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Review

4-Bromomethyl-7-methoxycoumarin and analogues as derivatization agents for high-performance liquid chromatography determinations: a review

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Abstract: A major part of modern analytical problem solving deals with the trace level determination of organic compounds and contaminants in biomedical, food and environmental samples. In the analysis of these samples chromatographic techniques play a predominant role. Unfortunately, however, even the combined force of an efficient separation plus a sophisticated mode of detection does not always create sufficient selectivity and/or sensitivity for the final goal to be attained. In such cases, special attention has to be devoted to derivatization or conversion of the analyte(s) of interest (for improved detection selectivity and/or sensitivity) and sample pretreatment (for trace enrichment and clean-up). The above is especially true when, as is often the case today, relatively polar drugs, endogenous compounds, additives or environmental pollutants and/or their (bio)-degradation products have to be determined. For such classes of compounds high-performance column liquid chromatography (HPLC) generally is the preferred method of separation. Reversed-phase HPLC with fluorescence detection is a powerful means of analysis for compounds which possess native fluorescence. They are, however, relatively few in number. In order to make the method useful for a much wider range of analytes, one can therefore resort to derivatization (labelling) or other means of analyte conversion to obtain highly fluorescent reaction products, which can then be detected with the required selectivity and sensitivity. 4-Bromomethyl-7-methoxycoumarin is often used as fluorescent label for the determination of compounds possessing a carboxylic group. About 8% of the biologically interesting analytes — ranging from polar amino acids and peptides to apolar fatty acids — possess such a group. In this paper a review of the applications of 4-bromomethyl-7-methoxycoumarin and its analogues as derivatization agents for HPLC determinations is given.

Keywords: *4-Bromomethyl-7-methoxycoumarin; carboxylic acids; pre-column derivatization; pyrimidine compounds; fatty acids; reversed-phase HPLC; drug analysis.*

Introduction

For the assay of biochemical or pharmaceutical compounds reversed-phase HPLC (RP-HPLC) is normally the analytical method of choice. Compared with other methods sample preparation procedures are usually simple and straightforward. Of all known organic compounds about 8% contain a carboxylic group [1] and for such compounds their analysis is often complicated due to a lack of specific interaction with LC detection systems. The UV absorbance of the carboxyl bond is not sufficient for acceptable sensitivity; carboxylic acids possessing natural fluorescence are rare. Thus in order to obtain the adequate sensitivity pre- or post-column derivatization is a necessity. Extensive reviews of derivatization as an acid to LC analysis have recently been published [2–4].

For the derivatization of carboxylic acids a large number of reagents have been described of which only a few are commercially available [2–4]. Most reactions are performed in non-aqueous media, in fact water hinders most of the reactions. At the time we started our research for an automated pre-column derivatization procedure for fatty acids with fluorescence labelling only 4-bromomethyl-7-methoxycoumarin (BrMMC, Fig. 1), also known as 4-bromomethyl-7-methoxy-2-oxo-2H-benzopyran, was commercially available and for this reason it was chosen as the label.

The synthesis of BrMMC was described by Baker and Collins in 1949 [5]. As a label it was first used by Dünges at the end of 1977 [6] and

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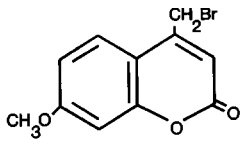

Figure 1
Structure of BrMMC.

further details were published in the following years [1, 7–10]. The applicability of the method was demonstrated for a great variety of compounds including aliphatic and aromatic monocarboxylic acids, acidic herbicides, barbiturates, drugs and prostaglandins. Most of the work was done with thin-layer chromatography (TLC). In this review the use of BrMMC as a fluorescent and UV label for HPLC detection will be discussed.

Characteristics of the Derivatization Reaction

The mechanism of the derivatization reaction of a carboxylic acid with BrMMC is given in Fig. 2. Basically a salt of the fatty acid, commonly the potassium salt, is formed in an aprotic solvent under reflux. This salt is then reacted with the label BrMMC. Lam and Grushka [11] introduced the use of a crown ether to solubilize potassium salts. The two procedures described by these workers which are commonly cited in the literature, illustrate the reaction mechanism very well. In the first procedure fatty acids (2–4 mg depending on the chain length) are neutralized to a phenolphthalein endpoint with a 10% methanoic potassium hydroxide solution, and the potassium salt recovered by removal of the solvent under vacuum. Crown ether (1 ml 18-crown-6, 0.7 mg ml^{-1} in acetonitrile) and BrMMC solution (5 ml 1.1 mg ml^{-1} in acetone, corresponding to a 10–20% molar excess relative to the fatty acid) are added and the mixture refluxed for 15 min. In the other procedure, the neutralization is effected by 20–30 mg anhydrous potassium carbonate and the reaction time is increased to 25 min. The esters are either purified over silica gel or injected directly. The first procedure is used mostly for preparative work, whilst the second one is used for analytical purposes.

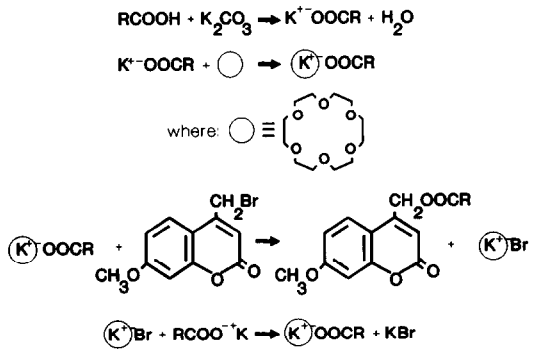

Figure 2
Proposed reaction scheme of the base catalysed derivatization reaction of BrMMC.

The choice of reaction solvent is not critical provided it is aprotic. Acetone and acetonitrile are the most widely used, followed by dimethylsulphoxide and N,N-dimethylformamide. Wolf and Korf [12] compared the dependence of the detector response using different mixtures of acetonitrile with methanol, 1,2-dichloroethane and ethyl acetate with that obtained from acetonitrile solution. Although a large number of solvents can be used, acetone and acetonitrile are the most frequently employed. In RP-HPLC it is advisable to reduce the solvent strength of the sample to be injected so that it is lower than that of the eluent in order to avoid peak distortion. In automated procedures direct injection of the reaction medium can give problems. Wolf *et al.* [12–15] used the combination of acetonitrile as reaction solvent and acetonitrile–water mixtures as mobile phase to avoid peak distortion upon injection. In this case only a slight peak reduction was observed, compared with that produced by an injection of a reaction mixture compatible with the mobile phase, furthermore, the peaks are found to be symmetrical. Other solvent combinations gave leading, tailing, double or 'strange' peak forms. The choice of base is more limited than the choice of reaction solvent. In their original publication Dünges *et al.* [8] showed that potassium carbonate and bicarbonate catalyse the reaction, while lithium, sodium, ammonium, calcium carbonate and barium and sodium bicarbonate do not. Of the amines tested only triethylamine [8, 11] is found to be effective as base catalyst, although the reaction mechanism is probably different from the one given in Fig. 2. For the derivatization of acidic herbicides caesium carbonate has been used [16, 17]. However, in the author's experience this base is not an efficient catalyst of the reaction with fatty acids. The introduction of the crown ether, 18-crown-6, accelerated the reaction, insofar as the potassium salts of the fatty acids are

solubilized by the crown ether [11]. For the reaction with prostaglandins McGuffin and Zare [18] used dibenzo-18-crown-6 in combination with potassium bicarbonate and sodium sulphate. Okuyama *et al.* [19] prepared sodium salts of bile acids in combination with 15-crown-5 at room temperature.

Generally, the reaction temperature used is always 60°C or higher except in two studies. At room temperature bile acids were derivatized in 1 h, whilst with the automated procedure developed by Wolf *et al.* [12–15], utilizing a suspension of potassium carbonate in a crown ether solution, the reaction of fatty acids was completed in 15 min at room temperature. Generally, fatty acids are derivatized within 30 min at 60°C [1, 6–8, 12, 20, 21], dicarboxylic acids at 80°C for 1 h [22], while the same period of time was used for gibberellins. For prostaglandins a reaction time of 10 h was used by McGuffin and Zare [18] to prevent the dehydration of the prostaglandins which normally takes place in non-aqueous reagents.

The esters formed are stable unlike the label BrMMC, which is highly sensitive to light [6, 23], and in solution decompose within a few days [24]. As a precaution, in TLC it is necessary to cover the starting position with dark material immediately after evaporation of the solvent. During the reaction a yellow precipitate is formed, which was identified by NMR spectroscopy to be 7-methoxycoumarin-4-carboxylic acid (Wolf and Lankhorst, unpublished results). The esters are stable in solution for a prolonged period [8], in a 5% trichloroacetic acid solution at least overnight [21] and upon heating [25].

BrMMC is not fluorescent due to the quenching effect of the bromine atom, however, when used in excess a strongly fluorescent compound, probably the methylhydroxy derivative, is formed by the reaction of BrMMC and water. Roseboom and Greve [17] reacted *p*-nitrobenzoic acid with the excess of BrMMC to form a non-fluorescent compound, whilst Roseboom *et al.* [16] employed glacial acetic acid to stop the reaction.

The derivatization procedure described by Horst *et al.* [26, 27] is worth mentioning. In an extensive study they investigated the use of micelles in the derivatization process. Compared with other studies the extraction step is avoided, but their procedure is rather laborious, critical with respect to reagent preparation and is not easily automated. It is doubtful if the method will find application for the reaction of BrMMC with fatty acids, but the concept could be applied to other forms of precolumn derivatization.

Separation, Spectral Properties and Detection of Methyl-methoxycoumarin-Derivatives

For the separation of the methyl-methoxycoumarin-derivatives (MMC-derivatives) of carboxylic acid most authors use RP-HPLC. The separation of MMC-derivatives of fatty acids is relatively simple and straightforward on C_8 or C_{18} columns. As a modifier, methanol or acetonitrile can be used. To improve the separation of short chain fatty acids Xie *et al.* [28] added cadmium nitrate and boric acid to the RP-HPLC mobile phase. Zelenski and Huber [29] separated the MMC-derivatives of fatty acids on a normal-phase silica column with a gradient from isooctane to acetonitrile–methylene chloride (10:15, v/v). Both separation systems, normal- and reversed-phase, were used by Crozier *et al.* [30] to separate gibberellins, and by Chakir *et al.* [20] to analyse glucuronic acid conjugates. For the normal-phase separation hexane–dichloromethane and hexane–methanol mixtures were used, respectively.

The spectral properties of MMC-derivatives have been extensively investigated by Lloyd [25]. The derivatives possess an absorption maximum at about 330 nm and a fluorescence maximum at about 390 nm. The fluorescence yields of the MMC-derivatives are strongly dependent on the mobile phase composition. The yields are highest in water, lower in hydrogen bonding solvents as methanol and lowest in non-hydrogen bonding solvents such as acetonitrile. With respect to the use of gradients in RP-HPLC this implies that the mobile phase composition determines in part the sensitivity of the assay. Generally, during a gradient elution the water content of the eluent decreases, whilst the modifier content increases and for fatty acids with chains longer than approximately 16–18 carbon atoms elution is effected by pure modifier. It is found that the fluorescence intensity in methanol is about four times that in acetonitrile, which can be attributed to the higher polarity of the methanol. As long as there is some water in the eluent no difference in intensity is detectable, however it is substantial when the fatty acids are eluted in pure modifier. In the authors'

automated derivatization procedure [12–15], optimum separation combined with a high sensitivity is achieved when the derivatization solvent acetonitrile is injected in acetonitrile–water mixtures. This resulted in the best peak shape (see above). By ending the gradient in methanol–acetonitrile 1:1 (v/v) instead of acetonitrile the sensitivity was enhanced.

A comparison of the detection limit between UV and fluorescence has been made using myristic acid, C14:0 as a model compound. For UV a detection limit of 160 pg was obtained for C14:0 at a signal to noise ratio of 2. On the other hand for fluorescence detection the limit was a factor of 30 lower. For fatty acids with longer chains this factor is lower because they require pure methanol–acetonitrile for elution. The lack of water results in a lower detector response, so UV detection might equally well be applied [25].

The system described by McGuffin and Zare [18] is different from the others with respect to separation and detection. Fatty acid and prostaglandin derivatives are separated in a capillary reversed-phase column using a gradient methanol–ethylacetate–water to methanol–acetonitrile–ethyl acetate and are detected by laser-fluorescence. In this way detection limits of 'a few femtograms' could be achieved. Another way to enhance the sensitivity is chemiluminescence in which light is emitted as a result of a chemical reaction. However, Grayeski and De Vasto [31] did not observe any chemiluminescence activity of MMC-derivatives.

Compounds Analysed with the Use of BRMMC

Dünges has investigated systematically the application of BrMMC to TLC [1, 8]. Although a wide range of compounds were tested few real applications, however, were developed.

Fatty acids

Fatty acids are the most frequently derivatized group of compounds with BrMMC [1, 8, 11–14, 18, 23, 25–29, 32–37]. A general derivatization procedure is described above. Separations are performed most often with reversed-phase eluents and with methanol or acetonitrile as modifier and with fluorescence detection. The acids have been analysed in serum or plasma [12, 14, 27, 33, 36], rat brain homogenates [12, 13], vegetable oils [11, 18], river water [37], atmospheric particulate matter [24] and lake sediments [32].

Dicarboxylic acids

The derivatization of dicarboxylic acids is interesting from a theoretical rather than from a practical point of view. According to Dünges [1, 8], dicarboxylic acids could not be derivatized. On the other hand, Grushka *et al.* [22] have showed that they can be derivatized with the addition of a crown ether, higher temperature (80°C) and longer reaction time (1 h). Gonnet *et al.* [35] showed that this procedure leads exclusively to mono-esters, but Elbert *et al.* [24] have demonstrated the formation of both mono- and di-esters. As an illustration of their procedure, they quoted the analysis of organic acids in atmospheric particulate matter.

Phenoxycarboxylic acid pesticides

This class of compounds to which 2,4-D, 2,4,5-T, 2,4-DP, MCPA, MTCPB, dicamba and mecoprop belong reacts easily under reflux in acetone with potassium carbonate and BrMMC as was shown by Dünges. Roseboom [16, 17] extended the procedure to HPLC analysis. They used caesium carbonate as the base catalyst but gave no reasons for its choice. GC analysis after derivatization as pentafluorobenzyl esters was compared with HPLC analysis as MMC-esters and after derivatization with 2-naphthacyl bromide. The MMC-esters, which enabled a higher sensitivity of detection than the GC analysis, were formed with the lowest number of side reactions and permitted a more specific detection.

Gibberellins

Gibberellins are plant hormones involved in the regulation of growth. Crozier *et al.* [30] synthesized 13 different mono-, bis- or tris-gibberellin esters in acetonitrile with crown ether, potassium carbonate and BrMMC in 2 h, at 60°C. After separation by reversed-phase chromatography the identity of the compouinds formed were confirmed by mass spectrometry. No applications were given.

Bile acids

The analysis of bile acids is of value in the study of several hepatobiliary diseases [38]. Free and glycine conjugated bile acids were analysed by Okuyama *et al.* [19]. Sodium salts of bile acids were treated with BrMMC and 15-

crown-5 in acetonitrile for 1 h. The detection limit was found to be 10 ng when a very poor separation system was used. A more promising study was that of Andreolini *et al.* [38]. After a separation of the free/glycine conjugated and the taurine conjugated bile esters, both fractions were derivatized in acetonitrile in the presence of potassium carbonate and crown ether. To achieve the required resolution they used a microbore LC column and subsequent detection with a He–Cd laser. Bile acid profiles for serum are given along with detection limits, which are in the low femtomol range.

5-Pyrrolidone-2-carboxylic acid

This compound is a cyclic derivative of glutamic acid and one of the most important compounds associated with the synthesis of glutathione, a ubiquitous compound of the living cell [21]. With triethylamine as base, this acid was reacted in acetonitrile–tetrahydrofuran (2:1, v/v) at 60°C for 30 min. Applications for the analyses in brain, liver and kidney tissues were given. The recovery of the analytes was studied with the ^{14}C-labelled compound.

Carboxybetaine amphoteric surfactants

This class of compounds (alkyldimethylammonio)-methane carboxylate (DAMC), are frequently used as surfactants in household and cosmetic products [40]. The commercial product is generally a mixture of a homologous series of alkyl chains composed of 8–18 carbon atoms. A methanolic DAMC solution is added to a phosphate buffer of pH 3.0 together with an internal standard in methanol. This solution is evaporated under a stream of nitrogen. BrMMC in N,N-dimethylformamide is added, the reaction mixture is heated at 70°C for 30 min and an aliquot is injected. A gradient of a 0.1 M sodium perchlorate in acetonitrile–water (45:55, v/v) to 0.1 M sodium perchlorate in acetonitrile–water (90:10, v/v) was used to separate the esters on a C_8 column.

Biotin and its analogues

Biotin or hexahydro-2-oxo-1-thieno-3,4-imidazole-4-valeric acid is a very potent growth factor for micro-organisms. This compound, and also biotin-1-sulphoxide, biotin-*d*-sulphoxide and biotin sulphone, were derivatized according to the procedure of Lam and Grushka [11] and separated by using a gradient of different mixtures of tetrahydrofuran–water by Desbene *et al.* The detection limit is in the low nmol range [41].

Glucuronic acid conjugates

Phenol, menthol, borneol, estrone and testosterone were dissolved in a mixture of N,N-dimethylformamide and acetone (8:50, v/v). BrMMC and crown ether in acetone were added together with potassium carbonate. This mixture was heated at 70°C for 30 min in a closed vial, afterwards cooled in ice and an aliquot injected. Different extraction procedures were compared. The esters were chromatographed on either a normal-phase column with hexane–ethanol mixtures as eluent or on a reversed-phase column with methanol–water. Ultraviolet detection at 328 nm was used. Detection limits were 5–30 $\mu g\ ml^{-1}$ [20]. To provide short elution times Nicolas and Leroy [42] used an eluent with a hydrophobic counter ion and a high methanol content. A sensitivity of 10 pmol for each conjugate injected was found. The method was used to follow the glucuronidation of aromatic steroid and terpene aglycons in rat and human hepatic microsomal preparations.

Pyrimidine compounds

Next to fatty acids BrMMC labelling is most widely applied to pyrimidine compounds [10, 43, 46–48]. Yoshida, Hirose and Iwamoto have described the derivatization and the applicability of BrMMC labelling in the trace analysis of pyrimidine nucleobases, nucleosides and related compounds [10, 43]. They found that dimethyl sulphoxide was a better solvent for the reaction than acetone. The reaction was completed within 5 min at room temperature in an excess of BrMMC and potassium carbonate: the crown ether could be omitted in this case. One should keep in mind that in the case of pyrimidine compounds, BrMMC derivatization is at the imide group. In this procedure the excess BrMMC could be treated with *p*-nitrobenzoic acid to give a non-fluorescent derivative. An application to serum analysis was given. Typical detection limits are 50 pg for inosine, 150 pg for uridine, 50 pg for uracil, 50 pg for thymine and 100 pg for fluorodeoxyuridine. Kindberg *et al.* described the analysis of 5-fluorouracil (5-FU), an antineoplastic agent, which has been used in cancer chemotherapy for many years [47, 48]. They proved that both heterocyclic nitrogen atoms of 5-FU were labelled [47]. For the labelling they used

the procedure of Yoshida *et al.* [10], although they did omit the use of *p*-nitrobenzoic acid to remove the excess reagent. The assay for the analysis of 5-FU was given in a second article [43]. After extraction with ethyl acetate, the extracts were dried under nitrogen and then dissolved in ethyl acetate containing 18-crown-6, subsequently potassium carbonate and BrMMC in acetone–acetonitrile (1:2, v/v) were added. After 2 h at 70°C or 40 h at room temperature the reaction was quenched with 5% acetic acid and the mixture was extracted twice with hexane. The hexane layers were discarded and portions of the remaining layers were injected into the HPLC. In this procedure a crown ether was used, whilst dimethyl sulphoxide was replaced by acetone–acetonitrile. The reason for this was that with dimethyl sulphoxide only satisfactory results for 10 ng or more 5-FU could be achieved, whilst with the method described it is possible to analyse down to 0.2 ng. The assay had a detection limit of 0.5 ng ml^{-1} plasma with a linearity of up to 20 $\mu g\ ml^{-1}$ in 0.5 ml of sample.

Prostaglandins

For the analysis of prostaglandins in biological matrices Turk *et al.* [51] followed the original procedure of Dünges. After extraction, the derivatization was completed in 10 min at 60°C in acetone with potassium carbonate as base. Separation was performed by normal-phase chromatography with different eluents. As little as 20 ng PGE_2 could be detected. Different derivatization agents, BrMMC among them, were used by Wintersteiger and Juan [49] for the quantitative determination of prostaglandins to find the optimum reaction conditions for this class of compounds. BrMMC gave the highest sensitivity at a low reaction temperature.

Lignin and related compounds

Lignin and humic substances are determined by HPLC after derivatization with BrMMC. The method was applied to the characterization of humic waters and for the differentiation between humic and lignin compounds in waste water, from pulp mills.

Analogue Labels of BrMMC Used for Derivatization

As discussed previously, the use of BrMMC does have some disadvantages, namely: (i) the fluorescence quantum yield is dependent on the mobile phase composition [25]; (ii) different fluorescence intensities of the carboxylic acid MMC esters are found with equimolar amounts of different carboxylic acids [25].

In attempts to overcome these disadvantages analogues of BrMMC have been synthesized and evaluated. Although the primary aim of this article is to give an overview of the use of BrMMC as a derivatization agent, it was thought that for completeness the analogues should be discussed. If possible their use will be compared with that of BrMMC. In Fig. 3 and Table 1 the names and structures of some analogues are given.

4-Bromomethyl-7-acetoxycoumarin (BrMAC)

This label was introduced by Tsuchiya *et al.* [53]. They investigated the fluorescence intensities of several coumarine derivatives and found that a substituent group at the 7-position greatly affects the fluorescence quantum yield, whereas one in the 4-position did not. They concluded "that the presence of an electron donating group such as a hydroxyl group tends to enhance the fluorescence intensity of the 7-hydroxy-derivative, which is increased by making the pH of its solution more alkaline". BrMAC was chosen as a post-column derivatization label. After derivatization, using a similar procedure to that with BrMMC, and HPLC separation, the eluent is mixed with a 0.1 M borate buffer, pH 11.0. In a reaction coil (10 m, 0.5 mm i.d. at 50°C) the esters are hydrolysed to 4-methyl-7-hydroxycoumarin. The fluorescence intensities of this compound are constant over the range acetonitrile–water 10:90 to 90:10 (v/v). A great variety of carboxylic acids can be determined by this procedure. The usefulness of the procedure was demonstrated by the analysis of prostaglandins in human seminal fluid [55]. With some minor modifications to the post-column mixing coil procedure, Kelly *et al.* [54] used the label for the determination of endogenous carboxylic acids, including arachidonic acid metabolites. Detection limits of 10 fmol were attained and, according to the authors, it should be possible for the first time to determine arachidonic acid metabolites, including the lipoxygenase products, in biological fluids.

4-Bromomethyl-6,7-dimethoxycoumarin (BrMDC)

Farinotti *et al.* [56] synthesized this com-

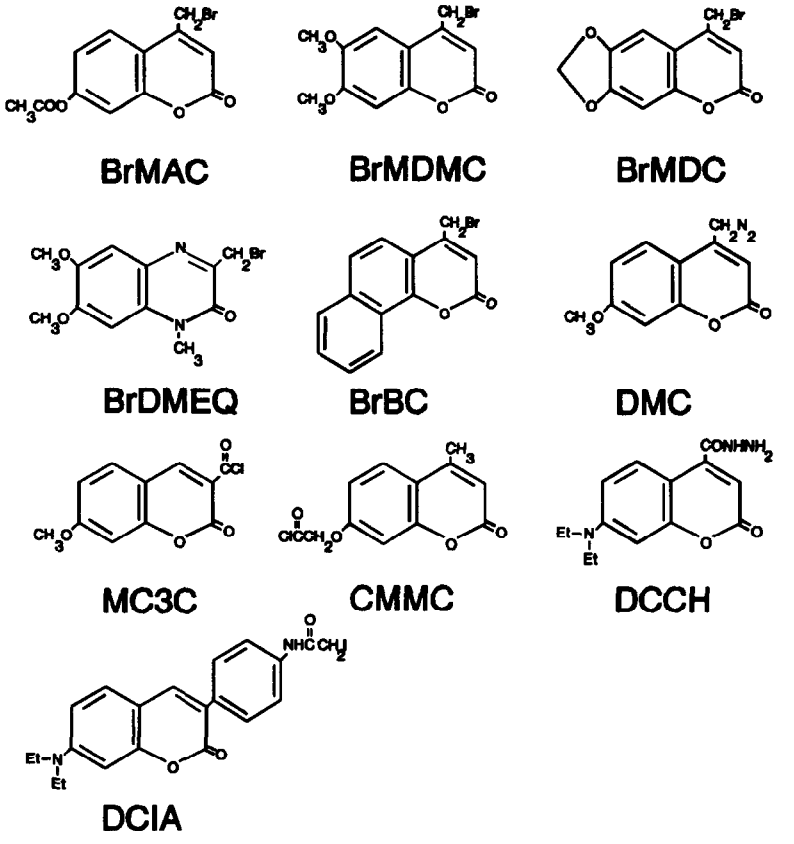

Figure 3
The chemical structures of the analogues of 4-bromomethyl-7-methoxycoumarin as mentioned in Table 1.

Table 1
Analogues of BrMMC used for derivatization

Compound	Abbreviation	References
4-Bromomethyl-7-acetoxycoumarin	BrMAC	53–55
4-Bromomethyl-6,7-dimethoxycoumarin	BrMDMC	56, 57
4-Bromomethyl-6,7-methylenedioxycoumarin	BrMDC	58, 59
3-Bromomethyl-6,7-dimethoxy-1-2(1H)-quinoxalinone	BrDMEQ	60–63
4-Bromomethyl-7,8-benzocoumarin	BrBC	64
4-Diazomethyl-7-methoxycoumarin	DMC	65
3-Chloroformyl-7-methoxycoumarin	MC3C	66
7-[(Chlororcarbonyl)methoxy]-4-methylcoumarin	CMMC	67
7-(Diethylamino)-coumarin-3-carbohydrazide	DCCH	31
7-(Diethylamino)-3-{4-[(iodoacetyl)amino]phenyl}-4-methylcoumarin	DCIA	31, 69

pound in their search for a more polar fluorescent label to overcome the problems of the low fluorescence yield as discussed by Lloyd [25]. Besides reaction conditions, which are identical to those for BrMMC, they discuss the UV and fluorescence properties, quantum yield and fluorescence lifetime of esters in different solvents, as well as the influence of chromatographic parameters such as pH and ionic strength. The label was used by Yoshida *et al.* [57] in the analysis of 5-FU in human serum.

4-Bromomethyl-6,7-methylenedioxycoumarin (BrMDC)

Naganuma and Kawahara [59] synthesized this compound because they expected that the 6,7-methylenedioxycoumarin moiety would possess a very fluorogenic group. The quenching of the fluorescence of this compound was

lowest in conventional mixed solvent systems, in comparison with BrMDC and BrMMC. The detection limit was just below 15 fmol; reaction conditions were the same as for BrMMC. They used the procedure for the determination of some acidic non-steroidal anti-inflammatory agents. The same authors demonstrated the use of the label in the analysis of loxoprofen and its diastereomeric alcohols in a pharmacokinetic study of this drug in human plasma and urine. Detection limits were 0.01 μg ml^{-1} for plasma and 0.05 μg ml^{-1} for urine.

3-Bromomethyl-6,7-dimethoxy-1-2(1H)-quinoxalinone (BrDMEQ)

By accident, Yamaguchi *et al.* [63] discovered that 6,7-dimethoxy-1-2(1H)-quinoxalinone derivatives possess intense fluorescence. As a result of this observation they synthesized the 3-bromomethyl derivative. Reaction conditions, fluorescent and chromatographic properties of the reaction products of this label with fatty acids as model compounds were investigated. The reactivity was very similar to BrMMC and BrMAC. The same group investigated the use of this label for the derivatization of fatty acids in serum [61, 62]. The analysis of phenylacetic acid, *p*- and *m*-hydroxyphenylacetic acid in human urine has also been described [60].

4-Bromomethyl-7,8-benzocoumarin (BrBC)

This label was used for an efficient separation of prostaglandins, fatty acids and other compounds containing carboxylic acids groups. The esters were prepared by methods as described for BrMMc.

4-Diazomethyl-7-methoxycoumarin (DMC)

Besides carboxylic acids, this label can also be used for the determination of alcohols. DMC reacts at room temperature with alcohols in dichloromethane, with fluoroboric acid as catalyst. DMC is non-fluorescent in solvents such as acetonitrile. For cholesterol the detection limit is 500 pmol ml^{-1}.

3-Chloroformyl-7-methoxycoumarin (MC3C)

Another label for alcohols is the one used by Hamada and Iwasaki [66]. They showed that the use of this label for the derivatization of 17-oxosteroids in urine gave detection limits of about 100 ng ml^{-1} urine. The label also reacts with aliphatic primary and secondary amines, but not with aromatic amines.

7-[(Chlororcarbonyl)methoxy]-4-methylcoumarin (CMMC)

This label is more specific than the labels mentioned previously and reacts only with hydroxy compounds. Karlsson *et al.* [67] used it to analyse prostaglandins and hydroxy steroids.

7-(Diethylamino)-coumarin-3-carbohydrazide (DCCH) and 7-(diethyl-amino)-3-{4-[(iodoacetyl)amino]phenyl}-4-methylcoumarin (DCIA)

Both these labels were used for the analysis of carboxylic acids with chemiluminescence detection by Grayeski and De Vasto for which BrMMC cannot be used. Detection limits in the low femtomol range can be achieved. Recently, Yoshida *et al.* [69] used DCIA for the analysis of pyrimidine compounds. The compounds react in acetone in the presence of crystalline anhydrous potassium carbonate and 18-crown-6. After separation by RP-HPLC they were detected using chemiluminescence. Detection limits were between 10–20 fmol per injection. The technique can be used for the determination of 5-fluoropyrimidines, which are used in chemotherapy, in small volumes of serum.

Conclusion

As shown, bromomethyl-7-methoxycoumarin as well as its analogues can be used to derivatize a large variety of compounds, including mono- and dicarboxylic acids, alcohols, pesticides and surfactants. In general compounds with a carbonyl group can be derivatized provided that the acids are soluble in the solvents used as the derivatization media. In other words, the label is suitable for lipophilic acids. For more hydrophylic acids other procedures should be employed, for example, as described by Korf *et al.* [68] who used a carbodiimide coupling of 2-amino-anthracene with small peptides in aqueous solutions.

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