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Review

Labeling of free carboxyl groups

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Abstract

The latest trends in the labeling of free carboxyl groups for high-performance liquid chromatography are reviewed. The labeling reagents for fluorescence detection are mainly discussed according to their reaction type (or functional group). Attention is also paid to the reagents used for ultraviolet detection and for enantiomeric separation. The reactivity and sensitivity of the reagents used for the labeling of carboxylic acids are described.

Contents

List of abbreviations

ABD-AP	4-(Aminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole
ABEI	N-(4-Aminobutyl)-N-ethylisoluminol
ADAM	9-Anthryldiazomethane
ANBA	2-[4-(1-Aminoethyl)naphthyl]-6-methoxy-N-methyl-2H-benzotriazolyl-5-amine dihydrochloride

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9-AP	9-Aminophenanthrene
9-APB	*p*-(9-Anthroyloxy)phenacyl bromide
APMB	2-[4-(1-Aminoethyl)phenyl]-6-methoxybenzoxazole
BP-OTf	4′-Bromophenacyl trifluoromethanesulphonate
Br-ADMC	3-Bromoacetyl-6,7-methylenedioxycoumarin
Br-ADMF	3-Bromoacetyl-1,1′-dimethylferrocene
Br-AMC	3-Bromoacetyl-7-methoxycoumarin
Br-DMC	4-Bromomethyl-6,7-dimethoxycoumarin
Br-DMEQ	3-Bromomethyl-6,7-dimethoxy-1-methyl-2(1H) quinoxalinone
Br-MA	9-Bromomethylacridine
Br-MAC	4-Bromomethyl-7-acetoxycoumarin
Br-MB	3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one
Br-MDC	4-Bromomethyl-6,7-methylenedioxycoumarin
Br-MMC	4-Bromomethyl-7-methoxycoumarin
Br-MMEQ	3-Bromomethyl-6,7-methylenedioxy-1-methyl 2(1H)-quinoxalinone
Br-PB	*p*-Bromophenacyl bromide
DAM-MC	4-Diazomethyl-7-methoxycoumarin
DBD-AP	4-(N,N-Dimethylaminosulphonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole
DBD-PZ	4-(N,N-Dimethylamino-sulphonyl)-7-N-piperadino 2,1,3-benzoxadiazole
2,4-DBE	1-(2,4-Dihydroxyphenyl)-2-bromoethanone
2,5-DBE	1-(2,5-Dihydroxyphenyl)-2-bromoethanone
DCCI	N,N′-Dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea
DEPC	Diethyl phosphorocyanide
DMBI-BH	4-(5,6-Dimethoxy-2-benzimidazoyl)benzohydrazide
DMEQ-PAH	6,7-Dimethoxy-1-methyl-2(1H)-quinoxalinone-3 propionylcarboxylic acid hydrazide
DMF	N,N-Dimethylformamide
DNB-OTs	3,5-Dinitrobenzyl *p*-toluenesulphonate
ED	Electrochemical detection
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FEA	1-Ferrocenylethylamine
FPA	1-Ferrocenylpropylamine
4-HBE	1-(4-Hydroxyphenyl)-2-bromoethanone
HCPI	2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole
HMA	9-(Hydroxymethyl)anthracene
MBPA	2-[*p*-(5,6-Methylenedioxy-2H-benzotriazol-2-yl)]phenethylamine
MDC	Monodansyl cadaverine
MMC	(7-Methoxycoumarin-4-yl)methyl
NBD-AP	4-Nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole
NB-OTs	*p*-Nitrobenzyl *p*-toluenesulphonate
NB-PS	Poly(4-nitrobenzyl *p*-styrenesulphonate)
NE-OTf	2-(2,3-Naphthalimido)ethyl trifluoromethane-sulphonate
NE-PS	Poly[2-(1-naphthyl)ethyl *p*-styrenesulphonate]
NPH	2-Nitrophenylhydrazine
PB	Phenacyl bromide
PDAM	1-Pyrenyldiazomethane
PE-OTf	2-(Phthalimido)ethyl trifluoromethanesulphonate
PE-OTs	2-(Phthalimido)ethyl *p*-toluenesulphonate
PE-PS	Poly[2-(phthalimido)ethyl *p*-styrenesulphonate]
PTM	5-(4-Pyridyl)-2-thiophenemethanol

1. Introduction

In general, derivatization with suitable labeling reagents is used for the sensitive HPLC determination of carboxylic acids which produce no strong absorption or fluorescence, owing to the weak UV-Vis absorption and lack of fluorescence by the carboxyl group itself. Because of the low reactivity of the carboxyl group, prechromatographic labeling is preferred to postchromatographic labeling, because the former offers greater freedom and flexibility in the selection of optimum reaction conditions regardless of HPLC. The great lack of reactivity of the carboxyl group in an aqueous matrix is caused by the solvation of the carboxylic moiety by water molecules. Therefore, the carboxylic acids have to be extracted from the aqueous matrix into a suitable aprotic solvent prior to labeling. In order to circumvent these generally tedious extraction procedures, a biphasic reaction system incorporating a phase-transfer agent [1] and micellar systems [2–5] are advantageously used. Both reaction systems also enhance the selectivity and reactivity in the labeling procedure and simplify sample handling.

Carboxylic acids are biomedically and environmentally important compounds and they usually occur in complicated matrices. Therefore, it is of great importance for labeling reagents to produce derivatives that have not only highly sensitive and selective detector responses but also good HPLC properties. A variety of chromophores, fluorophores and electrophores have been used for labeling in carboxylic acid analysis by HPLC. Particularly fluorogenic labeling is widely applied due to its high sensitivity and selectivity. A number of fluorescent labeling reagents have been developed for the carboxylic acid function and some of them are now commercially available from various sources.

This review discusses labeling reagents for free carboxyl groups according to their reaction type. The wavelengths used for UV and/or fluorescence detection of the derivatives labeled with the reagents are summarized in Table 1.

2. Labeling reagents

2.1. Alkylation

Carboxylic acids are most frequently labeled in the presence of potassium carbonate combined with 18-crown-6 as a catalyst to facilitate reactions with alkylating reagents. Potassium bicarbonate or fluoride are also used as catalyst in order to prevent the labeling reagents from being degraded by potassium carbonate. A weakly basic anion-exchange resin could be used instead of the catalysts to simplify the labeling [6]. The labeling reactions usually proceed faster in acetone, which has a medium dielectric constant (ϵ), than in acetonitrile, which has a higher ϵ [6,7]. The above-mentioned biphasic and micellar reaction systems are very useful for the labeling of carboxylic acids in aqueous matrices. The use of the latter offered a fully automated HPLC analysis of free fatty acids in plasma [5].

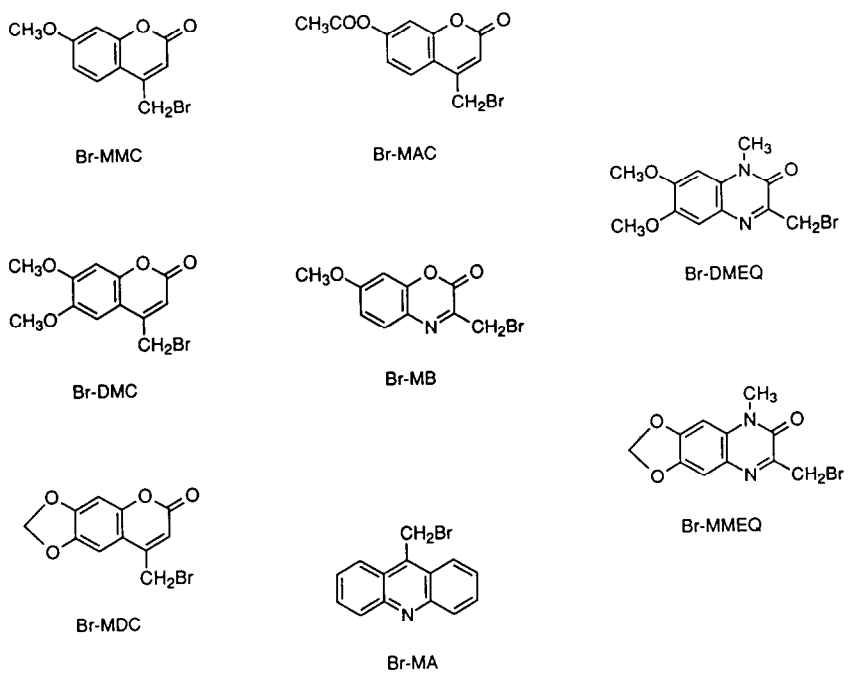
2.1.1. Bromomethyl group bearing reagents

The structures of the discussed bromomethyl group bearing reagents are shown in Scheme 1. 4-Bromomethyl-7-methoxycoumarin (Br-MMC) is one of the most frequently used labeling reagents [3,8–10]. It is known that the fluorescence properties of the (7-methoxycoumarin-4-yl)methyl (MMC) esters of fatty acids are strongly affected by their chain length and the eluent (solvent) composition [11]. The fatty acid-MMC derivatives have reportedly been determined at the fmol level in fish oil dietary supplements with laser-induced fluorescence detection [12]. Fatty acid derivatives labeled with 4-bromomethyl-6,7-dimethoxycoumarin (Br-DMC) exhibited higher fluorescence sensitivities than the homologous MMC derivatives [13]. In acetonitrile–water mixtures, the *n*-caproic acid derivatized with 4-bromomethyl-6,7-methylenedioxycoumarin (Br-MDC) exhibited a higher fluorescence than that derivatized with Br-DMC, and the detection limit was 15 fmol at a signal-to-noise ratio of 3 [14]. Br-MDC was successfully applied to the simultaneous analysis of some

Table 1
Wavelengths operated for UV and fluorescence detection of labeled derivatives

Reagent	Wavelength (nm)		
	UV detection	Fluorescence detection	
		λ_{ex}	λ_{em}
(+)-ABD-AP		470	585
ADAM		254, 255, 365	412, 415
S-(−)-ANBA		355	480
9-AP		303	376
9-APB		325, 375	470
(−)-APMB		320	375, 380
BP-OTf	254		
Br-ADMC		388	475
Br-AMC		365	417
Br-DMC	341–346	341–346	423–427
Br-DMEQ		370, 380	450, 455, 460
Br-MA	252	362, 365	418, 425
Br-MAC		365, 375	460, 475
Br-MB		345	440
Br-MDC		355	435
Br-MMC	318, 330, 340	318, 325, 330	378, 395, 396, 398, 410
Br-MMEQ		363	437
Br-PB	254		
DAM-MC		325	386
(+)-DBD-AP		470	580
DBD-PZ		440	569
DCCI		325	395
DMBI-BH		360	460
DMEQ-PAH		360, 365	435, 447
DNB-OTs	272		
HCPI		335	455
HMA		360	420
MBPA		333	372
MDC		340	518
(+)-NBD-AP		470	540
NB-OTs	232		
NB-PS	254		
NE-OTf	259	259	394
NE-PS		275	332
NPH	230, 400		
PB	242		
PDAM		340	376, 395
PE-OTf	219		
PE-OTs	222		
PE-PS	221		
PTM		300	360

acidic non-steroidal anti-inflammatory agents. The presence of a hydroxyl group at the 7-position in coumarin enhanced the fluorescence intensity in alkaline solutions [15]. Therefore, carboxylic acids labeled with 4-bromomethyl-7-acetoxycoumarin (Br-MAC) were hydrolyzed to



Scheme 1. Structures of bromomethyl group bearing reagents.

their 7-hydroxy derivatives [7,15,16]. In this system, the fluorescence intensity depends neither on the kind of carboxylic acid nor on the composition of the mobile phase. On the other hand, the coumarin ring is susceptible to base-catalyzed solvolysis. Consequently, the labeling reaction conditions must be carefully controlled to obtain reproducible results [17].

Recently, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MB) was synthesized. It is a more reactive labeling reagent than Br-MMC and Br-MDC. The labeling reaction of saturated aliphatic fatty acids with Br-MB in the presence of potassium carbonate and 18-crown-6 in acetonitrile could be completed within 10 min at 40°C. The detection limits of the fatty acids were in the range 2–10 fmol at a signal-to-noise ratio of 3 [18]. Br-MB was also applied to the quantitative analysis of labile fatty acids such as polyunsaturated hydroxy and hydroperoxy fatty acids by HPLC–mass spectrometry [19].

In N,N-dimethylformamide (DMF) or dimethylsulfoxide in the presence of tetraethylammonium carbonate as a base, 9-bromomethylacridine (Br-MA) completely labeled fatty acids within 10 min at room temperature [20]. Both Br-MA [5] and Br-MMC [2–4] were also used in micellar-mediated labeling reactions. Fatty acids [5] and valproic acid [2] could be determined in plasma with these methods.

It was reported that the sensitivity of the method with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ, whose detection limits for fatty acids were 0.3–1 fmol at a signal-to-noise ratio of 2), was at least 100 times higher than that with Br-MMC and *ca.* 10 times higher than that with Br-MAC [21,22]. Br-DMEQ was applied to the determination of benzoylecgonine (the main metabolite of cocaine) in human urine [23] and a cysteine protease inhibitor in mouse serum [24]. 3-Bromomethyl - 6,7 - methylenedioxy-1-methyl-2(1H)-quinoxalinone (Br-MMEQ) as a fluorescent labeling reagent gives a *ca.* 1.6 times higher sensitivity than Br-DMEQ [25]. Aqueous methanol was found to be suitable as an eluent in reversed-phase HPLC of fatty acid-MMEQ derivatives with gradient elution, because the fluo-

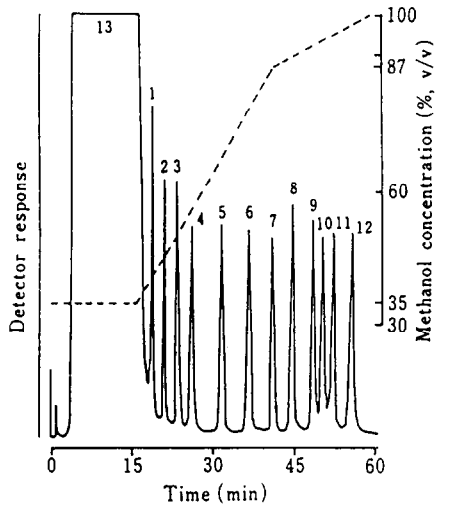


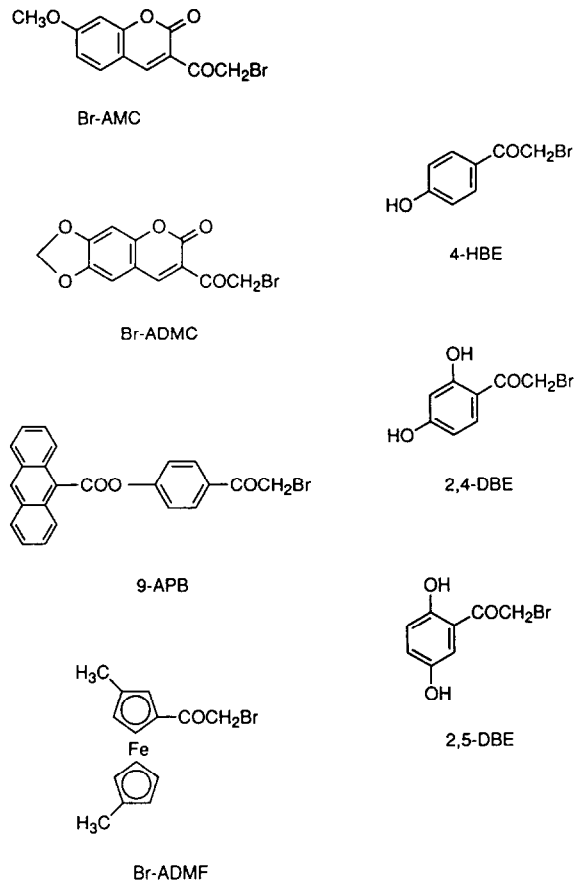
Fig. 1. Chromatogram of MMEQ derivatives of fatty acids. Column, Radial Pak C_{18} cartridge; mobile phase, aqueous methanol (35-100%); flow-rate, 2 ml/min. Peaks: 1 = propionic acid; 2 = butyric acid; 3 = valeric acid; 4 = caproic acid; 5 = caprylic acid; 6 = capric acid; 7 = lauric acid; 8 = myristic acid; 9 = palmitic acid; 10 = margaric acid; 11 = stearic acid; 12 = arachidic acid; 13 = reagent blank. Reproduced with permission from ref. 25.

rescence intensity only slightly decreased with an increase in the amount of water in the eluent. Fig. 1 shows a typical chromatogram of the MMEQ derivatives of twelve fatty acids completely separated within 60 min.

2.1.2. *Bromoacetyl group bearing reagents*

The structures of the bromoacetyl group bearing reagents are shown in Scheme 2. A bromoacetyl group shows a higher reactivity with fatty acids than the bromomethyl group. *p*-Bromophenacyl bromide (Br-PB) [26] and phenacyl bromide (PB) [27] are the most well-known UV-labeling reagents for carboxylic acids. A fast method for determining serum free fatty acids was developed by using a modified one-step Dole extraction and labeling with Br-PB [28]. The detection limit of this method was *ca.* 2 pmol per 100 μl of serum, and a series of 10–20 samples could be analyzed in about 2 h including extraction.

Recently, 3-bromoacetyl-7-methoxycoumarin (Br-AMC) has been synthesized as a highly reactive fluorescent labeling reagent [6]. Br-



Scheme 2. Structures of bromoacetyl group bearing reagents.

AMC readily reacted with carboxylic acids at room temperature in the presence of potassium bicarbonate and 18-crown-6 or Duolite A-375 (a weakly basic anion-exchange resin) as catalysts. The detection limits for the methods using the former and the latter catalysts were 0.4 and 0.5 pmol at a signal-to-noise ratio of 3, respectively. The use of potassium carbonate or strongly basic anion-exchange resins resulted in decomposition of the reagent. Comparison of chromatograms of free fatty acids in a normal human plasma and the plasma of a patient with diabetes exhibited a remarkably large difference. 3-Bromoacetyl-6,7-methylene-dioxycoumarin (Br-ADMC) was also synthesized as a highly reactive and sensitive fluorescent reagent [29]. The fluorescence quantum yields of lauric acid derivatized with Br-ADMC in various solvents were definitely large

compared with those obtained with bromomethyl-type Br-DMC and Br-MDC. (15*R*)- and (15*S*)-15-Methylprostaglandin E_2 in human plasma were labeled with *p*-(9-anthroyloxy)phenacyl bromide (9-APB) and determined down to 10 pg/ml at a signal-to-noise ratio of 5 with fluorescence detection [30]. 9-APB combined with laser-induced fluorescence detection also afforded a sensitive HPLC method for the analysis of alkylphosphonic acids at fmol levels [31].

A labeling method using ferrocene reagents was developed for the determination of fatty acids in human serum by HPLC with electrochemical detection (ED). Fatty acid esters labeled with 3-bromoacetyl-1,1′-dimethylferrocene (Br-ADMF) exhibited satisfactory sensitivity at +0.60 V *vs.* an Ag/AgCl reference electrode with a detection limit of 0.5 pmol at a signal-to-noise ratio of 5 using a twin-electrode system [32]. It took 60 min for Br-ADMF to effectively label the fatty acids in the presence of potassium fluoride and 18-crown-6 in DMF at 80°C. Fig. 2 shows a typical chromatogram of fatty acids in 50 μl of human serum extracted with hexane–chloroform and then labeled with Br-ADMF as described above. 1-(4-Hydroxyphenyl)- (4-HBE), 1-(2,4-dihydroxyphenyl)-(2,4-DBE) and 1-(2,5-dihydroxyphenyl)-2-bromoethanone (2,5-DBE) were also applied as electrochemical reagents in labeling drugs and metabolites with carboxylic acid groups [33]. Acetonitrile and benzene as reaction solvents were reported to give higher yields than DMF and acetone.

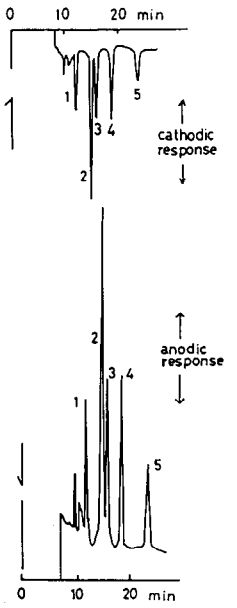


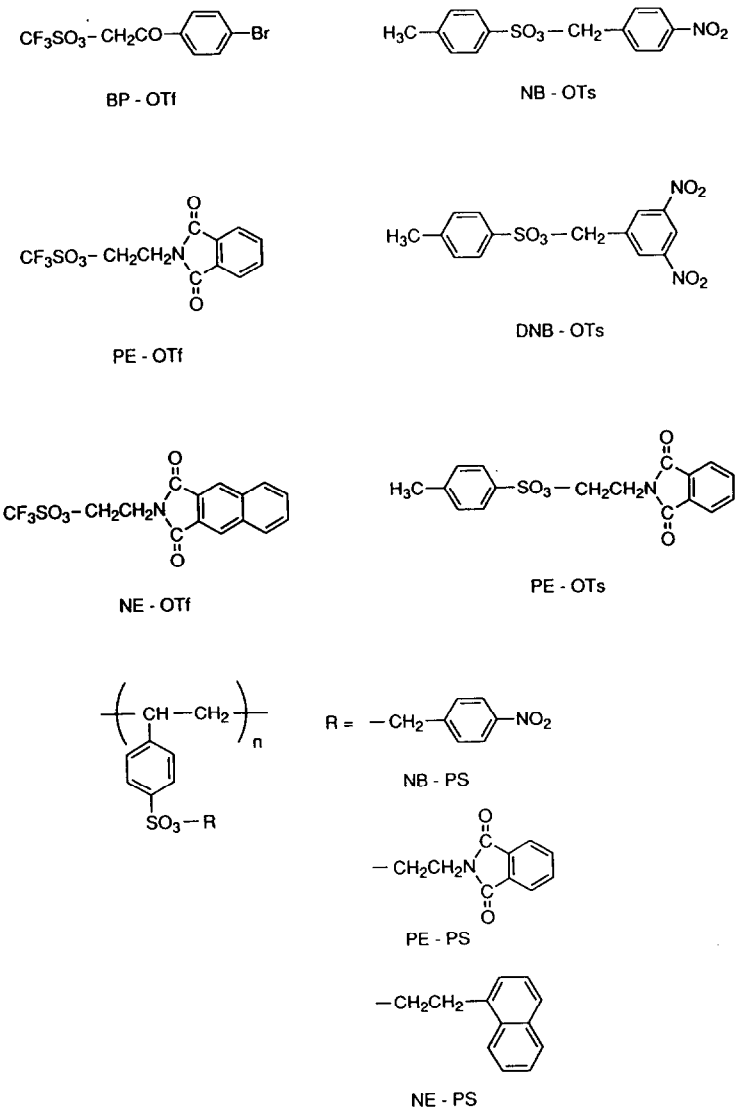
Fig. 2. Chromatogram of fatty acids in human serum. Column, TSKgel ODS-80TM; mobile phase, 0.1 *M* $NaClO_4$ in methanol–water (13:1, v/v); flow-rate, 1.0 ml/min. Peaks: 1 = linoleic acid; 2 = palmitic acid; 3 = oleic acid; 4 = margaric acid (internal standard); 5 = stearic acid. Reproduced with permission from ref. 32.

2.1.3. Sulphonate group bearing reagents

The structures of the sulphonate group bearing reagents are shown in Scheme 3. The powerful alkylating abilities of trifluoromethanesulphonates are well-known. 4′-Bromophenacyl trifluoromethanesulphonate (BP-OTf) was first used as a highly reactive UV-labeling reagent for carboxylic acids for HPLC [34]. Even reactions of sterically hindered carboxylic acids with BP-OTf in the presence of N,N-diisopropylethylamine in acetonitrile at room temperature proceeded to completion in 5 min. Similarly, as highly reactive labeling reagents, 2-(phthalimido)ethyl trifluoromethanesulphonate (PE-OTf) [35] and 2-(2,3-naphthalimido)-ethyl trifluoromethanesulphonate (NE-OTf) [36] were synthesized for UV and fluorescence detection, respectively. Both PE-OTf and NE-OTf were successfully applied to the determination of carboxylic acids in mouse brain. The detection limits for the method using NE-OTf with UV and fluorescence detection were 100 fmol and 4 fmol at a signal-to-noise ratio of 3, respectively. Fig. 3 shows a chromatogram of the determination of carboxylic acids in mouse brain.

Three *p*-toluenesulphonate-type UV-labeling reagents, *p*-nitrobenzyl, 3,5-dinitrobenzyl and 2-(phthalimido)ethyl *p*-toluenesulphonates (NB-OTs, DNB-OTs and PE-OTs, respectively) were compared in terms of their reactivity for fatty acids [37]. The reactivity increased in the order NB-OTs > DNB-OTs > PE-OTs according to the electrophilicity of their labeled groups.

After labeling, the excess of the low-molecular-mass reagents cannot be removed from the



Scheme 3. Structures of sulphonate group bearing reagents.

reaction solutions by simple operations. Therefore, the reagents must be co-injected with the derivatives onto the liquid chromatograph. This results in a large background. The most obvious advantage of polymeric reagents is their easy separability from low-molecular-mass compounds by a simple operation such as precipitation and/or filtration. Polymeric reagents, poly(4-nitrobenzyl *p*-styrenesulphonate) (NB-PS) and poly[2-(phthalimido)ethyl *p*-styrenesulphonate] (PE-PS) for UV detection [38] and poly[2-(1-naphthyl)ethyl *p*-styrenesulphonate] (NE-PS) for fluorescence detection [39] were developed. The large background tailing could be greatly reduced by using these polymeric reagents instead of the low-molecular-mass reagents. This is due to the fact that no large excess of the polymeric reagents is co-injected.

2.1.4. Diazomethyl group bearing reagents

The structures of the diazomethyl group bearing reagents are shown in Scheme 4. Diazo-

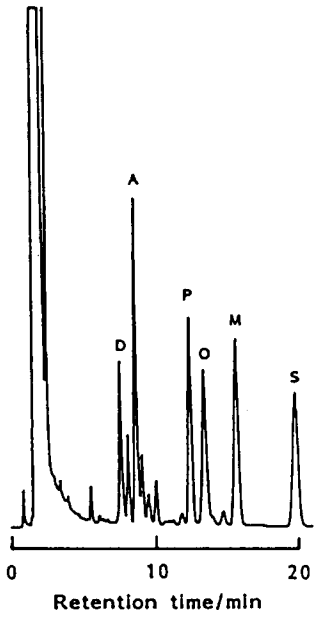
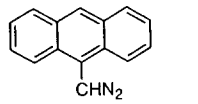
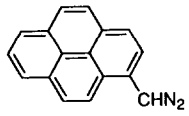


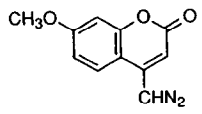
Fig. 3. Chromatogram of the determination of carboxylic acids in mouse brain.Column, Chemcosorb 5C8; mobile phase, methanol–water (87:13, v/v); flow-rate, 1.0 ml/min. Peaks: D = docosahexaenoic acid; A = arachidonic acid; P = palmitic acid; O = oleic acid; M = margaric acid (internal standard); S = stearic acid. Reproduced with permission from ref. 36.



ADAM



PDAM



DAM - MC

Scheme 4. Structures of diazomethyl group bearing reagents.

methane-type reagents can react with carboxylic acids at room temperature without the presence of a catalyst and even in the presence of water. In particular, 9-anthryldiazomethane (ADAM) has been widely used as a fluorescent labeling reagent for biologically important carboxylic acids such as those in serum, urine and tissue [40–42]. Imidapril and its active metabolite in human plasma and urine, for instance, could be simultaneously determined by HPLC with fluorescence detection [42]. The detection limits of imidapril and its active metabolite were 0.2 ng/ml in plasma and 10 ng/ml in urine at a signal-to-noise ratio of 3, respectively. However, ADAM is unstable and decomposes on storage. In order to overcome this drawback, a simple method for the preparation of ADAM *in situ* was established [43]. Fig. 4 shows a chromatogram of the determination of α-hydroxycarboxylic acids as their ADAM derivatives. Fatty acids labeled with ADAM have an excitation wavelength of 365 nm and are considered suitable for peroxyoxalate chemiluminescence detection [44].

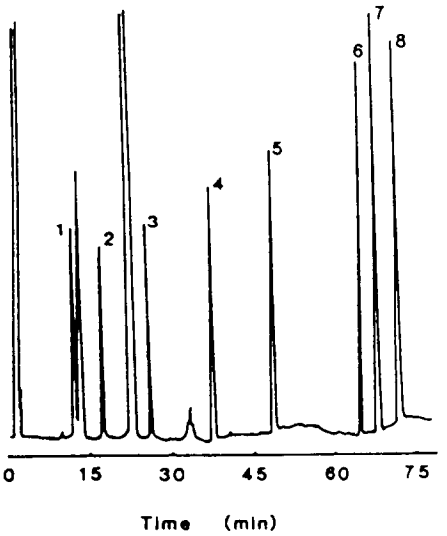


Fig. 4. Chromatogram of ADAM derivatives of α-hydroxycarboxylic acids. Column, TSKgel-120T; mobile phase, aqueous methanol (0–10 min, 55%; 10–75 min, 55–100%); flow-rate, 1.0 ml/min. Peaks: 1 = glycolic acid; 2 = lactic acid; 3 = α-hydroxyisobutyric acid; 4 = α-hydroxyisocaproic acid; 5 = α-hydroxycaprylic acid; 6 = α-hydroxynaphthoic acid; 7 = β-hydroxymyristic acid; 8 = α-hydroxystearic acid. Reproduced with permission from ref. 43.

The stability of aryldiazoalkanes generally depends mainly on the aromaticity of the aromatic ring and the electron-withdrawing activity of the ring substituents. 1-Pyrenyldiazomethane (PDAM) as a more stable diazomethane reagent than ADAM was developed by converting its anthryl group into a more aromatic pyrenyl group [45,46]. The fluorescence intensity of fatty acids labeled with PDAM was *ca.* 5 times stronger than those labeled with ADAM. The detection limits of the PDAM derivatives were 15–30 fmol at a signal-to-noise ratio of 3. However, PDAM took a longer reaction time than ADAM to reach a constant derivatization yield. Some short-chain dicarboxylic acids reacted with PDAM in an aqueous medium to form their monoesters with one free carboxyl residue [47]. The reaction was strongly dependent not only on the buffer used but also of its pH.

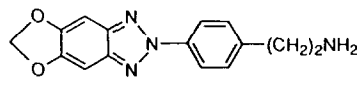
As already described, 4-diazomethyl-7-methoxycoumarin (DAM-MC) [48] may be promising as a highly sensitive labeling reagent for He-Cd laser-induced fluorescence detection, though the reactivity of DAM-MC is less than those of ADAM and PDAM.

2.2. Amidation

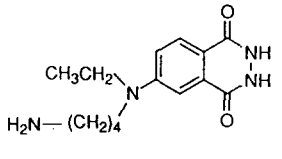
The structures of the reagents used for amidation are shown in Scheme 5. Fluorescent amines can also be utilized for the labeling of carboxyl groups. In general, activation of the carboxyl groups is required prior to reaction with the amines. For this purpose, 1,3-dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) has been widely used as an activation agent. The addition of 1-hydroxybenzotriazole or 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine is effective not only in suppressing the formation of N-acylisourea by-products but also in enhancing the reaction rates and yields. This is advantageous for the prevention of racemation of optically active analytes during their labeling.

2-[*p*-(5,6-Methylenedioxy-2H-benzotriazol-2-yl)]phenethyl amine (MBPA) was synthesized as a highly sensitive fluorogenic labeling reagent for carboxylic acids [49]. MBPA reacted with ibuprofen in the presence of 2-bromo-1-ethylpyridinium tetrafluoroborate to give the corresponding fluorescent amide at room temperature. The detection limit for ibuprofen in serum was 1.5 pg per injection (20 μl). The fluorescence intensity of MBPA derivatives in aqueous acetonitrile was almost constant at water concentrations of 0–50% (v/v). Ibuprofen in saliva was also reacted with N-(4-aminobutyl)-N-ethylisoluminol (ABEI) in the presence of 1-hydroxybenzotriazole as a pre-activator and EDAC [50]. The labeled derivative was detected by chemiluminescence at 389 nm and its detection limit was 0.7 ng at a signal-to-noise ratio of 3 per 0.5 ml of saliva.

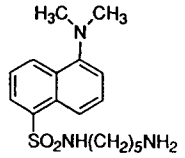
Monodansyl cadaverine (MDC) was proposed



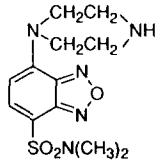
MBPA



ABEI



MDC



DBD-PZ

Scheme 5. Structures of reagents used for amidation.

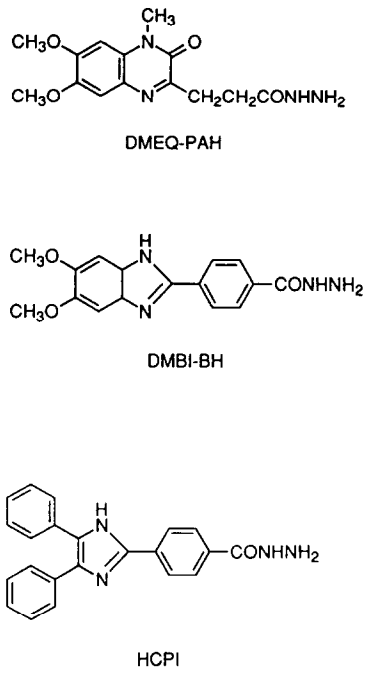
as a promising fluorogenic reagent for rapid labeling of fatty acids in the presence of diethyl phosphorocyanide (DEPC) as an effective activation agent [51]. The labeling reaction of arachidic acid with MDC and DEPC proceeded very quickly in DMF at room temperature, and the peak height of the derivative reached a plateau in 15 min. The detection limits of fatty acids were below 100 fmol at a signal-to-noise ratio of 3.

In the presence of DEPC at room temperature, 4-(N,N-dimethyl-aminosulphonyl)-7-N-piperadino-2,1,3-benzoxadiazole (DBD-PZ) also reacted with carboxylic acids to produce highly sensitive, fluorescent derivatives [52]. However, a long reaction time of 5 h, for instance, was required for obtaining a constant production of the derivative by reaction of DBD-PZ with arachidic acid in acetonitrile. In this case, the reaction in acetonitrile gave higher labeling yields than in DMF. The detection limits of fatty acids were in the range 3.9–4.7 fmol at a signal-to-noise ratio of 3. 4-(Aminosulphonyl)-2,1,3-benzoxadiazole derivatives were also reported by the same research group [53]. The detection limits of fatty acids labeled with these reagents were higher, compared with that for DBD-PZ, the dimethylaminosulphonyl-type reagent.

9-Aminophenanthrene (9-AP) was reported as a fluorescent labeling reagent for valproic acid which is widely used in epileptic therapy [54]. The acid was chlorinated with thionyl chloride in the presence of triethylamine. Then the resulting acid chloride was reacted with 9-AP to produce the amide derivative. The concentrations of thionyl chloride and triethylamine and the reaction temperature and time significantly affected this labeling. The detection limit of valproic acid was 9.4 pg at a signal-to-noise ratio of 3. This method was successfully applied for the determination of serum valproic acid levels in epileptic patients.

2.3. Reaction with hydrazines

The structures of the reagents with a hydrazino group are shown in Scheme 6. Most of the above-mentioned labeling reagents generally require dried aprotic solvents for labeling carboxylic acids to attain high reaction yields. On the other hand, the hydrazino group readily reacts with carboxylic acids in aqueous solution in the presence of EDAC. Several reagents of this type were reported.



Scheme 6. Structures of reagents with a hydrazino group.

2-Nitrophenylhydrazine (NPH) was successfully utilized as a UV labeling reagent in a fully automated pre-column derivatization unit for the determination of aliphatic monocarboxylic acids [55]. The acid hydrazides could be detected by their absorbance at 230 or 400 nm. Although the hydrazides were detected with a four-fold lower sensitivity than at 230 nm, a wavelength of 400 nm was recommended because the main interferences did not absorb at this wavelength. The detection limit of heptanoic acid at 400 nm was 1.3 ng at a signal-to-noise ratio of 3.

As a highly sensitive fluorescent reagent, 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-PAH) was synthesized. DMEQ-PAH readily reacted with fatty acids in aqueous solution in the presence of pyridine and EDAC at room

temperature. The reagent was applied to the labeling of some metabolites of arachidonic acids with detection limits of 3–6 fmol at a signal-to-noise ratio of 3 [56] and to the determination of free fatty acids in human serum with detection limits of 2.5–5 fmol [57].

4-(5, 6-Dimethoxy-2-benzimidazoyl)benzohydrazide (DMBI-BH) was also developed as a more sensitive labeling reagent for carboxylic acids (detection limits of which were 1–3 fmol at a signal-to-noise ratio of 3) than DMEQ-PAH [58]. Fig. 5 shows a typical chromatogram obtained for eleven fatty acids.

2-(4-Hydrazinocarbonylphenyl)-4, 5-diphenylimidazole (HCPI) was recently synthesized as a new fluorescent reagent and successfully applied to the assay of saturated free fatty acids in human serum with detection limits of 7–57 fmol at a signal-to-noise ratio of 3 [59].

2.4. Reaction with alcohols

Activation of the carboxyl groups is also required prior to their reaction with alcohols, in a similar way as for the amino groups described above.

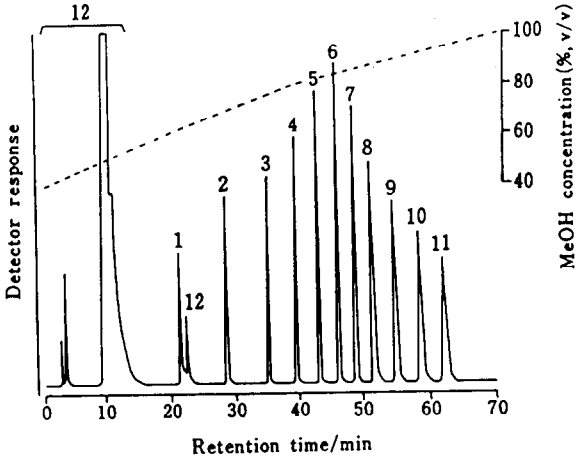
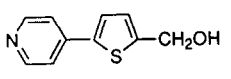


Fig. 5. Chromatogram of DMBI derivatives of linear saturated fatty acids. Column, ODS; mobile phase, aqueous methanol (0-40 min, 40-80%; 40-70 min, 80-100%); flow-rate, 1.0 ml/min. Peaks: 1 = C_6; 2 = C_8; 3 = C_{10}; 4 = C_{12}; 5 = C_{14}; 6 = C_{16}; 7 = C_{18}; 8 = C_{20}; 9 = C_{22}; 10 = C_{24}; 11 = C_{26}; 12 = reagent blank. Reproduced with permission from ref. 58.



PTM

Scheme 7. Structure of 5-(4-Pyridyl)-2-thiophenemethanol (PTM).

5-(4-Pyridyl)-2-thiophenemethanol (PTM) (for structure see Scheme 7) was used as a new fluorescent reagent [60]. Several carboxylic acids were labeled with PTM in chloroform in the presence of 1-isopropyl-3-(3-dimethylamino-propyl)carbodiimide by heating at 60°C for 3 h. The detection limits of octadecanoic and benzoic acids were 5 fmol and 50 fmol at a signal-to-noise ratio of 2, respectively. Fig. 6 shows typical chromatograms of the linear saturated fatty acids esters of PTM.

Reaction of carboxylic acids with 9-(hydroxymethyl)anthracene (HMA) [61] in the presence of 2-bromo-1-methylpyridinium iodide produced the corresponding anthrylmethyl esters (obtained by reacting the acids with ADAM). The method was applied to the analysis of plasma free fatty acids in normal and diabetic subjects. Ibuprofen in plasma was also labeled with HMA in the presence of EDAC as an activation agent and analyzed with fluorescence detection [62].

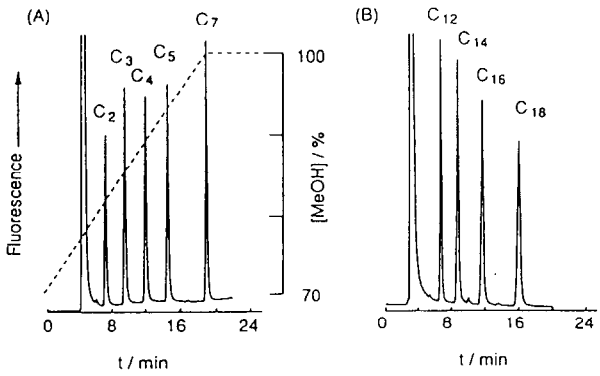
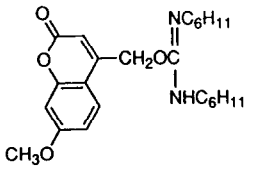


Fig. 6. Chromatograms of PTM esters of linear saturated fatty acids. Column, Shim-pack CLC-ODS; mobile phase, (A) aqueous methanol (70–100%), (B) methanol; flow-rate, 1.0 ml/min. Reproduced with permission from ref. 60.



DCCI

Scheme 8. Structure of N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea (DCCI).

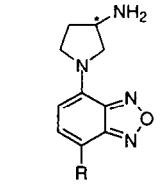
2.5. Reaction with isoureas

Early in the 1980s, two isourea-type reagents, N,N′-dicyclohexyl- and N,N′-diisopropyl-O-(7-methoxycoumarin-4-yl)methylisourea (DCCI and DICI, respectively) (for structure of DCCI see Scheme 8) were reported for carboxylic acid analysis by thin-layer chromatography with fluorescence detection [63,64]. Nicotinic acid was reacted with DCCI in acetone to give the corresponding fluorescent 4-hydroxymethyl-7-methoxycoumarin ester, which was then determined by HPLC with fluorescence detection [65]. The detection limit of nicotinic acid in serum was 0.2 nmol/ml at a signal-to-noise ratio of 2. However, to our knowledge, these isourea-type reagents have not been utilized in recent years.

2.6. Chiral labeling reagents

The structures of the chiral labeling reagents are shown in Scheme 9. The chiral derivatization procedure is one of the useful techniques for separating enantiomers by HPLC. Amino group bearing reagents are most frequently utilized for this purpose. *R*-(−)-2-Amino-1-propanol [66], *R*-(+)- and *S*-(−)-α-methylbenzylamine [67–69], and *S*-(−)-1-(naphthen-1-yl)ethylamine [70] were used for UV detection and L-1-(4-dimethylamino-1-naphthyl)ethylamine [71,72] for fluorescence detection. Several chiral reagents bearing amino acid groups were also reported such as L-leucinamide hydrochloride [73–75], D- and L-forms of O-(4-nitrobenzyl)-tyrosine methyl ester [76] and L-alanine-α- and -β-naphthylamide and L-phenylalanine-β-naphthylamide [77].

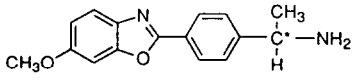
Recently, fluorescent chiral reagents having a 7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole structure were synthesized to permit separation of carboxylic acid enantiomers, *i.e.* 4-(N,N-dimethylaminosulfonyl)- (DBD-AP), 4-nitro-



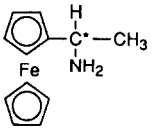
DBD-AP R = $SO_2N(CH_3)_2$

NBD-AP R = NO_2

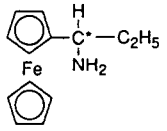
ABD-AP R = SO_2NH_2



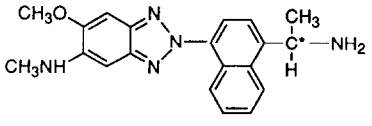
APMB



FEA



FPA



ANBA

Scheme 9. Structures of the chiral labeling reagents.

(NBD-AP) and 4-(aminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (ABD-AP) [78]. Racemic naproxen was reacted with these reagents at room temperature in the presence of 2,2′-dipyridyl disulphide and triphenylphosphine. Excellent resolution of racemic naproxen using a conventional ODS column and the lowest detection limit at the sub-fmol level at a signal-to-noise ratio of 3 with argon ion laser-induced fluorescence detection were obtained with the (+)-NBD-AP derivatives [79]. The diastereomers could also be detected with high sensitivity using peroxyoxalate chemiluminescence detection [80]. In this case, the lowest detection limit was 0.49 fmol at a signal-to-noise ratio of 2 for the (+)-DBD-AP derivatives. Fig. 7 shows chromatograms of DBD-AP derivatives of ibuprofen added to human urine.

S-(−)-2-[4-(1-Aminoethyl)naphthyl]-6-methoxy-N-methyl-2H-benzotriazolyl-5-amine dihydrochloride [*S*-(−)-ANBA] was developed and applied to the optical resolution of 5-(N,N-dimethylsulphamoyl)-6,7-dichloro-2, 3-dihydrobenzofuran-2-carboxylic acid, a new uricosuric diuretic [81]. The detection limit for each enantiomer was 15 fmol at a signal-to-noise ratio of 5.

Recently, (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole (APMB) was synthesized as a fluorescent labeling reagent [82]. Several enantiomeric carboxylic acids were labeled with APMB in the presence of 2,2′-dipyridyl disulphide and triphenylphosphine. The diastereomers showed good resolution in both the normal- and reversed-phase modes. The detection limit of the 2-phenylpropionic acid derivative was 10 fmol at a signal-to-noise ratio of 3.

Two chiral ferrocene reagents, 1-ferrocenylethylamine (FEA) and 1-ferrocenylpropylamine

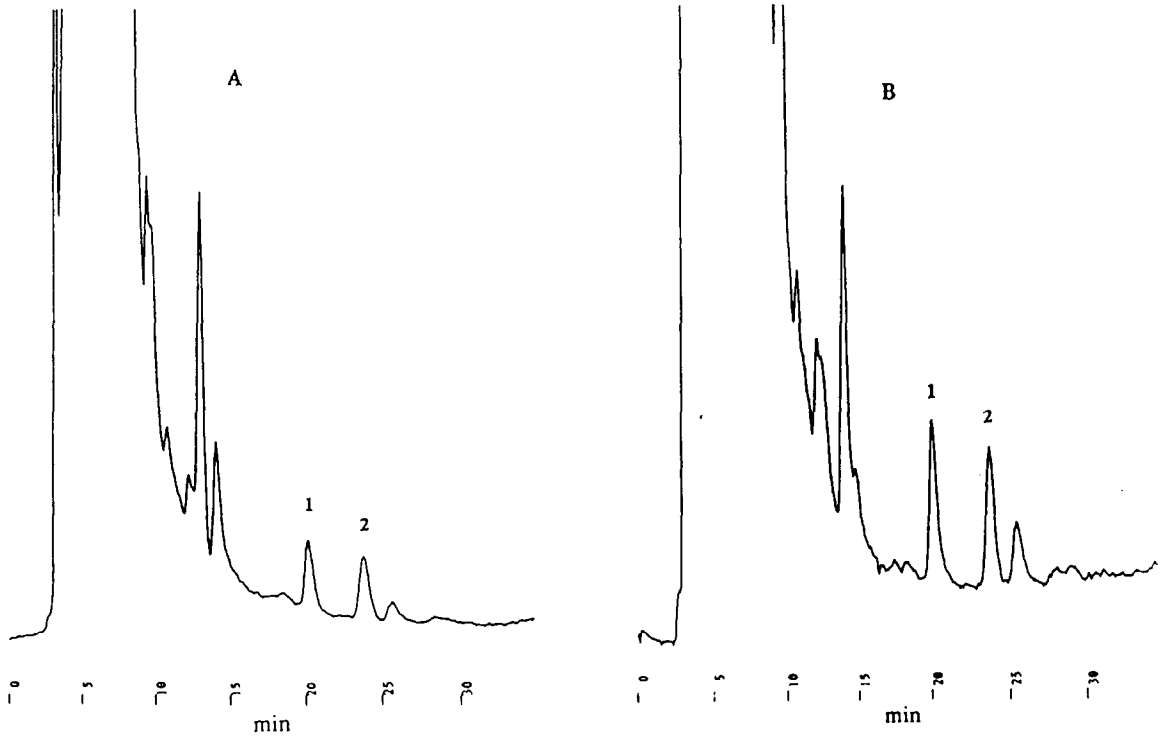


Fig. 7. Chromatograms of DBD-AP derivatives of racemic ibuprofen added to human urine (A) with bis(2,4,6-trichlorophenyl) oxalate–H_2O_2 and (B) with bis[4-nitro-2-(3,6,9-trioxadecyloxy)phenyl] oxalate–H_2O_2 chemiluminescence detection. Column, Inertsil ODS-2; mobile phase, (A) 0.1 *M* imidazole-HNO_3 (pH 7.0)–acetonitrile (2:3, v/v), (B) 0.1 *M* imidazole-HNO_3 (pH 6.5)–acetonitrile (2:3, v/v); flow-rate, 0.5 ml/min. Peaks: 1 = (+)-ibuprofen; 2 = (−)-ibuprofen. Reproduced with permission from ref. 80.

Table 2
Chiral labeling reagents for separation of enantiomers as diastereomers

Reagent	Analyte	Detection
(+)-ABD-AP, (+)-DBD-AP and (+)-NBD-AP	Naproxen	Fluorescence
	Ibuprofen, naproxen, loxoprofen and N-acetylamino acids	Chemiluminescence
L-Alanine-α-naphthylamide,L-alanine-β-naphthylamide and L-phenylalanine-β-naphthylamide	6-Methoxy-2-naphthylpropionic acid, α-phenylpropionic acid and α-phenyl-*n*-butyric acid	UV
R-(−)-2-Amino-1-propanol	Gossypol acetic acid	ED
S-(−)-ANBA	5-(N,N-Dimethylsulfamoyl)6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acid	Fluorescence
(−)-APMB	2-Phenylpropionic acid, ibuprofen, naproxen and flurbiprofen	Fluorescence
L-1-(4-Dimethylamino-1-naphthyl)ethylamine	Naproxen	Fluorescence
	Loxoprofen and its metabolites	Fluorescence
1-Ferrocenylethylamine and 1-ferrocenylpropylamine	Ibuprofen, naproxen and N-acetylamino acids	ED
L-Leucineamide	Ketoprofen	UV
	Caprofen	Fluorescence
	Flurbiprofen	UV
R-(+)-α-Methylbenzylamine	Pirprofen	UV
S-(−)-α-Methylbenzylamine	Flurbiprofen and its metabolites	Fluorescence
	Ibuprofen metabolites	UV
S-(−)-1-(Naphthen-1-yl)ethylamine	2-Phenylpropionic acid, ibuprofen, caprofen, pirprofen and pirprofen pyrrol	UV
O-(4-Nitrobenzyl)-tyrosine methyl ester	N-Protected amino acids	UV

(FPA), were also developed for the optical resolution of carboxylic acids by HPLC with ED [83]. The diastereomeric amides obtained by the reaction of ibuprofen with FEA, for instance, could be completely resolved by reversed-phase HPLC and exhibited good sensitivity at + 0.45 V *vs.* an Ag/AgCl reference electrode. The detection limit was 0.5 pmol at a signal-to-noise ratio of 5.

The chiral labeling reagents and their analytes resolved are summarized in Table 2.

3. Conclusions

Of the detectors utilized in HPLC, the fluorescence detector seems to be the most useful, considering its versatility, sensitivity, selectivity and ease of use. Therefore, most of the recent studies on the development of new labeling reagents have been focussed on reagents having highly sensitive fluorophores as well as showing a high reactivity under mild reaction conditions. Laser-induced fluorescence detection is usually expected to be much more sensitive than the conventional type using gas-discharge lamp excited fluorescence. 7-Methoxycoumarin derivatives as highly sensitive fluorescent labeling reagents have attracted attention again because of their maximum excitation wavelengths around 325 nm (using a He–Cd laser).

At present there is an increased interest in diode laser-induced fluorescence detection in HPLC [84]. These lasers are compact, easy to handle, highly efficient and relatively cheap. Most naturally occurring compounds do not show fluorescence in the red and near-infrared region in which diode lasers emit. Consequently, the most important feature of fluorescence detection using the red and near-infrared lasers as

excitation sources is the reduction of the background fluorescence. This means that an especially interesting application of the diode laser-induced fluorescence detection is trace analysis of biomedical samples, because of the large background fluorescence encountered in such samples when excitation is performed at shorter wavelengths. In order to utilize the potential of diode laser-induced fluorescence detection, it is essential to develop new labeling reagents with special fluorophores that absorb and fluorescence in the red and near-infrared region.

References

[1] S. Allenmark and M. Chelminska-Bertilsson, *J. Chromatogr.*, 456 (1988) 410.
[2] F.A.L. van der Horst, G.G. Eikelboom and J.J.M. Holthuis, *J. Chromatogr.*, 456 (1988) 191.
[3] F.A.L. van der Horst, *Trends Anal. Chem.*, 8 (1989) 268.
[4] F.A.L. van der Horst, J.M. Reijn, M.H. Post, A. Bult, J.J.M. Holthuis and U.A.Th. Brinkman, *J. Chromatogr.*, 507 (1990) 351.
[5] F.A.L. van der Horst, M.H. Post, J.J.M. Holthuis and U.A.Th. Brinkman, *J. Chromatogr.*, 500 (1990) 443.
[6] A. Takadatc, T. Masuda, C. Tajima, C. Murata, M. Irikura and S. Goya, *Anal. Sci.*, 8 (1992) 663.
[7] D.E. Richardson, J.B. Bremner and B.V. O'Grady, *J. Chromatogr.*, 595 (1992) 155.
[8] J.H. Wolf and J. Korf, *J. Chromatogr.*, 502 (1990) 423.
[9] E.H.J.M. Jansen and P. de Fluiter, *J. Liq. Chromatogr.*, 15 (1992) 2247.
[10] R.B. Gueddour, M. Matt and A. Nicolas, *Anal. Lett.*, 26 (1993) 429.
[11] J.B.F. Lloyd, *J. Chromatogr.*, 178 (1979) 249.
[12] J.S. Yoo and V.L. McGuffin, *J. Chromatogr.*, 627 (1992) 87.
[13] R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, *J. Chromatogr.*, 269 (1983) 81.
[14] H. Naganuma and Y. Kawahara, *J. Chromatogr.*, 478 (1989) 149.
[15] H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, *J. Chromatogr.*, 234 (1982) 121.
[16] J.K. Volkman, D.G. Holdsworth and D.E. Richardson, *J. Chromatogr.*, 643 (1993) 209.
[17] K.D. Ertel and J.T. Carstensen, *J. Chromatogr.*, 411 (1987) 297.
[18] A. Nakanishi, H. Naganuma, J. Kondo, K. Watanabe, K. Hirano, T. Kawasaki and Y. Kawahara, *J. Chromatogr.*, 591 (1992) 159.
[19] T. Kusaka and M. Ikeda, *J. Chromatogr.*, 639 (1993) 165.
[20] K. Akasaka, T. Suzuki, H. Ohrui, H. Meguro, Y. Shindo and H. Takahashi, *Anal. Lett.*, 20 (1987) 1581.
[21] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *Anal. Sci.*, 1 (1985) 295.
[22] M. Yamaguchi, S. Hara, R. Matsunaga, M. Nakamura and Y. Ohkura, *J. Chromatogr.*, 346 (1985) 227.
[23] K. Nakashima, M. Okamoto, K. Yoshida, N. Kuroda, S. Akiyama and M. Yamaguchi, *J. Chromatogr.*, 584 (1992) 275.
[24] W.-F. Chao, M. Kai and Y. Ohkura, *J. Chromatogr.*, 526 (1990) 77.
[25] M. Yamaguchi, O. Takehiro, S. Hara, M. Nakamura and Y. Ohkura, *Chem. Pharm. Bull.*, 36 (1988) 2263.
[26] H.D. Durst, M. Milano, E.J. Kikta, Jr., S.A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
[27] H. Chen and R.E. Anderson, *J. Chromatogr.*, 578 (1992) 124.
[28] M. Püttman, H. Krug, E. von Ochsenstein and R. Kattermann, *Clin. Chem.*, 39 (1993) 825.
[29] A. Takadate, T. Masuda, C. Murata, C. Haratake, A. Isobe, M. Irikura and S. Goya, *Anal. Sci.*, 8 (1992) 695.
[30] R.H. Pullen and J.W. Cox, *J. Chromatogr.*, 343 (1985) 271.
[31] M.C. Roach, L.W. Ungar, R.N. Zare, L.M. Reimer, D.L. Pompliano and J.W. Frost, *Anal. Chem.*, 59 (1987) 1056.
[32] K. Shimada, C. Sakayori and T. Nambara, *J. Liq. Chromatogr.*, 10 (1987) 2177.
[33] R.K. Munns, J.E. Roybal, W. Shimoda and J.A. Hurlbut, *J. Chromatogr.*, 442 (1988) 209.
[34] S.T. Ingalls, P.E. Minkler, C.L. Hoppel and J.E. Nordlander, *J. Chromatogr.*, 299 (1984) 365.
[35] Y. Yasaka, M. Tanaka, T. Matsumoto, J. Katakawa, T. Tetsumi and T. Shono, *Anal. Sci.*, 6 (1990) 49.
[36] Y. Yasaka, M. Tanaka, T. Shono, T. Tetsumi and J. Katakawa, *J. Chromatogr.*, 508 (1990) 133.
[37] K. Funazo, M. Tanaka, Y. Yasaka, H. Takigawa and T. Shono, *J. Chromatogr.*, 481 (1989) 211.
[38] Y. Yasaka, M. Tanaka, T. Matsumoto, K. Funazo and T. Shono, *Anal. Sci.*, 5 (1989) 611.
[39] M. Tanaka, Y. Yasaka, K. Funazo and T. Shono, *Fresenius Z. Anal. Chem.*, 335 (1989) 311.
[40] N. Nimura and T. Kinoshita, *Anal. Lett.*, 13 (1980) 191.
[41] Th.H.M. Roemen and G.J. van der Vusse, *J. Chromatogr.*, 570 (1991) 243.
[42] K. Tagawa, K. Hayashi, M. Mizobe and K. Noda, *J. Chromatogr.*, 617 (1993) 95.
[43] T. Yoshida, A. Uetake, H. Yamaguchi, N. Nimura and T. Kinoshita, *Anal. Biochem.*, 173 (1988) 70.
[44] N. Hanaoka, *J. Chromatogr.*, 503 (1990) 155.
[45] N. Nimura, T. Kinoshita, T. Yoshida, A. Uetake and C. Nakai, *Anal. Chem.*, 60 (1988) 2067.
[46] T. Yoshida, A. Uetake, C. Nakai, N. Nimura and T. Kinoshita, *J. Chromatogr.*, 456 (1988) 421.
[47] J. Schneede and P.M. Ueland, *Anal. Chem.*, 64 (1992) 315.

[48] A. Takadate, T. Tahara, H. Fujino and S. Goya, *Chem. Pharm. Bull.*, 30 (1982) 4120.
[49] S. Narita and T. Kitagawa, *Anal. Sci.*, 5 (1989) 31.
[50] O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde and D.A. Doornbos, *J. Chromatogr.*, 615 (1993) 97.
[51] Y.-M. Lee, H. Nakamura and T. Nakajima, *Anal. Sci.*, 5 (1989) 209.
[52] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu and K. Imai, *J. Chromatogr.*, 588 (1991) 61.
[53] T. Toyo'oka, M. Ishibashi, Y. Takeda and K. Imai, *Analyst*, 116 (1991) 609.
[54] M. Nakajima, A. Sato and K. Shimada, *Anal. Sci.*, 4 (1988) 385.
[55] A.J.J.M. Coenen, M.J.G. Kerkhoff, R.M. Heringa and Sj. van der Wal, *J. Chromatogr.*, 593 (1992) 243.
[56] M. Yamaguchi, T. Iwata, K. Inoue, S. Hara and M. Nakamura, *Analyst*, 115 (1990) 1363.
[57] T. Iwata, K. Inoue, M. Nakamura and M. Yamaguchi, *Biomed. Chromatogr.*, 6 (1992) 120.
[58] T. Iwata, M. Nakamura and M. Yamaguchi, *Anal. Sci.*, 8 (1992) 889.
[59] K. Nakashima, Y. Taguchi, N. Kuroda, S. Akiyama and G. Duan, *J. Chromatogr.*, 619 (1993) 1.
[60] R. Nakajima, K. Shimada, Y. Fujii, A. Yamamoto and T. Hara, *Bull. Chem. Soc. Jpn.*, 64 (1991) 3173.
[61] J.D. Baty, S. Pazouki and J. Dolphin, *J. Chromatogr.*, 395 (1987) 403.
[62] H. Lingeman, A. Hulshoff, W.J.M. Underberg and F.B.J.M. Offermann, *J. Chromatogr.*, 290 (1984) 215.
[63] S. Goya, A. Takadate, H. Fujino and T. Tanaka, *Yakugaku Zasshi*, 100 (1980) 744.
[64] S. Goya, A. Takadate and H. Fujino, *Yakugaku Zasshi*, 102 (1982) 63.
[65] Y. Tsuruta, K. Kohashi, S. Ishid and Y. Ohkura, *J. Chromatogr.*, 309 (1984) 309.
[66] D.-F. Wu, M.M. Reidenberg and D.E. Drayer, *J. Chromatogr.*, 433 (1988) 141.
[67] A. Sioufi, D. Colussi, F. Marfil and J.P. Dubois, *J. Chromatogr.*, 414 (1987) 131.
[68] M.P. Knadler and S.D. Hall, *J. Chromatogr.*, 494 (1989) 173.
[69] A.C. Rudy, K.S. Anliker and S.D. Hall, *J. Chromatogr.*, 528 (1990) 395.
[70] A.J. Hutt, S. Fournel and J. Caldwell, *J. Chromatogr.*, 378 (1986) 409.
[71] J. Goto, N. Goto and T. Nambara, *J. Chromatogr.*, 239 (1982) 559.
[72] H. Nagashima, Y. Tanaka, H. Watanabe, R. Hayashi and K. Kawada, *Chem. Pharm. Bull.*, 32 (1984) 251.
[73] R.T. Foster and F. Jamali, *J. Chromatogr.*, 416 (11987) 388.
[74] H. Spahn, I. Spah, G. Pflugmann, E. Mutschler and L.Z. Benet, *J. Chromatogr.*, 433 (19880 331.
[75] B.W. Berry and F. Jamali, *Pharm. Res.*, 5 (1988) 123.
[76] S. Görög, B. Herényi and M. Löw, *J. Chromatogr.*, 353 (1986) 417.
[77] Y. Fujimoto, K. Ishi, H. Nishi, N. Tsumagari, T. Kakimoto and R. Shimizu, *J. Chromatogr.*, 402 (1987) 344.
[78] T. Toyo'oka, M. Ishibashi and T. Terao, *Analyst*, 117 (1992) 727.
[79] T. Toyo'oka, M. Ishibashi and T. Terao, *J. Chromatogr.*, 625 (1992) 357.
[80] T. Toyo'oka, M. Ishibashi and T. Terao, *J. Chromatogr.*, 627 (1992) 75.
[81] S. Narita and T. Kitagawa, *Anal. Sci.*, 5 (1989) 361.
[82] J. Kondo, T. Imaoka, T. Kawasaki, A. Nakanishi and Y. Kawahara, *J. Chromatogr.*, 645 (1993) 75.
[83] K. Shimada, E. Haniuda, T. Oe and T. Nambara, *J. Liq. Chromatogr.*, 10 (1987) 3161.
[84] A.J.G. Mank, H. Lingeman and C. Gooijer, *Trends Anal. Chem.*, 11 (1992) 210.