N-methylaniline, pyrrolidine, and benzylamine derivatives were separated by using HPLC with electrochemical detection and an eluent of 50:50 Methanol-phosphate buffer pH 8.

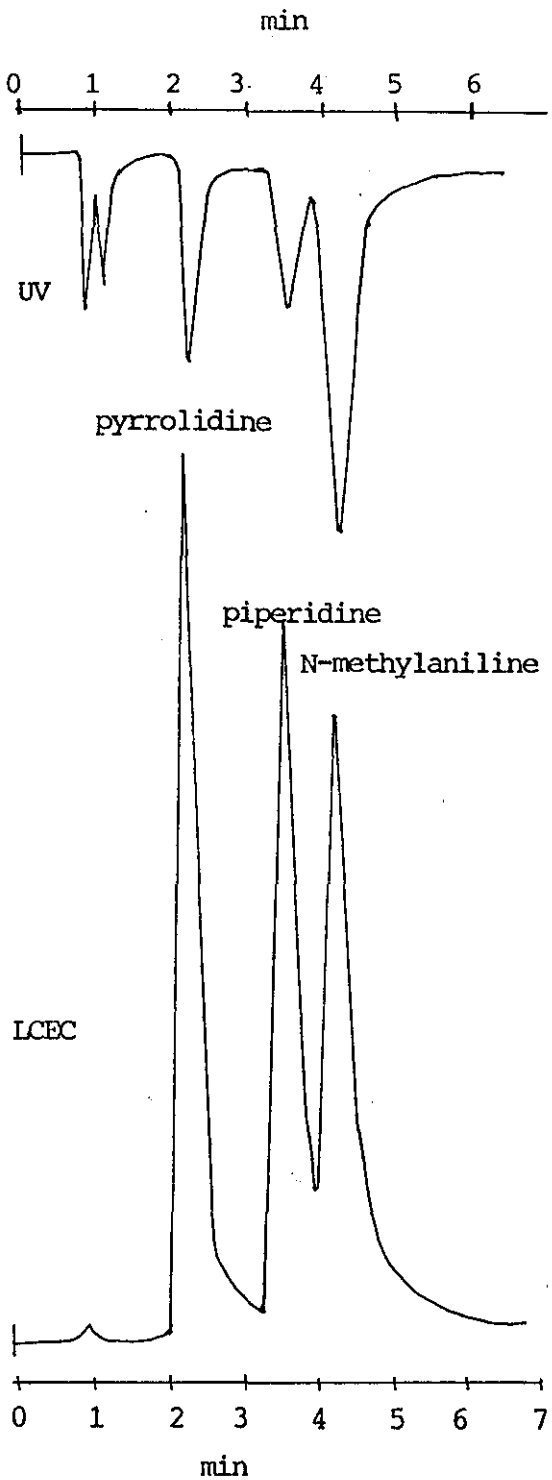
Fig. 20 - Chromatogram of 2-Hydroxybenzamide of Amines using HPLC with Electrochemical Detection

Table 2 - Retention Times of the 2-Hydroxybenzamide of Amines

$k' = (t_R - t_o) / t_o$ where t_R = retention time for the 2-hydroxybenzamide derivative and t_o = retention time for solvent.

Derivative	t_R (min)	t_o (min)	k'
pyrrolidine	2.5	1.0	1.5
piperidine	3.7	1.0	2.7
N-methylaniline	4.5	1.0	3.5
benzylamine	12.5	1.0	11.5

Fig. 20. Chromatograms of 1 x 10^{-3} M 2-Hydroxybenzamide of Amines in MeOH-H_2O



HPLC conditions:
Column: ODS-Hypersil
(10 cm x 5 mm)
M/phase: 50:50 MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
Detection:
EC: +0.9 V vs Ag/AgCl
20 µA range
UV: 254 nm, 0.1 AUFS

3.3. Effect of Applied Potential vs Peak Height for 2-Hydroxybenzamide of Amines

To determine a suitable applied potential for electrochemical detection, the 2-hydroxybenzamide of piperidine was examined at different potentials and the detector response was measured (Fig. 21).

Fig. 21 - Relationship between Applied Potential and Peak Response of 10^{-3} M 2-Hydroxybenzamide of Piperidine

3.4. Calibration Curves for 2-Hydroxybenzamide of Amines

Calibration curves were measured for the pure derivatives using *HPLC with electrochemical detection*

Fig. 22 - Calibration Curve for the pure 2-Hydroxybenzamide of Piperidine in Methanol

Fig. 23 - Calibration Curve for the pure 2-Hydroxybenzamide of N-Methylaniline in Methanol

Fig. 24 - Calibration Curve for the pure 2-Hydroxybenzamide of Benzylamine in Methanol

Fig. 21 - Relationship between Applied Potential and Peak Response of 1 x 10^{-3} M 2-Hydroxybenzamide of Piperidine

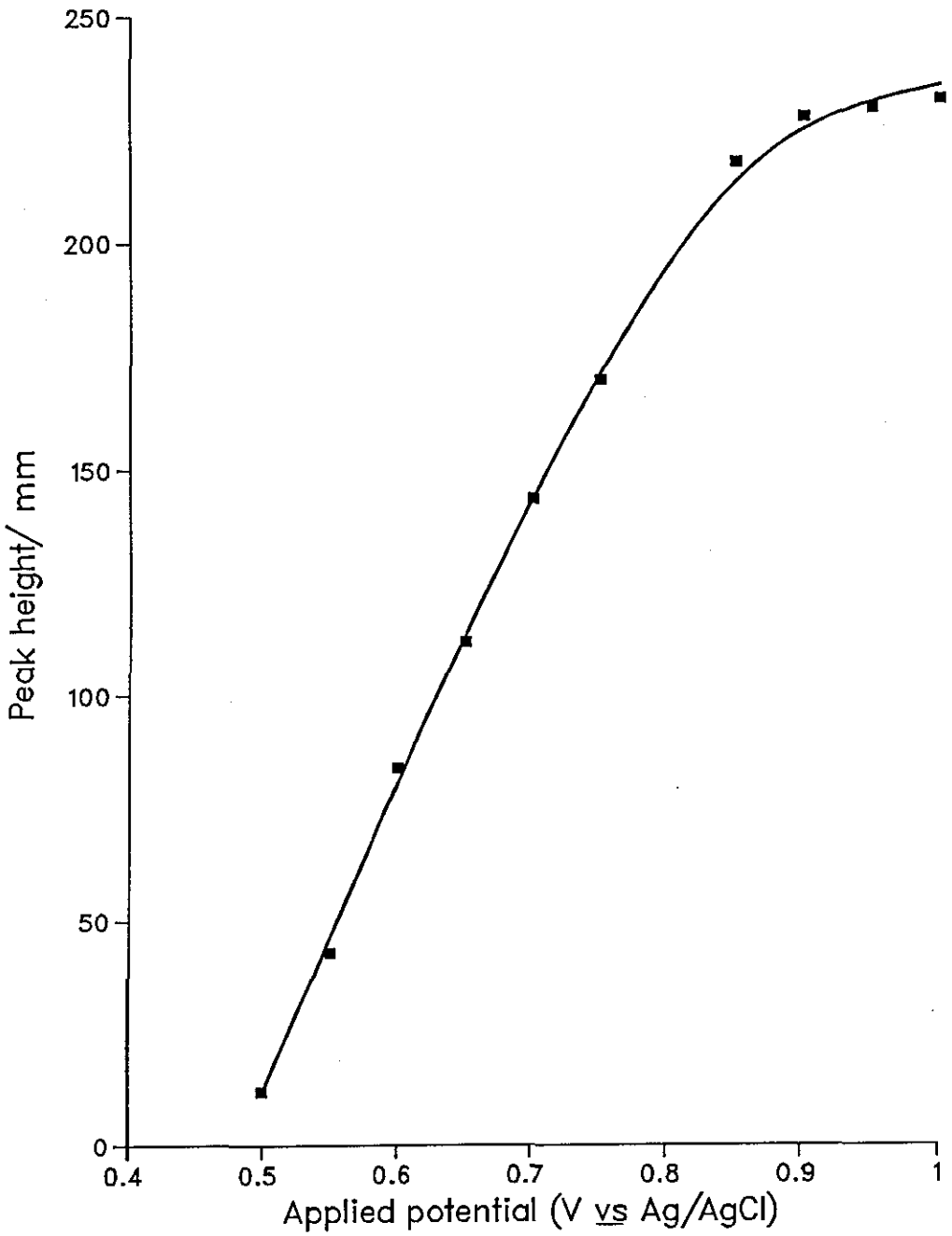


Fig. 22 - Calibration Curve for 10^{-7} M Pure 2-Hydroxybenzamide of Piperidine in Methanol with Electrochemical Detection at 0.05 μA

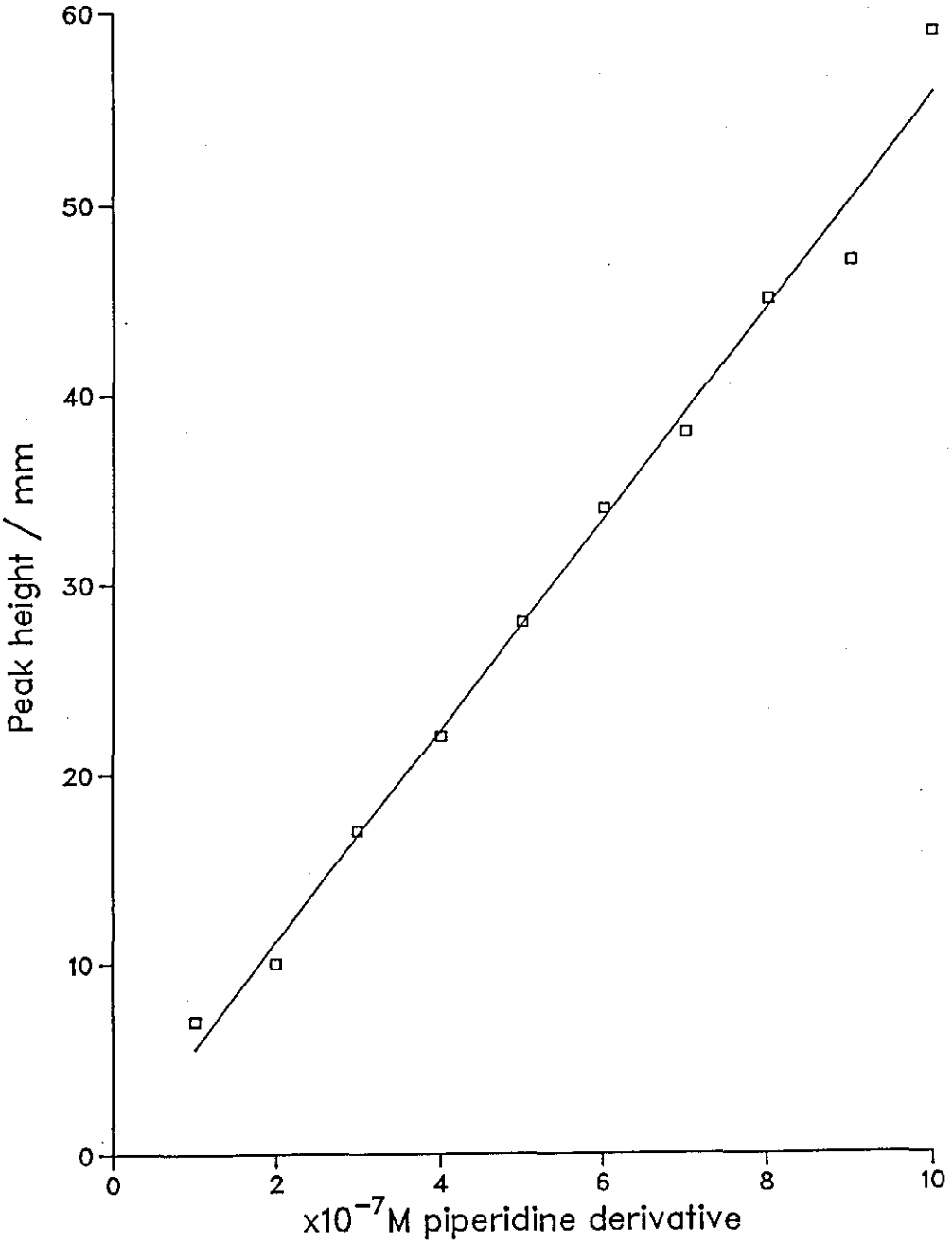


Fig. 23 - Calibration Curve for 10^{-6} M Pure 2-Hydroxybenzamide of N-Methylaniline in Methanol with Electrochemical Detection at 0.1 μA

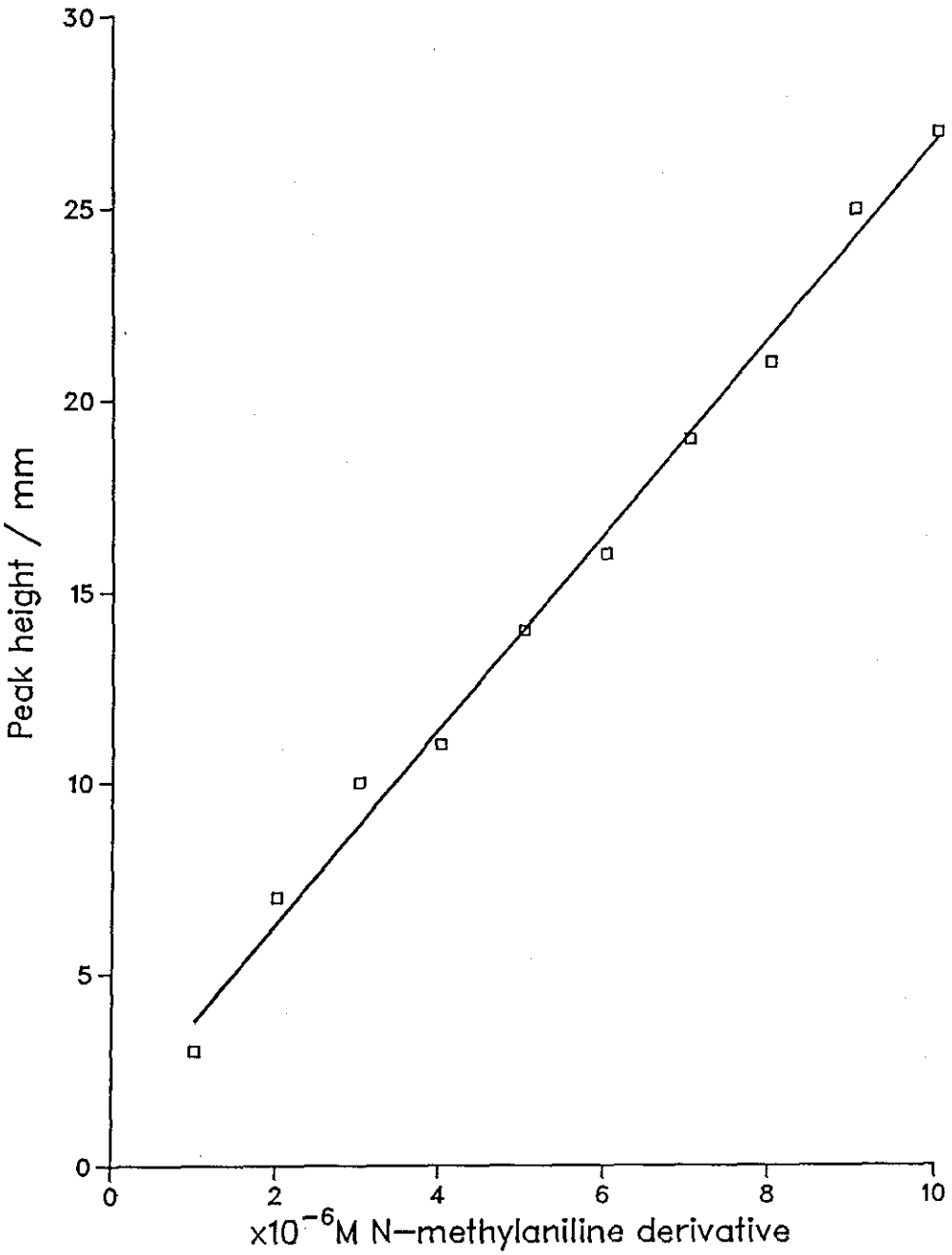
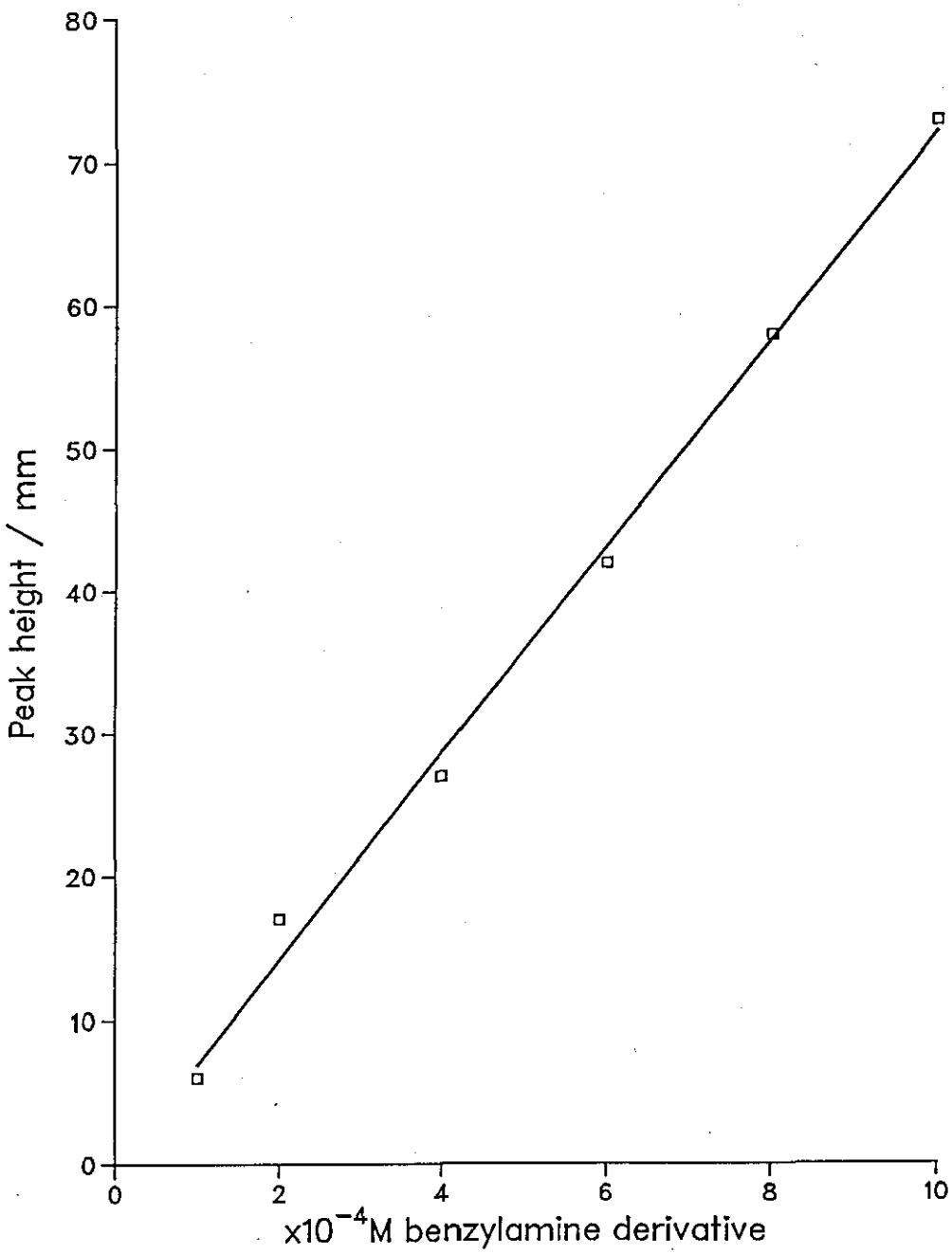


Fig. 24 - Calibration Curve for 10^{-4} M Pure 2-Hydroxybenzamide of Benzylamine in Methanol with Electrochemical Detection at 20 μA



3.5. 2-Hydroxybenzamide of Piperidine using O-Acetylsalicyloyl Chloride

A series of experiments were carried out to measure the yield of the derivative prepared from a dilute solution of piperidine in 0.1 M NaOH (10 ml) with O-acetylsalicyloyl chloride (10 ng - 200 ng) to determine the quantity of reagent required to give satisfaction reaction.

Fig. 25 - Percentage Yield of Derivatisation of 1×10^{-4} M Piperidine in 0.1 M NaOH with increasing Amounts of O-Acetylsalicyloyl Chloride

Fig. 26 - Percentage Yield of Derivatisation of 1×10^{-5} M Piperidine in 0.1 M NaOH with increasing Amounts of O-Acetylsalicyloyl Chloride

Fig. 27 - Calibration Curve for Derivatisation of 10^{-5} M Piperidine in 0.1 M NaOH

Fig. 28 - Calibration Curve for Derivatisation of 10^{-6} M Piperidine in 0.1 M NaOH

Fig. 29 - Chromatograms of 1×10^{-4} M 2-Hydroxybenzamide of Piperidine from Reaction and O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH

Fig. 30 - Chromatogram of 6×10^{-7} M 2-Hydroxybenzamide of Piperidine from Reaction with O-Acetylsalicyloyl Chloride (200 mg) in 0.1 M NaOH

Fig. 25 - Percentage Yield of Derivatisation of 1 x 10^{-4} M Piperidine in 0.1 M NaOH with increasing Amounts of O-Acetylsalicyloyl Chloride

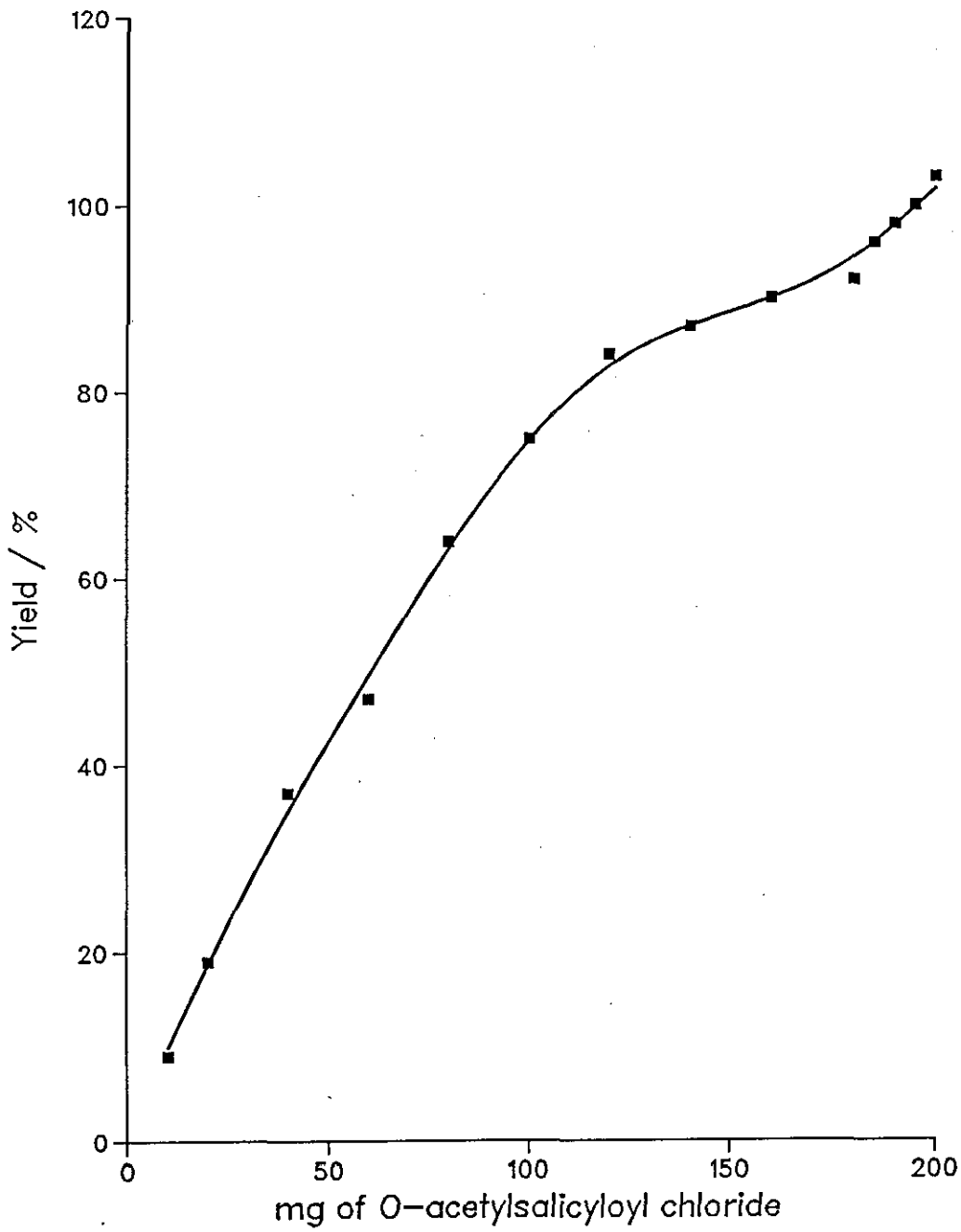


Fig. 26 - Percentage Yield of Derivatisation of 1 x 10^{-5} M Piperidine in 0.1 M NaOH with increasing Amounts of O-Acetylsalicyloyl Chloride with Electrochemical Detection at 1.0 µA

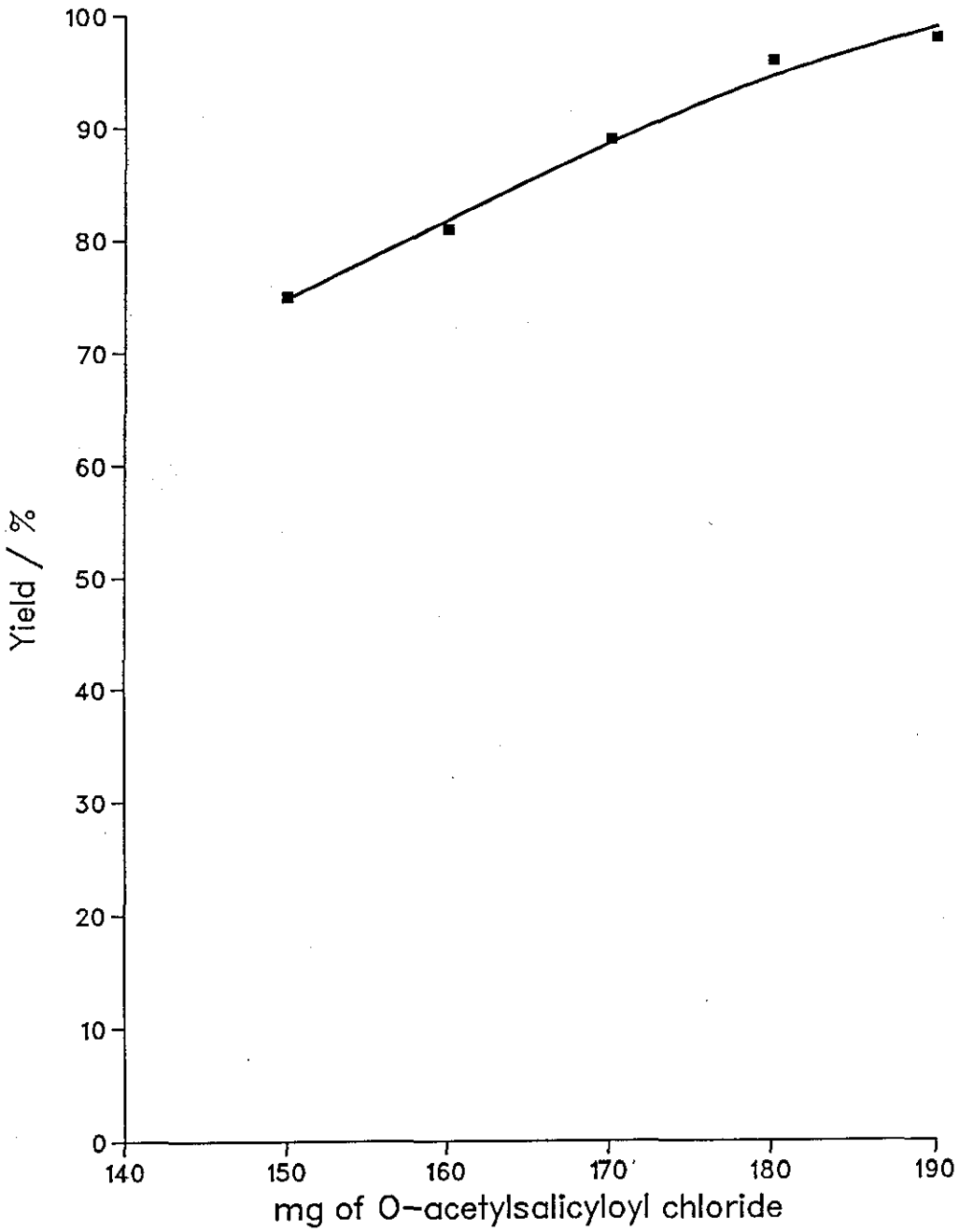


Fig. 27 - Calibration Curve for Derivatisation of 10^{-5} M Piperidine in 0.1 M NaOH with Electrochemical Detection at 1.0 μA

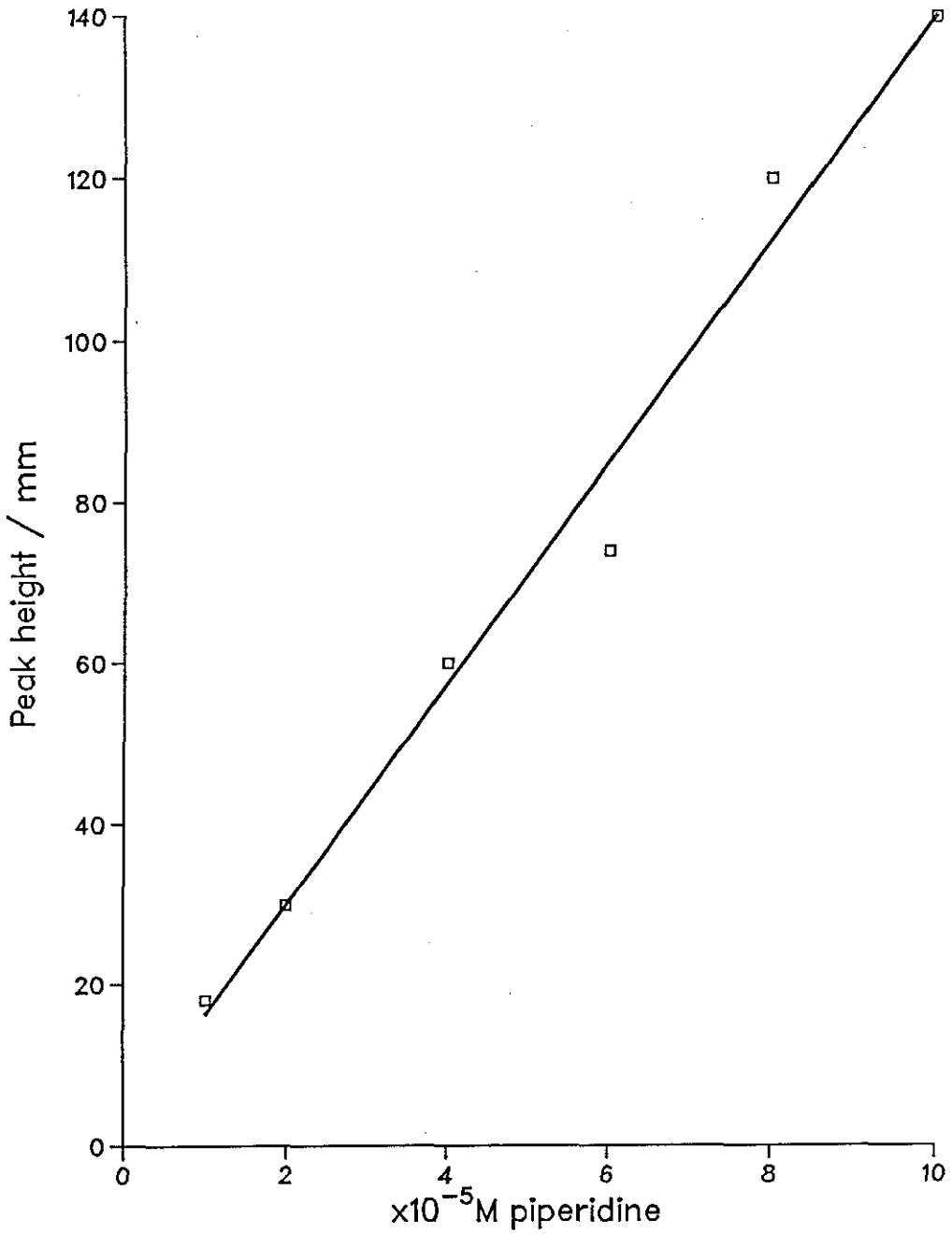


Fig. 28 - Calibration Curve for Derivatisation of 10^{-6} M Piperidine in 0.1 M NaOH with Electrochemical Detection at 0.5 μA

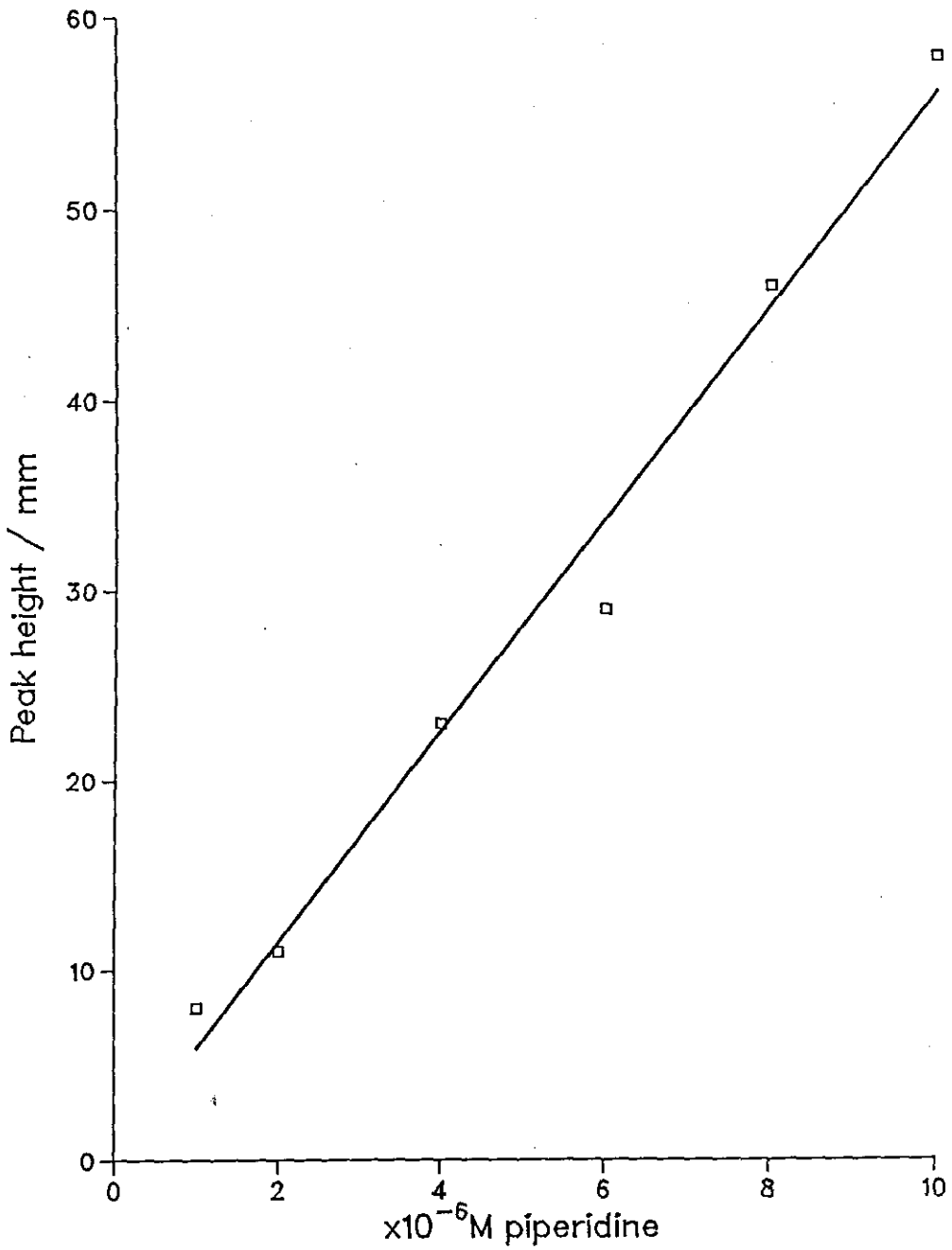
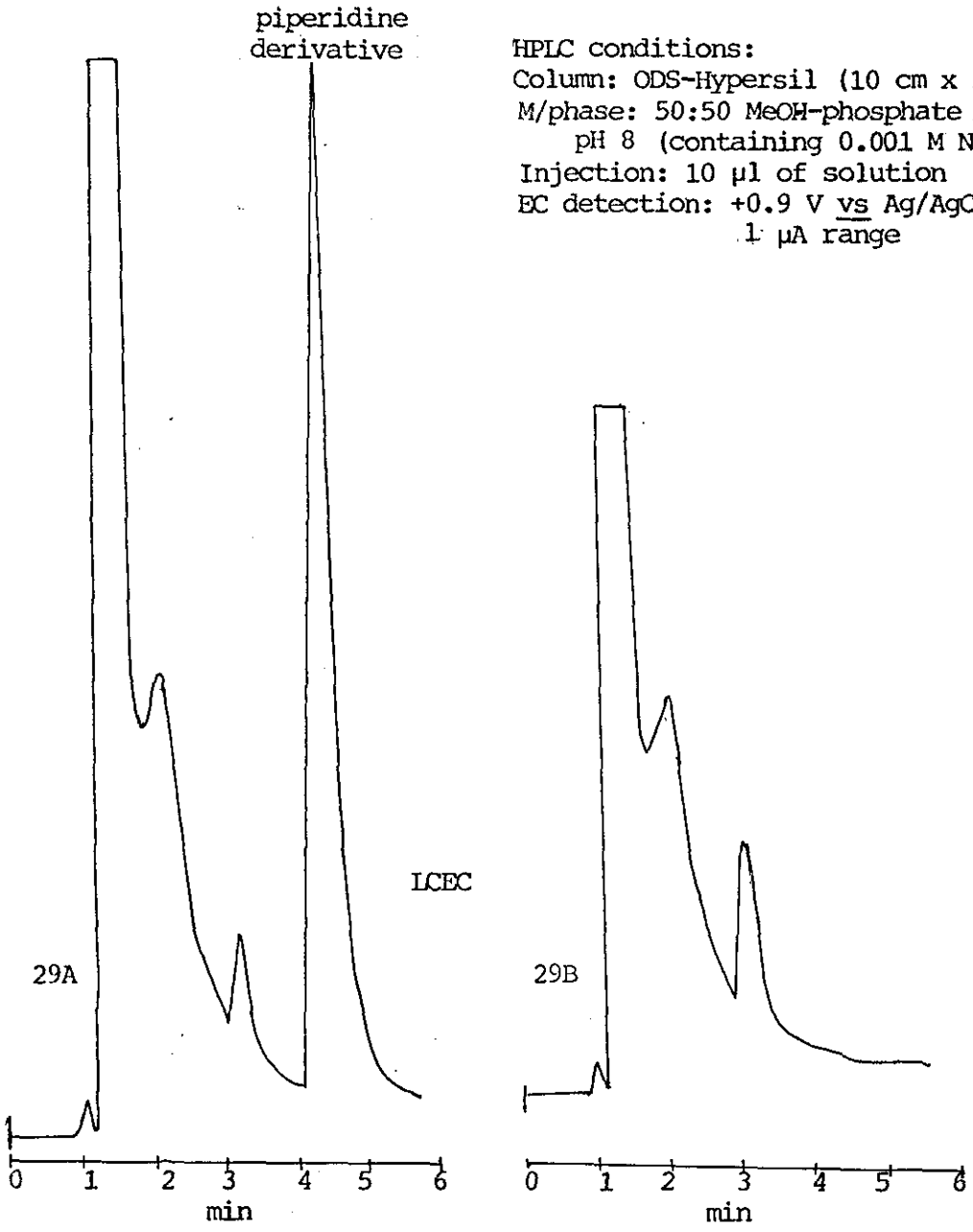
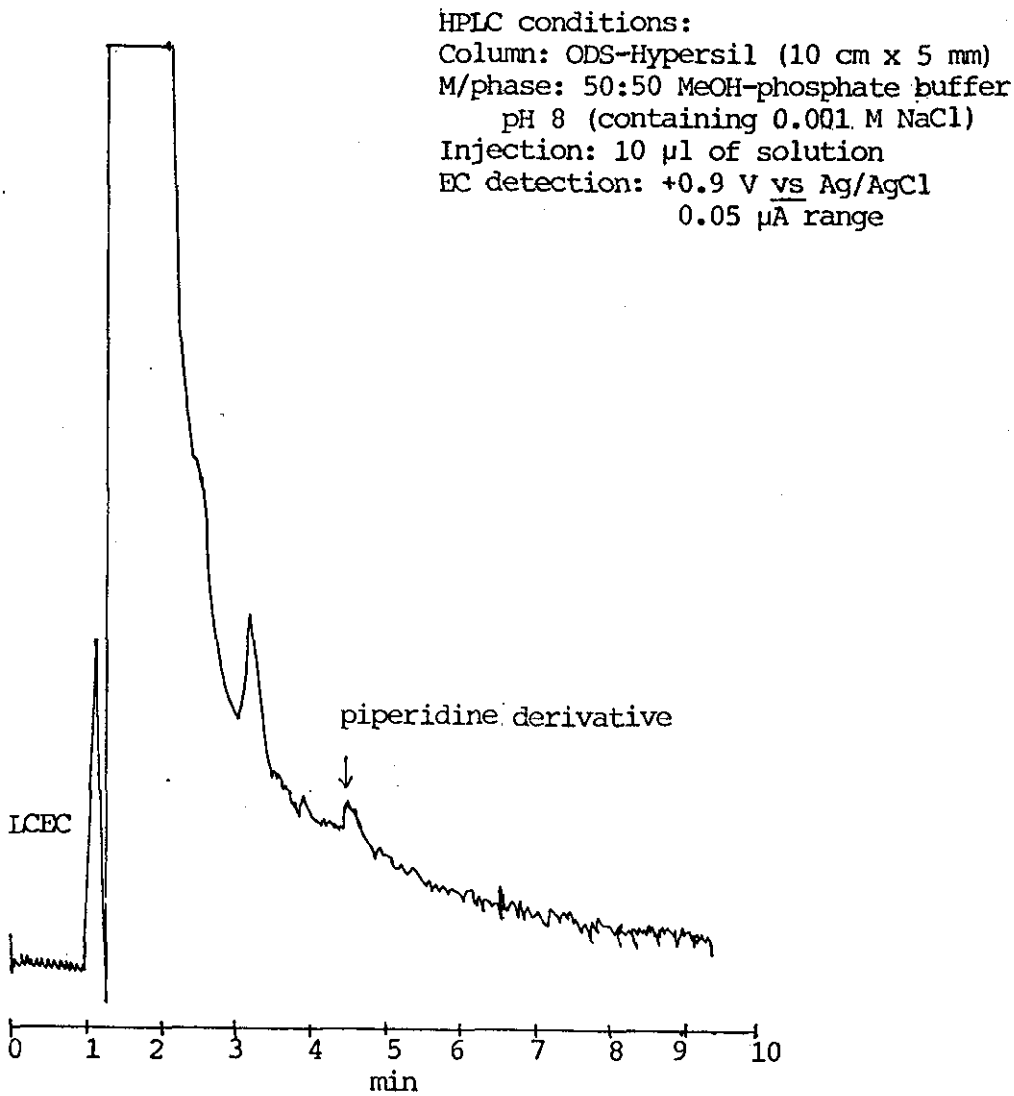


Fig. 29. Chromatograms for 1 x 10^{-4} M 2-Hydroxybenzamide of Piperidine from Reaction and O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH



29A - Derivative from 1 x 10^{-4} M Piperidine in 0.1 M NaOH
29B - O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH

Fig. 30. Chromatogram of 6 x 10^{-7} M 2-Hydroxybenzamide of Piperidine from Reaction with O-Acetylsalicyloyl Chloride (200 mg) in 0.1 M NaOH



Derivative from 6 x 10^{-7} M Piperidine in 0.1 M NaOH

3.6. N-Methylaniline and Benzylamine Derivatives in 0.1 M NaOH

Both amines also examined for the potential of derivatisation in dilute aqueous solution with O-acetylsalicyloyl chloride (200 mg).

Fig. 31 - Calibration Curve for Derivatisation of 10^{-4} M N-Methylaniline in 0.1 M NaOH

Fig. 32 - Chromatograms of 1 x 10^{-4} M 2-Hydroxybenzamide of N-Methylaniline from Reaction

Fig. 33 - Chromatograms for Derivatisation of N-Methylaniline using the Valdez and Reier Method

Fig. 34 - Calibration Curve for Derivatisation of 10^{-4} M Benzylamine in 0.1 M NaOH

Fig. 35 - Chromatograms of 1 x 10^{-4} M 2-Hydroxybenzamide of benzylamine from Reaction and O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH

3.7. Studies of Reaction Yield using O-Acetylsalicyloyl Chloride (200 mg)

Table 3. - Percentage reaction yield of derivatisation

Amine Derivative	% Yield		
	10^{-4} M	10^{-5} M	10^{-6} M
piperidine	100	100	100
N-methylaniline	42.5	54.6	-
benzylamine	99.2	-	-

Fig. 31 - Calibration Curve for Derivatisation of 10^{-4} M N-Methylaniline in 0.1 M NaOH with Electrochemical Detection at 10 μA

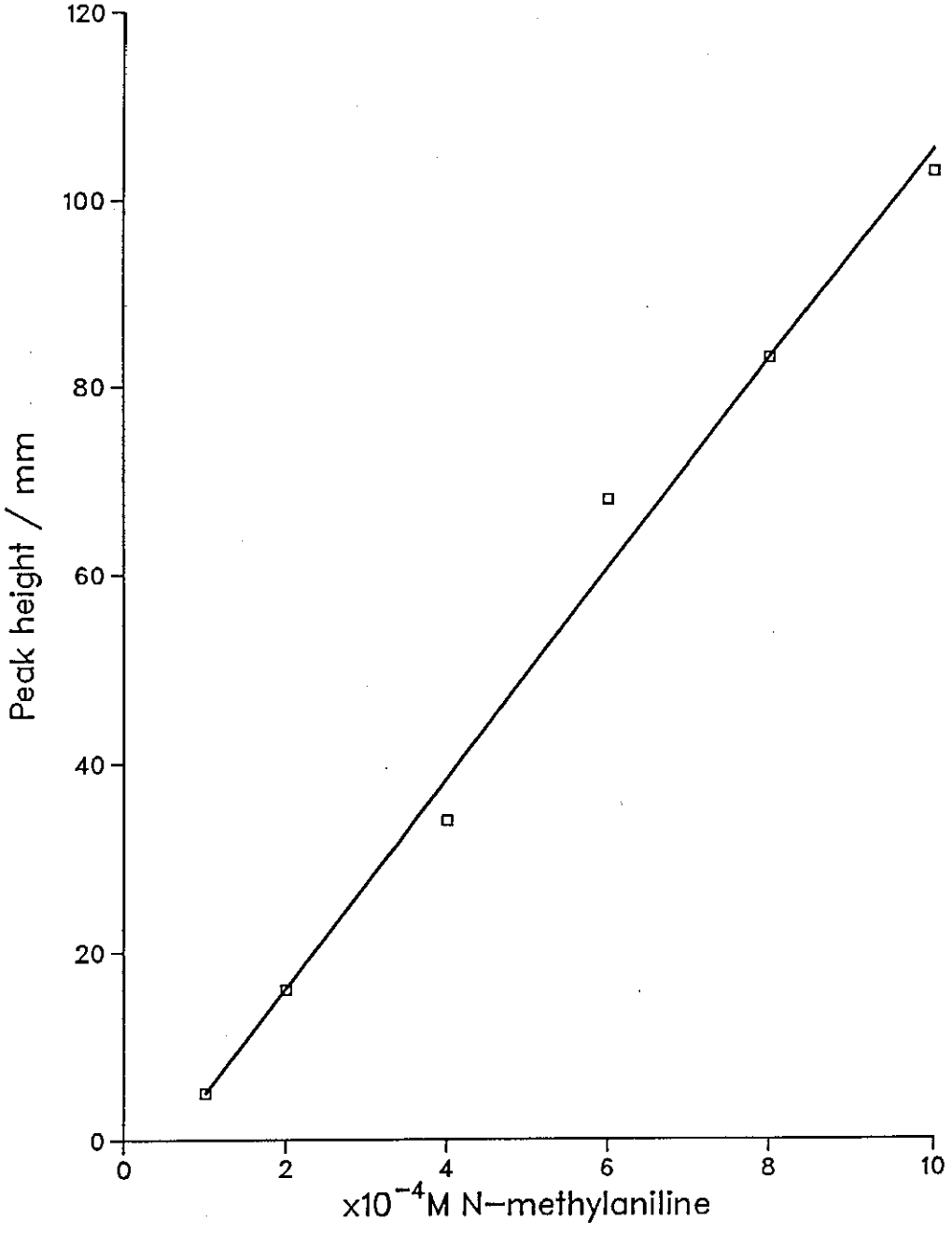
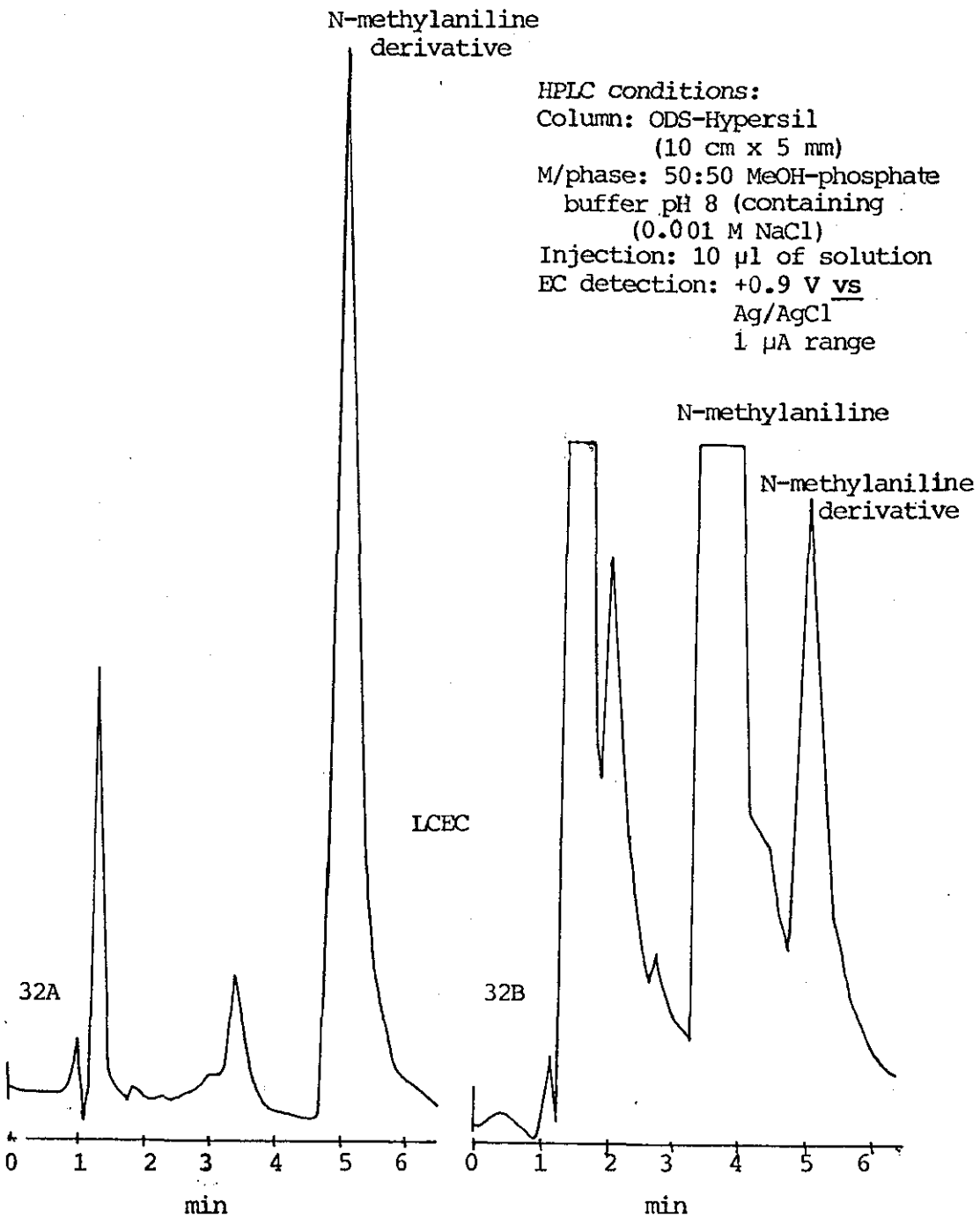
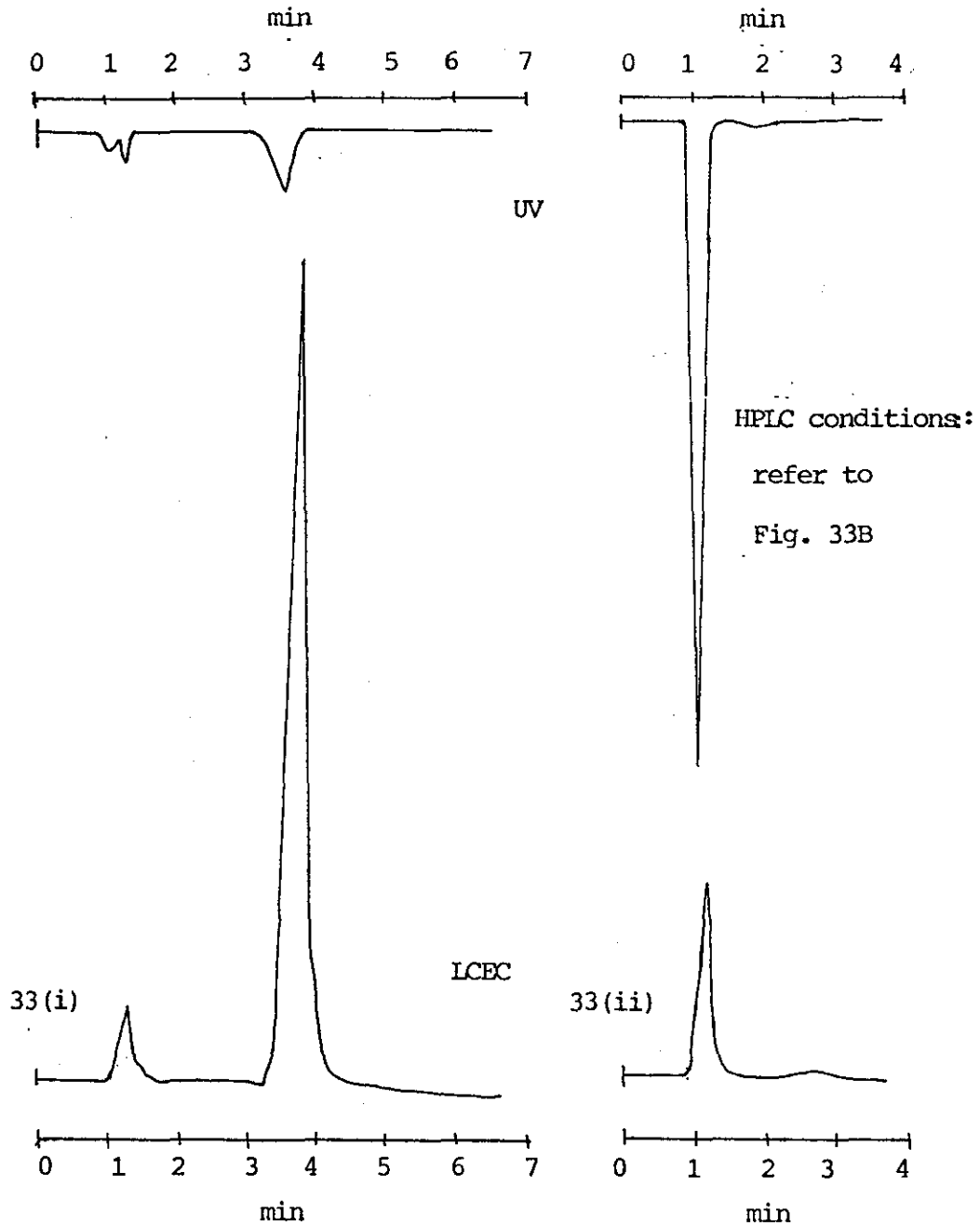


Fig. 32. Chromatograms of 1 x 10^{-4} M 2-Hydroxybenzamide of N-Methylaniline from Reaction



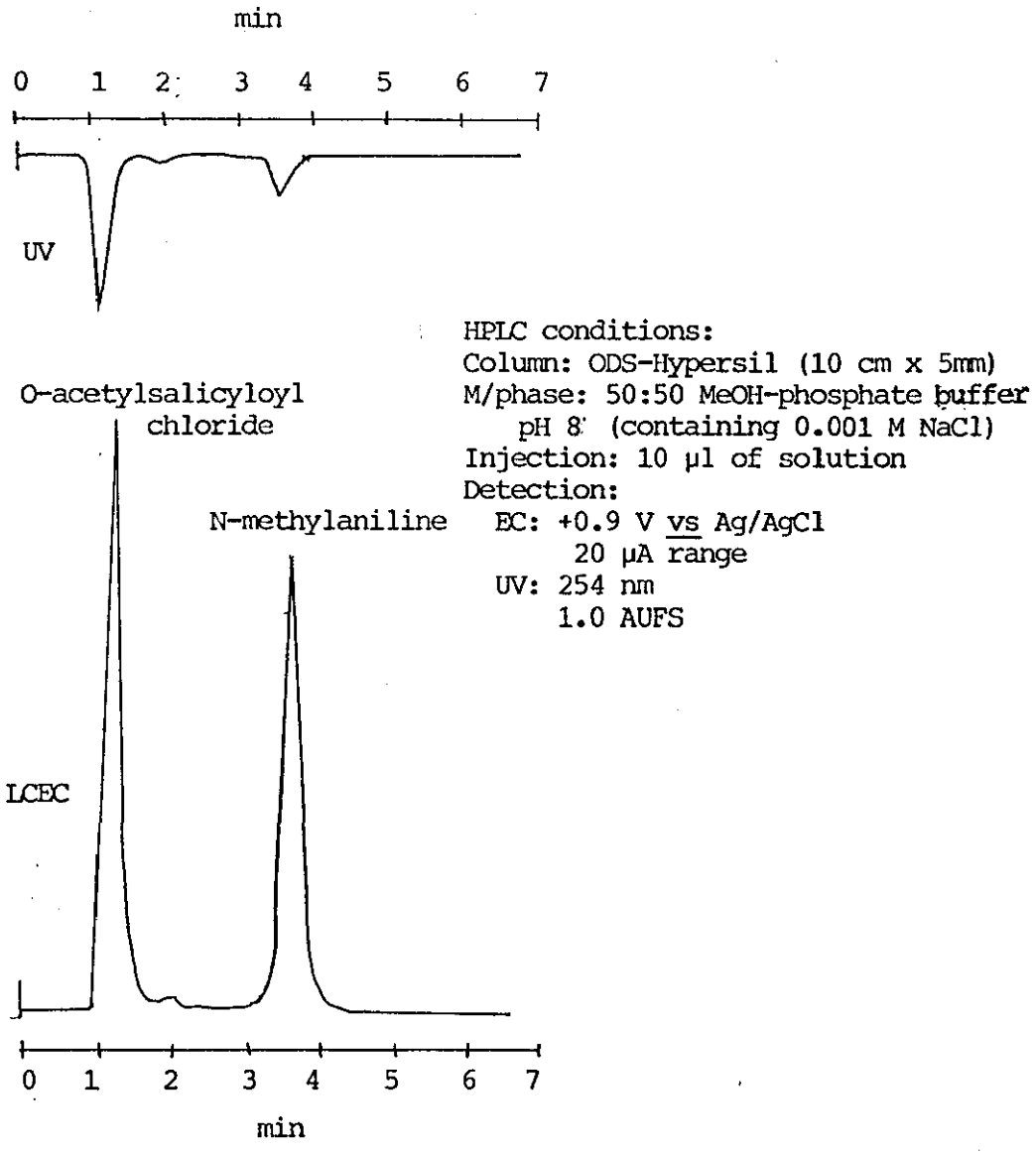
32A - 1 x 10^{-4} M Pure N-Methylaniline Derivative in 0.1 M NaOH
32B - 1 x 10^{-4} M N-Methylaniline after Reaction using 200 mg of O-Acetylsalicyloyl Chloride prepared in 0.1 M NaOH

Fig. 33A. Chromatograms for Derivatisation of N-Methylaniline using the Valdez and Reier Method



33(i) - 1000 µl O-Acetylsalicyloyl Chloride in CH_3CN
33(ii) - 1000 ppm N-Methylaniline in CH_3CN

Fig. 33B. Chromatograms for Derivatisation of N-Methylaniline using the Valdez and Reier Method



33(iii) - 1000 ppm N-Methylaniline + 1000 µl O-Acetylsalicyloyl Chloride in CH_3CN

Fig. 34 - Calibration Curve for Derivatisation of 10^{-4} M Benzylamine in 0.1 M NaOH with Electrochemical Detection at 10 μA

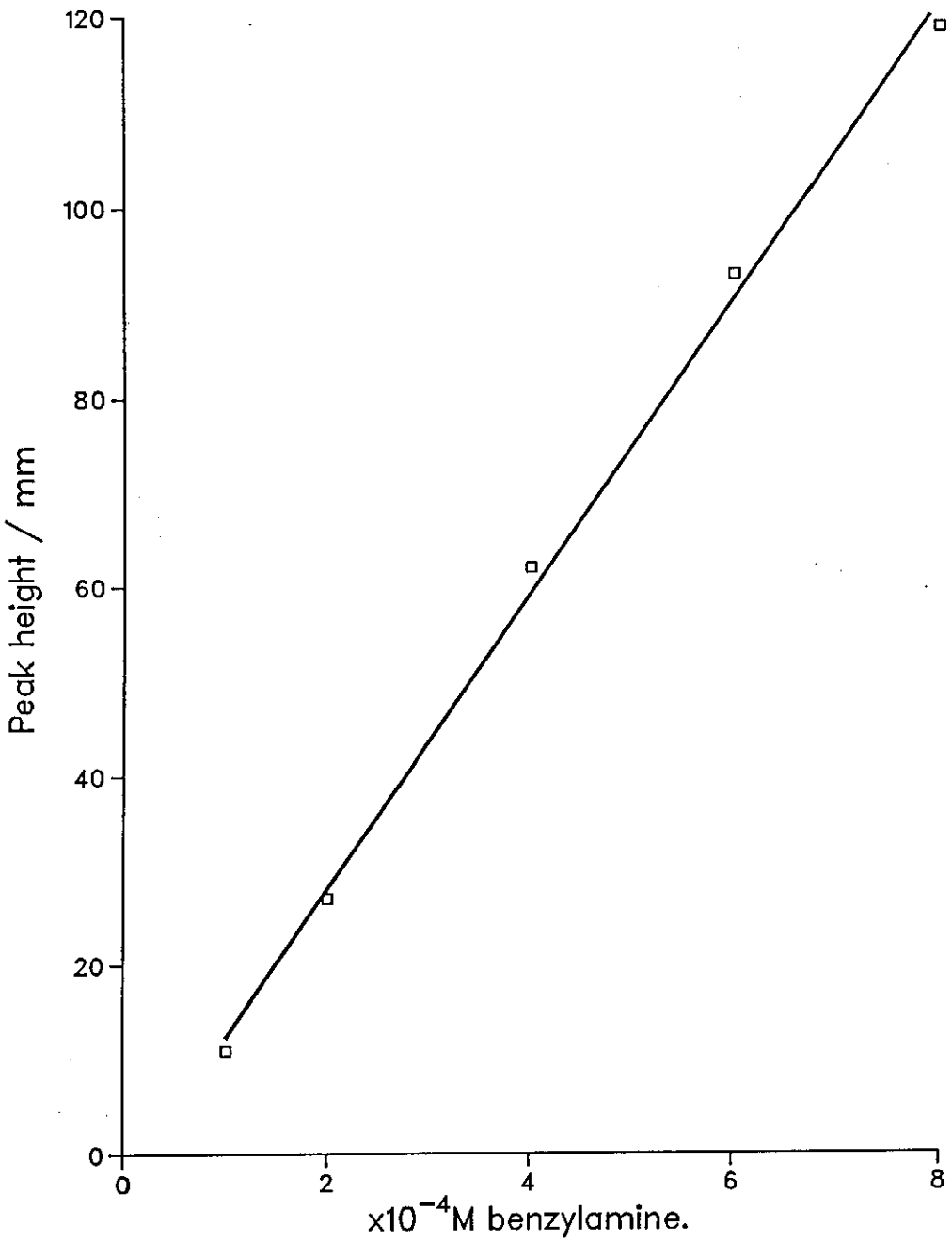
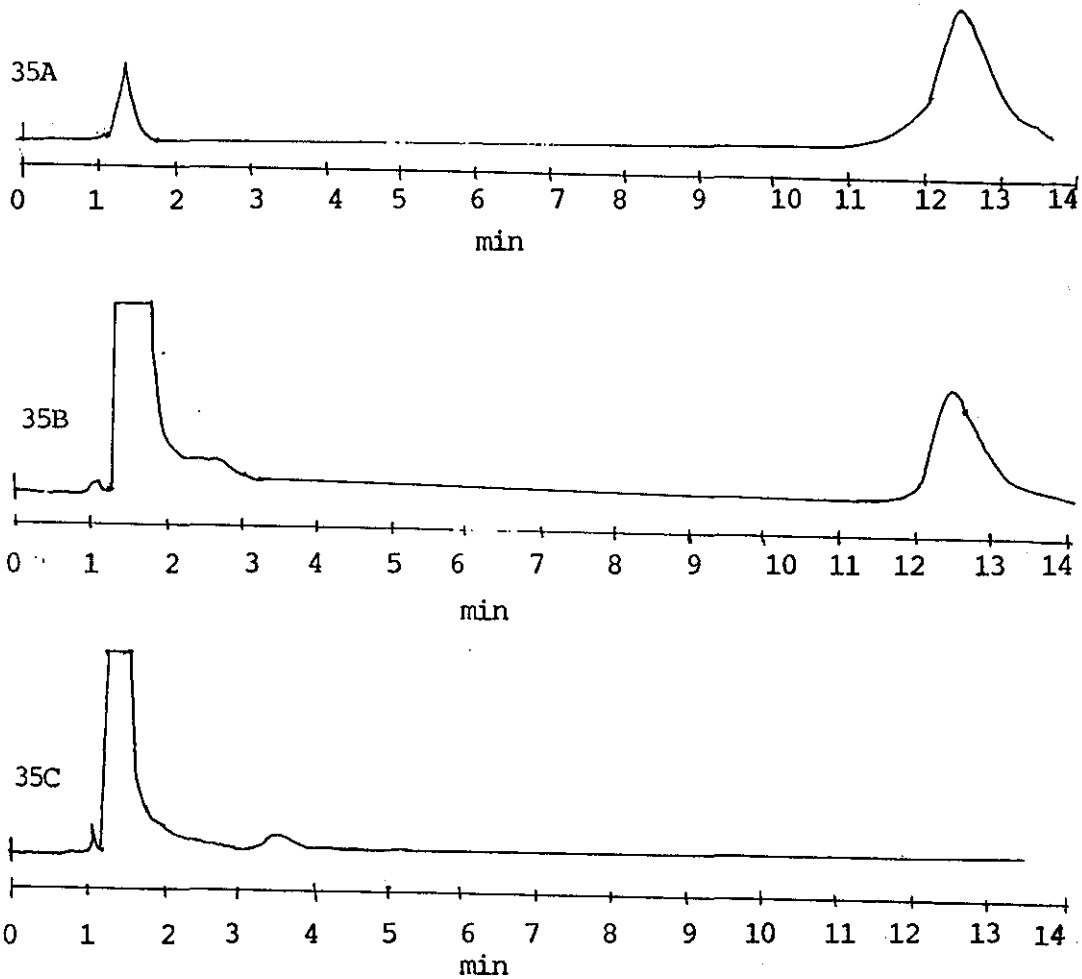


Fig. 35. Chromatograms of 1 x 10^{-4} M 2-Hydroxybenzamide of Benzylamine from Reaction and O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH

HPLC conditions:
Column: ODS-Hypersil (10 cm x 5 mm)
M/phase: 50:50 MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
EC detection: +0.9 V vs Ag/AgCl
10 µA range

35A - 1 x 10^{-4} M Pure Benzylamine Derivative in 0.1 M NaOH
35B - Derivative from 1 x 10^{-4} M Benzylamine in 0.1 M NaOH
35C - 1 x 10^{-4} M O-Acetylsalicyloyl Chloride Blank in 0.1 M NaOH



3.8. Precision Studies of Derivatisation of Amine with O-Acetylsalicyloyl Chloride

Data of N-methylaniline excluded because N-methylaniline gave a low yield.

Table 4. - Reproducibility of Amine Derivatives from Reaction in 0.1 M NaOH

Amine	Injection	Peak height, Mean, mm	CV
piperidine (1×10^{-5} M)	4	61.3	1.4
benzylamine (1×10^{-4} M)	4	10.5	5.5

Coefficient of variation = 100 x (SD/X)

3.9. Derivatisation of Amines using Bolton-Hunter Reagent

Three amines were derivatised using Bolton-Hunter Reagent. The derivatives obtained after extraction using chloroform.

a) 3-(4-Hydroxyphenyl)propionamide of Cyclohexylamine

The cyclohexylamine derivative was white crystals in 25% yield; m.p. 88°C - 90°C; IR spectrum (in nujol on KBr discs): 3320 cm^{-1} (H-bonded -OH) and gave three bands between 1560-1620 cm^{-1} (C = O and N-H of amide). ^{1}H-NMR ($CDCl_3$): δ_H = 6.7 - 7.3 (m, 4H, for aromatic ring), δ_H = 2.4 - 3.2((t, t, 4H, $-CH_2-CH_2-$), and δ_H = 0.9 - 2.0 (m, 11H, hexyl ring). The thin layer chromatography on silica gel in ethyl acetate gave a single spot at R_f = 0.84.

b) 3-(4-Hydroxyphenyl)propionamide of Benzylamine

The benzylamine derivative was white crystals in 30% yield; m.p. 74°C - 77°C; IR spectrum (in nujol on KBr discs): 3310 cm^{-1} (H-bonded -OH) and gave three bands between 1540 - 1640 cm^{-1} (C = O and N-H of amide). ^{1}H-NMR ($CDCl_3$/DMSO): δ_H = 6.4 - 7.1 (m, 9H, for aromatic rings), δ_H = 4.2 (d, 2H, $-\underline{CH_2}-C_6H_5$), and δ_H = 3.2 (s, 4H, $-CH_2-CH_2-$). The thin layer chromatography on silica gel in ethyl acetate gave a single spot at R_f = 0.81.

c) 3-(4-Hydroxyphenyl)propionamide of Piperidine

The piperidine derivative was brown gum in 25% yield. The melting point was not measured. IR spectrum (in nujol on KBr discs): 3200 cm^{-1} (H-bonded -OH) and 1600 cm^{-1} (C = O of amide); ^{1}H-NMR ($CDCl_3$): δ_H = 6.4 - 7.1 (m, 4H, for aromatic ring) and δ_H = 0.7 - 1.6 (m, 10H, $-\overline{N-CH_2(CH_2)_3CH_2}$). The thin layer chromatography on silica gel in ethyl acetate gave a single spot at R_f = 0.89.

3.9.1. The HPLC Study of Pure 3-(4-Hydroxyphenyl)propionamides of Amines

The pure amine derivatives were examined by LCEC using the same HPLC conditions as employed for the 2-hydroxybenzamides of amines.

Fig. 36 - Chromatograms of 3-(4-Hydroxyphenyl)propionamide of Cyclohexylamine in MeOH-H_2O

Fig. 37 - Chromatogram of 3-(4-Hydroxyphenyl)propionamide of Benzylamine in MeOH-H_2O

Fig. 38 - Chromatogram of 3-(4-Hydroxyphenyl)propionamide of Piperidine in MeOH-H_2O

Table 5 - Retention Time for the 3-(4-Hydroxyphenyl)-propionamides of Amines

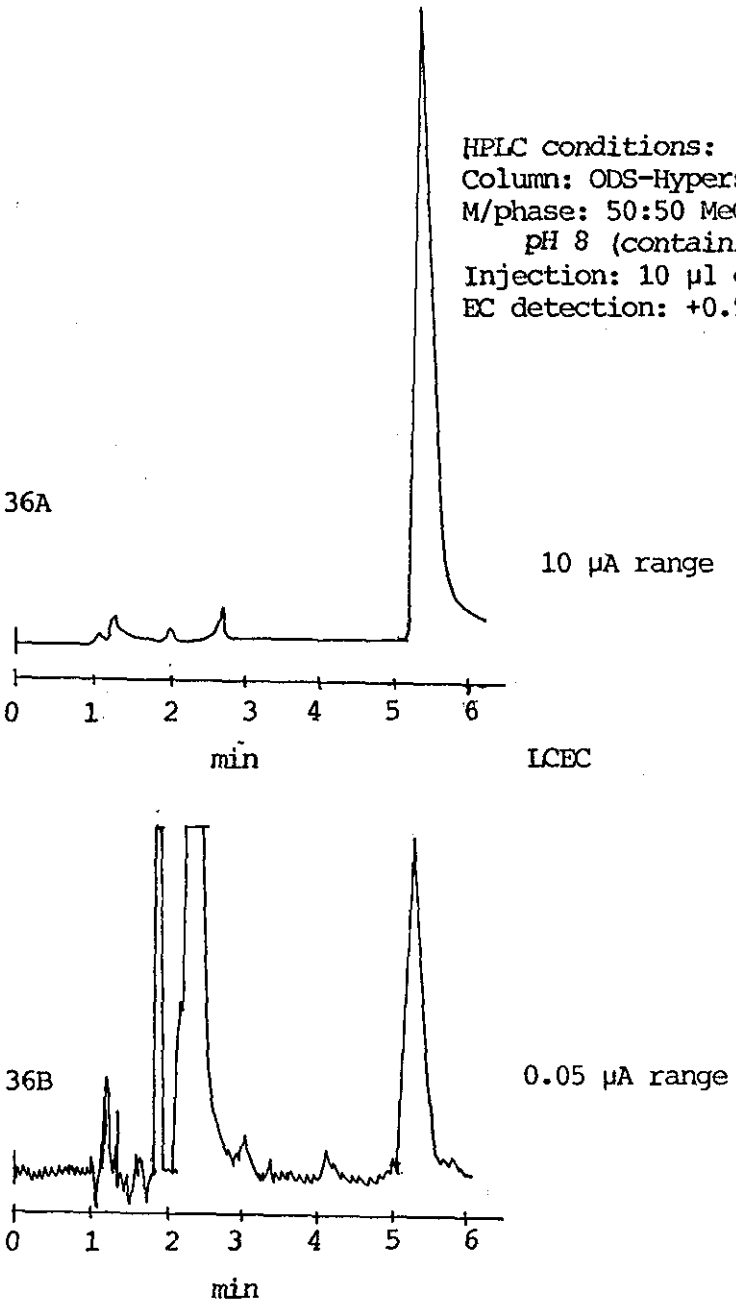
$k' = (t_R - t_o) / t_o$ where t_R = retention time for the amine derivative and t_o = retention time for solvent

Amine derivative	Capacity factor, k'
cyclohexylamine	4.3
benzylamine	2.3
piperidine	5.7

The applied potential of 3-(4-Hydroxyphenyl)propionamide of amine was also carried out and the detector response was measured.

Fig. 39 - Applied Potential vs Peak Height for 1 x 10^{-3} M 3-(4-Hydroxyphenyl)propionamide of cyclohexylamine

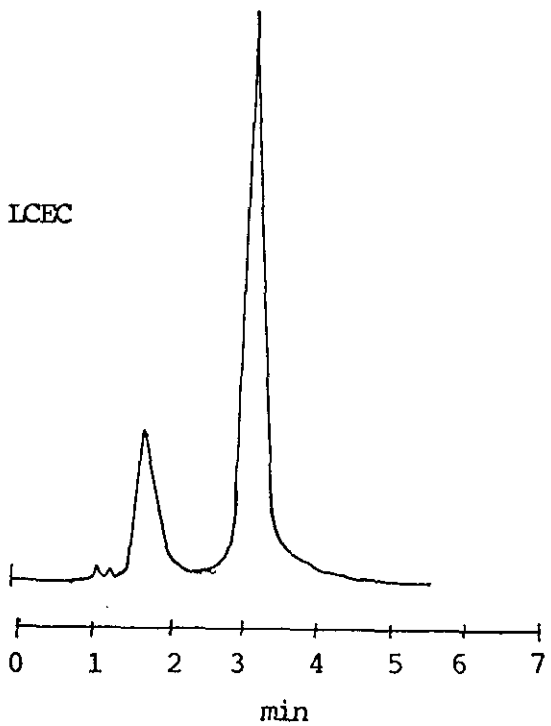
Fig. 36. Chromatograms of 3-(4-Hydroxyphenyl)propionamide of Cyclohexylamine in MeOH-H_2O



36A - 1 x 10^{-3} M Cyclohexylamine Derivative
36B - 1 x 10^{-7} M Cyclohexylamine Derivative

Fig. 37. Chromatogram of 1 x 10^{-3} M 3-(4-Hydroxyphenyl)-propionamide of Benzylamine in MeOH-H_2O

HPLC conditions:
Column: ODS-Hypersil (10 cm x 5 mm)
M/phase: 50:50 MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
EC detection: +0.9 V vs Ag/AgCl
20 µA range



1 x 10^{-3} M Pure Benzylamine Derivative in MeOH-H_2O

Fig. 38. Chromatogram of 1×10^{-4} M 3-(4-Hydroxyphenyl)-propionamide of Piperidine in MeOH-H_2O

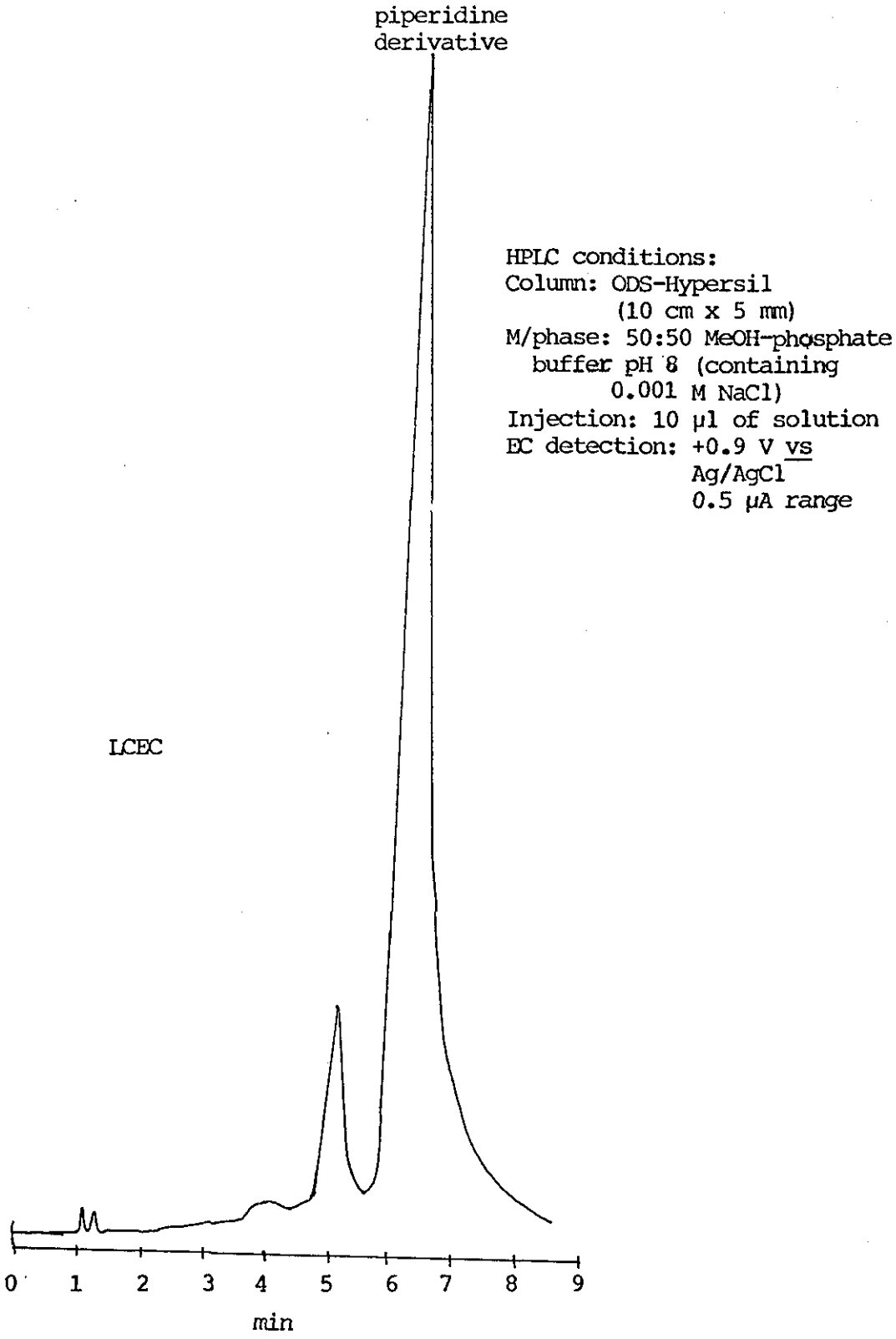
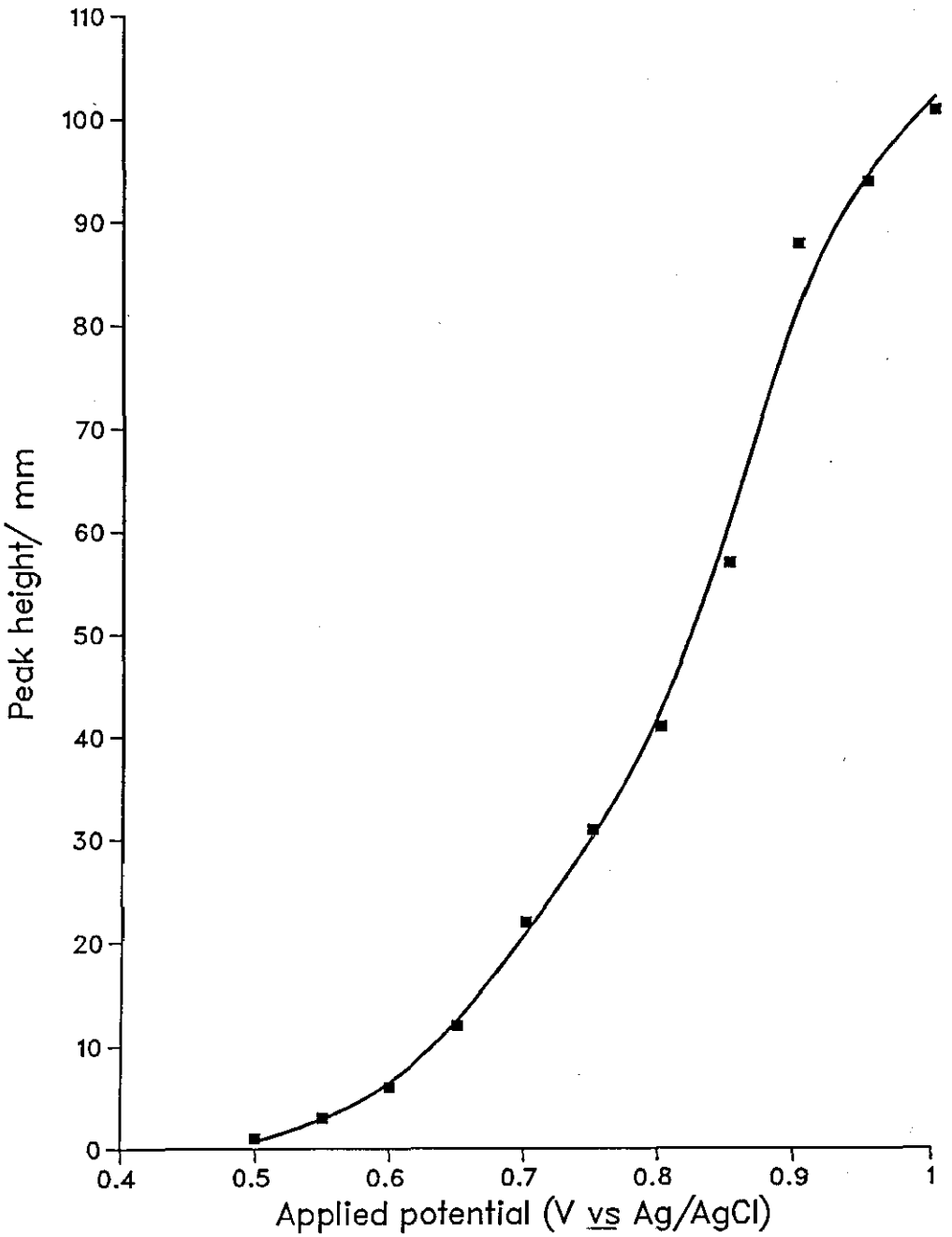


Fig. 39 - Relationship between Applied Potential vs Peak Height for 1 x 10^{-3} M 3-(4-Hydroxyphenyl)propionamide of Cyclohexylamine



3.9.2. Derivatisation of Cyclohexylamine in Aqueous Solution using Bolton-Hunter Reagent

The calibration curve for pure 3-(4-hydroxyphenyl)propionamide of cyclohexylamine was firstly measured using LCEC.

Fig. 40 - Calibration Curve for 1 x 10^{-6} M - 1 x 10^{-5} M 3-(4-Hydroxyphenyl)propionamide of Cyclohexylamine

Then, derivatisation of cyclohexylamine was carried out in dilute aqueous solution using bicarbonate solution.

Fig. 41 - Chromatograms for Cyclohexylamine derivative after reaction and Bolton-Hunter Reagent Blank

Table 6 - Comparison of Yield for Derivatisation of Cyclohexylamine in 0.1 M $NaHCO_3$ with Electro - chemical Detection at 0.9 V vs Ag/AgCl, 0.5 µA range

Concentration (M)	Peak height (mm)		% Yield
	pure derivative	in-situ derivative	
1 x 10^{-5}	67	61	91
1 x 10^{-6}	18	21	116

3.9.3. Derivatisation of Benzylamine in Aqueous Solution using Bolton-Hunter Reagent

Because of decomposition of pure benzylamine derivative, the calibration curve was not measured. However, the in-situ derivatisation was carried out to examine the efficiency of derivatisation in aqueous solution.

Fig. 40 - Calibration Curve for 10^{-6} M 3-(4-Hydroxyphenyl)-propionamide of Cyclohexylamine with Electrochemical Detection at 0.2 μA

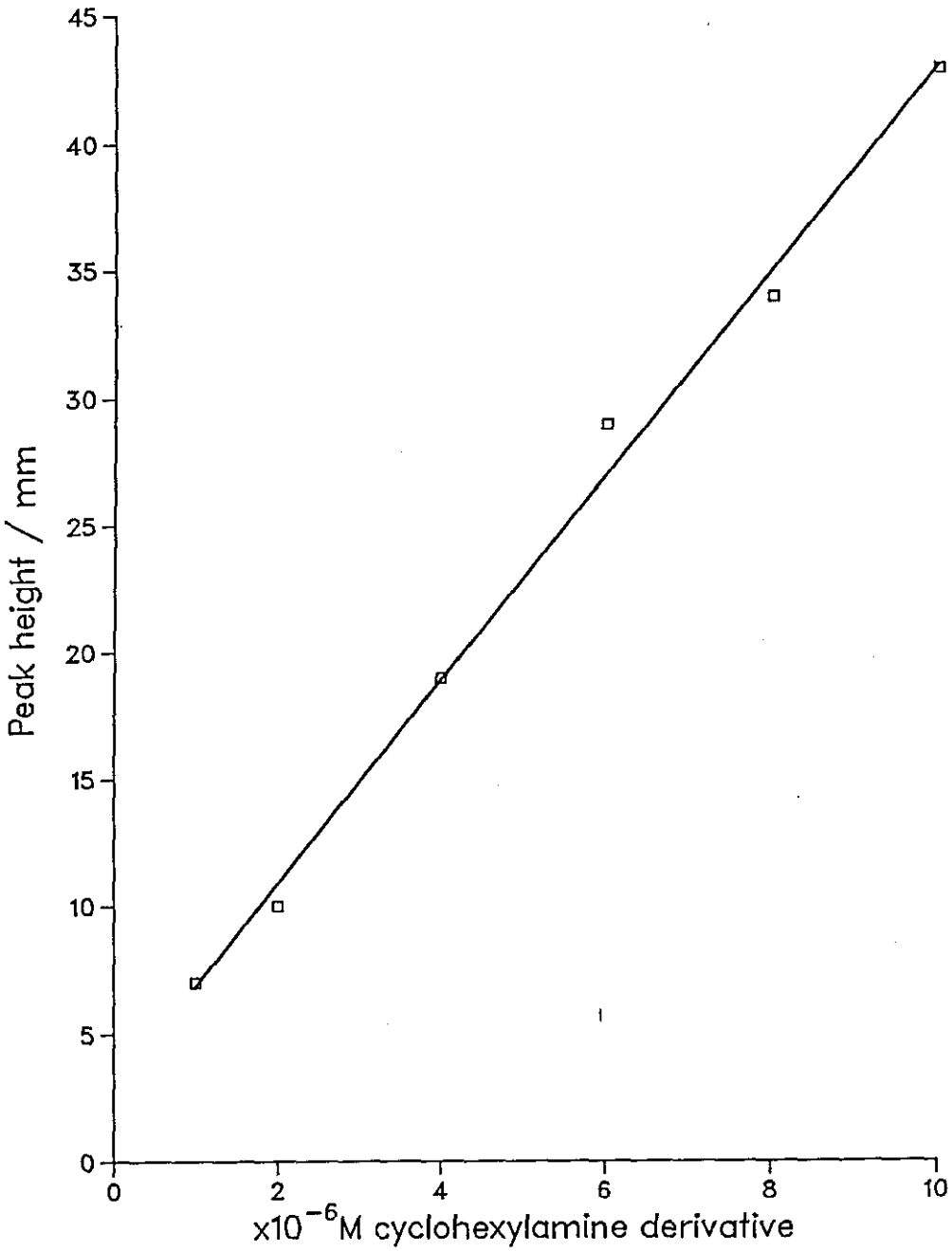
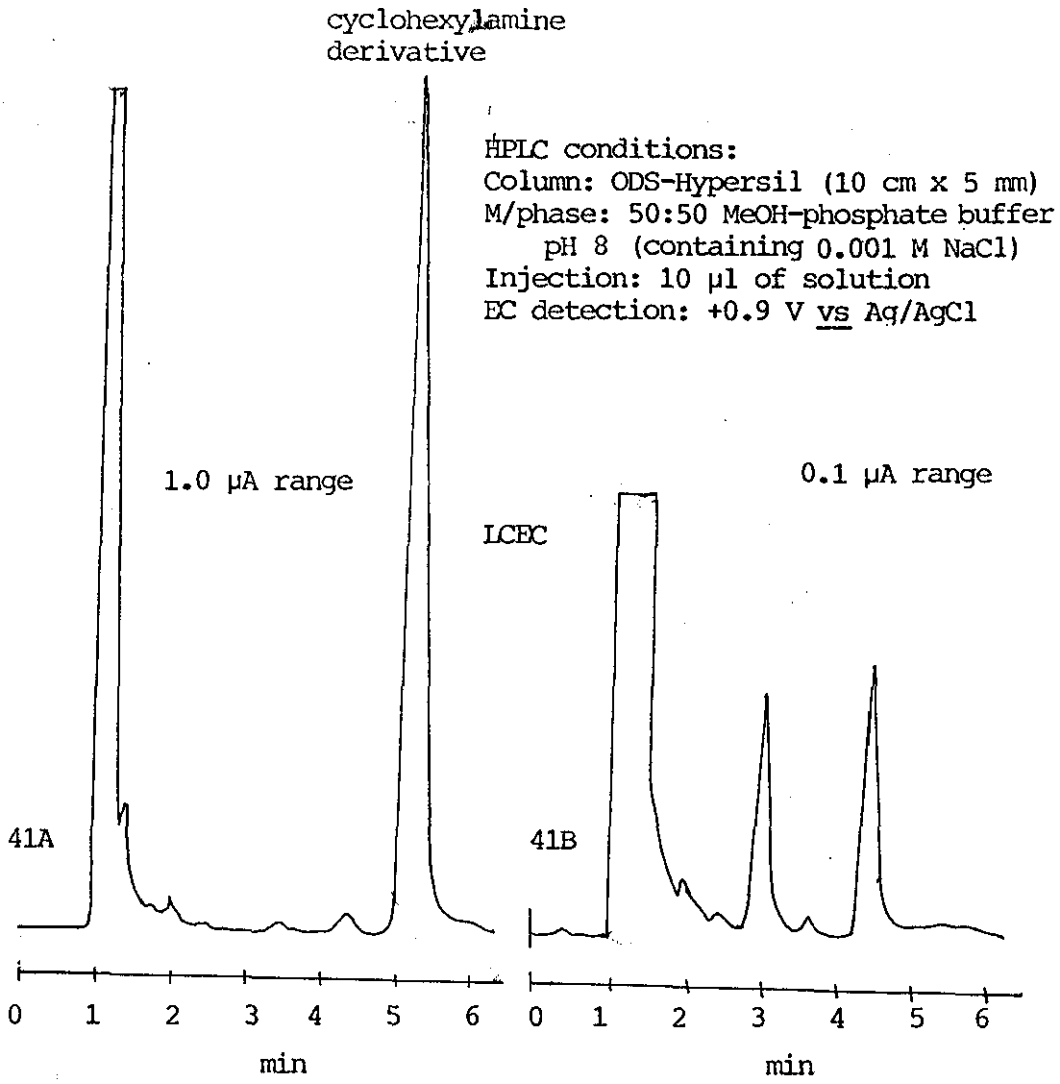


Fig. 41. Chromatograms of Cyclohexylamine Derivative and Bolton-Hunter Reagent Blank in 0.1 M $NaHCO_3$



41A - 10^{-4} M Cyclohexylamine Derivative after Derivatisation with Bolton-Hunter Reagent

41B - Bolton-Hunter Reagent Blank

Fig. 42 - Chromatograms of Benzylamine Derivative after Reaction and Bolton-Hunter Reagent Blank

3.10. Derivatisation of Amines using Wood's Reagent

Cyclohexylamine was selected as a model for derivatisation using Wood's reagent.

Fig. 43 - Chromatograms of Cyclohexylamine Derivative with and without Ion-pair Reagent (1-hexanesulphonic acid) and Wood's Reagent Blank

3.11. Electrochemical Studies of Dansyl Derivatives

Some dansyl derivatives of amino-acids were examined using LCEC.

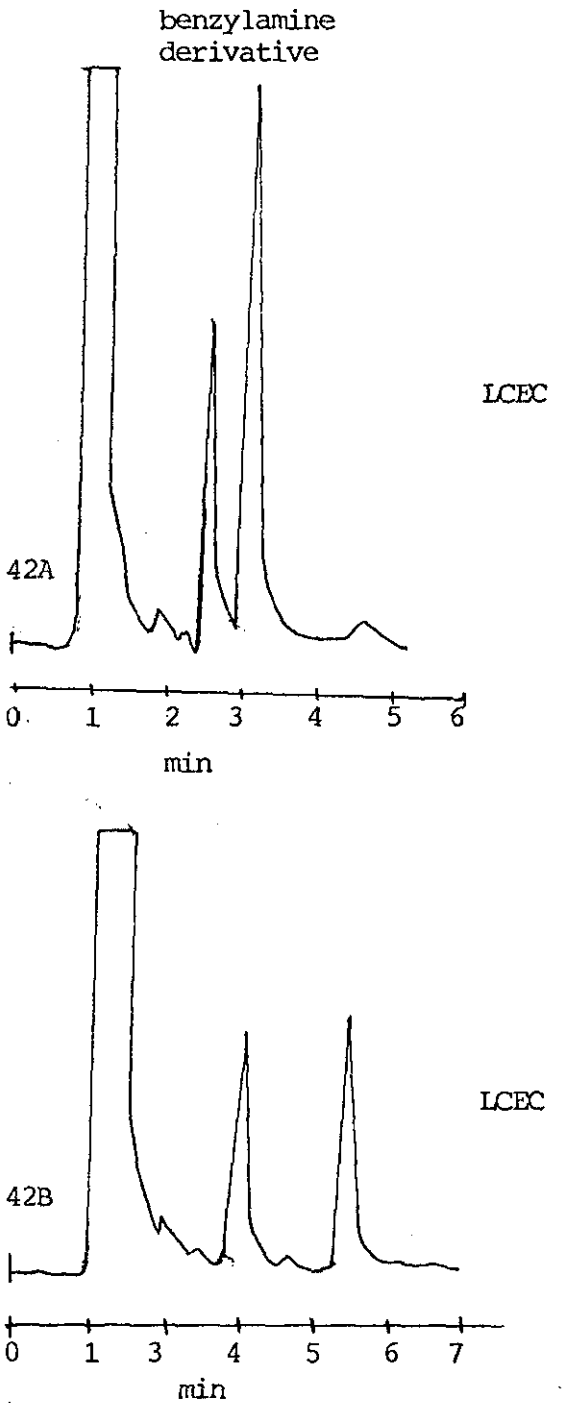
Fig. 44 and 45 - Chromatograms of Dansyl Proline in 50:50 MeOH-phosphate buffer

Table 7 - k' Values for Dansyl Derivative of amino-acids

$k' = (t_R - t_o) / t_o$ where t_R = retention time for the dansyl derivative and t_o = retention time for solvent

Dansyl derivative	k'
Glycine	1.2
Threonine	1.4
Aspartic acid	0.1
Lysine	3.2
Proline	2.8
Valine	4.2
Phenylalanine	3.3

Fig. 42. Chromatograms of Benzylamine Derivative and Bolton-Hunter Reagent Blank in 0.1 M $NaHCO_3$

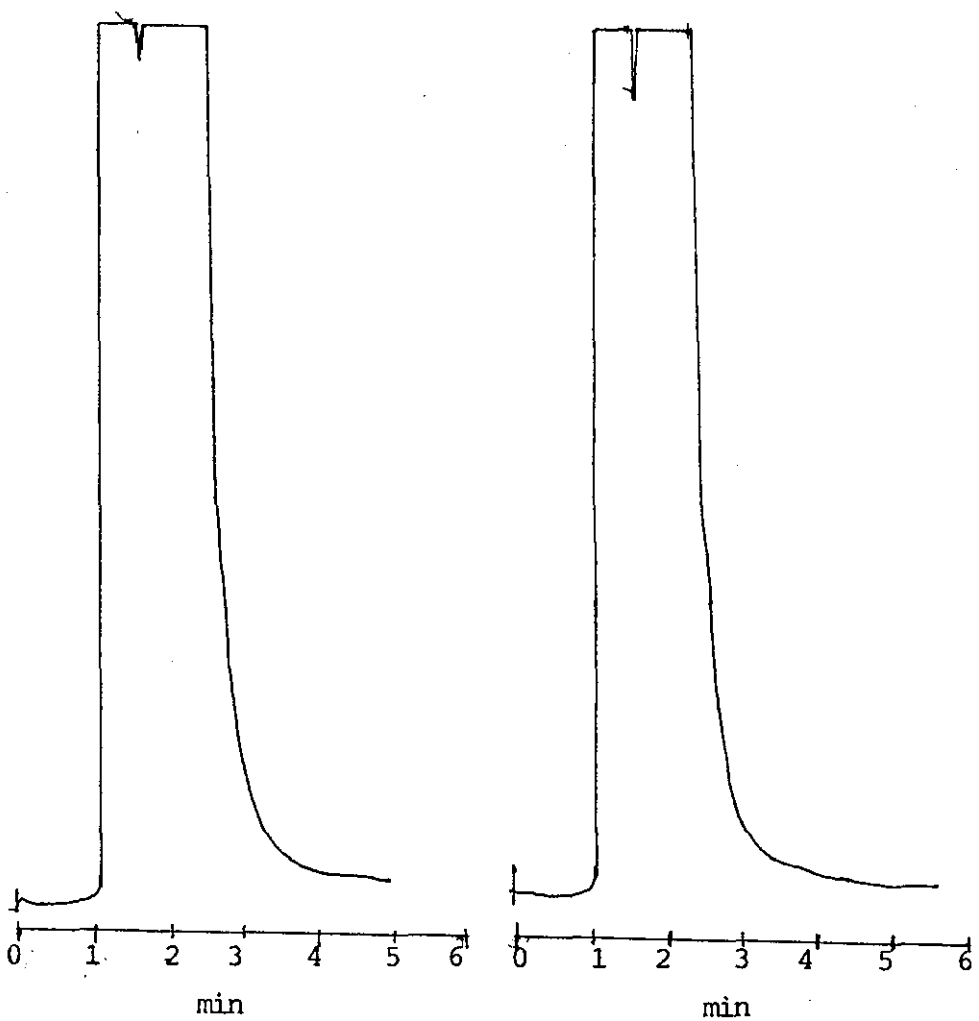


HPLC Conditions:
Column: ODS-Hypersil (10 x 5 mm)
M/phase: 50:50 MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
EC detection: +0.9 V vs Ag/AgCl
0.1 µA range

42A - 1 x 10^{-5} M Benzylamine Derivative after Derivatisation with Bolton-Hunter Reagent
42B - Bolton-Hunter Reagent Blank

Fig. 43A. Chromatograms of 2 x 10^{-4} M Cyclohexylamine Derivative and Wood's Reagent Blank without Ion-pair Reagent

HPLC conditions:
Column: ODS-Hypersil (10 cm x 5 mm)
M/phase: 70-30 MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
EC detection: +0.9 V vs Ag/AgCl
20 µA range



Cyclohexylamine derivative

Wood's Reagent blank

Fig. 43B. Chromatograms of 2 x 10^{-4} M Cyclohexylamine Derivative after Derivatisation and Wood's Reagent Blank with Ion-pair Reagent

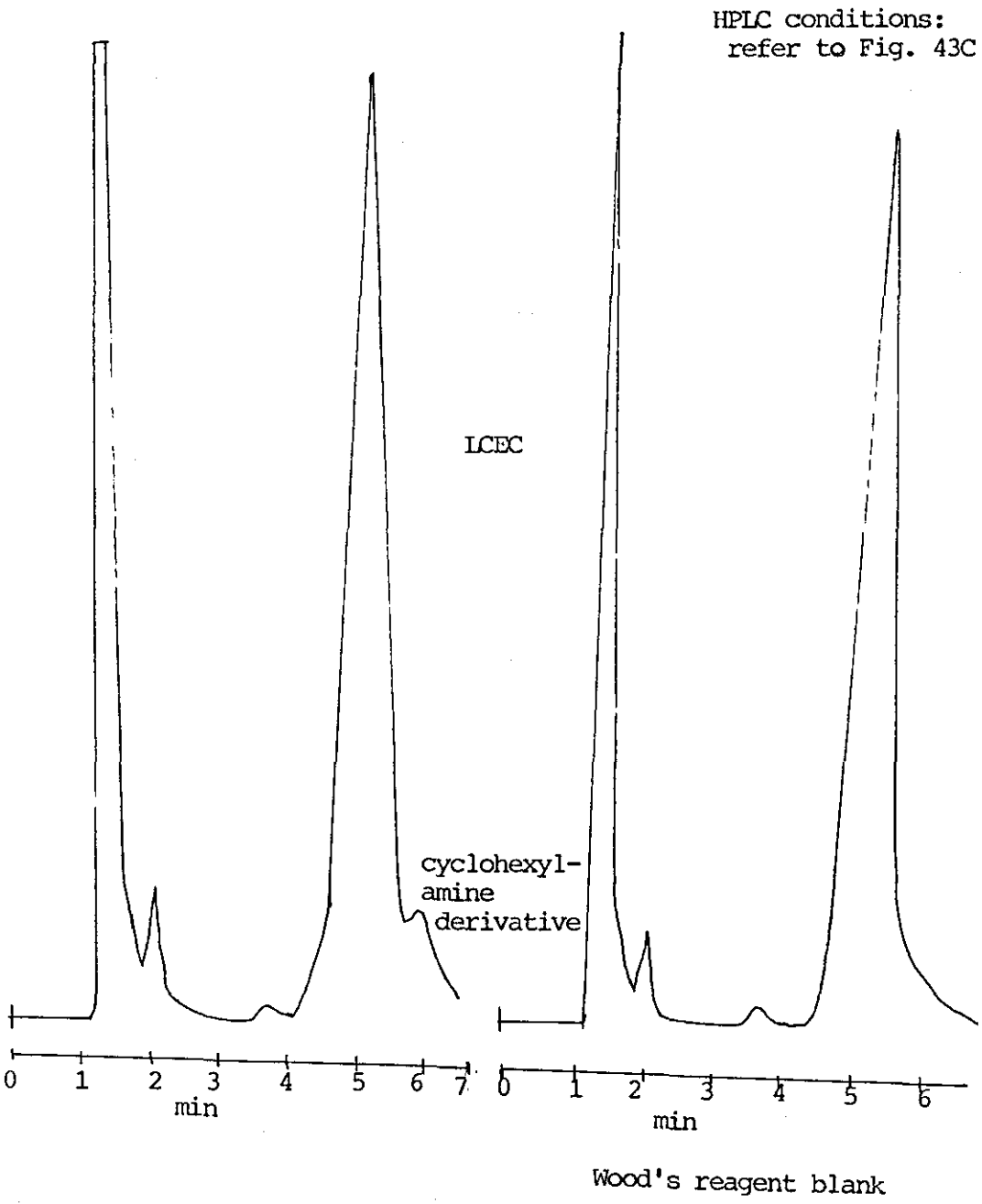
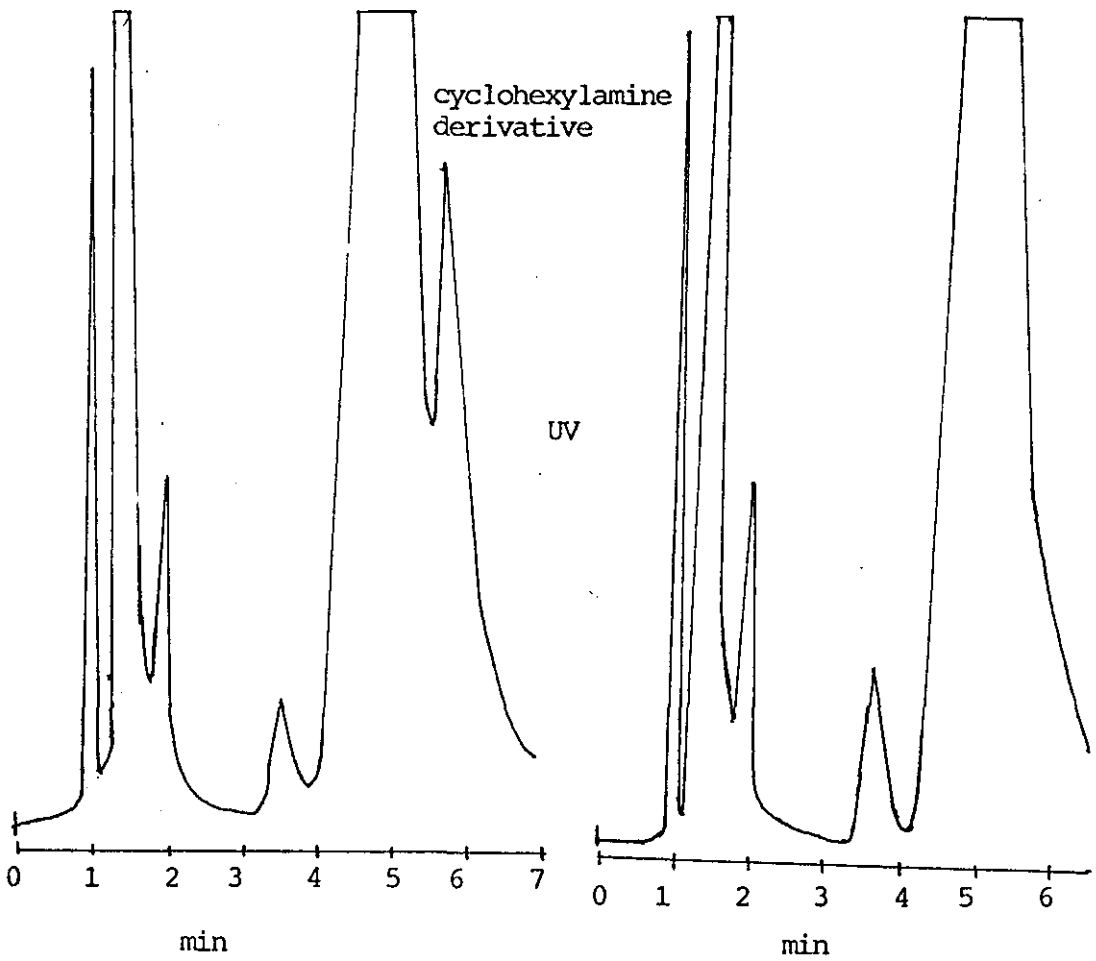


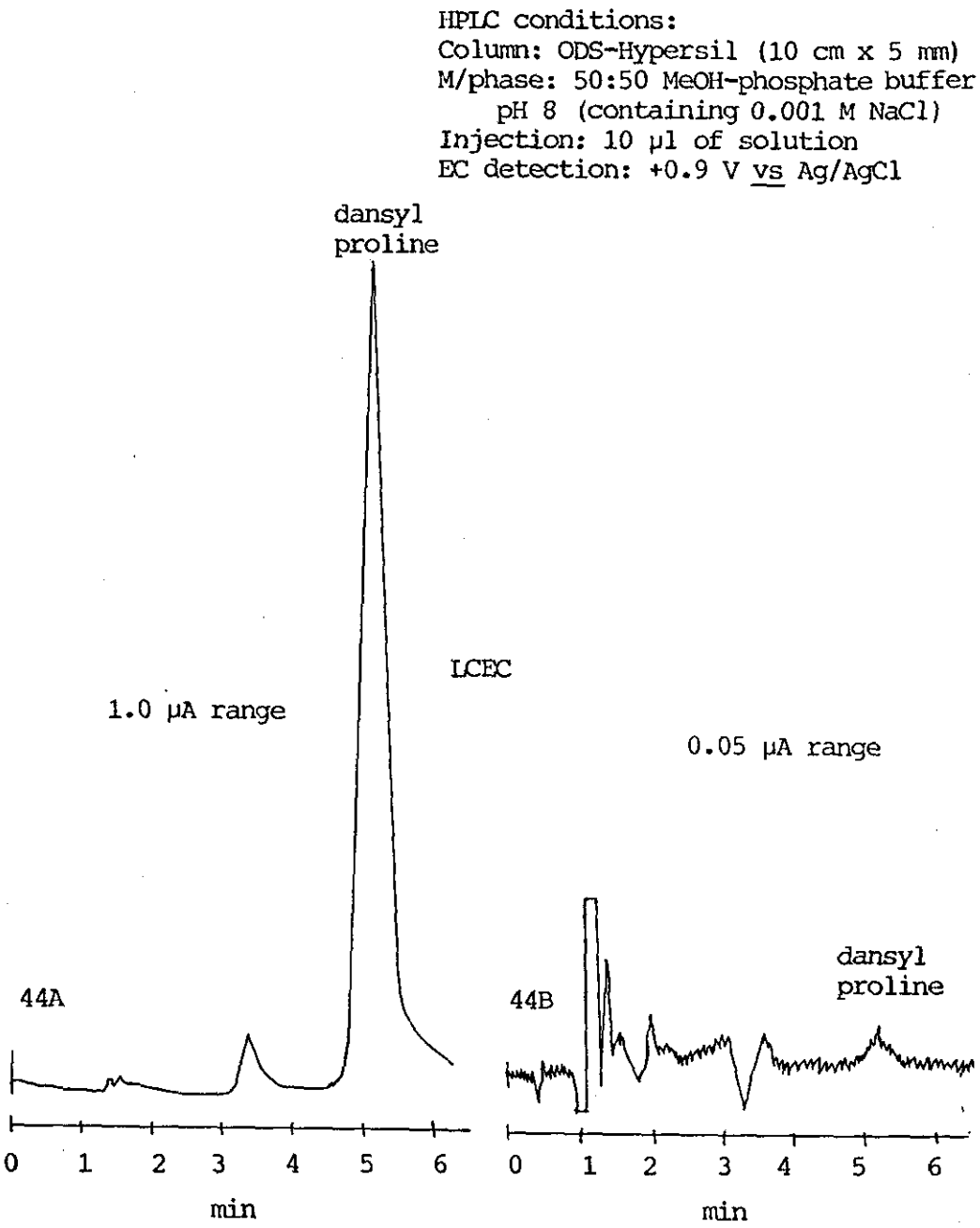
Fig. 43C. Chromatograms of 2 x 10^{-4} M Cyclohexylamine Derivative after Derivatisation and Wood's Reagent Blank with Ion-pair Reagent

HPLC conditions:
Column: ODS-Hypersil (10 cm x 5 mm)
M/phase: 70:30 MeOH-phosphate buffer (containing 0.05 M 1-hexane-sulphonic acid and 0.001 M NaCl)
Injection: 10 µl of solution
Detection:
EC: +0.9 V vs Ag/AgCl, 20 µA range
UV: 254 nm, 0.2 AUFS



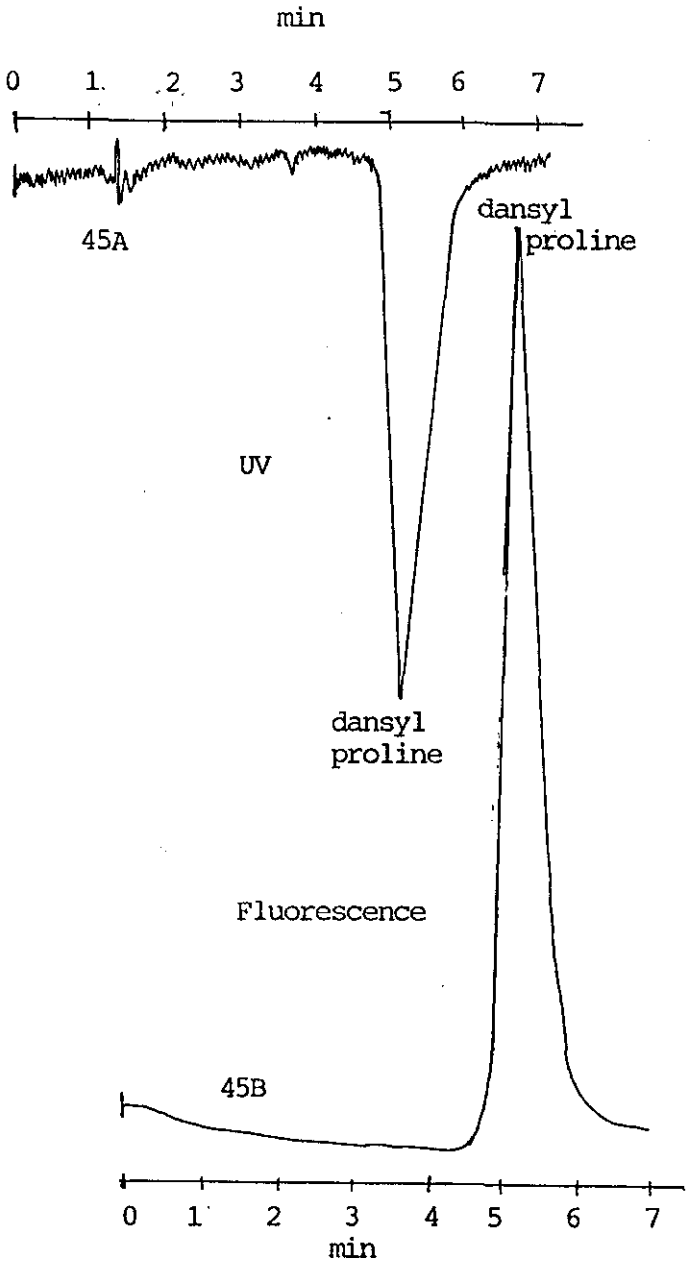
Wood's reagent blank

Fig. 44. Chromatograms of Dansyl Proline in 50:50 MeOH-phosphate Buffer



44A - 1 x 10^{-3} M Dansyl Proline in MeOH-phosphate Buffer pH 8
44B - 1 x 10^{-6} M Dansyl Proline in MeOH-phosphate Buffer pH 8

Fig. 45. Chromatograms of Dansyl Proline in 50:50 MeOH-phosphate Buffer



HPLC conditions:
Column: ODS-Hypersil (10 cm x 5 mm)
M/phase: MeOH-phosphate buffer pH 8 (containing 0.001 M NaCl)
Injection: 10 µl of solution
Detection:
UV: 254 nm, 0.1 AUFS
Fluorescence:
λ_{ex} = 340 nm
λ_{em} = 530 nm

45A - 1 x 10^{-3} M Dansyl Proline in MeOH-phosphate Buffer pH 8
45B - 1 x 10^{-3} M Dansyl Proline in MeOH-phosphate Buffer pH 8

CHAPTER IV

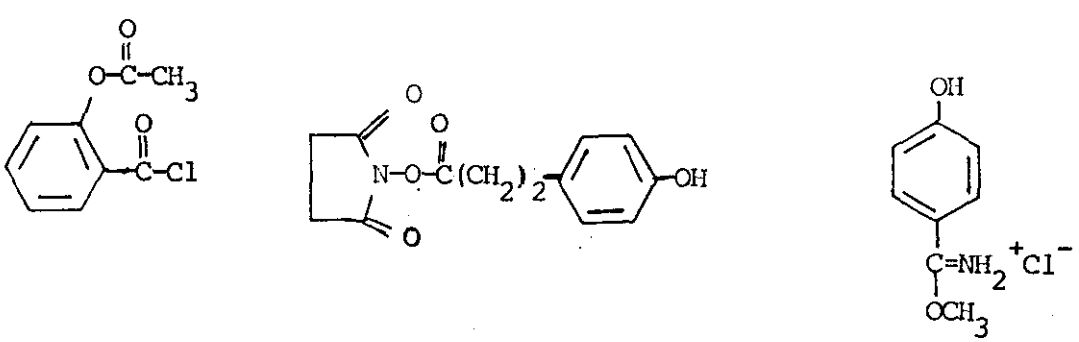
DISCUSSION

Since LCEC is suitable for the analysis of phenols, research in this field has been aimed mainly at the development of derivatisation techniques which introduce a phenolic group. As described earlier, polar compounds such as amines, which are difficult to analyse using GLC, can be easily derivatised to give products that can be separated and detected by HPLC using suitable detectors including UV, fluorescence, and electrochemical detectors. In my research, an electrochemical detector has been selected in conjunction with HPLC because of its sensitivity and selectivity compared to others for the analysis of electroactive compounds.

Recently, pre-column derivatisation with phenolic compounds has become popular in order to improve the detection of analytes in HPLC with electrochemical detection. Although, there are many reports describing the utility of LCEC for the determination of electroactive compounds, only few reagents can be used and are suitable for derivatisation of aliphatic amines. Many acid chlorides such as tosyl chloride [80], and benzoyl chloride [81,82] have been used to derivatise aliphatic amines and polyamines for HPLC separation and detection with ultra-violet spectroscopy. The tendency to use acid chlorides as the derivatising reagents,

led us to develop a potential derivatising reagent by employing salicyloyl chloride, i.e. O-acetylsalicyloyl chloride, for pre-column derivatisation of aliphatic amines for LCEC. A phenolic electrochemical active group from the derivatising reagent has been formed by using the Schotten-Baumann alkaline reaction conditions. This compound has the advantage that the phenolic group is protected during reaction and then the acetyl group is removed by hydrolysis. This phenolic group is easily detected by oxidative electrochemical detection. Other phenolic compounds such as Bolton-Hunter reagent (3-(4-hydroxyphenyl)-propionic acid-N-hydroxysuccinimide ester) and Wood's reagent (methyl p-hydroxybenzimidate.HCl) are also possible for use with LCEC. These two reagents are widely studied as derivatising reagents for aliphatic amines. Fig. 46 shows the structure of the reagents named above.

Each of these reagents and the detection of their derivatives using LCEC are discussed in more detail.



O-acetylsalicyloyl chloride

Bolton-Hunter reagent

Wood's reagent

Fig. 46. Structure of O-acetylsalicyloyl chloride, Bolton-Hunter, and Wood's reagents

4.1. Reaction of Amines with O-Acetylsalicyloyl Chloride

Preparation of pure 2-hydroxybenzamides of amines were carried out by reacting the amines with O-acetylsalicyloyl chloride. The amine was firstly dissolved in 2 M NaOH and O-acetylsalicyloyl chloride in diethyl ether was added. The mixture was shaken. The two layers were separated and the aqueous layer was acidified with 2 M HCl down to pH 7.0. The product was extracted. The crystalline solid derivatives then needed further recrystallisation to give a pure amine derivative. The yields were almost 70%. The schematic diagram of reaction is shown on Fig. 47.

The identity of the products from derivatisation of amines (see Section 3.1) were confirmed by spectroscopic (IR and NMR) and TLC analysis. The main characteristics such as the dominant three-band for an amide group on the IR spectrum has shown the products are amide derivatives. A single spot on the TLC plate had indicated that the products are pure amine derivatives.

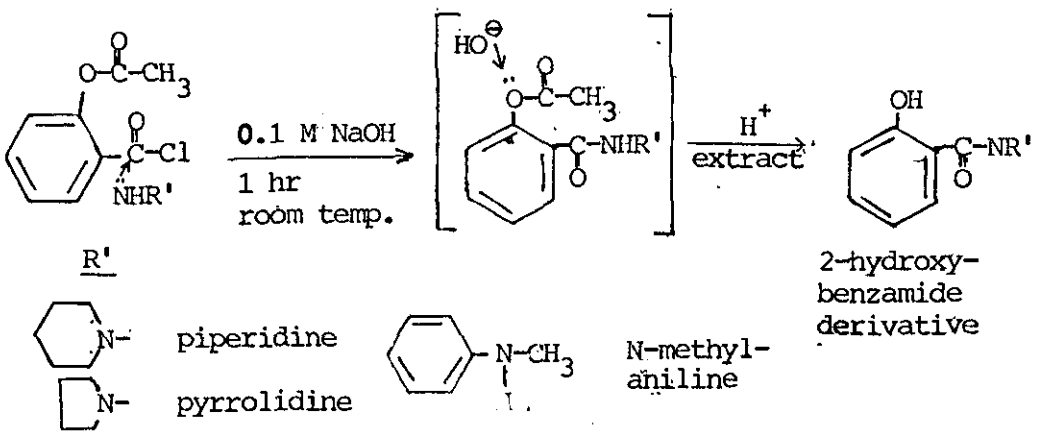


Fig. 47. Derivatisation of amines with O-acetylsalicyloyl chloride

4.1.1. Chromatographic Conditions

The HPLC conditions for separation of 2-hydroxy-benzamides of amines were selected including the choice of solvents for mobile phase, pH, composition of eluent, and temperature. The optimisation of the electrochemical detector for detection of 2-hydroxybenzamide of amines should also be considered. The previous student, [77] who had initially carried out the experiment, suggested the most suitable mobile phase composition for analysis of amine drivatives to be a solution of methanol-0.025 M phosphate buffer pH 8. Kissinger [83] has reported buffered mobile phase in the concentration range 0.01 M to 0.1 M. In addition, phosphate buffer can be used as a modifier, which reduces the peak tailing, increasing the efficiency of separation, and also acts as the supporting electrolyte for the electrochemical detector [84].

4.1.2. Optimisation of Electrochemical Detector Potential

In the analysis of amine derivatives, the electrochemical detector has been used in conjunction with HPLC. Basically the detection is at a constant applied potential, while the current is measured as a function of time. As explained before, the applied potential relates to the minimum potential at which the current reaches its limiting current plateau, so that, the maximum current response to the analyte can be maintained at all times. In this study, the signal of detector response is measured in terms of peak height. A determination of the

limiting current plateau was carried out and Fig. 21 shows the peak heights measured at the different applied potentials for a series of injections of the 2-hydroxybenzamide of piperidine. The detector gave a response above the applied potential of +0.5 V and the measurement was limited to applied potential of +1.0 V. Further increase beyond this applied potential, would probably cause a significant increases in background current and noise levels. From this work, an applied potential of +0.9 V was chosen for the detection of amine derivatives.

4.1.3. Chromatographic Separation of 2-Hydroxybenzamide of Amines

Using the suggested conditions [77], each amine derivative was well separated by LCEC. Fig. 20 shows the separation of three amine derivatives: 2-hydroxybenzamide of pyrrolidine, 2-hydroxybenzamide of piperidine, and 2-hydroxybenzamide of N-methylaniline. The capacity factors (k') for the amine derivatives were less than 12 and are shown in Table 2 which agreed in order of polarity with the results of R_f obtained by the TLC analysis (see Section 3.1). Fig. 20 also shows the chromatogram obtained from the UV detection at 254 nm in comparison to electrochemical detection. Therefore, these amine derivatives can also be detected using the UV detector. However, the sensitivity of the UV detector was limited in further applications so that use of the electrochemical detector

for the determination of these amine derivatives was fully considered.

4.1.4. Standardisation

In HPLC analysis, calibration can be achieved using either internal or external standardisation [85]. Analysis using an internal standard requires the addition of a compound that elutes close to the analyte, meanwhile when external standard is considered the actual compounds of interest are used in the preparation of calibration curves.

Calibration curves for electrochemical detection of peak height vs concentration were prepared for three pure amine derivatives, 2-hydroxybenzamides of piperidine, N-methylaniline, and benzylamine. All standard solutions were prepared in methanol. The peak heights of the amine derivatives were calculated from the LCEC response. Fig. 22 shows a linear calibration curve was obtained for the 2-hydroxybenzamide of piperidine in the range studied, from 1×10^{-7} M to 1×10^{-6} M with a good correlation coefficient of 0.9950. For the 2-hydroxybenzamide of N-methylaniline, linear calibration curves were also obtained and the level of 10^{-6} M concentration was detectable with correlation coefficient of 0.9964 (Fig. 23).

In the case of benzylamine, calibration curve was only plotted at 10^{-4} M range with correlation coefficient of 0.9979 (Fig. 24) and a linear calibration as low as the other

two amine derivatives should also be possible. Therefore, these pure amine derivatives can be used as external standards to monitor the in-situ derivatisation of amines in dilute aqueous solution.

4.1.5. Stability of 2-Hydroxybenzamide Amine Derivatives

The pure amine derivatives either in dry state or in methanol solution appeared to be stable without decomposition for a long time at room temperature as they gave comparable results on repeating injections or preparing new sample solutions after a period of time.

4.2. The Study of Amine Derivatives in Dilute Aqueous Solution

To demonstrate the usefulness of amine derivatives, the in-situ derivatisation of amines in dilute solution using O-acetylsalicyloyl chloride was undertaken. As demonstrated earlier, the amounts of pure amine derivatives detected by LCEC were linear at low concentrations.

For preparation of samples, a sodium hydroxide solution was used as a derivatisation media. To guarantee complete reaction for all amines studied an excess of reagent would probably be required. For example, Wellons and Carey [86] employed an excess of m-toluoyl chloride in the derivatisation of amines for complete reaction.

In the present study, the reaction with three amines, piperidine, N-methylaniline, and benzylamine, has been studied in detail. For the general preparation of amine derivatives the amine was firstly dissolved in 0.1 M NaOH. Then, the derivatising reagent, O-acetylsalicyloyl chloride was added as a solid to the solution. The derivatisation was allowed to react under room temperature for 1 h. Then, 10 µl of solution was injected directly onto the column and detected by electrochemical detection at +0.9 V vs Ag/AgCl. No washing or extraction steps were necessary before injection.

4.2.1. 2-Hydroxybenzamide of Piperidine in 0.1 M NaOH

Firstly, the efficiency of derivatisation was studied to examine the percentage yield of derivatisation in 0.1 M NaOH and the amount of O-acetylsalicyloyl chloride that was required to complete the reaction has been determined. As shown in Fig. 25, the percentage derivatisation of 1×10^{-4} M piperidine increased as the weight of O-acetylsalicyloyl chloride added increased. At 190 mg of O-acetylsalicyloyl chloride, 100% derivatisation has taken place for 1×10^{-4} M piperidine in 0.1 M NaOH. However for satisfactory derivatisation, O-acetylsalicyloyl chloride (200 mg) was used in further studies. To determine the percentage yield, the peak heights of derivatised piperidine were compared with the pure piperidine derivative calibration curve. The retention time of the derivatised piperidine has also been compared with the standard.

Fig. 26 shows a similar shape for the percentage yield with increasing amounts of O-acetylsalicyloyl chloride with 1 x 10^{-5} M piperidine. Once again, under the same conditions, the reaction proceeded to 100% derivatisation. Therefore, both graphs indicate that the O-acetylsalicyloyl chloride is available for reaction with the amine in dilute aqueous solution. It is noted that small amount of the reagent was also hydrolysed at dilute levels in aqueous solution.

Fig. 29 shows the chromatograms of derivatised piperidine using O-acetylsalicyloyl chloride in 0.1 M NaOH and of O-acetylsalicyloyl chloride in 0.1 M NaOH for comparison. A single peak was obtained indicating that no by-product of derivatisation was present other than the hydrolysed reagent.

From the chromatogram in Fig. 30, the limit of detection that was achieved with the electrochemical detector at 0.05 μA sensitivity is 6 x 10^{-7} M of piperidine. The limit of detection was defined as the concentration of analyte that will produce a signal to noise ratio (S/N) of 2 and is considered to be the minimum concentration that can be detected [87]. The main factor which limited the detection at the lowest sensitivity was the noise present in the chromatogram. White [87] has categorised three types of noise including short-term noise, long-term noise and drift. The main causes of short-term noise are pump pulsation, recorder and detector itself (stability). Meanwhile temperature and pressure fluctuation can affect the long-term noise. Finally, drift

results from mobile phase and temperature variations. However, most of these factors were eliminated to minimise the sources of noise during experiments, for example, effective degassing of the eluent was found to be important for increasing the stability and reducing the noise.

For the detection of pure piperidine derivative, the electrochemical detector showed a good linear response. The study was also carried out to ensure the electrochemical detector response was linear with concentration for the in-situ derivatisation of amines. A series of piperidine solutions for calibration were prepared and O-acetylsalicyloyl chloride was added to each solution. After injection onto the column and detection by the electrochemical detection, a graph was prepared by plotting the peak height of the response against the concentration (Fig. 27) and was found to be linear over the concentration range of 1×10^{-5} M of piperidine to 1×10^{-4} M with correlation coefficient of 0.9920. In addition, the derivatisation has been extended into lower level concentrations. As shown in Fig. 28, the derivatisation produced a linear calibration plot from 1×10^{-6} M to 1×10^{-5} M of piperidine with correlation coefficient of 0.9950. This result suggests this method should be useful for the trace analysis of amines in dilute aqueous solution when the sample has been required to be analysed in a trace quantity.

4.2.2. Derivatisation of N-Methylaniline in Dilute Aqueous Solution

In order to demonstrate that a variety of amines could be derivatised in dilute aqueous solution, N-methylaniline has been studied using a similar method to the derivatisation of piperidine. Assuming that the excess of O-acetylsalicyloyl chloride (200 mg) should be maintained to ensure complete reaction, the experiment was carried out in 0.1 M NaOH. A sample of N-methylaniline at various concentration (from 10^{-4} M to 10^{-6} M) was firstly derivatised. After allowing to react for 1 h, the solution was directly injected into the HPLC system. The applied potential was also set at +0.9 V vs Ag/AgCl for detection.

Using the pure 2-hydroxybenzamide of N-methylaniline as a standard, the retention time of peak height has been compared. The example of chromatogram for derivatisation of N-methylaniline is shown in Fig. 32. However, the chromatogram shows an additional peak higher than the peak for the N-methylaniline derivative. This peak was further checked against the N-methylaniline blank and found to correspond to N-methylaniline in both UV and LCEC detectors.

The main factors that affected the reaction were steric effects of N-methylaniline and its reduced nucleophilicity owing to the electron-withdrawing effect of aromatic ring. The similar problem has arisen in the derivatisation of primary and secondary amines by Chou et al. [66]. They explained the

reactivity of a secondary amine was 2 - 3 times lower than a primary amine caused by the steric effect.

In the study of linearity of detection at 10^{-4} M concentration level, the electrochemical detector was still giving a linear response with good correlation coefficient of 0.9949 (Fig. 31). However, the precise determination of calibration curve at lower concentration could not be achieved because the derivatisation was incomplete.

In order to improve the yield of derivatisation, an alternative procedure was carried out to derivatise N-methylaniline in which followed the Valdez and Reier [74] method. The authors have reported a successful method for derivatisation of alcohols in aqueous samples. Because their procedure for derivatisation of alcohols in aqueous solutions used an acetonitrile-based solvent system, the method was modified by changing the solvents for N-methylaniline and O-acetyl-salicyloyl chloride, respectively. The reaction was allowed to stand overnight. Unfortunately, no reaction was observed on the chromatogram as shown in Fig. 33. The present procedure was shown to give a better result than the Valdez and Reier method even though 100% derivatisation efficiency was not achieved.

4.2.3. Derivatisation of Benzylamine in Dilute Aqueous Solution

For the preparation of 2-hydroxybenzamide of benzylamine in dilute aqueous solution (0.1 M NaOH) the same procedure for piperidine was taken. The derivatisation efficiency of benzylamine was also good. Only a single peak was obtained after derivatisation. Fig. 35 shows a comparison between the yield of benzylamine derivative in dilute aqueous and the pure benzylamine derivative. The O-acetylsalicyloyl chloride blank and products were detected at the higher sensitivity of the electrochemical detector (10 μA range). The applied potential was set up at +0.9 V vs Ag/AgCl.

A series of benzylamine concentrations has also been prepared for a calibration curve. A linear result was obtained for detector response in the range studied from 1×10^{-4} M to 1×10^{-3} M benzylamine in 0.1 M NaOH with correlation coefficient of 0.9985 (Fig. 34).

4.2.4. Efficiency of Derivatisation in Dilute Aqueous Solution

All amines studied have shown a good response to the LCEC detector. Piperidine and benzylamine can be easily derivatised in dilute aqueous solution and high yields were achieved. Table 3 shows a comparison of derivatisation yield for three amines. As explained, N-methylaniline could only be derivatised to nearly 55%. At a concentration of 1×10^{-6} M N-methylaniline, the peak of interest could not be detected from the tailing peak of underivatised N-methylaniline.

The precision studies of the HPLC method using the LCEC detector were investigated by injecting four samples of each amine derivative prepared separately in dilute aqueous solutions. The calculation of coefficient of variation of the LCEC detector for piperidine derivative was 1.4% at 1 x 10^{-5} M, meanwhile for the benzylamine derivative was slightly higher than piperidine derivative which the LCEC detector gave 5.5% at 1 x 10^{-4} M as shown in Table 4. Thus, the reproducibility was considered good for the pre-column derivatisation of aliphatic amines using O-acetylsalicyloyl chloride in dilute aqueous solution and LCEC.

4.3. Derivatisation of Amines using Bolton-Hunter Reagent

Bolton-Hunter reagent has been selected as a derivatising reagent since it had advantages for the derivatisation of amines. Firstly, the carboxyl group is activated as the N-hydroxy-succinimide towards amine groups. The Bolton-Hunter reagent has also been used as a radiolabelling reagent to introduce radioactive iodine atoms (^{125}I) into peptides [88,89], because of its high reactivity toward peptides and other nucleophilic groups. Meanwhile, water-soluble sulpho analogue of Bolton-Hunter reagent (sulphosuccinimidyl-3-(4-hydroxyphenyl)propionate) has also used to determine amines in body fluids (mammalian brain) using LCEC [62-64]. The derivatisation occurred more efficient by using the sulpho Bolton-Hunter reagent.

N-Hydroxysuccinimide esters were firstly used to syntheses peptides [90]. Then, Bolton-Hunter [88] developed a new method for labelling proteins with the acylating reagent, N-succinimidyl-3-(4-hydroxyphenyl)propionate (called Bolton-Hunter reagent) which reacts with free amino groups in the protein to form amides. The amidation conditions were 1.0 M borate buffer containing protein at pH 8.5 and the reaction took 15 min at 0°C. They reported the yield was only 13% - 53%. In another study, Shing and Ruoho [89] have also used the Bolton-Hunter reagent and reacted it with polypeptides to convert them to the amide derivatives. The protein was dissolved in 0.1 M $NaHCO_3$ at pH 8.7 and then reacted with ^{125}I-labelled Bolton-Hunter reagent for 3 h at 23°C.

Based on these methods, the direct reaction of the Bolton-Hunter has been employed as a derivatising reagent in the pre-column derivatisation of aliphatic amines. In a preliminary study, an experiment was carried out to examine the reaction of Bolton-Hunter reagent with amines. The above procedure was modified with 0.1 M $NaHCO_3$ at pH 8.5. The amine was dissolved in 0.1 M $NaHCO_3$ and then the Bolton-Hunter reagent was added to the solution. After 3 h reaction, the mixture was extracted with chloroform. The extract was injected into the HPLC system and the peaks were detected using ICEC detector. The chromatogram was compared with the Bolton-Hunter blank. When this extracted solution (containing cyclohexylamine derivative) was treated with 0.1 M HCl and injected onto the

column, the observed peak disappeared from the chromatogram. Hence, the peak obtained after the first extraction could be the peak of amine derivative, which was hydrolysed after treated with acid and could not give response to the LCEC detector. Thus, the Bolton-Hunter reagent has been utilised for the derivatisation of amines.

Firstly, the pure amine derivatives have been prepared from cyclohexylamine, benzylamine, and piperidine. The derivatisation with Bolton-Hunter reagent was carried out at pH 8.5 at room temperature. The reaction was terminated after 3 h. The schematic diagram of reaction is shown in Fig. 48.

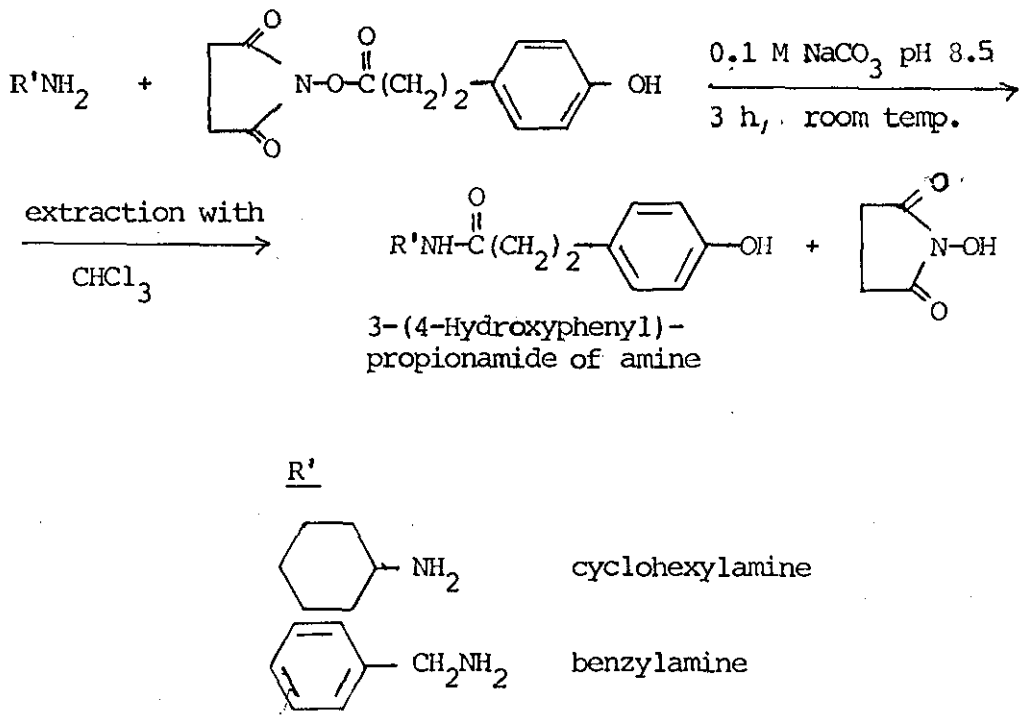


Fig. 48. Derivatisation of aliphatic amines with Bolton-Hunter reagent

To obtain the pure derivatives, the by-product, probably p-hydroxyphenylpropionic acid, was removed by employing a column containing silica. Then the TLC analysis confirmed that only a single compound was present. The products are 3-(4-hydroxyphenyl)propionamides of amines.

4.4. Chromatographic Studies of 3-(4-Hydroxyphenyl)propionamide of Amines

The chromatographic conditions used methanol-0.025 M phosphate buffer pH 8.0 as an eluent. Using the electrochemical detector with applied potential set at +0.9 V vs Ag/AgCl, the prepared pure amine derivatives were separated by HPLC. Fig. 36 shows the chromatogram of 3-(4-hydroxyphenyl)propionamide of cyclohexylamine, which gave a single peak on detection. However, in the case of 3-(4-hydroxyphenyl)propionamide of benzylamine, two peaks were observed in the chromatogram as demonstrated in Fig. 37. The additional peak is probably caused by the breakdown of benzylamine derivative in the methanol-water solution. The same problem has been reported by Gusovsky et al.[64], in the study of sulpho Bolton-Hunter reagent as a derivatising reagent. Meanwhile, Fig. 38 shows the chromatogram of 3-(4-hydroxyphenyl)propionamide of piperidine derivative in methanol-water. The capacity factors (k') for the amine derivatives were 4.3, 3.3, and 5.7 for cyclohexylamine, benzylamine, and piperidine derivatives, respectively (Table 5).

In the study of detector response with the applied potential potential, Fig. 39 shows a plot of the peak height vs applied potential for 1×10^{-3} M 3-(4-hydroxyphenyl)propionamide of cyclohexylamine. The LCEC detector only gave response above +0.5 V applied potential which increased with applied potential. The limiting current of cyclohexylamine probably reached a plateau about +0.9 V vs Ag/AgCl. Thus, the LCEC detector was set at 0.9 V vs Ag/AgCl for the detection of amine derivatives.

For the pure cyclohexylamine derivative, the calibration curve was firstly plotted by preparing a series of cyclohexylamine solutions. Because the sensitivity of electrochemical detector gave no problems at 10^{-6} M cyclohexylamine derivative level, the experiment for preparation of calibration curve was tested at this level. Fig. 40 shows a calibration curve of pure cyclohexylamine derivative giving a linear response over the range 1×10^{-6} M to 1×10^{-5} M of concentration with correlation coefficient of 0.9970. The in-situ derivatisation of amines using Bolton-Hunter reagent was then carried out in 0.1 M $NaHCO_3$. In each case, 5 mg of reagent was used to react with amines. The efficiency of derivatisation was compared with pure standards of 3-(4-hydroxyphenyl)-propionamide of amines.

For in-situ derivatisation of cyclohexylamine, the sample was derivatised under the conditions described in the experimental section, and the peak obtained from the LCEC detector showed the same retention time as the pure cyclohexylamine derivative.

Fig. 41 shows the chromatograms of cyclohexylamine derivative after reaction in aqueous solution with the Bolton-Hunter reagent blank also in aqueous solution (5 mg / 10 ml $NaHCO_3$).

Under the present conditions, the yield of in-situ derivatisation of cyclohexylamine was nearly 100% at the concentration of 1×10^{-5} M cyclohexylamine (Table 6). The peak height was calculated by comparison with the pure amine derivative. However, for 1×10^{-6} M cyclohexylamine, the yield was above 100%. The main reason is the peaks of interest and peaks in the blank were very close and had nearly the same retention time.

In addition, the detection was carried out at the higher sensitivity (0.5 μA range) which gave the ambiguous peak between the cyclohexylamine derivative and Bolton-Hunter blank.

For in-situ derivatisation of benzylamine with Bolton-Hunter reagent in dilute aqueous solution, the derivatisation was not fully studied because its pure derivative slowly decomposed and was difficult to plot a calibration curve at low concentration. However, Fig. 42 shows the chromatograms of benzylamine derivative after reaction showing that the in-situ derivatisation is still possible.

In conclusion, the experiment could not be further studied since the Bolton-Hunter reagent has given problems such as poor resolution, the reagent peak interfered with the derivative peak, the product was not very sensitive to LCEC, and instability of the derivatives.

4.5. Derivatisation of Amines using Wood's Reagent

Since the Wood's reagent, methyl p-hydroxybenzimidate.HCl has had a similar role to the Bolton-Hunter reagent for the selective labelling of proteins with radioactive iodine (^{125}I), it was selected as a derivatising reagent for amines and which can be detected using LCEC. The Wood's reagent is less reactive and moderately stable in aqueous solution compare to the Bolton-Hunter reagent [76]. So far, the main purpose of Wood's reagent has mainly been used to form an iodinated imidoester product and which then reacts with amino groups of the protein to give the amidine linkage [76,91 - 96]. The schematic diagram of reaction is shown in Fig. 49.

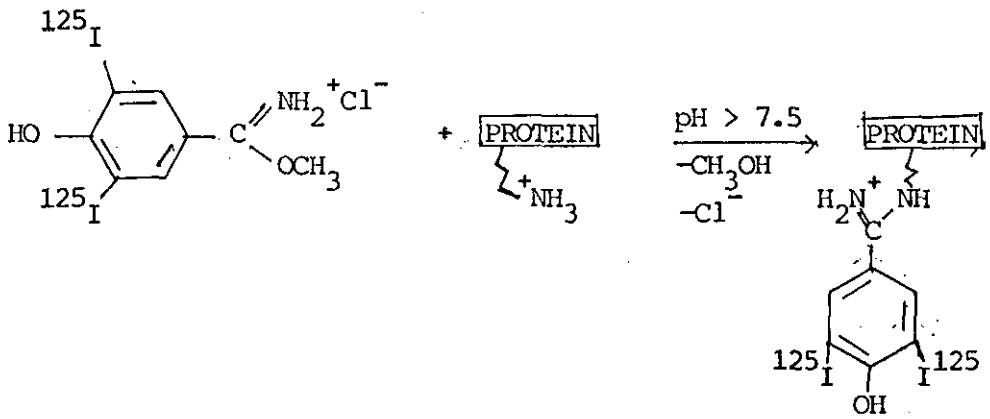


Fig. 49. Amidination of Proteins with ^{125}I Labelled Wood's Reagent

For example, Wood et al. [76] have discussed the use of Wood's reagent to prepare a radioactive labelled protein. The iodinated reagent is dissolved in slightly alkaline pH (0.005 M borate buffer, pH 9.5) to react with protein amino groups at 37°C, to which it couples through an amidine linkage.

To study additional possible reagents for LCEC trial experiments were carried out on Wood's reagent. The phenolic group of Wood's reagent is believed to be electrochemically active. To test its application, the derivatisation was carried out with aliphatic amines. With cyclohexylamine as a model, the derivatisation was studied using the modified procedure from Wood et al. [76]. The derivatisation reaction is shown in Fig. 50.

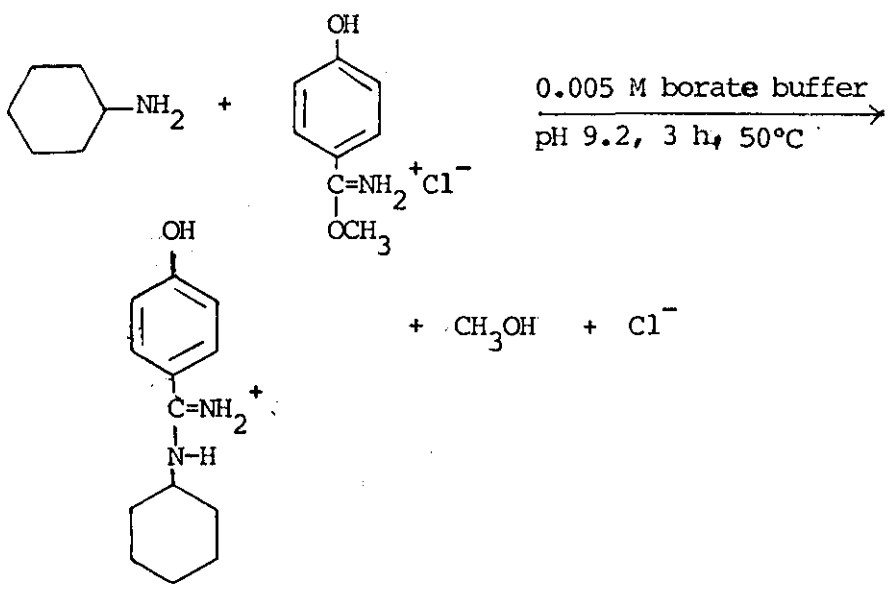


Fig. 50. Derivatisation of cyclohexylamine with Wood's reagent

Because the positive charge is preserved in the product, ion-pair chromatography has been used to improve the retention time of cyclohexylamine derivative (Fig. 43B). An eluent consisting of methanol-0.025 M phosphate buffer pH 8.0 1-hexanesulphonic acid (0.05 M) was selected for use in the mobile phase. The amine derivative was detected with the applied potential of ICEC detector set at +0.9 V vs Ag/AgCl. Fig. 43A, 43B, and 43C show the chromatograms of cyclohexylamine derivative using Wood's reagent. Without ion-pair, the retention time of the derivative was very short and peaks were eluted together with the solvent front (Fig. 43A). From the small peak (Fig. 43B and 43C) observed in the chromatograms, the result was suggested that the Wood's reagent also has potential as a derivatising reagent for electrochemical detection. But, the disadvantage was the amine derivative needs the use of ion-pairing for separation.

4.6. Electrochemical Studies of Dansyl Derivatives

So far, the dansyl derivatives have been widely studied as a derivatising reagent for fluorescence detection [97 - 99]. In this preliminary study, the dansyl derivatives of amino-acids have been examined as potential electrochemical derivatives because of dimethylamino group. To show that dansyl derivatives can be studied by the ICEC detection, the separation of dansyl proline is illustrated in Fig. 44. The level of 1×10^{-6} M of dansyl proline was detected by ICEC

detector. By comparison, UV and fluorescence detectors were also connected for detection of dansyl proline. The UV detector was set at 254 nm whereas the excitation and emission wavelengths of fluorescence detectors were 340 nm and 530 nm. Fig. 45 shows both chromatograms obtained from these detectors.

For other dansyl amino-acids studied, the retention times have been summarised in Table 7. From the results of capacity factor (k'), most dansyl derivatives were well separated by ICEC.

In wider application, the effectiveness of ICEC detection can be further studied and the fluorescence detection can be used as comparison. The studies should be examined in terms of linearity, selectivity, and reproducibility by both detectors. Because the ICEC detection is more sensitive down to sub-nanogram level, the dansyl derivatives would also be studied in these detection levels.

CHAPTER V

CONCLUSION

Since LCEC detectors are available for the analysis of compounds containing a phenolic group, one of the goals of the research was to develop a new derivatising reagent for pre-column derivatisation of aliphatic amines in reversed-phase HPLC with electrochemical detection. Because the LCEC offers an attractive method, the application of the detector should also increase sensitivity, selectivity, and simplicity of detection for amine derivatives.

O-Acetylsalicyloyl chloride has been proved to be the most suitable derivatising reagent for derivatisation of aliphatic amines. The derivatisation procedure is remarkably simple and easy to perform. All pure amine derivatives were stable crystalline solids and can be kept at room temperature. The separation of amine derivatives can be achieved by LCEC within 12 min and most samples give detection down to sub-nanogram level. The LCEC detector response is also linear down to 10^{-6} M.

The ability of O-acetylsalicyloyl chloride to react with aliphatic amines has been studied in the dilute aqueous solution. Taking piperidine as a model for aliphatic amines, the efficiency of in-situ derivatisation is high with 100% recovery for all concentrations studied. The detector response is also linear down to 10^{-6} M concentration level of piperidine derivatised.

However, for aromatic amines which are weakly basic, the derivatisation was not completed in the dilute aqueous solution.

The derivatisation of aliphatic amines with Bolton-Hunter and Wood's reagents in aqueous solution have also been carried out. The results obtained for these reagents suggested that HPLC with electrochemical detection can be monitored by using the amine derivative from Bolton-Hunter and Wood's reagent but there are practical difficulties of resolution and because of the need of an ion-pair reagent.

Finally, the dansyl derivatives should be further studied using electrochemical detection , which can be used together with fluorescence detection.

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