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AN INVESTIGATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF AMPHETAMINES

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SUMMARY

Three pre-column derivatization reagents, namely *o*-phthalaldehyde, 4-chloro-7-nitrobenz-2,1,3-oxadiazole, sodium naphthaquinone-4-sulphonate and two ion-pair reagents, namely, naphthalene-2-sulphonate and sodium dodecylsulphate have been investigated for their suitability for the qualitative and quantitative analysis of urine and plasma samples containing amphetamines. The derivatization method employing sodium naphthaquinone-4-sulphonate was found to be selective and sufficiently sensitive for the routine determination of amphetamine and methylamphetamine in urine and plasma samples at the ng/ml level.

INTRODUCTION

The continual abuse of amphetamine for its central stimulant effects has led to thorough studies concerning their metabolism, distribution and excretion [1—4]. Many analytical procedures have been developed for qualitative and quantitative purposes especially in toxicology and forensic science. They include colour tests [5, 6], physicochemical methods [7, 8], UV spectrophotometry [9, 10], spectrofluorimetry [11, 12], microcrystallography [13], immunological methods [14], and all forms of chromatography: thin-layer chromatography (TLC) [15, 16], gas—liquid chromatography (GLC) [17, 18], GLC—mass spectrometry (MS) [19, 20] and high-performance liquid chromatography (HPLC) [21, 22]. The latter technique has not been widely applied to the analysis of amphetamine because its low specific extinction value (9.7 at 257 nm in water) renders the UV detector of limited value, and also amphetamine has very little natural fluorescence.

The purpose of this study was to overcome these detection difficulties and various techniques have been compared for their practical applicability for the monitoring, screening and analysis of amphetamines and related sym-

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pathomimetic amines having low UV absorptivities. One of the most important advantages of HPLC over GLC [23] is its ability to analyse aqueous samples, especially plasma and urine, with minimum sample preparation. Improvements in the detection of amphetamines would therefore permit rapid and selective methods to be devised having adequate sensitivity.

EXPERIMENTAL

Reagents and materials

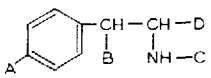
Acetonitrile, chloroform, cyclohexane, ethyl acetate, hexane, methanol, propan-2-ol and tetrahydrofuran of HPLC grade, and pentan-1-ol of laboratory reagent grade, were supplied by Fisons (Loughborough, Great Britain). Mercaptoethanol and methyl isobutyl ketone (MIBK) were from BDH (Poole, Great Britain). The water used for the preparation of aqueous mobile phases was double distilled from an all-glass still and deionised by passage through an Elgastat deioniser (Elga Products, Buckingham, Great Britain). The derivatizing reagent 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD-Cl) was supplied by Phase Separations (Queensferry, Great Britain), *o*-phthalaldehyde (OPA) by BDH, and sodium β-naphthaquinone-4-sulphonate (NQS) by Kodak Eastman (Liverpool, Great Britain).

The following column packing materials were used: Hypersil-ODS (Shandon Southern, Runcorn, Great Britain), LiChrospher Si 100 and LiChrosorb Si 100 (Merck, Darmstadt, G.F.R.), Spherisorb ODS (Phase Separations) and Partisil (Whatman, Maidstone, Great Britain). Columns were packed using the upward slurry technique described by Bristow et al. [24], employing methanol—chloroform (20:80, v/v). The HPLC instrumentation used was assembled from commercially available components. Both constant flow-rate (Constametric III and Milton Roy Minipump, LDC, Stone, Great Britain, with pulse dampener) and constant pressure (Haskel Model 27502, Olin Energy Systems, Sunderland, Great Britain) pumps were used.

The structures of the amphetamines studied are given in Table I.

TABLE I

FORMULAE OF THE AMPHETAMINES AND RELATED COMPOUNDS USED



Compound	Source*	A	B	C	D
Phenethylamine	c	H	H	H	H
Amphetamine	a	H	H	H	CH_3
Methylamphetamine	a	H	H	CH_3	CH_3
Ephedrine	c	H	OH	CH_3	CH_3
Norephedrine	b	H	OH	H	CH_3
p-Hydroxyamphetamine	a	OH	H	H	CH_3
p-Hydroxynorephedrine	a	OH	OH	H	CH_3
p-Hydroxymethylamphetamine	a	OH	H	CH_3	CH_3

*a = Gift from Smith, Kline and French (Welwyn Garden City, Great Britain); b = May and Baker (Dagenham, Great Britain); c = BDH (Poole, Great Britain).

Whenever practical the temperatures of the mobile phase reservoir, the column and injector were controlled by immersion in a heated water bath. The output to the detector was recorded on a potentiometric chart recorder (Servoscribe IS RE 541.20, Smith Industries, London, Great Britain). The output was also monitored by a Perkin-Elmer (Beaconsfield, Great Britain) Sigma X Data Station. Samples were introduced on to the column by means of a loop valve (Rheodyne 7125, Jones Chromatography, Llanbradoach, Great Britain).

Two types of fluorimeter were used: Perkin-Elmer Fluorescence Spectrophotometer fitted with a xenon arc lamp and an LDC Fluoromonitor III, Model 1311, fitted with a standard low-pressure mercury and phosphor lamp, 370 nm excitation filter and 418—700 nm emission filter. The UV detectors were either a Cecil Model 2012 or a 212 variable-wavelength UV monitor using deuterium lamps (Cecil Instruments, Cambridge, Great Britain). Spectroscopic measurements were made using a Perkin-Elmer 550-S UV-VIS spectrophotometer.

Extraction

Samples of urine (20 ml) and plasma (10 ml) were made alkaline with 5 *N* sodium hydroxide to pH 11.4. The solutions were then introduced to columns (5-ml graduated pipettes) each containing about 1.5 g of clean XAD-2 resin (Amberlite, BDH). The columns were then washed with 10 ml water and the adsorbed solutes were eluted with 40 ml (for urine) or 20 ml (for plasma) of chloroform—isopropanol (3:1). Ethanolic hydrochloric acid (6 *N*, 100 μl) was added to the organic eluate which was then evaporated to dryness using a rotary evaporator (Buchi Rotavapor). The residue was dissolved in the appropriate solvent required for direct injection or derivatization.

The column for straight phase ion-pair HPLC was prepared using both methods described in detail by Crommen et al. [25]. The injection technique was preferred.

Ion-pair formation

Solutes for reversed-phase ion-pair formation with sodium dodecylsulphate (SDDS) were dissolved in mobile phase and chromatographed directly. The solutes for straight-phase ion-pair analysis were dissolved in aqueous stationary phase and then extracted into an equal volume of mobile phase and this was used for chromatography.

Derivatization

For UV detection [26]: Sample extracts were dissolved in 8% sodium hydrogen carbonate (1 ml) and an equal volume of 0.5% NQS solution was added. After heating in an oven at 70°C for 20 min, the aqueous solution was extracted (vortex mixed for 1 min) with an equal volume of chloroform, and the organic layer used for chromatography.

For fluorescence detection [27—29]: For reversed-phase: The OPA derivatizing reagent was prepared by dissolving OPA (200 mg) in methanol (2 ml) and undiluted mercaptoethanol (0.4 ml) was added. This solution was added to boric buffer (1 g boric acid in 38 ml water) adjusted to pH 10.4 with 4 *M*

potassium hydroxide. This reagent is stable for five days when protected from light and stored in a refrigerator. The biological sample extracts were taken up in ethanol (1 ml) and OPA derivatizing reagent (1 ml) was added. This solution was filtered prior to injection on to the column.

For straight-phase [30, 31]: Sample extracts were dissolved in 0.1 *M* sodium hydrogen carbonate (4 ml) and 1% NBD-Cl in MIBK (2 ml) was added. After shaking briefly the reaction vessel was heated at 80°C for 30 min. The upper (MIBK) layer was used for chromatography.

Definitions

The following expressions have been employed in order to assess various chromatographic parameters used in this study.

Resolution, R_s, of two adjacent solute bands is defined as being equal to the distance between the two band centres, divided by the average band width (W). Thus:

$$R_s = \frac{t_2 - t_1}{\frac{1}{2}(W_2 + W_1)} \tag{1}$$

where t is the elution time or chart distances of solutes 1 and 2. The selectivity factor, α, for two solutes with capacity ratios, κ_1 and κ_2 is given by:

$$\alpha = \frac{\kappa_2}{\kappa_1} \tag{2}$$

The functional group contribution towards retention may be defined as:

$$\tau = \log \alpha = \log \frac{\kappa_2}{\kappa_1} \tag{3}$$

where solutes 1 and 2 differ by a functional group and the reference solute 1 is taken as the parent compound.

RESULTS AND DISCUSSION

Reversed-phase ion-pair HPLC

The effect of changing the pairing-ion concentration in the mobile phase on retention (κ) was studied. The observed relationship (Fig. 1A) has been attributed to the retention mechanism in surfactant ion-pair HPLC [32] being a combination of ion-pair distribution and in situ ion exchange [33].

The initial sigmoidal effect can be attributed to an initial depletion of pairing-ion at low concentrations such that here retention is related to solute concentration and column load capacity. The influence of these side reactions is confirmed by the relationship between column efficiency and pairing-ion concentration (Fig. 1B) in which the plate height reaches a constant minimum at higher pairing-ion concentrations. This latter region produces reproducible κ values, whilst the maximum concentration is indicated by Fig. 1A suggesting a concentration range of approximately 2 to $12 \cdot 10^{-4}$ *M*. For the calibration

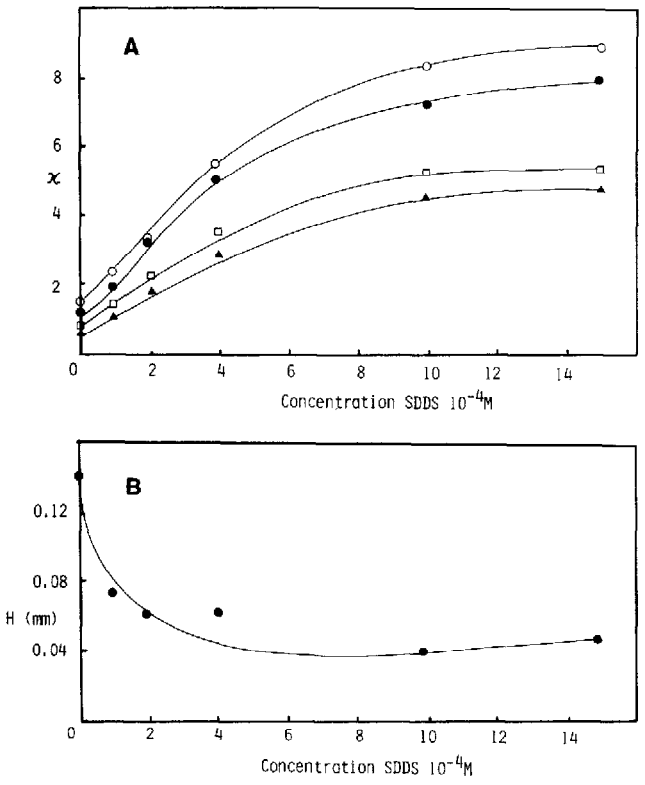


Fig. 1. Relationship between pairing-ion concentration and (A) the capacity ratio (κ) and (B) the plate height (H). Amphetamine (•), methylamphetamine (○), norephedrine (□) and ephedrine (▲). Conditions as in Table II for SDDS.

of amphetamine and hydroxyamphetamine, a pairing-ion concentration of $1 \cdot 10^{-3}$ M SDDS was used since this resulted in ideal capacity ratios and gave good column efficiencies ($h \approx 4$).

The estimations were made on the basis of peak height ratio using an internal standard and comparison with known standard solutions. The linearity of response was checked by a calibration curve of solutes dissolved in mobile phase in the concentration range 20—250 μg/ml. The correlation coefficient for amphetamine and hydroxyamphetamine using 5 points was 0.999 with coefficients of variation of the slopes of 0.8% and 1.8%, respectively. The peaks obtained (Fig. 2) were sharper than those in the other reversed-phase (OPA derivatization) method (see Fig. 7). There was some tailing but resolution was nevertheless 1.5 or greater, indicating complete separations. The limit of detection was approximately 5 μg/ml although this is lowered when samples of urine are processed (see Table III).

In general, metabolites of drug compounds are more polar than the parent compound since this facilitates urinary excretion by the kidney. In the reversed-phase mode, metabolites are generally eluted before the parent drug, which is an advantage when searching for metabolite peaks. The example in Fig. 2 is a good illustration of this point. Retention is due to the combined effects of ion-pair formation plus functional group contributions. The former

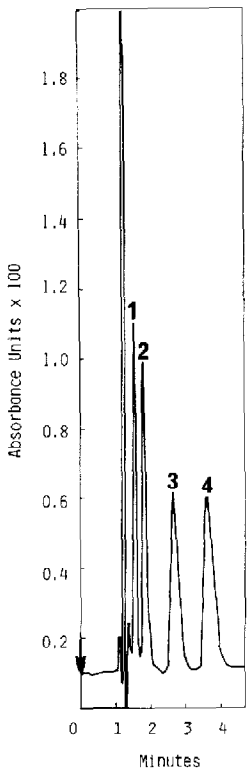


Fig. 2. Separation of amphetamine and three of its metabolites, dissolved in mobile phase. Conditions as in Table II, for SDDS. Peaks: 1 = *p*-hydroxynorephedrine; 2 = *p*-hydroxyamphetamine; 3 = norephedrine; 4 = amphetamine. Selectivity between adjacent peaks is 1.8, 2.1 and 1.6, respectively.

is a common factor, due to the protonated primary amino group in the four solutes. Differences in retention are due solely to differences in functional groups, which permit functional group contribution values, τ, to be calculated.

Aromatic hydroxylation of amphetamine and norephedrine produced falls in their κ values that may be expressed in terms of τ values as −0.52 and −0.51, respectively. The β-hydroxylation of amphetamine to norephedrine had less effect in reducing κ (τ = −0.21) probably due to steric hindrance of the hydroxyl group. Interestingly, the combined effect of aromatic hydroxylation plus β-hydroxylation as illustrated by the metabolism of amphetamine to *p*-hydroxynorephedrine, produced a fall in κ value equivalent to a τ value of −0.71, clearly demonstrating the additive effect of functional groups that do not interfere with one another. Functional group contribution values are relative and vary with solute type and reversed-phase material used. The percentage of carbon loading has a major influence on τ values, increasing the range of values obtained as carbon content is increased. The values obtained here are compatible with those of Riley et al. [34], who obtained −0.45 for the introduction of 4-OH into benzoic acid using a lower carbon-loaded material (Spherisorb-ODS).

A 24-h urine sample from a 22-year-old male subject was analysed using the developed assay. The urine was obtained 8 h after an unknown amount

of illegally obtained amphetamine sulphate had been taken. The pH of the urine was normal. Standard urine extracts containing 12.5, 25 and 50 μg/ml of amphetamine sulphate were used to compare peak height response to that of the clinical sample. The concentration of amphetamine in the urine was found to be 8.5 μg/ml corresponding to an original dose of 55 mg of amphetamine sulphate.

Straight-phase ion-pair HPLC

In this mode, highly UV-absorbing or fluorescing ions can be used as pairing-ions which permits extremely sensitive detection of non-UV-absorbing or non-fluorescing solutes. The ion-pair formed is lipophilic, increasing the solubility of the solute in the mobile phase.

Crommen et al. [25] published a method taking advantage of these factors for the resolution of amino acids, dipeptides and alkylamines. Using naphthalene-2-sulphonate (NS) as the highly UV-adsorbing pairing-ion contained in the stationary phase, they were able to separate and detect these compounds down to the 1-ng level. They compared their results with batch extraction data and showed that the retention of the cations, except the most hydrophobic, was due mainly to liquid—liquid distribution. The capacity ratio (κ) of the solute A^+ is given by:

$$\kappa_A = \frac{1}{E_{A,B}\,[B^-]} \cdot \frac{V_s}{V_m} \tag{4}$$

Where $E_{A,B}$ is the conditional extraction constant of the ion-pair, B^- is the pairing-ion and V_s/V_m is the phase volume ratio. In practice the capacity ratio can be varied to some extent by the concentration of the pairing-ion, by changing the nature of the pairing-ion, or by the regulation of $E_{A,B}$ by altering the composition of the organic mobile phase. The latter variable is the simplest method of varying κ values.

The choice of column packing material was based on surface area and pore size characteristics. In general, pore size is inversely proportional to surface area and for a support material to hold a maximum amount of stationary base, it should ideally have a large pore volume. LiChrospher Si 100 has spherical particles, 100 μm pore diameter and 1.2 ml/g pore volume.

Ion-pairs between naphthalene sulphonate and alkylamines have a high molar absorptivity (about 1400 at 254 nm). Naphthalene sulphonate is almost aprotic and can be used as a pairing-ion at a low pH (2.0) where distribution of many solutes, e.g. alkylamines, in non-charged form is negligible. This pairing-ion is very soluble in water and has a very low distribution to the organic mobile phase. The blank obtained with a mobile phase of chloroform and 1-pentanol depends on the amount of 1-pentanol present and the pH of the aqueous phase, both of which need to be determined before chromatographic conditions may be chosen. The curve showing the influence of pH (Fig. 3) indicates the narrow range providing minimal absorbance.

Solutions of NS in citrate buffer (disodium citrate and sulphuric acid, ionic strength 0.1) to be used as stationary phases were purified by repeated extraction with chloroform—pentanol (9:1) until the extract had a constant

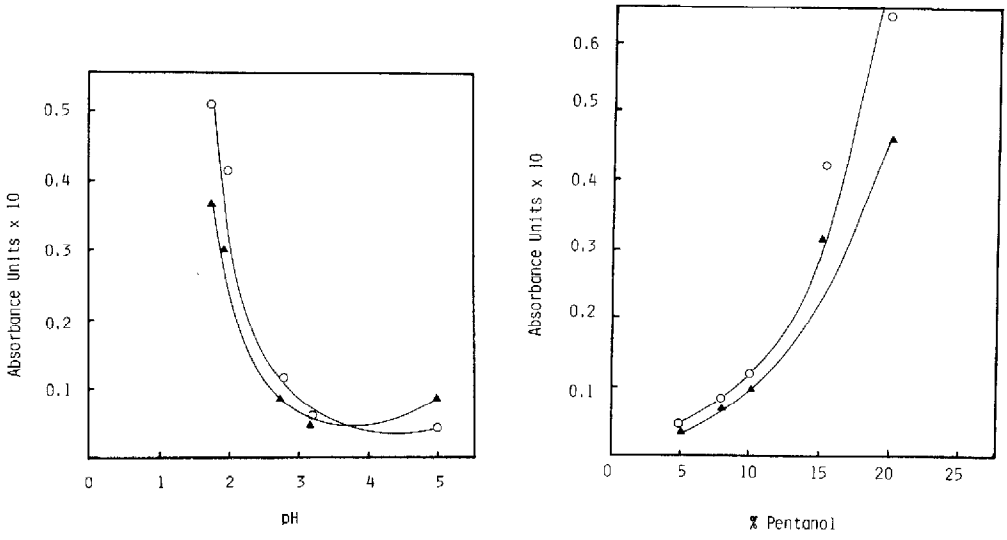


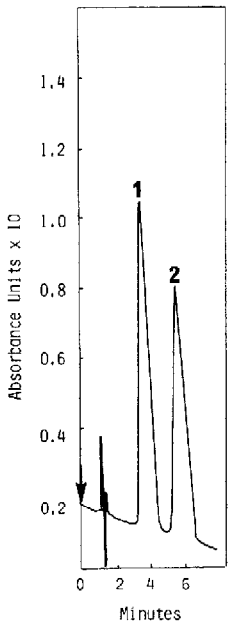
Fig. 3. Relationship between the absorbance of NS and the pH of buffer at 254 nm (▲) and 274 nm (○) at 10% pentanol and 0.1 *M* NS.

Fig. 4. Relationship between the absorbance of NS and the percentage of 1-pentanol in the mobile phase at 254 nm (▲) and 274 nm (○) at pH 3.0 and 0.1 *M* NS.

absorbance. The influence of 1-pentanol concentration on absorbance is shown in Fig. 4. From these graphs it was decided to use 10% 1-pentanol in the mobile phase and that the stationary phase should be buffered at pH 3.0.

The aqueous phase on the silica support material had a volume of approximately 1.0 ml/g of support, which is close to the specific pore volumes given by the manufacturer (1.2 ml/g). The complete filling of the pores is also illustrated by the fact that the fraction of the column volume occupied by the mobile phase after coating is 0.38—0.40, i.e. almost equal to the interstitial volume [25]. In order to ensure stable conditions during the recycling, the reservoir always contained 500 ml of mobile phase with a layer of stationary phase on the top. The mobile and stationary phase (on the column) was kept at a constant temperature (25°C) in a thermostatted water bath. This was necessary to maintain a constant solubility (and hence a constant background signal) of the NS in the mobile phase. The detector cell was kept at a higher temperature (35°C) than the mobile phase to ensure complete solubility of the stationary phase in it.

The log k values for amphetamine and methylamphetamine were found to be directly proportional to the logarithm of the molar concentration of pentanol and a mobile phase containing 10% pentanol was chosen as it gave a selectivity factor of 2 and resolution of 1.56 for amphetamine and methylamphetamine (Fig. 5). With a stable system, there was very little background signal, sensitivity was high and the columns gave good symmetrical peaks without tailing, due to the silica surface being inactivated by the aqueous stationary phase. Unfortunately, a persistent lack of stability arose from the loss of aqueous stationary phase from the columns, causing an increase in



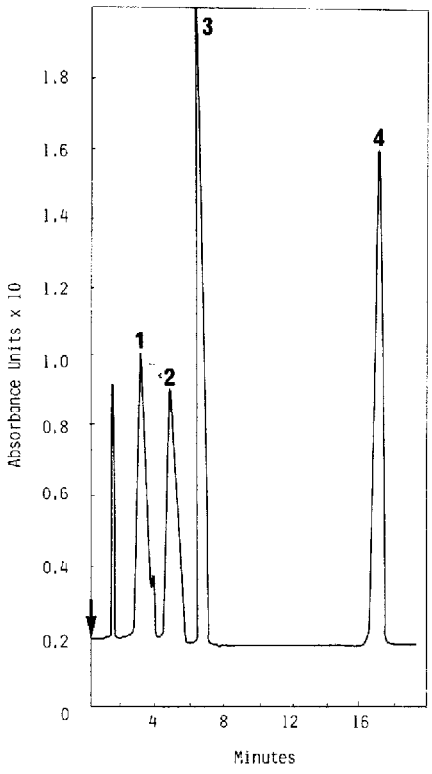


Fig. 5. Separation of methylamphetamine (1) and amphetamine (2) dissolved in mobile phase. Conditions as in Table II, for NS.

Fig. 6. Composite chromatogram of hydroxymethylamphetamine (1), phenylethylamine (2), hydroxyamphetamine (3) and hydroxynorephedrine (4) dissolved in mobile phase. Conditions as in Table II for NS.

background UV absorbance. Additionally, it was found that NS adhered to the quartz windows of the UV cell, eventually causing a full-scale deflection despite the precaution of increasing the temperature of the cell.

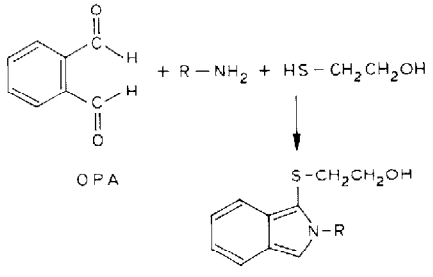
However, the results illustrated in Figs. 5 and 6 clearly indicate the excellent sensitivity of the method. Methylamphetamine and amphetamine were easily separated (α = 2) in 6 min, whilst the separation of three amphetamine metabolites and phenylethylamine gives some indication of the potential available. The elution order is clearly that expected in a straight-phase system, i.e. least polar compound eluted first. The addition of a β-hydroxy group produced a large increase in κ value, as shown by the retention of hydroxynorephedrine (Fig. 6, peak 4) compared with hydroxyamphetamine (Fig. 6, peak 3). Aromatic hydroxylation, as illustrated by hydroxyamphetamine compared to amphetamine gave a smaller increase in κ value. This difference in selectivity for β-hydroxy and aromatic hydroxy substituents is the opposite of that encountered in reversed-phase ion-pair HPLC. Secondary amines, i.e. methylamphetamine and hydroxymethylamphetamine (Fig. 6, peak 1), gave significantly lower κ values than their corresponding primary amines, i.e.

amphetamine and hydroxyamphetamine, the order of decrease being identical in both cases.

The method has been shown to be applicable to the amphetamines and potentially has both selectivity and sensitivity but further work is required to achieve column stability.

Reversed-phase HPLC with derivatization

The reaction of OPA in the presence of a strong reducing agent such as 2-mercaptoethanol was described by Roth [27] in 1971 for the fluorimetric detection of α-amino acids. Benson and Hare [28] found that: (a) it is five to ten times more sensitive than fluorescamine which is, in turn, more sensitive than ninhydrin used for UV detection of primary amines; (b) it has the advantage of being soluble and stable in aqueous buffers; (c) because of its aqueous solubility it is compatible with biological fluids and gives a stable baseline when mixed with an aqueous mobile phase; (d) OPA is considerably less expensive than fluorescamine and is therefore more economical for routine or automated use. Davies et al. [29] used it to detect nanogram quantities of biogenic amines. They compared their HPLC method to that of a GLC analysis and found it more sensitive. The fluorophore is produced as follows:



The excitation and emission maxima of OPA-amphetamine were found to be 345 and 445 nm, respectively. It also shows a UV-absorption maximum at 254 nm and spectrophotometric analysis showed that the absorbance is quantitative and sensitive at that wavelength. After studying the effect of varying methanol concentrations in the mobile phase, a composition of 73% was chosen as it gave a short overall analysis time (15 min) with resolution of 1.3 between peaks 1 and 2 and 2.6 between peaks 2 and 3 (Fig. 7). The stability of the derivatives was investigated by measuring the peak area ratios of three amphetamines injected every 30 min for 8 h. The results showed that maximum fluorescence occurs almost immediately and remains stable for at least 60 min. Thereafter the peak area decreases to a lower level where it is stable from 3.5 to 7 h. The calibration of amphetamine, hydroxyamphetamine and norephedrine produced a linear response with correlation coefficients of 0.997, 0.996 and 0.999, respectively, over the range 0.5—8 μg/ml. The percentage recovery of the drugs from biological fluids was high, ranging from 87% for hydroxyamphetamine to 98% for amphetamine.

Straight-phase HPLC with derivatization

UV detection. In 1922 Folin described a method for the determination of amino acid nitrogen which depended upon the combination of the amino

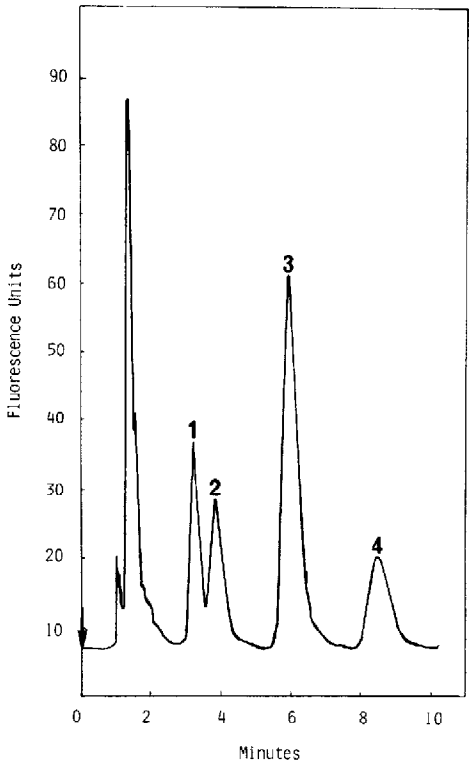
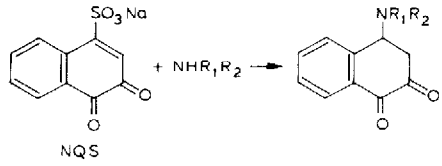


Fig. 7. Typical chromatogram of the OPA derivatives of norephedrine (1), hydroxyamphetamine (2), benzylamine (internal standard) (3) and amphetamine (4), prepared from underivatized standards added to urine. Conditions as in Table II. Unidentified peaks also occur in a urine blank. Suitable for plasma samples.

groups with 1,2-naphthaquinone-4-sulphonate (NQS) in alkaline solution to form highly coloured compounds which could be determined colorimetrically. Gürkan [35] applied the reaction to the investigation of sympathomimetic amines, including amphetamine. Absorbance maxima occurred in the visible region (447 nm) and in the UV region (245 nm). He found that sympathomimetic amines which contain a hydroxyl group, especially in the α-position, did not give a quantitative reaction. Also, after reduction with sodium borohydride, the coloured reaction products yielded an intense blue fluorescence. The fluorimetric analysis was more sensitive than UV detection but the fluorescent products were stable only for 10 min at room temperature. In 1978 Hashimoto et al. [16] used NQS for the quantitative analysis of phenylethylamine derivatives including amphetamine. The reaction products were isolated by TLC and subjected to elemental analysis, nuclear magnetic resonance (NMR), infra-red spectroscopy (IR) and MS. The results suggest that compounds have the general structure shown below.



Endo et al. [26] applied the method to the determination of amphetamine and methylamphetamine in urine by HPLC. They were able to detect 2 ng of drug at 280 nm, which was comparable to the sensitivity obtained by GLC. The mobile phase employed by us was similar to that used by Endo et al. and was a mixture of chloroform, ethyl acetate and hexane, saturated with water and one part of ethanol added after saturation. The water in the mobile phase "caps" the most active silanol groups on the silica packing material and the peaks produced are narrow and symmetrical. Retention was adjusted by varying the amount of ethyl acetate in the mobile phase. The separation of NQS-methylamphetamine from three of its metabolites and an internal standard under the chromatographic conditions listed in Table II is shown in Fig. 8.

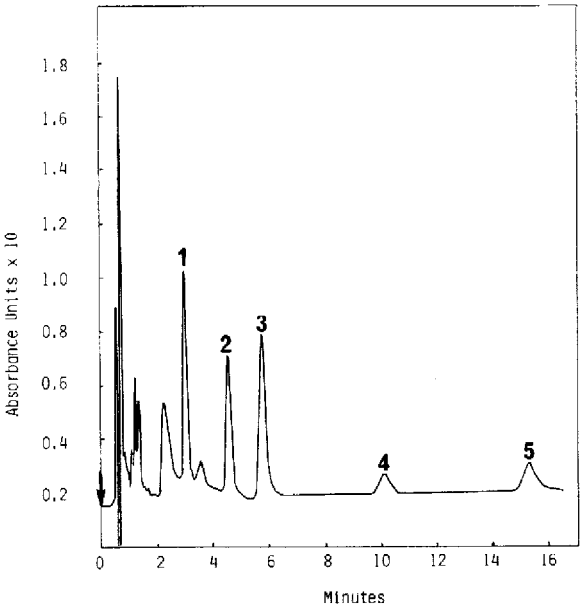


Fig. 8. Separation of the NQS derivatives of methylamphetamine (1), amphetamine (2), phenylethylamine (internal standard) (3), norephedrine (4) and hydroxyamphetamine (5) prepared from underivatized standards added to urine. Conditions as in Table II. Unidentified peaks also occur in a urine blank. Selectivity between adjacent drug peaks is 1.65, 1.31, 1.85 and 1.59. Suitable for plasma samples.

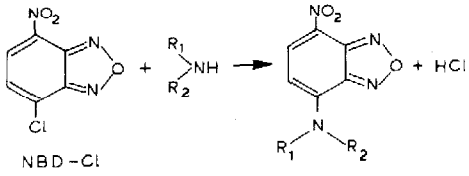
Hydroxyamphetamine and norephedrine were not included in quantitative analyses as they both contain a hydroxy group and according to Hashimoto et al. [16] do not react completely with NQS or form stable derivatives. The stability of other derivatives with time was investigated by injecting the samples into the chromatograph immediately after heating and extraction into chloroform and thereafter every 30 min for 8 h. The results using spiked plasma and urine extracts show that 30 min after removal of the reaction vessels from the oven, the derivatives had a stable maximum absorbance for at least 4 h. Injections were continued at 24-h intervals and the derivatives still showed good response after 60 h. The calibrations of NQS-methylamphetamine and NQS-amphetamine in water, plasma and urine produced linear

relationships between 0.5 and 5 μg/ml. All correlation coefficients were above 0.998. The limit of detection was 2 ng and confirmed that reported by Endo et al. [26]. The recovery of the drugs from biological fluids for methylamphetamine and amphetamine was 98 and 109% respectively (urine) and 88 and 95% respectively (plasma).

Fluorescence detection. Primary and secondary aliphatic amines react with NBD-Cl to produce intensely fluorescent derivatives. Although anilines, phenols and thiols also react with NBD-Cl, the derivatives produced have weak or no fluorescence, thus making the reaction more selective for amines. In 1968 Ghosh and Whitehouse [36] compared NBD-Cl with dansyl chloride and found that the former was more stable and soluble in aqueous solutions. Dansyl chloride and lutidine reagents are fluorescent and so produce significant blanks, whereas NBD-Cl is non-fluorescent. Another disadvantage of dansyl chloride and lutidine reagents is that they are non-specific and react with many naturally occurring amines in extracts of biological samples to produce interfering fluorophores.

Monforte et al. [37] used NBD-Cl for the analysis of amphetamines in blood and urine following TLC. MIBK was chosen as a solvent for NBD-Cl because the coupling reaction is faster in polar solvents such as alcohols or ketones but a solvent with a high boiling point was necessary since the reaction is carried out at 80°C (MIBK boiling point is 117°C) to reduce the reaction time for derivative formation.

Hopen et al. [38] chemically characterised the NBD-Cl derivative of amphetamine by NMR. They confirmed that the reaction with amphetamine proceeds as follows:



NBD-Cl

The production of hydrogen chloride requires the presence of a buffer to obtain a high product yield. The possibility of a hydrolysis reaction at the chlorine position of the reagent is prevented by the highly basic medium of the 0.1 *M* sodium bicarbonate buffer.

The excitation and emission maxima of NBD-amphetamine occur at 465 nm and 575 nm, respectively. Monforte et al. [37] showed that although the emission spectrum is not different from that of NBD-methylamphetamine, significant differences do exist in the excitation spectra and these may be used to distinguish between the primary and secondary amines. Chromatographic conditions for the separation and quantification of amphetamine and methylamphetamine are summarised in Table II. Using methylamine as an internal standard, the total elution time was 7 min. Resolution between the three solutes was 1.2 and 2.6 and selectivity was 5 and 3. The stability of the derivatives in biological fluid extracts with time was investigated over an 8-h period. Both were found to be extremely stable. Calibration of methyl-

TABLE II

SUMMARY OF CHROMATOGRAPHIC CONDITIONS EMPLOYED

	SDDS	NS	OPA	NQS	NBD-Cl
Internal standard	norephedrine 50 μg/ml	—	benzylamine 7.5 μg/ml	phenylethylamine 5 μg/ml	methylamine 10 μg/ml
Stationary phase	Partisil ODS-2 10 μm	LiChrospher Si 100, 10 μm, containing 0.1 *M* NS in citrate buffer *I* = 0.1, pH 3	Partisil ODS-2 10 μm	Partisil 5	Partisil 5
Column dimensions (mm)	250 × 5	150 × 5	250 × 5	150 × 5	200 × 5
Mobile phase	$1 \cdot 10^{-3}$ *M* SDDS in 0.01% H_2SO_4 (pH 2)—methanol (50:50)	10% pentan-1-ol in chloroform	0.2% EDTA in 73% methanol	chloroform—ethyl acetate—hexane—ethanol (25:35:50:1)	ethyl acetate—cyclohexane (40:60)
Injection volume (μl)	20	20	50	50	20
Detection wavelength (nm)	254	274	345 (ex) 445 (em)	248	465 (ex) 515 (em)
Temperature (°C)	20	25 35 (UV cell)	20	20	20
Flow-rate (ml/min)	1.0	1.0	1.8	2.5	2.0

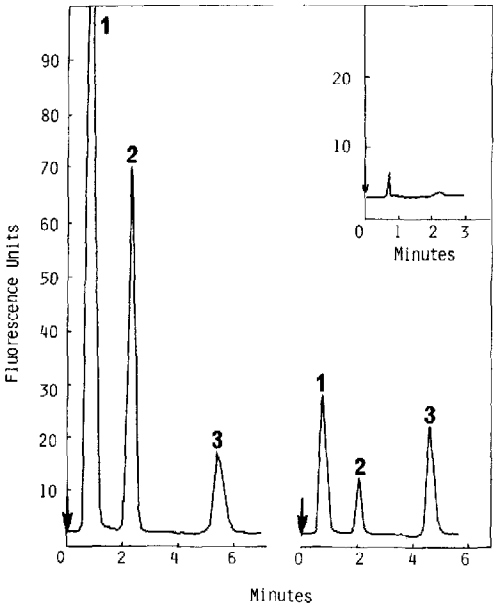


Fig. 9. Typical calibration chromatograms of the NBD derivatives of amphetamine (1), methylamphetamine (2) and methylamine (internal standard) (3). Conditions as in Table II. Inset: NBD blank. Suitable for urine and plasma samples.

amphetamine and amphetamine in water, plasma and urine over the range 0.5 to 5.0 μg/ml were found to be linear with correlation coefficients better than 0.996. Recovery of the derivatives from biological fluids was high. A typical chromatogram is shown in Fig. 9.

CONCLUSION

Both straight- and reversed-phase modes of HPLC have demonstrated their suitability for the resolution of methylamphetamine, amphetamine and their metabolites. Straight-phase columns are inherently highly efficient, elute the drug followed by the more polar metabolites, but require protection from irreversible adsorption that causes a fall in column efficiency. Reversed-phase columns can be highly efficient, elute the more polar metabolites before the drug, and are more tolerant towards the injection of complex biological solutions.

The starting point of this study was the reversed-phase ion-pair system employing SDDS, which clearly demonstrates the excellent chromatographic properties available and for which an improved detection system is required. Table III summarises some of the features of the HPLC systems studied to provide improved detection. The minimum detectable amount of drug for each method has been given for various injection solutions. For standard solutions the quantity represents the amount injected and expresses the sensitivity of the detection system. The levels recorded for urine and plasma samples depend upon the volumes of these materials available. For urine, 20-ml samples enable a concentration factor of ten times to be readily ob-

TABLE III

SUMMARY OF SOME OF THE FEATURES OF THE FIVE HPLC SYSTEMS STUDIED

Consideration	SDDS ion-pair	NS ion-pair	OPA derivatization	NQS derivatization	NBD-Cl derivatization
1. Sensitivity					
standards (ng)	100	20	30	2	5
Urine (20 ml; ng/ml)	250	not	60	4	25
Plasma (1 ml; ng/ml)	—	determined	600	20	125
2. Reliability	excellent	poor	good	good	poor
3. Application	primary and secondary amines non-specific	primary and secondary amines non-specific	primary amines only specific	primary and secondary amines specific	primary and secondary amines specific
4. Versatility	(a) chromatographic conditions easily changed	(a) chromatographic conditions not easily changed	(a) chromatographic conditions easily changed	(a) chromatographic conditions easily changed	(a) chromatographic conditions easily changed
	(b) UV detection	(b) UV detection	(b) UV and fluorimetric detection	(b) UV and fluorimetric detection	(b) fluorimetric detection
5. Speed of analysis	(a) preparation fast and simple	(a) preparation lengthy	(a) preparation relatively fast and simple	(a) preparation relatively simple	(a) preparation fairly lengthy
	(b) no heating, derivatization or extraction necessary	(b) extraction, but no derivatization or heating	(b) derivatization but no extraction or heating	(b) derivatization, heating and extraction	(b) derivatization, heating and extraction
	(c) chromatography 5 min	(c) chromatography 7 min	(c) chromatography 10 min	(c) chromatography 10 min	(c) chromatography 8 min
6. Stability	excellent	poor	derivatives stable for 1 h	derivatives stable for 4 h	derivatives stable for 7 h
7. Practical operation	very simple	complex	simple	simple	hazardous and prone to contamination

tainable using the XAD-2 resin extraction process. Plasma samples (1 ml) can, with care, be processed in the same way to obtain an injection solution of 1 ml, and for the NQS and NBD methods a concentration step is possible by evaporation of the final organic solvent (1 ml) and solvation of the residue in 250 μl solvent. For all the HPLC systems, sensitivities can be improved by increasing the volume injected, up to 100 μl, although the loss in resolution will depend upon the column efficiency.

After an oral dose of 10—15 mg amphetamine sulphate, peak plasma concentrations of 40—50 ng/ml are attained in 1—2 h, falling to about 2 ng/ml after 8—10 h [39]. This study has shown that only the method employing derivatization with NQS is able to provide the required sensitivity. It was found to be a simple, reliable and rapid method, suitable for the routine determination of amphetamine and methylamphetamine in plasma or urine. It is not suitable for hydroxylated metabolites, but these form a very minor percentage of the excreted forms. The reagent employed in the NBD method was found to accumulate in the injection valve and glassware, despite repeated and thorough rinsing. The reaction products were extremely darkly coloured and left a sticky residue upon evaporation. Although linear calibrations were obtained, the derivatization reaction was found to be unreliable for routine use. The OPA method was simple, reliable and rapid, but did not provide us with the sensitivity required. The straight-phase system employing NS had better sensitivity but we were unable to maintain column stability. Our studies on the methods of improving the detection of amphetamines and related compounds are continuing.

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