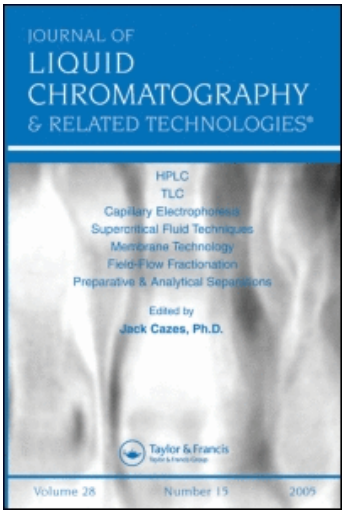

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Amphetamine and Methamphetamine Determinations in Biological Samples by High Performance Liquid Chromatography. A Review

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AMPHETAMINE AND METHAMPHETAMINE DETERMINATIONS IN BIOLOGICAL SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. A REVIEW

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Abstract: This critical review shows the different high performance liquid chromatography methods proposed for amphetamine and methamphetamine determinations. It is directed mainly towards sample clean up and derivatizations steps, because of their significance in such determinations.

INTRODUCTION

Amphetamines are psychoactive substances that have been abused both in society in general and in sports. However, a number of isomeric forms of these sympathomimetic amines have found therapeutic applications as analeptics, stimulants, anorexigens, in combination with antipyretics, etc.

The psychostimulant amines are all synthetic in origin, and possess phenylisopropylamine as common basic structure. Amphetamine is a phenolic derivative with a ramified aliphatic

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amine lateral chain. Amphetamine and methamphetamine both possess an asymmetric carbon which confers a number of isomeric forms (d, l and dl).

The half-life of amphetamine is about 20 hours *in vivo*. The compound is partially hydrolyzed by microsomal liver enzymes, and 30-40% of it is excreted in active form in urine within 48 hours of administration. Urine also contains p-hydroxiamphtamine, 3% as conjugated benzyl methyl ketone, benzoic acid and traces of conjugated 1-phenyl propan-2-ol. Methylamphetamine is fundamentally excreted without modification, with only a small proportion being demethylated to amphetamine (1). The excretion of amphetamines is markedly affected by urine pH (2).

A number of methods have been proposed to assay these analytes. Thus, in 1985 Rasmussen et al. (3) reviewed the analytical techniques developed to detect and identify amphetamines and amphetamine-like substances in non-biological samples. They concluded that infrared and ultraviolet spectroscopy are the most frequently used spectroscopic methods, while thin layer chromatography (TLC) and gas chromatography (GC) are the workhorse separation methods. Budd (4) contrasted the advantages and disadvantages of GC, enzyme multiplied immunoassay technique (EMIT) and radioimmunoassay (RIA). GC was found to be the best method, although the number of analyses obtained was lower than that afforded by the other techniques.

Figure 1 shows the number of publications on amphetamine determination in the literature in the past 10 years (information obtained from Chemical Abstracts). The analytical techniques most used are GC, EMIT, RIA and high-performance liquid chromatography (HPLC). The situation is similar in the case of methamphetamine. These analytical procedures have been applied to pharmaceutical samples and, to a lesser degree, to biological specimens. Most publications to date have employed GC.

In the last few years new methods have been proposed to assay methamphetamine and related

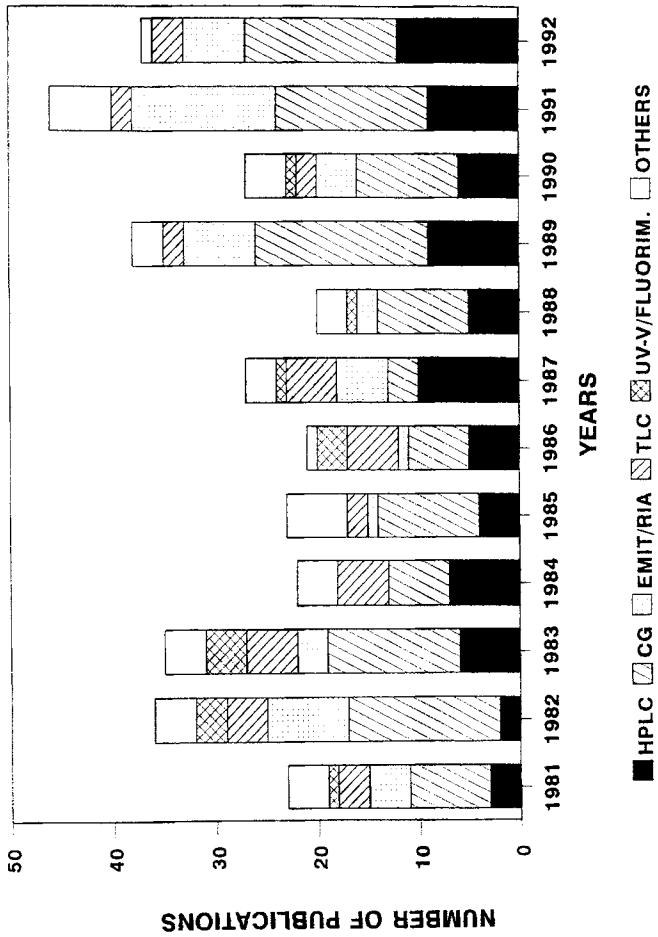

FIGURE 1. Bibliographic evolution of the published papers about the analysis of amphetamine by different techniques during last decade.

compounds by HPLC, this in turn being reflected by an increase in the number of publications. However, only 25% of HPLC papers involve biological samples.

The aim of the present review was to evaluate the different HPLC methods proposed in the literature for assaying amphetamine and methaphetamine samples, in an attempt to establish the most suitable technique.

SAMPLE CLEAN-UP

Table 1 shows that liquid-liquid extraction is used for sample clean-up before HPLC determination. Solvents such as diethyl ether (Et_2O) in strong alkali pH (5-8), and n-hexane (9) have been used in liquid-liquid extraction. Amphetamine and related compounds have pKa values of about 9.9, and an alkaline medium is required.

Ion-pair extraction has been employed by Hoogewijs et al. (10) to analyze basic drugs through the direct injection of extracts into the liquid chromatography column. The extraction efficacy of Na-n-octylsulfate as ion pairing reagent was compared with that of bis (2-ethylhexyl) phosphate. Direct injection of the ion-pairing extracts into the column was possible because the retention behaviour was independent of whether the basic drugs were injected as an ion-pair or as a base.

Reference (11) shows a clean-up procedure based on the precipitation and extraction of methamphetamine with acetone (Table 1).

In recent years, a trend to use phase-solid extraction has been observed. Sekine et al. (12) quantitatively extracted amphetamine, methamphetamine, methylephedrine and p-hydroxymethamphetamine in urine with a Sep-Pak C_{18} cartridge. Each drug could be almost quantitatively extracted at pH 8 and with sample flow rates of between 2.5 and 5.0 ml/min. These authors (13) proposed automatic extraction using an ODS-minicolumn (25 mm x 9 mm i.d.) for analyzing drugs of abuse in biological fluids. Patel et al. (14) isolated amphetamine and methamphetamine from urine using polymer-based C_{18} extraction cartridges. The eluent

was injected directly into the HPLC column for analysis. The extraction principle involved hydrophobic interaction using ion pairing with hexanesulfonic acid before sample application. The extraction was linear between 5.0 and 25 μg/ml.

A simple and reproducible column extraction procedure, using a silica based mixed phase bonded chromatographic column was described for the screening and confirmation of drugs in horse urine by TLC and gas chromatography-mass spectrometry (GC-MS), respectively. Amphetamine and methamphetamine exhibited < 25% recovery by liquid-liquid extraction when dichloromethane (DCM)-isopropanol (1:3 v/v) were used as extraction solvents; in turn, a recovery of over 85% was achieved by column extraction (15).

Farrell and Jefferies (16) used solid-phase extraction for sample clean-up in an investigation of different HPLC methods for analyzing amphetamines (Table 1).

Recently, Helmlin and Brenneisen (17) extracted phenylalkylamine derivatives, such as methylenedioxymethylamphetamine (MDMA) and methylenedioxyamphetamine (MDA), from urine samples on an Adsorbex SCX (100 mg) cation exchange solid-phase extraction column. Recoveries higher than 98% were obtained (Table 1).

In other procedures the sample was subjected to analyte derivatization, followed by liquid-liquid extraction of the reaction products to remove excess reagent and undesired compounds present in the sample (18,19). Suitable detection limits are achieved by these procedures (Table 1).

Some authors (20) have used urine samples containing methamphetamine directly without either derivatization or extraction.

Other procedures have been described (21-23) to determine amphetamine in physiological fluids using on-line solid phase derivatization and reversed-phase liquid chromatography. The samples were filtered (21-22) or diluted (23) prior to injection in the system. Koning et al. (34) also used dilution of the sample and injection into an on-line derivatization system with naphthalenedialdehyde as fluorigenic label.

TABLE 1. Analytical properties of the different methods proposed for the analysis of amphetamine and methamphetamine by HPLC.

Drug separated	Source	Sample Clean-up	Derivatization	Stationary phase	Mobile phase	Detector	Detection limit	Note	Ref.
Methamphetamine	Urine and Plasma	----------	NQS	μBondasphere C_{18} 5 μm	acetonitrile-methanol-0.01M sulfuric acid (20:20:70)	EC	1 ng/mL	The amine-derivatives was extracted with (n-hexane-ethyl acetate) 1:1. Spiked urine samples.	19
Methamphetamine	Urine	----------	----------	Asahipak GS-320M	0.02M $NaHCO_3$ 0.02M Na_2CO_3(8:2) 40°C 1.0 mL/min	UV 207 nm	--------	Linear interval 1-10 μg/mL.	20
Amphetamine	Urine	Filtration at pH=10	Polymeric reagents contained an activated ester linkage to the 9-fluororenyl group (FMOC-Cl).	LichroSpher C_{18}, 5μm	50/50, 80/20, acetonitrile/water	UV 254 nm Fluorescence (265/320nm)	0.1 μg/mL	% of derivatizations are 90%-70% for 1st and 2nd amines. Spiked urine samples.	21
Dl-amphetamine	Urine	Filtration at pH=10	FMOC-L-proline	C_{18}	(40-48%) acetonitrile-water	UV, Fluorescence	50 ng/mL	The linearity of the overall measurement was 3-4 orders of magnitude of concentration. Spiked urine samples.	22
Amphetamine	Plasma	Dilution	9-fluoreneacetyl-tagged (FA)	Supelcosil LC-ABZ, 5 μm	Step gradient from 1 mM SDS in ACN-water(10:90 v/v) to 1 mM SDS in ACN-water (55:45 v/v) for analytical separation	Fluorescence (254/313 nm)	0.2 μg/mL in plasma	On line derivatization in ACN:water (10:90 v/v). Linear range 2-40 μg/mL with 1 mM SDS at 75°C for 8 min. Spiked plasma samples	23
Amphetamine	Human and horses urine	Concentration in precolumns switching technique	----------	Spherisorb ODS-2, 5μm	0.001M perchloric acid-0.1M sodium perchlorate in water/MeOH	UV 210 nm	20 ng/mL	Microbore HPLC gradient. Determination limit 0.1 μg/mL. Spiked urine samples.	24
Amphetamine, Methamphetamine	Urine	Switching technique with two precolumns	----------	C_8 column and silica column	Buffer pH=6.5 and acetonitrile	UV 210 and 235 nm	0.2 μg/mL	Linear range 0.3-10 mg/L. Suspected urine samples MA: 8.2 mg/L; A: 1.8 mg/L.	25
Amphetamine	Standard	--------	Naphthalene-2,3-dialdehyde, Anthracene-2,3-dialdehyde.	Lichrosorb RP-18 5μm	Acetonitrile-water(70:30) containing 2.5mM imidazole	Fluorescence, Chemiluminescence with peroxyoxalate	$>10^{-8}$M	The derivates were extracted with toluene:hexane (1:1) and diluted with dichloromethane	33
Amphetamine	Plasma	Diluted as much as 200- fold	Naphthalenedialdehyde	ODS-2-Chromospher C_{18}, 5μm	Gradient elution A- THF-potassium phosphate buffer (pH=6.8 0.05M) (5:95) B-acetonitrile-MeOH-potassium phosphate buffer (pH=6.8 0.05M) (55:10:35)	Fluorescence	50 fmol of A.(50 μL sample injected)	Automated precolumn derivatization. This reaction is carried out in aqueous solution at pH 9.3 in 15 min. Linear range 10 fmol-100 pmol injected.	34
Amphetamine, methamphetamine	Standards	---------	9-fluorenyl methyl chloroformate (FMOC-Cl)	Nucleosil C_{18} (NM) 5μm	acetonitrile-water (58:42, v/v)	UV, 265 nm	---------	On line preconcentration and derivatization. Linear in the range $2x10^{-9}$ - $1x10^{-7}$ M	36

TABLE 1 (continued).

Drug separated	Source	Sample Clean-up	Derivatization	Stationary phase	Mobile phase	Detector	Detection limit	Note	Ref.
Amphetamine (A)	Urine	Liquid-liquid (L/L) 1) Et_2O 2) HCl	o-phthaldialdehyde (OPA)	RP-18 (10μm)	MeOH:H_2O (75:25)	Fluorescence 450/350nm	Detection limit 0.2μg/mL	Linear interval 0.25-3μg/mL Spiked urine samples	5
Amphetamine, methamphetamine (MA)	Urine	L/L Et_2O, alk. pH	different reagents: dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole, naphthalene-2,3-dicarbaldehyde.	Inertsil ODS-2	1.0x10^{-3} M imidazole in acetonitrile-water.	Fluorescence Chemiluminescence after reaction with Bis(2,4,6-trichlorophenyl) oxalate hydrogen peroxide in acetonitrile.	For Dns-derivatives 3x10^{-15}-4x10^{-15} mol	Dns-Cl was the most suitable. Recovery with derivatization Dns 99.3$\pm$0.7% for (MA). Spiked urine samples	6
Amphetamine, methamphetamine and piperidine	Urine	L/L Et_2O, alk pH	Dansyl chloride	Inertsil ODS-2	Acetonitrile-water(7:3), 1mM imidazole pH 7	Chemiluminescence after reaction with bis(2,4,6-trichlrophenyl) oxalate and hydrogen peroxide	2x10^{-10}M (MA) in urine	Sensitivity was higher than that of Simon's reagent test and CG. Suspected urine samples MA: 1.3x10^{-5} to 3.6x10^{-8}M; A: 3.5x10^{-5} to 3.3x10^{-6}M.	7
Amphetamine, methamphetamine	Urine	L/L Et_2O	ABEI N-(4-amino-butyl)-N-ethyl-isoluminol plus N,N'-disuccinimidyl carbonate in presence of methanol and trimethylamine	RP-C_{18}	MeOH:water (54:64) containing 30mM Na-octanesulfonate	Chemiluminescence after reaction with K-ferricianide, NaOH, H_2O_2 and β-ciclodextrin	Detection limit 20 fmol(MA) and 100 fmol(A). 10^{-7}M in urine for (A)	Linear interval until 5 pmol (MA) and 10 pmol(A). Spiked urine samples.	8
Amphetamine, Methamphetamine	Urine Plasma	L/L n-hexane	Sodium β-naphthoquinone-4-sulphonate (NQS)	Wakogel LC 5H 5μm LiChrosorb SI 100 10μm	$CHCl_3$:ethyl acetate:ethanol:n-hexane(25:10:1:50)	UV 450 and 280 nm	MA: 464 nm 5ng 280 nm 2 ng	Linear range in the visible 0.25-2 μg for both A and MA. Suspected urine samples.	9
Methamphetamine	Serum	Precipitation with acetone	ABEI N-(4-aminobutyl)-N-ethylsoluminol plus N,N'-disuccinimidyl carbonate in presence of methanol and trimethylamine. Heat 80° 30'	Shimpack CLC-C_{18}	MeOH:water (54:64) containing 30mM Na-octanesulfonate	Chemiluminescence after reaction with K-ferricianide, NaOH, H_2O_2 and β-ciclodextrin	Detection limit 20 fmol (100 μL of serum were taken)	Linear range 0.05-5 pmol. Comparable sensitivity CG-MS and Fluorescence-HPLC. Recovery for MA was 99%. Addict serum sample, MA: 3.8 μg/mL.	11
Amphetamine	Urine and plasma	Solid phase: XAD-2	OPA, NBD-Cl, NQS, NS, SDDS.	Partisil 5, LiChrospher Si 100, Partisil ODS-2.	Several	UV/ Fluorescence	Urine 4 ng/mL Plasma 20 ng/mL	Addict urine sample, A: 8.5 μg/mL with SDDS derivatization. The best results were obtained with NQS.	16
Phenylalkylamines (amphetamine, methamphetamine etc...)	Biological matrix (urine and plants material)	Solid phase: Adsorbex SCX(100 mg) cation exchange	-------	3μm Spherisorb ODS-1	Acetonitrile:water (72:928 v/v), 5mL ortophosphoric acid (85%), 0.28 mL hexyl amine per 1000 mL	Photodiode array UV 198 and 205 nm		Separation isocratic. Linear graphs	17
Basic Drugs (Amphetamine, Methamphetamine etc...)	Biological fluids	---------	NQS	μBondasphere C_{18}	MeCN-methanol-0.01M sulfuric acid	Electrochemical detection (EC)	< 10 ng/mL	Only 50 μL of biological fluid containing these drugs at > 1μg/mL was required for determination with good accuracy. Spiked urine samples.	18

Slais et al. (24) have screened amphetamines in human and horse urine. Urine samples can be screened for amphetamines without off-line pre-treatment or derivatization by combining the use of a two pre-column series for sample clean-up and enrichment with a period of selective solute displacement. The reproducibility of the peak areas is acceptable for quantifying the amphetamine below the 1 μg/ml level (the detection limit in urine was 20 ng/ml)(Table 1). Another switching system was proposed by Binder et al. (25) for the automated analysis of basic drugs in urine. The authors used two polymeric pre-columns to isolate the drugs, and a reversed-phase column coupled to a silica column produced the analytical separation. A urine specimen which was positive for amphetamines by TLC was analyzed by this procedure. Concentrations were determined by comparison with urine samples supplemented with known concentrations of drugs: concentrations were amphetamine 1.8 mg/l and meth-amphetamine 8.2 mg/l.

A new on-line method for HPLC using zone electrophoretic sample treatment has recently been introduced (26-27).

DERIVATIZATION

Derivatization has long been accepted as an effective modification technique in HPLC, improving overall specificity, chromatographic performance, and sensitivity of the original trace analysis.

Kinberger (5) incubated amphetamine with o-phthalaldehyde (OPA) at room temperature prior to chromatographic analysis. The precolumn fluorescence derivatization with OPA allowed a sensitive HPLC determination of amphetamine in urine samples, as seen in Table 1.

Derivatization of sympathomimetic drugs with OPA in the presence of different thiols (2-mercaptoethanol, ethanethiol and tert-butylmercaptan) has also been used (28). Some structural assignments of the OPA-amino drug adducts were proposed and the electroactive

properties of these substituted isoindolic products were investigated by voltamperometry. HPLC with chemiluminescence detection (CL) is highly sensitive and selective for fluorescent compounds (29). The CL detection thresholds of several fluorescent compounds were 10-100 times lower than those obtained by fluorescence detection.

For the determination of trace levels of amphetamine-related compounds Hayakawa et al. (6) studied fluorigenic derivatization. Bis (2,4,6-trichlorophenyl) oxalate and hydrogen peroxide in acetonitrile were used as post-column chemilumigenic reagent. As precolumn derivatization reagents, dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole (NBD-F) and naphthalene-2,3-dicarbaldehyde (NDA) fluorigenic reagent, which reacts only with primary amines in the presence of cyanide, were compared by these authors. NBD-F reacts with both primary and secondary amines; the reaction is very rapid, which constitutes an advantage over NBD-Cl (30,31). The main disadvantage of NBD derivatives was that the sensitivity by chemiluminescence detection was less than that by fluorescence detection. NDA derivatives could be detected with 10 times greater sensitivity than dansyl derivatives, and over 50 times greater than NBD-F derivatives. However, Dns-Cl was the best derivatization agent for simultaneously determining primary and secondary amines. Only diethyl-ether extraction was necessary as clean treatment before Dns-derivatization. The detection limits of Dns-derivatives were 3×10^{-15} - 4×10^{-15} M (Table 1) with chemiluminescence detection (6). The method was more sensitive than GC-MS by a factor of 70 for NDA derivatives and by 3.5 for Dns derivatives. Methamphetamine was detected as low as 1.0×10^{-7} M in urine without any interfering peak at the corresponding retention time. In another paper (7), these authors identified the dansyl derivatives of these compounds by mass spectrometry in the corresponding peaks from suspected human urine. The concentrations of amphetamine, methamphetamine and piperidine in 6 suspected human urine samples were detected in the range 3.5×10^{-5} - 3.3×10^{-6} M, 1.3×10^{-3} - 3.6×10^{-8} M and 7.3×10^{-5} - 2.4×10^{-6} M, respectively. The detection limit for methamphetamine was 2×10^{-10} M in urine (Table 1).

Nakashima et al. (11) determined methamphetamine concentrations in human serum using N-(4-aminobutyl)-N-ethylisoluminol (ABEI) as precolumn labelling chemiluminogenic reagent. The greatest reaction was obtained at 80°C for 30-60 min. This method was very sensitive and could detect as little as 20 fmol of methamphetamine when 100 μl of serum sample were employed (Table 1). A serum sample from a methamphetamine addict, which contained 3.6 μg/ml methamphetamine as determined by GC, was analyzed. The amount of methamphetamine determined was 5.05 $\pm$ 0.07 μg/ml. The sensitivity was comparable that of the GC-MS (32) and HPLC-fluorescence method (6), but slightly lower than that afforded by the HPLC-peroxyoxalate chemi-luminescence method (6). Recently, these authors (8), have used ABEI reagent for the determination of methamphetamine and its metabolite amphetamine in human urine. The reported detection limit is higher for amphetamine, as seen in Table 1.

Naphthalene-2,3-dialdehyde (NDA) and anthracene-2,3-dialdehyde (ADA) as pre-column reagents for the peroxyoxalate chemiluminescence detection of primary amines using reversed and normal-phase liquid chromatography were used by Kwakman et al. (33). The derivatization reaction time was 20 min. at room temperature for NDA and ADA; however, NDA derivatives were stable, while ADA derivatives were unstable - probably owing to oxidation of one of the aromatic rings. A serious disadvantage was the formation of cyanide-induced side-products which were major interferences in reversed-phase chromatography. Unfortunately, for both NDA and ADA amphetamine derivatives in reversed-phase HPLC, the interfering peaks begin to dominate the chromatogram at the 10^{-8} M even with standard solutions, so that real trace - level analysis cannot be carried out.

Koning et al. (34) proposed an automated precolumn derivatization of amino acids, small peptides, brain amines and drugs with primary amino groups (amphetamine in urine or plasma) by reversed phase-HPLC using naphthalenedialdehyde (NDA) as fluorigenic label. The NDA-cyanide combination was transparent to the fluorescence detector in the absence

of analytes, so removal of the excess of reagent was not required after derivatization.

Another fluorescent labelling reagent (4-(N,N-dimethylamino-sulfonyl)-7-fluoro-2,1,3-benzoxadialzole (DBD-F) and a mixture of hydrogen peroxide and bis (4-nitro-2-(3,6,9-trioxadecyloxy-carbonyl) phenyl) oxalate in acetonitrile as postcolumn chemiluminogen reagent were used to detect methamphetamine and related compounds in urine samples by chemiluminescence. The detection limits were 27 and 100 fmol for methamphetamine and amphetamine, respectively (35).

Sulphonate group displacement in an aromatic reagent can be the basis of a derivatization procedure for the determination of low concentrations of amines. The amino groups with Na-naphthoquinone-4-sulphonate (NQS) in alkaline solution form highly colored compounds that could be determined colorimetrically. Endo et al. (9) applied the method to the determination of amphetamine and methamphetamine in urine by normal-phase HPLC. The absorption maxima for the amphetamine and methamphetamine derivatives were 451 nm and 464 nm, respectively. Derivatization resulted in an approximately 25-fold increase in sensitivity in the visible range. Farrell et al. (16) investigated three pre-column derivatization reagents: o-phthalaldehyde (OPA), 4-chloro-7-nitrobenz-2,1,3-oxadiazole (NBD-Cl), sodium naphthoquinone-4-sulphonate (NQS), and two ion-pair reagents, i.e., naphthalene-2-sulphonate (NS) and sodium dodecylsulfate (SDDS). A 24-hour urine sample from a 22-year-old male was analyzed using SDDS derivatization. The urine was obtained 8 hours after an unknown amount of illegally obtained amphetamine had been taken. Urine pH was normal. The concentration of amphetamine was found to be 8.5 μg/ml, corresponding to an original dose of 55 mg of amphetamine sulfate. However, since after an oral dose of 10-15 mg amphetamine sulfate, peak plasma concentrations of 40-50 ng/ml are attained in 1-2 hours, followed by a decrease to about 2 ng/ml after 8-10 hours, only the method employing derivatization with NQS was able to afford the required sensitivity for the quantitative

analysis of urine or plasma samples containing amphetamines (Table 1). It was not suitable for hydroxylated metabolites.

Nakahara et al. (18,19) used NQS to analyze urine and plasma without tedious extraction procedures by HPLC-electrochemical detection. The use of NQS as an electrochemical label for amines satisfies the requirements of a good labelling reagent as regards sensitivity, selectivity, short reaction time, reproducibility, simple sample pretreatment and low background. The detection limit was 1 ng/ml, as indicated in Table 1.

Polystyrene-divinylbenzene-based, o-nitrobenzophenone-attached labelling reagents containing o-acetylsalicyl or fluorenyl tags were designed for derivatization of primary and secondary amines, on line and off line in HPLC with UV/fluorescence detection (21). These particular polymeric reagents had exhibited good thermal and aqueous stability, high percentage derivatization, low detection limits for amines (low-parts-per-billion range), fewer interferences in the final HPLC-UV/FL chromatograms compared with the analogous solution reactions, and faster estimation of nucleophilic analytes via the on-line approach. The method was applied to urine samples, with a minimum of sample preparation prior to direct injection into the on-line derivatization-HPLC system (Table 1).

Maeder et al. (36) proposed an on-line precolumn derivatization method for the determination of low concentrations of amphetamine using an UV detector, and 9-fluorenylmethyl chloroformate (FMOC-CL) as the derivatizing reagent. Quantitative determination of amphetamines as low as $2x10^{-8}$ mol/l (Table 1) could be obtained using this on-line method with preconcentration. The sensitivity of the technique is about 50 times greater than the equivalent off-line method. The technique has been only applied to standards.

The use of 9-fluoreneacetyl (FA) tag on a controlled-pore substrate, for direct injection analysis of amphetamine in plasma has been described by Zhou et al. (23). The derivatized 9-fluoreneacetyl amphetamine was separated by reversed-phase HPLC with a step gradient

and determined by fluorescence detection. This solid-phase reagent combined with a surfactant containing mobile phase provided a sensitive and simple procedure for on-line derivatization in the direct injection analysis of biological fluids.

In recent years, a significant number of advances have been made in chromatographic separation techniques for the resolution of enantiomers, particularly in gas chromatography (37).

D-methamphetamine is a drug of common abuse, while l-methamphetamine is found in nasal spray. Traditional methods for quantification of enantiomers, e.g., chemical resolution or rotation of polarized light, are not adequate for the determination of trace amounts of enantiomers in biological fluids. The methods for the chromatographic resolution of enantiomers fall into three categories. The first involves the conversion of the enantiomers to diastereomers by reaction with a chiral derivatizing agent. The second makes use of the differences in rates of interaction of enantiomers with chiral stationary materials. A less commonly employed third method utilizes an achiral stationary phase and a mobile phase which contains a chiral eluent.

An analytical approach has been developed (22) for on-line solid-phase derivatizations in HPLC with UV-fluorescence detection. The method involves derivatization with a polymeric 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-proline) chiral reagent. The detection limit was 50 ng/ml (Table 1).

Miller et al.(38) separated enantiomers of amphetamines with four chiral reagents: (R)-(+)-1-phenylethyl isocyanate (PEIC), (-)α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPA.Cl), 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). Reactions were accomplished under mild conditions (25-70°C) and were complete for all substrates within 60 min. The diastereomeric derivatives were separated by reversed-phase HPLC (C_{18}) with methanol-

water mobile phase. In general, HPLC resolution of the diastereomeric reaction products of GITC or AITC and MTPA.Cl with amine substrate was more complete than products of PEIC.

Noggle et al. (39) applied precolumn derivatization of methamphetamine and amphetamine with phenylisothiocyanate. The thiourea products formed have good reversed-phase chromatographic properties and high UV molar absorption. Using similar derivatization procedures, the enantiomeric compounds can be determined for those amines containing a chiral centre. The separation of the diastereoisomeric products of chiral derivatization can be accomplished using achiral C_{18} stationary phases. The enantiomers of ephedrine, pseudoephedrine, amphetamine and methamphetamine were separated using sugar isotyiocyanate 2,3,4,6-tetra-o-acetyl-β-glucopyranosyl isothiocyanate as a chiral derivatizing agent. When (GITC) was used as derivatizing reagent for amphetamine and related compounds, the separation of amphetamine enantiomers was difficult (39-40). This difficulty for separating amphetamine enantiomers was also seen when 4-nitrophenylsulfonyl-l-prolyl chloride (NPSP) chiral derivatizating reagent was used (41).

The enantiomers of primary, secondary and some tertiary amines were resolved as carbamate derivatives formed by reaction with β-naphthyl chloroformate on an available Pirkel-type HPLC chiral stationary phase consisting of (R)-N-(3,5-dinitrobenzoyl) phenylglicine covalently bonded to silica, by using a mobile phase consisting of mixtures of iso-PrOH in hexane (42).

N-(trifluoroacetyl)-l-prolyl-(N-TFA-l-prolyl-) d- and l-amphetamine diastereoisomers were separated by HPLC and confirmed by an interfaced mass spectrometer system, using the commercially available N-3,5-(dinitrobenzolyl)phenylglycine chiral column (43). The use of chiral derivatizing reagent and chiral LC column achieved a better resolution of d- and l-amphetamine in comparison with those previously reported in the literature.

Nagai and Kamiyama (44) assayed the optical isomers of methamphetamine and amphetamine in rat urine using HPLC with chiral cellulose-based columns. Methamphetamine isomers excreted in rat urine were analyzed by the combined use of Chiralcel OB and OJ columns, which offered good peak resolution, l/d chira ratio and retention time. Mixtures of amphetamine and methamphetamine could be separated simultaneously. The analytical time was less than 25 minutes, and the minimum detection limit was 25 ng per 20 μl of urine (1.7 μl/ml).

Conclusions: A review has been made of the procedures proposed for samples clean-up and derivatization steps for the HPLC determination of amphetamine and methamphetamine. Both off-line and on-line procedures are described and generally those steps required for successful determination. Three types of analytical columns are proposed: normal-phase, reversed-phase and chiral columns for enantiomers resolution. Ultraviolet, fluorescence, chemiluminescence and electrochemical detectors have been used. Most of the procedures described in the literature have employed spiked samples with concentrations in the μg/ml range; others (7,11,16,25) had applied procedures to suspected urine samples, the concentrations encountered being very different - probably due to factors such as pH, urine collection time after administration of the drug, etc., which all affect the concentration present in the sample. The lower detection limits encountered are a few ng/ml in biological fluids (Table 1).

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