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Review

Derivatization reactions for neurotransmitters and their automation

Michael E. Bovingdon*, Roy A. Webster

Pharmacology Department, University College London, Gower Street, London WC1E 6BT, UK

Abstract

Many reagents suitable for the derivatization of neurotransmitters are selective for the amino function. Others, however, are selective for the carboxyl-, thiol- and hydroxyl function, and recently, reagents selective for more than one function have been produced. Interest persists in the established reagents, with their well understood behaviour which assists automation of analysis as much as new technology. Workers appear reluctant to tackle the optimization of many novel reagents. Chiral reagents may become important if d-amino acids are shown to be significant from a physiological point of view. Solid-phase reagents offer better regulated chemistry and combined derivatization/solid-phase extraction, which make them an exciting prospect.

Contents

* Corresponding author.

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List of abbreviations

ACh	Acetyl choline
ADAM	9-Anthryldiazomethane
APBA	2-(*p*-Aminomethylphenyl)-N,N-dimethyl-2H-benzotriazolyl-5-amine
APMB	(−)-2-[4-(1-Aminoethyl)phenyl]-6-methoxybenzoxazole
ARM	Aryl methylamine
asp	Aspartate
BQCA	3-Benzoyl-2-quinoline carboxaldehyde
BrAMN	2-Bromoacetyl-6-methoxynaphthalene
BrMMC	Bromomethylmethoxycoumarin
CBI	1-Cyano-2-substituted-benz[f]isoindole
CBQCA	3-(4-Carboxybenzoyl)-2-quinoline carboxaldehyde
COMT	Catechol-O-methyltransferase

C.V.	Coefficient of variation
DA	Dopamine
Dabsyl-Cl	Dimethylaminoazobenzene sulphonyl chloride
DBD-Apy	4-(Aminosulphonyl)-7-(3-aminopyrodin-1-yl)-2,1,3-benzodioxazole
DICLOX	2-Chloro-4,5-diphenyloxazole
DIFOX	2-Fluoro-4,5-diphenyloxazole
DIOX	Diphenyloxazole (derivative of DICLOX)
DMEQ	3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride
DNP	3,5-Dinitrophenol
DNPO	bis(2,4-Dinitrophenyl)oxalate
DNP-poly	DNP reagent bonded to copolymer solid matrix
DOMA	Dihydroxymandelic acid
DOPA	Dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
DPDS	2,2′-Dipyridyl disulphide
DPE	1,2-Diphenylethylenediamine
E	Epinephrine
ED	electrochemical detection
FDAA	1-Fluoro-2,4-dinitrophenyl-5-alanine
FDNDEA	N,N-Diethyl-2,4-dinitro-5-fluoroaniline
FITC	Fluoresceinisothiocyanate
FLEC	1-(9-Fluorenyl)ethyl chloroformate [in (+)-, and (−)-forms]
FMOC(Cl)	9-Fluorenylmethylchloroformate
gaba	γ-Aminobutyric acid
glu	Glutamate
gly	Glycine
HD	Histidine
5HIAA	5-Hydroxyindoleacetic acid
HT	Histamine
5HT	5-Hydroxytryptamine (serotonin)
LIFD	Laser-induced fluorescence detection
MA	Monoamine
MAO	Monoamine oxidase
Marfey's reagent	1-Fluoro-2,4-dinitrophenyl-5-alanine amide (FDAA)
MBNCC	(−)-2-Methyl-1,1′-binaphthalene-2′-carbonyl cyanide
2-ME	2-Mercaptoethanol (β-mercaptoethanol)
3-MMPA	d-3-Mercapto-2-methylpropionic acid
3-MPA	3-Mercaptopropionic acid
M-phisylchloride	2-Methoxy 5-(N-phthalmidyl) benzene sulphonyl chloride
MSQ-Cl	6-Methoxy-2-methylsulfonylquinoline-4-carbonyl chloride
NBD	7-Chloronitrobenzooxadiazole
NBD-Cl	4-Chloro-7-nitrobenzofuran
NDA	Naphthalene dialdehyde (naphthalene-2,3-dicarboxaldehyde)
NE	Norepinephrine
NIPTC	4-Nitrophenylisothiocyanate
NT	Neurotransmitter
ODS	Octadecyl silica
OPA	*o*-Phthalaldehyde (phthaldialdehyde)
PDA	Photodiode array
PDAM	1-Pyrenyldiazomethane
phisyl	Phthalmidyl benzoyl chloride
PITC	Phenylisothiocyanate
PTC	Phenylthiocarbamyl- (a derivative formed by PITC)
PTH	Phenylthiocarbamyl- (the other derivative formed by PITC)
RP	Reversed-phase
R.S.D.	Relative standard deviation
SAOX	2-Chloro-4,5-bis(*p*-N,N-dimethylaminosulfonyl)oxazole

SFPC	s-Flunoxapren chloride
SPE	Solid-phase extraction
SPR	Solid-phase reagent
TEA	Triethylammonium
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TP	Tryptophan
TPP	Triphenylphosphine
t_R	Retention time
VMA	Vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid)

1. Introduction

1.1. Neurotransmitters reviewed

A neurotransmitter (NT) is a chemical released from one neurone to affect the activity of another in a variety of ways, from a dramatic, rapid change in polarization and firing lasting only milliseconds, to more subtle, less marked and more prolonged changes in potential. In some instances it can even affect the neurone from which it is released. To achieve this diversity of function, the nervous system uses a number of chemicals of different structural complexity. Not surprisingly the methods used for detection and estimation vary with structure, and other features such as location (*i.e.* tissue, body fluids or perfusate), endogenous concentration and manner of degradation.

With the exception of most peptides, all NTs are preformed and stored in nerve terminals, ready for release. Thus, they can be present in quite high concentrations in brain extracts (10 μmol/g), but once released their degradation is generally rapid (within seconds): the concentrations in perfusates (and body fluids such as plasma and urine) can fall to fmol levels. In some instances, as with the monoamines, distinct metabolites may be recovered and measured in perfusates, but with others (*e.g.* acetylcholine) it is necessary to stop their metabolism with drugs which may themselves interfere with the estimation.

The chemical structures of the substances that can be considered as major neurotransmitters are shown in Fig. 1. They include the following substances.

1.1.1. Acetyl choline (ACh); the acetate ester of choline

Widely distributed in both peripheral nerves and the central nervous system (CNS) its concentration varies considerably, but is highest in the striatum and parts of the cortex (10 nmol/g). On release it is rapidly broken down by cholinesterases to its precursor choline which is quickly taken back up by the releasing nerve terminal and reacetylated to ACh. Thus, it is virtually impossible to measure ACh in perfusates without using an anticholinesterase such as physostigmine. Choline levels could be indicative of released ACh but not all the choline may be derived from that source, and it is itself quite quickly removed.

1.1.2. Monoamines (MAs)

These include the catecholamines dopamine (DA), norepinephrine (NE), and epinephrine (E) as well as the indoleamine 5-hydroxytryptamine (5HT, serotonin), and possibly tryptamine (TP). Histamine (HT) is also found in the CNS.

The precursors are amino acids and may also be of interest. Tyrosine (tyr) is the starting point for catecholamine synthesis (tyrosine–dopa–dopamine–norepinephrine–epinephrine), tryptophan for 5HT, *via* 5-hydroxytryptophan, and histidine (HD) for HT. The primary metabolism of all the amines is oxidative deamination by monoamine oxidase (MAO), generally after intraneuronal uptake of the amine, to give a carboxylated derivative. Thus, DA is transformed to 3,4-dihydroxyphenylacetic acid (DOPAC), NE to 3,4-dihydroxymandelic acid (DOMA), and 5HT to 5-hydroxyindoleacetic acid (5HIAA). DOPAC and DOMA also undergo O-methylation by catechol-O-methyl transferase (COMT) to form 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA), and

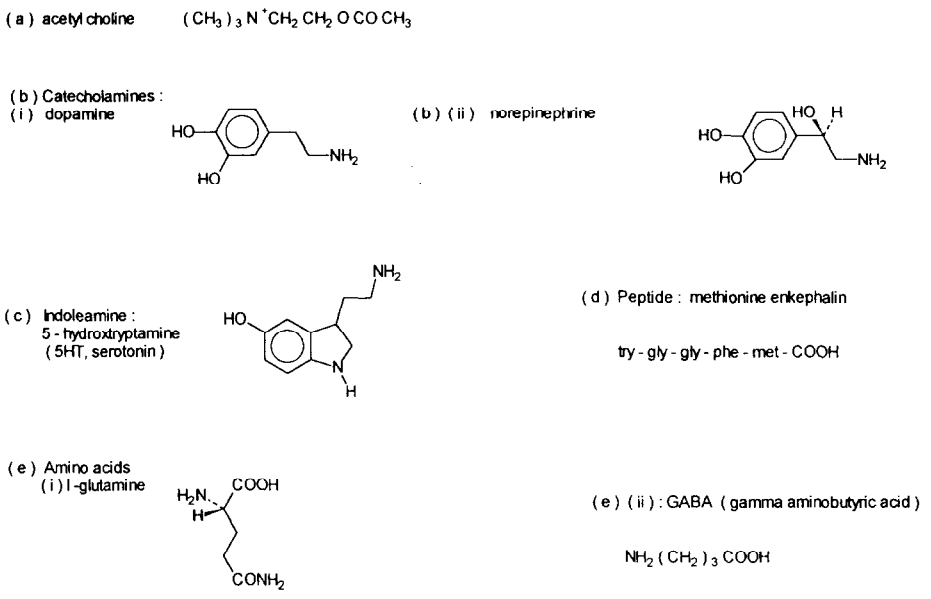

Fig. 1. Structures of some representative neurotransmitters.

3-methoxy-4-hydroxymandelic acid (VMA) respectively, whilst HT is methylated and then deaminated to methylimidazole acetic acid. Which metabolite predominates depends on circumstances such as method of collection and time after release.

Thus monoamines range chemically from amino acid precursors, through amine neurotransmitters, to acid metabolites. HPLC may make it possible to detect them all with the analytical conditions being arranged to favour the form that may be of most interest. Although all monoamines are initially removed by neuronal uptake after release, their metabolites are not re-used or modified further chemically, and their levels may be considered to reflect those of the neurotransmitters themselves.

Concentrations vary considerably: DA, NE and 5HT are released throughout the cortex from the terminals of diverging axons of neurones whose cell-bodies are concentrated in specific subcortical nuclei. Their concentrations are generally low in the cortex (1 nmol/g), and consequently difficult to measure in release studies, apart from DA in the striatum (50 nmol/g), where it is concentrated. Levels in the nuclei are higher.

1.1.3. Amino acids (AAs)

Unlike acetyl choline and the monoamines, which have mixed excitatory and inhibitory effects on neuronal activity, the AAs are either excitatory or inhibitory. The excitatory ones are dicarboxylic acids, like glutamate (glu) and aspartate (asp). The inhibitory ones include γ-aminobutyric acid (gaba) and glycine (gly).

Since they are also involved in metabolic processes, it is not easy to locate the neurotransmitter component, and their concentrations in brain tissue are much higher (10 μmol/g) than those of any other neurotransmitter. On release they appear to be rapidly removed by a critically-controlled uptake into the glia and neurones, so that in perfusion studies, although spontaneous background levels of glu, asp, gly (but rarely gaba) are detectable, this is not augmented by increased, or even excessive neuronal activity, unless this is accompanied by extreme chemical stimulation with, for example, kainic acid or cobalt application.

No specific metabolites are produced, the released amino acids being re-incorporated into various biochemical cycles.

1.1.4. Peptides

Although composed of amino acids, their utilization and role are quite different. The active peptide is often split from a larger peptide precursor whilst it is transported down the axon and then stored. Concentrations of peptides can be very low (200 pmol/g). Since they are quickly broken down on release by peptidases, the extracellular levels for detection are even lower, and the breakdown products often not adequately identified for estimation.

Some twenty or more peptides have been found in neural tissue, but the action and transmitter status of some are uncertain. Some, such as β-endorphin, have been analysed by HPLC without derivatization using electrochemical detection [1], and fluorescence [2].

1.1.5. Purines

Many neurotransmitters are known to stimulate (work through) adenosine triphosphate (ATP) production, but an action of ATP alone on neuronal activity, its established role in sympathetic nerve function, and in the so-called non-adrenergic, non-cholinergic inhibition of smooth muscle may have elevated it to neurotransmitter status.

1.2. Requirements for derivatization

Improved sensitivity is the major benefit of derivatization, but separation of mixtures can also be improved. An ideal pre-column derivatization would take place at ambient temperature, require only minimal sample clean-up, leave no unreacted reagent or by-products to interfere with chromatography, and produce adducts (derivatives) which are stable on the autosampler. Alternatively, valve switching may be used in conjunction with a solid-phase extraction (SPE) column prior to injection, to concentrate analytes and remove interfering compounds in what is now an automatable sequence.

Post-column derivatization requires an online reactor downstream of the analytical column. Short derivative half-life is therefore less of a problem than with pre-column derivatization, but analytes are inevitably diluted by mixing with the reagent carrier fluid for which a separate metering pump is required.

Sample clean up has often required dedicated apparatus or some manual bench work before untended injection is possible, but refinements in solid-phase extraction (recently reviewed [3]), derivatization chemistry and autosampler operation have reduced this. Modern reagents and instrumentation have brought fmol or amol detection limits with freedom from interference with chromatography and detection. There is often a choice of detector and a reaction occurring at ambient temperature with minimal sample preparation.

For confidence in results good resolution is essential, but high sample throughput demands shorter run-times. In general, reversed-phase (RP) columns, often run with mobile-phase gradients, can be used to optimize these requirements and have proved most popular, but also microcolumn separations have been reported [4,5].

1.3. Derivatization procedures

Despite the advances promised by logically designed novel reagents like naphthalene-2,3-dialdehyde (NDA, Fig. 1, Tables 4–6), further refinements in the use of well-tried reagents like *o*-phthalaldehyde (OPA, Fig. 1, Tables 1–3) and 9-fluorenyl methylchloroformate (FMOC, Fig. 3, Table 2) continue to appear. New reagents might form more stable derivatives, but the use of modern autosamplers and mobile-phase gradient formers can compensate for short life after pre-column derivatization by achieving consistent retention times. Also, the chromatography of the derivatives of classical reagents is well understood.

Neurotransmitter separations are largely confined to HPLC at present, but the reagent NDA has been used with conventional and microbore columns [5], and with open-column tubular col-

Table 1
OPA and/or FMOC amino-selective derivatizing reagents: selected performance data for neurotransmitter derivatives

Reagent Reference	OPA plus FMOC 44	OPA 57 (I)	OPA 57 (II)
Neurotransmitters	amino acids*	monoamines	amino acids*
Reaction			
Time (min)	3	2	as I
Temperature (°C)	ambient	ambient	as I
Yield (%)	not given	not given	as I
Derivative stability	***	**	as I
Chromatography			
Range	17 amino acids* including neurotransmitters	dopamine and 5HT systems	gaba plus other amino acids*
Run-time (min)	40	20	30
Column	S5ODS2	NBS C_{18} 5-μm	as I
Gradient	tertiary	isocratic	as I
Mobile phase	phosphate-propionate-MeCN-DMSO-water	sodium phosphate-HSA-MeOH (pH 3.0)	sodium phosphate-MeCN-MeOH (pH 6.8)
Detection method	fluorimetric	electrochemical array	as I
Limit of detection	not given	2.5 pg/20 μl sample	15 pg/20 μl sample
S/N ratio		3	3
Linearity	"linear"	$r \geq 0.9969$	$r = 0.9975$
Given range	5–2000 μmol/ml	0.25–20 ng/ml	0.25–20 ng/ml for gaba
Peak reproducibility (R.S.D.%)	(1.8–10.3 within-run; 2.8–15.1 between-runs)	(1.04–9.24 within-run; 0.45–6.45 between-runs)	(1.24–1.89 within-run; 1.49 between-runs)
t_R Reproducibility (R.S.D.%)	not given	not given	not given

Key to abbreviations: dabsyl = dimethylaminmoazobenzene sulphonyl chloride; DMEQ = 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline carbonyl chloride; FMOC = 9-fluorenymethyl chloroformate; HSA = heptanesulphonic acid; MeCN = acetonitrile; MeOH = methanol; NDA = naphthalene-2,3-dicarboxaldehyde; OPA = *o*-phthalaldehyde; phisyl = phthalmidyl benzoyl chloride; PITC = phenylisothiocyanate; THF = tetrahydrofuran.
Notes: (1) Assessment of derivative stability (based on data and/or degree of automation possible) is given by star rating (*–*****). (2) Peak and t_R data are quoted as R.S.D. except where otherwise stated. (3) Neurotransmitter references marked with asterisk thus–*e.g.* amino acids*–indicate that all or some of the compounds investigated are only generically related to actual neurotransmitters.

umns [6,7]. Capillary electrophoresis has also utilized derivatizing agents (Figs. 1–3) [8], but not supercritical fluid techniques thus far [9].

In neurotransmitter applications, fluorescence, electrochemical detection (ED), or UV absorption of derivatives formed by amino selective reagents is often employed. Utilization of the carboxyl function has been less successful. Some hydroxyl- or sulphydryl-selective reagents have been reported, while some novel, multi-selective reagents exist. Tables 1–6 summarise the more important recent advances in amino-selective

Table 2
OPA and/or FMOC: selected performance data for neurotransmitter derivatives

Reagent Reference	OPA 37	OPA 35	FMOC 28
Neurotransmitters	amino acids	amino acids*	amino acids*
Reaction			
Time (min)	not given	2.5	1.5
Temperature (°C)	not given	ambient	ambient
Yield (%)	not given	not given	not given
Derivative stability	not given	**	***
Chromatography			
Range	18 amino acids* including gaba gaba	17–23 amino acids* including neuro-transmitters	16 hydrolysate amino acids*
Run-time (min)	not given	15–35	25 at 43°C
Column	MicroBondapak ODS	S5ODS2	C_8 or C_{18}, 3 or 5 μm
Gradient	binary	binary	binary
Mobile phase	MeOH-THF-acetate (pH 7.2)-MeOH-acetate	phosphate (pH 7.2)-3% MeCN–phosphate–50% MeCN	aqueous aceate buffer–acetonitrile
Detection method	fluorimetric	fluorimetric	fluorimetric
Limit of detection S/N ratio	not given	0.8 pmol 2.5.	not given
Linearity	not given	not given	$r \geq 0.9976$ for neuro-transmitters
Given range			2.6–260 pmol
Peak reproducibility (R.S.D.%)	not given	C.V. 4.2–6.8 for 25 standards, 1.8-9.8 for 20 repeats	for neuro-transmitters: 0.15–1.5 at 500 pmol
t_R Reproducibility (R.S.D.%)	not given	not given	not given

For abbreviations used see footnote Table 1.

reagents, and significant refinements to established ones, and the chemical structures appear in Figs. 2 and 3. Other reagents are referred to in Tables 7 and 8 or in the text. At present chiral amino acid neurotransmitters appear to be confined to laevo forms, but there are numerous stereoisomeric forms of drug molecules with differing potencies and the separation of derivatives both chiral and non-chiral is expanding. Some discussion of chirality is included to cover the possible need to separate endogenous amino acids and peptides from experimentally added stereoisomers. There is also the tantalising prospect of as yet uncharacterized systems involving d-amino acids. Although the case has been made for natural systems to conserve homochirality [10], some peptides containing d-amino acids are known [11].

Solid-phase derivatizing reagents (SPRs, Fig. 4) offer some special advantages of their own, in both chiral and non-chiral separations [12–14].

2. Approaches to derivatization

Having described the requirements for derivatization, we can consider their application to neurotransmitters.

Table 3
OPA and/or FMOC amino-selective derivatizing reagents: selected performance data for neurotransmitter derivatives

Reagent Reference	OPA 22	OPA plus FMOC 45
Neurotransmitters	gaba with other other amino acids*	amino acids*
Reaction		
Time (min)	2	not given
Temperature (°C)	25	not given
Yield (%)	not given	not given
Derivative stability	**	***
Chromatography		
Given range	gaba from a mixture of aminoacids*	primary and secondary amino acid analysis*
Run-time (min)	< 20	30
Column	Wakosil ODS	Hypersil RP 5 μm
Gradient	isocratic	tertiary
Mobile phase	citrate-MeCN-MeOH	0.15 *M* acetate (pH 6.8)–MeOH–0.01 *M* acetate (pH 6.8)
Detection method	fluorimetric	photodiode array
Limit of detection	0.5 pmol	not given
S/N ratio	–	–
Linearity	$r \geq 0.9988$	$r > 0.99$
Given range	2–500 pmol	31.25–500 pmol/l
Peak reproducibility (R.S.D.%)	3.0, $n = 22$ at 20 pmol for gaba	C.V.% 0.78–2.84, mean 0.26, S.D. = 0.067
t_R reproducibility (R.S.D.%)	not given	C.V.% 0.02–0.976, mean 0.26, S.D. = 0.21

For abbreviations used see footnote Table 1.

2.1. Neurotransmitters and their detection

The actual or potential uses of different functional groups in detecting the various neurotransmitters are outlined below.

2.1.1. Amino acids

Amino acids (Fig. 1) have received much attention, the amino function presenting by far the most popular route for derivatization (Tables 1–8). Fluorescence, and latterly laser-induced fluorescence detection (LIFD) and electrochemical detection (ED) have been prominent.

2.1.2. Monoamines

Monoamines (Fig. 1) were first derivatized for fluorescence, but ED then promised more sensitivity. Derivatization *via* the amino function, for fluorescence and ED, has now returned to avoid the many problems of interference encountered in ED of conjugates and metabolites.

2.1.3. Peptides

Peptides (Fig. 1): UV absorption of the native compound is still a common detection method, but amino function derivatives have also been used. The appearance of reagents selective for

Table 4
NDA amino-selective derivatizing reagent: selected performance data for neurotransmitter derivatives

Reagent Reference	NDA 33	NDA 78	NDA 31
Neurotransmitters	amino acids*	catecholamines	amino acids*, peptides*
Reaction			
Time (min)	15	30	not given
Temperature (°C)	ambient	ambient	not given
Yield (%)	not given	not given	not given.
Derivative stability	not given	not given	not given
Chromatography			
Range	hydrolysate* aminoacids and peptides	dopamine, norepinephine dihydroxy bezylamine	18 amino acid and peptide standards* only
Run-time (min)	60	30	60
Column	Hypersil, Ultrasphere ODS5	TSK ODS-120 T, 5 μm	Ultrasphere ODS
Gradient	binary	isocratic	binary
Mobile phase	phosphate–MeCn–THF–MeOH	MeCN–THF–potassium phthalate (pH 2.5), 36:4:60	phosphate–THF–MeCN–MeOH–phosphate
Detection method	UV Abs at 420 and 246 nm	chemilumin-escence	selective electrochemical
Limit of detection	< 3 pmol/420 nm < 200 fmol/246 nm	1 fmol	not given
S/N ratio		15	
Linearity	$r = 0.999$	$r \geq 0.999$	$r \geq 0.999$
Given range	5-50 pmol	1-10 fmol	5-50 pmol
Peak reproducibility (R.S.D.%)	not given	not given	not given
t_R Reproducibility (R.S.D.%)	not given	not given	not given

For abbreviations used see footnote Table 1.

hydroxyl, sulphydryl and N-acetyl amino functions should prove invaluable.

2.1.4. Choline and acetyl choline

Choline and acetyl choline (Fig. 1) still rely on post-column oxidative reactors for indirect ED, and derivatization has not been reported [15,16]. The use of ED to quantify monoamines and metabolites, and Ach and choline from the same cerebrospinal fluid (CSF) samples has been described [17,18].

2.1.5. Purines

The purines implicated as the putative neurotransmitter system are ATP, ADP and AMP. Most existing methods rely on UV absorption, but a post-column, heat-induced reaction with bromoacetaldehyde has been used to determine a spectrum of ATP metabolites [19]. An isocratic

Table 5
NDA amino-selective derivatizing reagent: selected performance data for neurotransmitter derivatives

Reagent Reference	NDA 79 (I)	NDA 79 (II)
Neurotransmitters	amino acids*	monamines, peptides
Reaction		
Time (min)	not given	not given
Temperature (°C)	not given	not given
Yield (%)	not given	not given
Derivative stability	****	****
Chromatography		
Range	13 amino acids*, including neuro- transmitters	3 peptides, 6 biogenic amines, drugs*
Run-time (min)	amino acids: 65 peptides: 20	amines: 65 drugs: 70
Column	Chrompack ODS2, 5 μm	as I
Gradient	binary	binary
Mobile phase	phosphate (pH 7)– MeOH–THF–water	phosphate–THF- MeCN–MeOH–phosphate
Detection method	fluorimetric	as I
Limit of detection	asp 10 fmol, tyr 50 fmol	glygly 25 fmol, amphetamine 50 fmol
S/N ratio	2	2
Linearity	not given	not given
Given range	–	–
Peak reproducibility (R.S.D.%)	asp ($n = 8$), S.D. = 0.113 at 10 pmol	not given
t_R Reproducibility (R.S.D.%)	not given	not given

For abbreviations used see footnote Table I.

mobile phase containing sodium chloride, bromoacetaldehyde as reagent, and 15% acetonitrile was used with Hitachi gel No. 3013 or Asahipak GS-320 H columns. Analysis takes *ca.* 26 min. Detection limit for ATP is *ca.* 20 pmol.

2.2. Chromatography of derivatives

Frequently neurotransmitters must be separated from numerous similar compounds, and metabolites and/or conjugates [17–19] and this affects not only the utility of derivatizing agents, but also the optimization of the separation. Detail changes to the derivatization reaction, mobile-phase gradients and column can all aid successful resolution. In validation of methods, authors often quote a number of parameters, including: (a) detection limits/molar (or other) quantity at quoted signal-to-noise ratio (S/N)

(b) Linearity of response over a given molar

Table 6
NDA amino-selective derivatizing reagent: selected performance data for neurotransmitter derivatives

Reagent Reference	NDA 32 (I)	NDA 32 (II)
Neurotransmitter	amino acids*	catecholamines, peptides
Reaction		
Time (min)	60	as I
Temperature (°C)	ambient	as I
Yield (%)	not given	as I
Derivative stability	***	***
Chromatography		
Range	amino acid standards* only	monoamine and peptide standards only
Run-time (min)	60	as I
Column	Ultrasphere ODS, 5μm	as I
Gradient	binary	as I
Mobile phase	phosphate–MeCN–MeOH–THF	as I
Detection method	electrochemical	fluorimetric
Limit of detection detection	lys 100 fmol	catecholamines; "fmol" leu-enk; 200 pmol/ml
S/N/ ratio	–	–
Linearity Given range	not given	not given
Peak reproducibility (R.S.D.%)	not given	not given
t_R Reproducibility (R.S.D.%)	not given	not given

For abbreviations used see footnote Table 1.

range (the correlation coefficient r is often quoted)

(c) Reproducibility of t_R and/or peak size/ relative standard deviation (R.S.D.) %

These data are quoted in Tables 1–8 when they are given in the original reference.

2.3. Capillary electrophoresis

While HPLC separations are most numerous, capillary electrophoresis has been used to separate 17 amino acids to detection limits of 10–70 amol and short peptides to 4.6 and 13.8 amol at S/N = 3 [8] as 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA, Fig. 3) derivatives. This reagent was described by the authors as being the best suited of four evaluated to capillary electrophoresis.

A comparison has been carried out of fluorescamine, fluorescein isothiocyanate (FITC), 9-fluorenylmethylchloroformate (FMOC) (Fig. 3) and *o*-phthalaldehyde (OPA) (Fig. 2). All derivatizations were carried out pre- and post-electrophoresis, and a number of excitation sources

Table 7
Other amino-selective derivatizing reagents: selected performance data for neurotransmitter derivatives

Reagent Reference	NBD Cl 29	PITC 42	BQCA 47
Neurotransmitters	amino acids*	amino acids*	amino acids*
Reaction			
Time (min)	3; 20	20	30–60
Temperature (°C)	60°C; 25°C	ambient	ambient
Yield (%)	95	not given	not given
Derivative stability	***	***	**
Chromatography			
Range	primary and secondary aminoacids*	aminoacids* including asp, gaba, gln, glu, gly	15 aminoacid standards* only
Run-time (min)	not given	not given	150
Column	C_{18}, 5-μm	Dynamax C_{18}	S5ODS2
Gradient	isocratic	binary	binary
Mobile phase	phosphate (pH 1.9)–20% MeOH	sodium acetate-TEA–acetic acid-MeCN–water	MeCN–acetic acid–water–TEA
Detection method	fluorimetric	UV absorption	fluorimetric
Limit of detection S/N ratio	not given	not given	gly, 17 fg 3
Linearity	not given	$r \geq 0.9993$ for gly, $r \geq 0.9999$ for gaba	not given
Peak reproducibility (R.S.D.%)	(5 at 400 μmol/l)	within-day C.V.% 13.7–27.8, between-day C.V.% 10.8–19.4	not given
t_R Reproducibility (R.S.D.%)	not given	not given	not given

For abbreviations used see footnote Table 1.

were used [23]. The authors concluded that tunable laser light sources were essential for greatest detector sensitivity, because of variations in native fluorescence and absorption, and that reactor tube and column should be maintained at the same temperature. No mention was made of automation, but advantages and disadvantages for each reagent as regards separation were listed. It was concluded that OPA was the reagent of choice for post-column derivatization giving a limit of detection of 60 ng/ml for OPA–gly, and separating 6 amino acids in 16 min. The favoured pre-column reagent was FMOC. The buffer used contained 20 m*M* sodium phosphate (pH 2.5) and 50 m*M* hexanesulfonic acid, and the capillaries ranged from 50 to 100 μm I.D.

3. Functional groups and reagents

3.1. The amino function

Beginning twenty years ago with ninhydrin, the amino function has shown itself to be amenable to a wide range of agents, such as di-

Table 8
Other amino-selective derivatizing reagents: selected performance data for neurotransmitter derivatives

Reagent Reference	Dabsyl 26	DMEQ 48	Phisyl 53
Neurotransmitters	amino acids*	amino acids*	amino acids*
Reaction			
Time (min)	15	60	15
Temperature (°C)	70	ambient	50
Yield (%)	not given	84	not given
Derivative stability	***	*****	*****
Chromatography			
Range	protein hydrolysates*	17 alpha aminoacids*	20 aminoacid standards* only
Run-time (min)	25–75	not given	75
Column	Novapak 4-μm C_{18}	silica gel	ERC ODS 3-μm
Gradient	binary	isocratic	binary
Mobile phase	phosphate–MeCN	chloroform–acetone	phosphate (pH7.3)–MeOH
Detection method	UV absorption	fluorimetric	fluorimetric
Limit of detection S/N ratio	not given	12.4–18.1 fmol 3	<0.2 pmol 2
Linearity	$r>0.9$, except for pro, tryp pro, tryp	"linear", 20 fmol–100 pmol	"linear" up to 10 pmol
Peak reproducibility (R.S.D.%)	2.1 at 100 pmol	1.5–2.0 at 100 pmol; $n=30$	1.25–3.5; $n=8$
t_R Reproducibility (R.S.D.%)	not given	not given	not given

For abbreviations used see footnote Table 1.

methylaminoazobenzene sulphonyl chloride (dabs/dabsyl) (Table 8) [13,24–26], 9-fluorenylmethyl chloroformate (FMOC-Cl) (Fig. 3, Tables 1–3) [27,28]; 7-chloronitrobenzoxadiazole (NBD-Cl) (Fig. 3, Table 7) [29,30]; naphthalene-2,3-dicarboxaldehyde (NDA) (Fig. 2, Tables 4–6) [5,31–33]; *o*-phthalaldehyde (OPA) (Fig. 2, Tables 1–3) [20,22,34–38] and phenylisothiocyanate (PITC) [39,40]. The last reagent, although long-established, has been used recently, both for protein sequencing [41], and for estimation of neurotransmitter amino acids from rat brain [42] with detection by UV absorbance, although automation was not attempted in the latter case.

The use of FMOC to augment OPA by derivatizing secondary amines has also proved popular [28,43–45], and NBD-Cl has also been used [30,46]. Parallel evaluations of more than one amino-selective reagent have been reported [5,23(capillary zone electrophoresis),30].

Novel, logically-designed improvements on existing amino-selective reagents include 3-benzoyl-2-quinoline carboxaldehyde (BQCA; Fig. 2, Table 7) [47]; 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA; Fig. 3) [8]; 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl, Table 8) [48,49]; fluorosceinisothiocyanate (FITC) [50]; 6-methoxy-2-methylsulfonylquinoline-4-carbonyl chloride (MSQCl) [51]; naphthalene-2,3-dicarboxaldehyde (NDA; Fig. 2, Tables 4–6) [5,31,32]; 7-chloronitrobenzoxadiazole (NBD) (Fig. 3, Table 7) [29,30], phthalmidyl compounds [52], and phisyl (Table 8) and M-phisyl [53]. Improved detection limits and rapid, am-

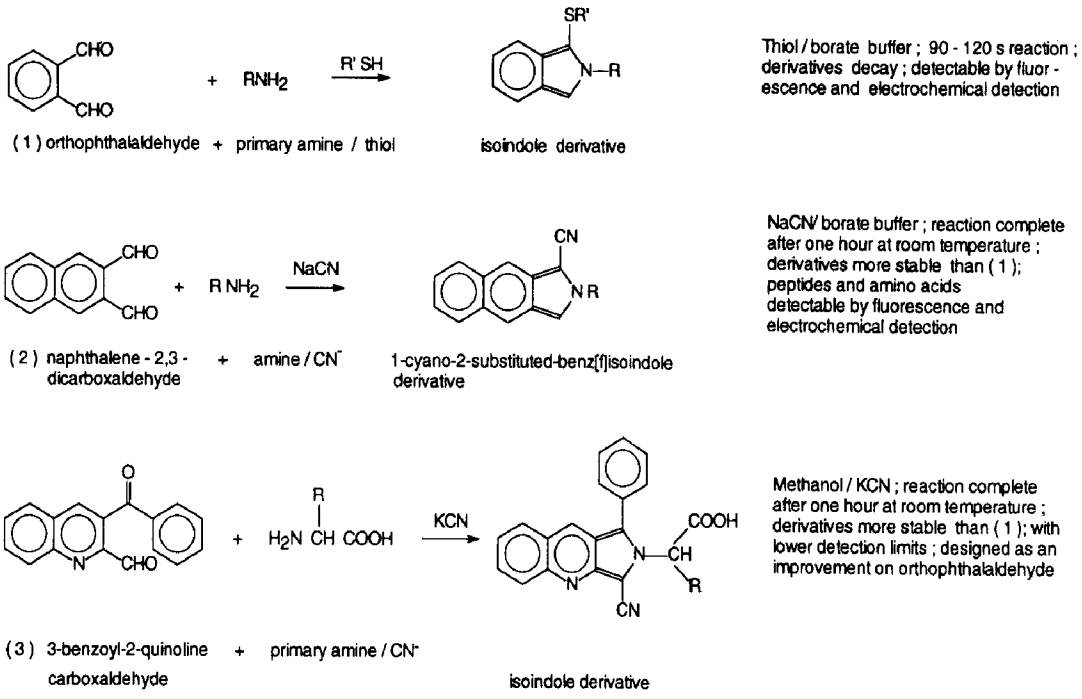

Fig. 2. The evolution of superior reagents for primary amines from *o*-phthalaldehyde (OPA).

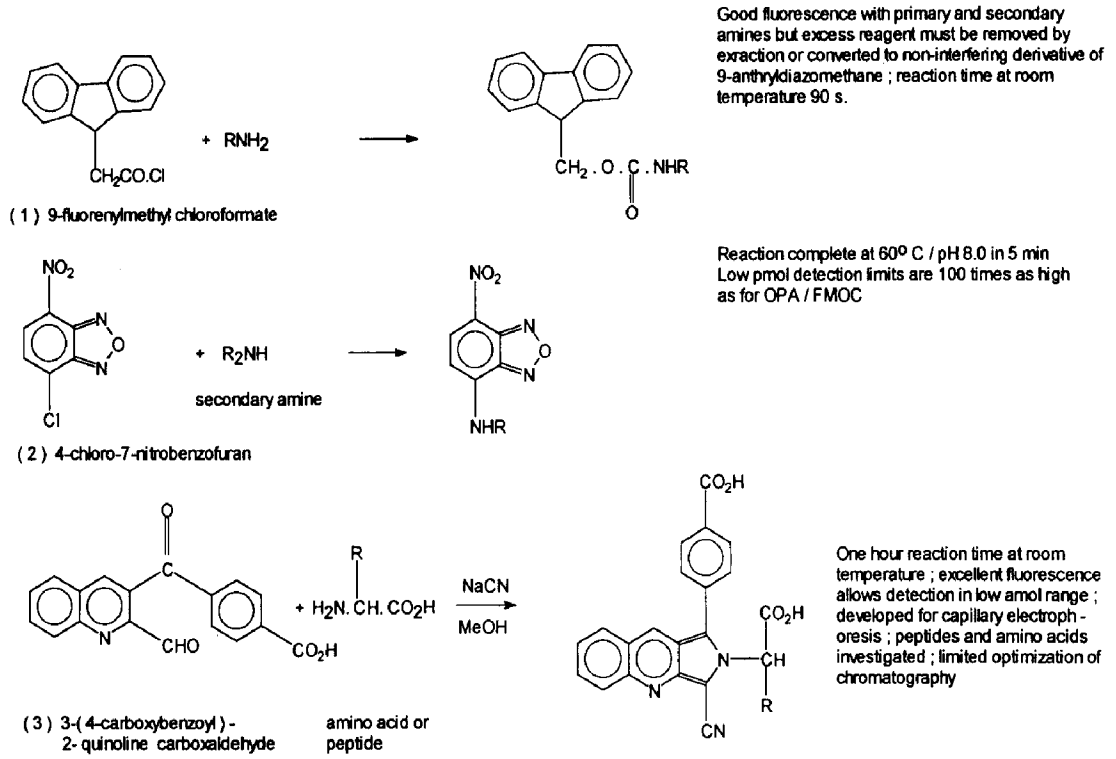

Fig. 3. Amino-selective reagents.

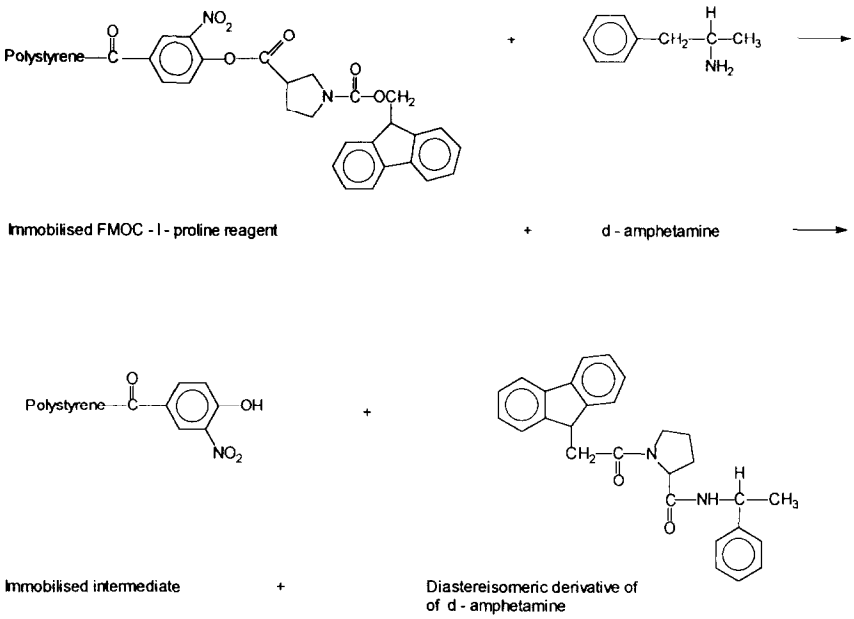

Fig. 4. Solid-phase chiral reagent: immobilised 9-fluorenylmethylchloroformate-I-prolyl (FMOC-I-prolyl).

bient temperature reactions which avoid production of interfering peaks are cited. Recently, the oxazole reagents have shown promise as amino-, hydroxyl-, and sulphydryl-selective reagents [54], but OPA (Fig. 2, Tables 1–3) and modifications persist, presumably because the chromatography of the derivatives is well optimized.

An example of a target compound in a complex mixture is gaba in cerebrospinal fluid (CSF) [20,36,37]. Successful quantification can be achieved by cleaning up samples beforehand, or limiting the scope of the separation to a small number of compounds, for example, to estimate the gaba alone [22].

Sensitivity can be limited by the S/N ratio obtainable under laboratory conditions. The purity of the reagent has been shown to be crucial where 2-mercaptoethanol (2-ME) is employed as the thiol with OPA–methanol–borate [22]. It has been noted that the use of NDA requires higher standards of glassware cleanliness if its potential detection limits are to be reached [33].

The amino function in N-acetylated compounds has been successfully derivatized using 9-anthryldiazomethane (ADAM) and the derivatives separated on an RP column with acetate–acetonitrile mobile phase. Problems were encountered with purity of commercial ADAM, and precipitation during derivatization [55].

3.1.1. o-Phthalaldehyde (OPA) and refinements (Fig. 2, Tables 1–3)

Considerable attention was paid to the optimization of the OPA system on its introduction in 1979 with 2-ME as thiol, borate buffer at pH 9.5, and methanol as reagent solvent [38] and has continued ever since (Fig. 1, Tables 1–3). Some workers have considered it necessary to allow the reagent to age [30,56], and maintain potency by continual additions of fresh thiol [38], while others found the reagent acceptably stable for up to one week [56,57].

The automation of OPA derivatization of amino acids can now be performed with commercially available equipment [20,36,37]. A recent report describes the use of OPA as a broad-spectrum derivatizing agent for biological fluid samples containing both amino acid and monoamine neurotransmitters, using a switching valve arrangement to split the sample for analysis

under different detection electrode conditions [57].

One report [22] describes isocratic HPLC of gaba, but gradients are still necessary to resolve and quantify complex mixtures of OPA derivatives of amino acids [20,37,57].

Various thiol co-reagents have been studied. A reduction in analysis times by increased adduct hydrophobicity compared with 2-ME accompanied better resolution of gly- and thre-OPA derivatives when 3-mercaptopropionic acid (3-MPA) was used as the thiol [35]. In a combined OPA/FMOC system (Tables 1 and 3), the use of 3-MPA lead to a more stable OPA reagent [45].

Choice of carrier solvent for OPA has been investigated [22,38,57]. Methanol was popular but acetonitrile has been used in a combined OPA/FMOC system [27], and it shortens retention times compared with methanol when used in the mobile phase [35,38].

OPA has also been used with the chiral reagent d-3-mercapto-2-methylpropionic acid (3-MMPA) to produce chiral separations on conventional reversed-phase columns, in a similar manner to non-chiral separations with OPA and/or FMOC [58]. The established OPA/2-ME combination has been used with a chiral stationary phase for chiral separations [56].

o-Phthalaldehyde has been compared directly with other reagents:

(1) with FMOC (Fig. 3, Tables 1–3), NBD Cl (Fig. 3, Table 7), and dabsyl [30];

(2) with N,N-diethyl-2,4-dinitro-fluoroaniline (FDNDEA) [59];

(3) with NDA (Fig. 2, Tables 4–6) [5];

(4) with FMOC, PITC (Table 7) and dansyl [60]. This was a comprehensive comparison of dansyl, FMOC, OPA and PITC as derivatizing agents for amino acids (and, for PITC, short peptides) from clinical sources, using HPLC, and narrow- and micro-bore LC.

Some conclusions were reached:

(1) All the methods would measure 22–24 amino acids within 25–35 min
(2) Despite short derivative life, OPA was clearly favoured by its automation potential
(3) FMOC was recommended where secondary amino acids were of interest, but the necessary removal of excess reagent was laborious
(4) PITC might be of interest in some clinical work, but its detection limits were 50-fold higher than the others, and the samples, though very stable, involved separate preparation
(5) Dansyl was recommended if cystine or peptides containing it were of interest
(6) All the reagents encountered problems with one or more of the amino acids.

A coulometric array detector has been used in the analysis of monamines and amino acids [57].

Sample preparation and OPA reagent stability are still improving: one report describes a four fold improvement in stability if nitriloacetic acid is included [61], which obviously assists automation.

Ion-pairing RP separation of 15 amino acids in 35 min has been carried out using post-column derivatization with N-acetyl-L-cysteine as thiol, and with NaOCl to oxidise imino acids for detection. Detection limits ranged from 2 to 9 pmol for protein hydrolysates [62].

3.1.2. Improvements on o-phthaladehyde (OPA)

The limitation of OPA to primary amines has lead to the development of a mixed system of OPA and FMOC-Cl (Tables 1 and 3) [43–45]. OPA/2-mercaptoethanol (2-ME) as thiol, with FMOC, has been used with the ASTED automatic sample handling device for fully automated analyses [44]. Good resolution and column life have been reported, but complex gradients were necessary. Fluorimetry and photodiode array (PDA) detection have been used to quantify clearly both OPA/3-mercaptopropionic acid (3-MPA) and FMOC derivatives: again, complex gradients were necessary [45].

Interference by unreacted FMOC was resolved by solvent extraction [27] or the use of a quenching reagent [28], and later 9-anthryldiazomethane (ADAM) to react with the excess [45]. However, ADAM scavenges only unreacted FMOC, not the by-product, and the two reagents must therefore be present within 5 min of each other. The low recoveries of hydro-

phobic amino acids following pentane extraction are compensated for if extra time is allowed for derivatization [63].

Monoamines have been quantified using FMOC-Cl with fluorescence detection: this is claimed to be 17 to 350 times as sensitive as electrochemical detection [64].

The analysis of a spectrum of amino acids from animal and plant samples using FMOC has been described [63]. Following hydrolysis with methanesulphonic acid, a Varian ODS 80 AminoTag column with a gradient of citrate buffer, acetonitrile, THF and tetramethylammonium achieved resolution. A UV-Vis photodiode array (PDA) detector was used.

A chiral modification of FMOC, namely 1-(9-fluorenyl)ethyl chloroformate (FLEC), has been used for chiral separations on RP columns [65]. 4-Chloro-7-nitrobenzofuran (NBD-Cl) has been used as reagent for secondary amines (Table 7) [29,30,40,66].

3.1.3. Other amino-selective reagents

These include bansyl, dabsyl (Table 8), dansyl, 3,5-dinitriphenol (DNP), 1,2-diphenylethylenediamine (DPE), fluorescamine, mansyl, ninhydrin and phenylisothiocyanate (PITC, Table 7).

Diethylaminoazobenzenesulphonyl chloride (dabsyl) [67], 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl) [68] and phenylisothiocyanate (PITC) [40,41,69] are still in use. Dabsyl recently gave a detection limit below 1 pmol for both primary and secondary amino acids, using a conventional 15-cm RP column (Supelco LC-DABS) and a binary gradient involving phosphate, acetonitrile, and methanol [67]. Derivatization took 10 min at 70°C, followed by evaporation and centrifugation steps, with only 1% decomposition per day at room temperature and a storage life at −20°C of several months. Good linearity was given over a wide concentration range, using absorption at 436 nm, when applying the technique to an amino acid mixture. Good reproducibility between columns is claimed.

The analysis of dansyl amino acids has been fully automated [68], including sample dissolution in bicarbonate–EDTA, mixing and pre-column incubation with dansyl chloride in acetonitrile, excess reagent hydrolysis with soda and injection. The reaction is complete in 30 min at ambient temperature, and the processing is carried out by a Gilson 231 autosampler linked to a 401 diluter module, with overall keypad-control. Recoveries exceed 95%, and low detector baseline noise is assured by automated hydrolysis. Peptide hydrolysis amino acids were separated on a Beckman Ultrasphere RP 18 5-μm column at 48°C, with a gradient of TFA–acetonitrile–2-propanol. At 1.2 ml/min, run-time is 34 min, and 4000 analyses can be carried out before columns show deterioration. In six successive runs t_R variation gave C.V. $\leq 0.2\%$. Twenty-two peaks appear in 32 min, including the neurotransmitter amino acids asp, glu and gly.

Phenylisothiocyanate (PITC) derivatives of a wide range of amino acids have been separated on RP columns while the Waters Pico-Tag ion-exchange type has been used in a fully automated method. Linearity over a 100-fold concentration range was obtained, t_R reproducibility = $\pm 0.2\%$ or better with a peak repeatability R.S.D. of $\pm$ 5–10%, when applied to feed formula analysis, even with a complex matrix in the sample [41]. Acetate–phosphate–acetonitrile binary gradients on an RP column followed by electrochemical detection can avoid many of the possible interferences from other amines and conjugates [40]. Derivatives from plasma and urine samples, prepared in 5 min at room temperature, showed no loss after 2 months dry storage at −20°C, and 5–10% loss at 4°C in buffer over 7 days.

Application of PITC to neurotransmitter amino acids using a Dynamax C_{18} column and a binary gradient containing sodium acetate, triethylamine (TEA) and acetonitrile followed by UV absorption was limited to manual injection [42]. Stabilities during storage of the PTC derivatives varied: asp and gaba recoveries increased, while gly and gln showed losses, even at −70°C.

The modern development 4-nitrophenylisothiocyanate (NPITC) has been used with a Waters M712 WISP to carry out toluene extraction rather than vacuum removal of excess

reagent [70]. This excess is considerably less than with PITC, reducing interference from reagent peaks. While unsuitable for sequencing work (unlike PITC), NPITC produces more stable derivatives (4-nitrophenylthiocarbamyls; NPTCs) which are better suited to automation. These are equally well detected at 340 nm, which avoids interferences at 254 nm. Five analyses were carried out using the derivative asp–NPTC. Peak area variation gave C.V. = 5.6%, and the C.V. for t_R was 0.35%. The figures for glu–NPTC were 7.2 and 0.33%. The observed ranges overall were 1.2–5.9% for area (apart from cys at 22.6), with 0.03–0.35% for t_R. Using a PicoTag column and a binary gradient of sodium acetate buffer–TEA–acetonitrile, normal hydrolysate amino acids were run in 25 min.

A specific method for assaying 3-methylhistidine from human urine using a phenyl RP column and electrochemical detection (ED) has been reported [71].

3.1.4. Novel amino-selective reagents

A list of some newer reagents follows. Except where stated, separations take place on conventional RP columns.

3.1.4.1. Aromatic methylamine (ARM) reagents and 1,2-diarylethylenediamines (DAD). Derivatization reactions involving potassium hexacyanoferrate [III] are used to produce specific and sensitive ARM reagents [72] and DAD reagents for monoamines [73–76], detectable by fluorescence. Where the reaction was post-column [74], periodate was used as post-column oxidant.

Following separation of 5-hydroxytryptamine and other indoleamines on a 5-μm TSKgel ODS-8 column using an acetate–acetonitrile mobile phase, ARM derivatives were produced by an optimized, on-line reaction at 70°C. This avoids the complications of electrochemical detection and extraction/column-switching. Detection limits of 140–470 fmol were reported at S/N = 3, with good linearity ($r = \geqslant 0.998$) up to 2.5 nmol [72].

Following solid-phase extraction, fourteen types of DAD derivative were prepared by reaction at 37°C for 40 min. Separation on a TSK-gel ODS-120T column with a mobile phase containing acetonitrile–methanol–Tris-HCl (pH 7.0) gave detection limits of 1, 1, and 2 pmol for norepinephrine, epinephrine and dopamine. Five compounds were resolved within 12 min [73].

Catecholamines have been quantified, using the selective fluorogenic reagent 1,2-diphenylethylenediamine (DPE) [74,75], (also detectable electrochemically) [74]. Derivatization was completed in 60 min at 20°C, and decay in the dark was $\leqslant 14\%/10$ h. An acetate–acetonitrile–methanol binary gradient was used with a 3-μm Microspher C_{18} column to separate 5 compounds, with a limit of detection at S/N = 2 for epinine of 0.6 pg [75].

3.1.4.2. 3-Benzoyl-2-quinoline carboxaldehyde (BQCA; Fig. 2, Table 7). This quinoline-type reagent gave femtogram detection limits, with derivatives more stable than those of *o*-phthalaldehyde (OPA), and absorption maxima better placed for laser output than those from benzenenoid reagents [47]. Fifteen amino acids, including asp, glu and gly were studied.

3.1.4.3. 3-(4-Carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA, Fig. 3). This reagent allows quick, ambient-temperature chemistry, freedom from excess reagent problems, and high sensitivity with laser-induced fluorescence detection, but as yet has been used only with capillary electrophoresis [8].

3.1.4.4. 3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCL; Table 8). This gives fmol detection limits for a range of amino acids, with a 60-min reaction time at ambient temperature. The adducts are stable in daylight for 120 h at ambient temperature, and conversion exceeds 84%. Linearity was shown over the 20 fmol to 100 pmol range for 17 amino acids [48,49].

3.1.4.5. N,N-Diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA). This reagent was found to produce more stable derivatives of amino acids than OPA, especially towards light, and to react with

both primary and secondary amino groups [59] but detection limits are higher, and only 30 rather than 38 amino acids were separated. Using a binary gradient of water–sodium propionate buffer–acetonitrile–methanol, with a Beckman Ultrasphere ODS column, total run times were *ca.* 100 min for OPA derivatives whereas DNDEA amino acids (derivatives of FNDEA) take a little longer. Between-day precision figures (C.V.%, $n = 7$) were 8.0 ± 3.0 for OPA and 4.0 ± 2.0 for FDNDEA.

3.1.4.6. 6-Methoxy-2-methylsulfonylquinoline-4-carbonyl chloride (MSQ-CL). The MSQ derivatives of several straight- and branched-chain alkylamines have been separated on a TSKgel ODS-80 column, using an aqueous acetonitrile mobile phase (R.S.D. = 3.1–7.3%, $n = 10$). For phenylethylamine in plasma, a detection limit of 1.0 pmol/20 μl injected (S/N = 3) was given. The 5-min reaction time was independent of temperature [51].

3.1.4.7. 4-Chloro-7-benzofuran (NBD-CL) (Fig. 3, Table 7). This reagent reacts at pH 8.0/60°C/5 min. Although the derivatives give poorer sensitivity than OPA and FMOC-Cl, they are more stable, and by contrast with FMOC-Cl, the tryp and cys derivatives do not suffer from intramolecular quenching and loss of sensitivity [30]. Isocratic chromatography of amino acids showed a ten times higher reactivity towards secondary amines than primary ones.

3.1.4.8. Naphthalene-2,3-dicarboxaldehyde (NDA) (Fig. 2, Tables 4–6). Evolved from *o*-phthaladehyde, this reagent forms 1-cyano-2-substituted-benz[f]isoindole (CBI) derivatives, detectable with electrochemical detection to a limit of 100 fmol for amino acids, 200–500 fmol by fluorescence [5] or 160–430 amol with laser-induced fluorescence detection (LIFD) [32]. The range of adduct oxidation potential optima could be used selectively in electrochemical detection [31]. For peptides, greater sensitivity by fluorescence detection than with OPA is claimed; 100 fmol detection limit for leu–enk by LIFD, and 200 pmol by conventional fluorescence [32,35,76]. The aspartate separation was typical in using an ODS-2 5-μm column with binary gradients containing phosphate buffer–THF–acetonitrile–water [37]. The detection limit was 10 fmol and S.D. for eight 100 pmol injections was 0.113.

Some chromatographic optimization with amino acid CBI derivatives has been carried out [32,33], and enantiomeric separations of amino acids have been performed using β-cyclodextrin-bonded columns [77]. Chemiluminescence detection has been used to estimate dopamine and norepinephrine following derivatization with NDA, separation on a TSK ODS 120T column with acetonitrile–THF–phthalate buffer mobile phase, and post-column oxidation with bis(2,4-dinitrophenyl)oxalate and peroxide [78]. The limit of detection is 1 fmol at S/N = 15, and response is linear from 1 to 600 fmol.

In what the authors believed to be the first attempt to automate with NDA (Tables 4–6), 13 amino acids (60-min run) and 3 peptides (20-min run) were quantified by fluorescence detection [79]. A Chrompack ODS-2 5-μm column was used, with gradients including phosphate–methanol–THF, and, for peptides, acetonitrile.

Derivatization for open tubular LC (OTLC) with gradient formation gave a detection limit of 36 amol at S/N = 3 for CBI–asn. Eighteen amino acids, including the neurotransmitters asp, glu, gln and gly were quantified using electrochemical detection. The electrode required pulse cleaning between runs but the development of a nanolitre injection device opens the prospect of analysing very small samples. Repeatability over a 1 week period gave R.S.D. = 1.20–4.69% with no t_R shifts interfering with identification [6,7].

3.1.4.9. Oxazoles. Oxazoles can react with amino, hydroxyl and sulphydryl functions (see section 3.3.4. below) [54].

3.1.4.10. Other derivatizing agents. Other derivatizing agents include phisyl-Cl and M-phisyl-Cl [53] and phthalmidyl benzoyl methoxybenzoyl chloride [52], which gives a detection limit of 10 fmol at S/N = 2 for aminoacid derivatives [48]. These showed more rapid derivatization re-

actions, greater derivative stability and larger detector responses than OPA.

3.1.4.11. Fluorosceinisothiocyanate (FITC). Fluorosceinisothiocyanate (FITC) has been used as a derivatizing agent for capillary zone electrophoresis [50], but the scope of the separation is limited compared with HPLC.

3.2. The carboxyl function

Suitable reagents to react with the carboxyl function have been harder to produce. Such reactions require higher temperatures for longer periods, and detection limits are higher. The same applies to the few carbonyl-linking reagents. The use of several halogenomethyl-, halogenoacyl-, and aryldiazomethane reagents, notably 2-bromoacetyl-6-methoxynaphthalene (BrAMN, Fig. 5), has been reported, and valproic acid (but no neurotransmitters) has been used to establish detection limits and linearity data [80].

The complete pre-column automation of derivatization with bromomethylmethoxycoumarin (BrMMc, Fig. 5), using solid potassium carbonate in suspension as a catalyst, with 18-crown-6 as reaction accelerator, all in acetonitrile solution, has been demonstrated [81] with fluorimetric detection, but not applied to amino acids. This reagent, both in automated or manual techniques, produces double peaks with stearate, possibly due to side-reactions.

The diazo reagent 9-anthryldiazomethane (ADAM) was reported to react less well than expected with amino acids via the carboxyl group [82]. However, the newer development 1-pyrenyl-diazomethane (PDAM, Fig. 5), has been used with neurotransmitter amino acids, and carboxylic acids [83]. The reaction is complete in 80 min and there are no excess reagent problems, but ethyl acetate is required as reagent solvent.

Derivatives of the fluorescent triazole, 2-(*p*-aminomethylphenyl)-N,N-dimethyl-2H-benzotriazolyl-5-amine (APBA), have shown linearity over the approximate range 10 to 500 pmol and detection limits of around 15 fmol for several carboxylated drugs and fatty acids [84]. The reagent yields 84–86% conversion after 30 min at ambient temperature, but sample clean-up and co-reagents would complicate automation.

Chiral separations have been performed with derivatives of anthrylethylamines [85], 4-(amino-

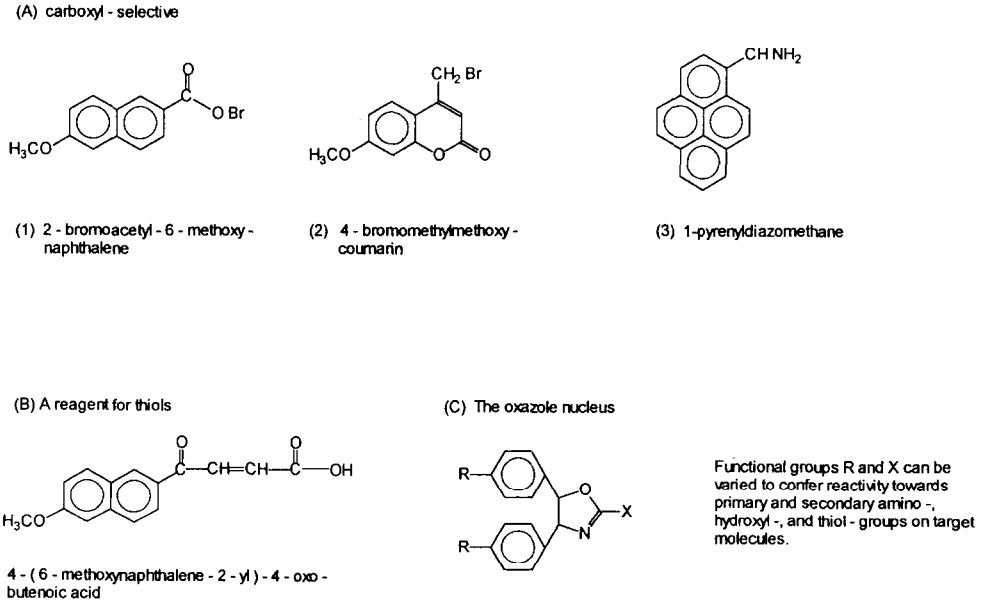

Fig. 5. Other reagents potentially suitable for neurotransmitters.

sulphonyl)-7-(3-aminopyridin-1-yl)-2,1,3-benzodioxazole (DBD-A-Py) [86] and (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole (APMB) [87], with attention focussed mainly on drugs.

3.3. Other functions

3.3.1. Sulphydryl-selective

A fluorogenic sulphydryl-selective reagent, 4-(6-methoxynaphthalene - 2 - yl)-4-oxo-butenoic acid, reacted quantitatively with cysteine in 10 min at ambient temperature [88]. Spherisorb or Hypersil RP-8 columns was used with methanol–phosphate–triethylamine at pH 3.0 (optimum). Limit of detection using fluorescence detection was 0.01 μg/ml at S/N = 3.

3.3.2. Hydroxyl-selective

A chiral reagent, (−)-2-methyl-1,1′-binaphthalene-2′-carbonyl cyanide (MBNCC) [89], has been reported in the analysis of popranolol.

3.3.3. Carboxyl-selective

Anthryl ethylamines have been used in the separation of carboxylic acids enantiomers, including N-acetyl amino acids [85].

3.3.4. Multi-selective

Oxazole reagents have been used to derivatize thiols, amines, and alcohols to produce derivatives from standards, [54] making this an interesting prospect for some peptides. Three reagents are described:

(1) 2-chloro-4,5-diphenyloxazole (DICLOX). This forms diphenyloxazole (DIOX) derivatives
(2) 2-fluoro-4,5-diphenyloxazole (DIFOX)
(3) 2-chloro-4,5-bis(*p*-N,N-dimethylaminosulfonyl)oxazole (SAOX-Cl).

Both DIFOX and SAOX-Cl react with thiols in aqueous media, with SAOX-Cl being more selective and giving maximum yield after 90 min incubation at 60°C. The reaction with DIFOX reached maximum after 60 min at room temperature.

Proline and N-acetylcysteine were derivatized via the amino group. In room light, DIOX and SAOX derivatives showed < 5% decomposition in 2 h at 60°C at pH 9.3 in sodium borate–EDTA, and similar in acetonitrile at the same temperature. No decomposition at pH 1 at room temperature was seen for any sample. Prolonged exposure to UV light did, however, produce decomposition.

Unlike NBD and dansyl amine derivatives, oxazoles show no effect of solvent polarity on fluorescence quantum efficiency. The DIOX and SAOX derivatives with N-acetylcysteine give constant fluorescence intensity over a wide pH range, while their by-products did not interfere with chromatography.

Amine derivatives were separated on a Supelco LC-8 octyl silica gel column, using phosphate–acetonitrile mobile phases. DIOX-pro and -ala derivatives gave limits of detection of 5.8/8.7 fmol and 10.9/14.6 fmol respectively (depending on mobile phase pH) at S/N = 2, while SAOX-pro and -ala gave 5.3/5.3 fmol and 9.0/7.3 fmol. Thiols were separated similarly, with SAOX- reduced glutathione (GSH), 2-mercaptopropionyl glycine (MPG) and N-acetylcysteine derivatives detectable to limits of 1.4/1.2, 1.4/1.9 and 1.3/1.5 fmol respectively, depending on pH.

3.4. Specific to monoamines

Derivatization with meso-1,2,-diphenylethylenediamine has been used specifically for catecholamines and their metabolites [74,75] and a number of other 1,2-diarylethylene diamines have also been evaluated [73], although without extensive automation (see section on ARM reagents and DAD above).

4. Approaches to chiral separations

Current interest centres on the differing activities and side-effects of d- and l-forms of drug molecules, notably beta-blocking agents [89]. There is less immediate and obvious relevance to neurotransmitters. The recognised amino acids are all l-forms, but there is some evidence of d-forms in neuropeptides [11,90], and present

knowledge may be revised as new techniques become more practical [65].

Chiral columns are currently unable to match conventional RP ones for peak shape, but there is interest in optimizing the resolution of some drugs active at known receptor sites [91–93]. Chiral phases also tend to be specific to particular analytes.

Resolution of enantiomers by achiral-phase chromatography has been reviewed [94].

4.1. Amino function reactions

4.1.1. Chiral reagents

The separation of racemic mixtures of amines derivatized with a reagent similar to FMOC, 1-(9-fluorenyl)ethyl chloroformate (FLEC), has been carried out on RP columns. Its use in separating l-dopa amongst other compounds is known [65,95], and its use with RP columns has been compared favourably with Pirkle column resolution of chiral oxazolidin-2-ones [96]. A newer reagent, [(1s)-endo]-(−)-borneol has been claimed to offer better separations for secondary amines [96].

Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-alanine, FDAA) has been used with amino acids and with peptides and their amino acid fragments in assessing racemization [97–99]. For peptides, ion-exchange and reversed-phase columns have been used to assess racemization [98]. Using a μBondapak C_{18} column and acetate–acetonitrile mobile phase, detection limits of 100 pmol with 0.1% racemization were obtained, monitoring at 340 nm.

With the combination of OPA with the chiral thiol d-3-mercapto-2-methylpropionic acid (3-MMPA), 17 pairs of amino acids plus glycine were separated isocratically [58] on a Nucleosil RP column at 40°C with an acetate buffer–methanol mobile phase. Fluorescence detection gave for repeated injections of 67 pmol of the valine derivative R.S.D. $< 2\%$ ($n = 5$) and $< 4\%$ for 5 pmol, with linearity at $r = 0.9999$.

Better detection limits have been obtained with s-flunoxapren chloride (SFPC), but it requires pre-conversion of the amino compounds to esters with HCl-2-propanol, plus solvent extraction and evaporation steps [100]. Various mobile phases containing *n*-hexane–methylene dichloride–chloroform–ethanol or propanol were used with a Zorbax-Sil 5 μm column to give $r = 0.995$ for ala, and an intra-day C.V. of 8.1%, lowering by 1.2% with a closely eluting amine as internal standard. Detection limits are quoted as 0.1–0.5 ng for compounds eluting within 30 min (approximately 1 pmol): run time is approximately 45 min.

4.1.2. Chiral stationary phases

Comparative studies of different cyclodextrin-bonded phases have been carried out, using, as test analytes, dansyl-, 3,5-dinitro-2-pyridyl-, dabsyl- and 3,5-dinitrobenzoyl-derivatives of racemic amino acids [101–103]. The effects on elution of derivatizing agent [101], cyclodextrin phase composition [101,102], mobile phase [101] and the chiral recognition mechanism [103] have been discussed.

The naphthalene-2,3-dicarboxaldehyde derivatives (CBIs) of amino acids and their amides have been separated on the β-cyclodextrin-bonded phase Cyclobond I, and the effects of the mobile-phase composition on enantioselectivity and the chiral recognition mechanism discussed [77].

4.1.3. Chiral component in the mobile phase

Enantiomeric separation of dansyl amino acid derivatives on a Develosil microcolumn packed with 5-μm ODS, and with the chiral compound γ-cyclodextrin included in the aqueous ammonium acetate–acetonitrile mobile phase, has been described. Detection is by UV absorption [4].

4.1.4. Solid-phase reagents

The solid-phase reagent (SPR) FMOC–l-prolyl (Fig. 4) was used, after functioning as a solid-phase extraction medium, to derivatize amphetamine (Fig. 4) [14]. A 9-fluorenylmethyl-SPR has been used with amino acids and amino alcohols [104] and an esterified form of the FMOC–l-prolyl reagent has also been investi-

gated [105], but this suffers from an interfering peak deriving from water.

4.2. Carboxyl function reactions

Anthryl ethylamine compounds give detection limits of approximately 100 fmol N-acetyl amino acids using a hexane–ethyl acetate mobile phase with a Resolve 5-μm RP column, and fluorescence detection [85]. The reagent is added as solid to the acid in pyridine and diluted with ether. The reaction takes 1 h at room temperature after which the solution must be acid- then alkali-washed and dried before injection, hindering automation.

So far, only a selection of drugs have been derivatized with (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole) (APMB). Derivatization is complete at room temperature after 5 min with nitrogen evaporation in the presence of 2,2′-dipyridyl disulphide (DPDS) and triphenylphosphine (TPP). A TSK gel Silica 60 column with *n*-hexane–ethyl acetate–2-propanol mobile phase was used, followed by fluorescence detection. Good linearity and reproducibility were quoted [87]. A series of compounds, including some N-acetyl amino acids have been derivatized with 4-(aminosulphonyl)-7-(3-aminopyridin-1-yl)-2,1,3-benzodioxazole (DBD-A-Py) to give NBD-A-Py- derivatives [86]. The reaction takes 2 h at ambient temperature, and an Inertosil ODS column is used with acetonitrile–water at 40°C (an Inertosil normal-phase column proved less robust and was abandoned). Fluorescence detection gave detection limits of 15–45 fmol for several d- and l-pairs of compounds, including N-acetyl amino acids, while the use of an argon laser gave 29 fmol for another carboxyl compound.

4.3. Hydroxyl function reactions

Propranolol has been quantified to a detection limit of 100 pg by fluorescence detection following solid-phase extraction, with (−)-2-methyl-1,1′-binaphthalene-2′-carbonyl chloride (MBNCC) [89]. Linearity from 500 pg/ml to 100 ng/ml, and recoveries $\geq$ 89.7% are reported.

4.4. Prospects

Various strategies in achiral-phase separations were reviewed in 1992 [92], and discussed elsewhere [56,65]. Chiral separations have not yet approached the performance of classical reagents with RP-HPLC, and their ultimate usefulness remains an open question. Fewer reports exist specifically describing relevant chiral separations.

5. Solid-phase reagents (SPRs)

5.1. Range of reagents

In SPRs, reactive derivatizing moieties are incorporated into polymers or copolymers of, for example, divinylbenzene (DVB) or ethylvinylbenzene (EVB) or copolymers such as 1:1 *p*-nitrobenzyl–3,5′-DNP. Those evaluated thus far include: (i) *o*-acetylsalicylyl-; (ii) 3,5′-DNP-; (iii) fluorenyl-; (iv) naphthyl-; (v) *p*-nitrobenzoyl-; (vi) various related to FMOC (see section 4.1.4.).

5.2. 3,5′-Dinitrophenol and the advantages of solid-phase reagents

A study of the 3,5-dinitrophenol (DNP) solid-phase reagent (SPR) has been made, and the following advantages over conventional reagents are put forward [12]:

(1) There is no excess reagent to interfere later
(2) Efficiency of reaction remains higher throughout
(3) Greater reaction selectivity gives simpler chromatograms
(4) High local concentration of reagent aids trace analysis
(5) Immobilized co-reagents avoid undesired reactions

Thus, SPRs are most suitable for chiral derivatization prior to conventional reversed-phase separations. Moreover, the use of a separate solid-phase extraction step is avoided, which with the use of an on-line SPR cartridge facilitates automation [12]. It has also been pointed out that regeneration of spent polystyrene–di-

vinyl benzene-bound FMOC reagent (non-chiral) is possible by repeating the last preparation step [106]. Other advantages for chiral solid-phase reagents are quoted [104]:

(1) Wide range of substrates (including amino acids, amides, catecholamines, peptides, nucleosides and nucleotides)
(2) Usable with conventional RP columns
(3) Usable with low detector-noise, non-chiral mobile phases
(4) Superior detector properties through use of chromo-, fluoro- or electrophores
(5) Potential to automate by using SPR in an on-line pre-column
(6) Cheaper than chiral system
(7) Disposable off-line cartridges could be developed
(8) Highly-loaded, on-line cartridges give long life

5.3. Prospects

There is every prospect of SPRs becoming more important, as there appears to be scope for development of both matrix and derivatizing moieties. Such developments could facilitate further automation of neurotransmitter separations, by combining sample clean-up, better control of on-, or off-line chemistry, and simpler chromatograms.

6. Conclusions

A high proportion of established neurotransmitters are amenable to derivatization, notably for HPLC. Some work has also been carried out with microcolumns and capillary electrophoresis. Amino-selective reagents have proved most successful, and now make possible quantification of amino acids from complex mixtures in biological fluids. This can be fully automated where *o*-phthaladehyde and/or 9-fluorenylmethylchloroformate are used and naphthalene-2,3-dicarboxaldehyde is approaching this degree of utility. Newer reagents exist which produce derivatives with superior properties for detection, but their chromatography has not been optimized for comprehensive separations, perhaps because of the effort this would involve. Carboxyl-, hydroxyl- and thiol-selective reagents are less numerous: oxazole reagents, recently developed, are reactive towards amines and thiols.

Autosamplers allow the use of stable derivatives produced by manual chemistry, but complete automation is now possible in some cases, using solid-phase extraction. Sample preparation is also possible with solid-phase derivatizing reagents: this could facilitate chiral derivatizations. Measurements of the choline/ACh system still use indirect electrochemical detection and purines are largely detected by UV absorption. Chiral separations may widen the knowledge of amine and peptide neurotransmitter systems.

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