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Review

An overview of applications of pre-column derivatization reactions for the liquid chromatographic analysis of pharmaceuticals and other compounds

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The applications of various pre-column derivatization reactions are presented in this review. The reactions are particularly applicable for various detection techniques [ultraviolet (UV), fluorescence, chemiluminscence, enantiomeric determination and mass spectrometry (MS)] in liquid chromatographic analysis of drugs and related compounds in various matrices. This review discovered that an improvement in reaction conditions, reagent suitability and detection limits have evolved over the last 60 years. The challenge however, still remains in ensuring that a particular pre-column derivatization step satisfies the requirement of high sensitivity, short reaction time, short work-up procedures and in particular selectivity for the drug(s) of interest. The evolution of high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as an emerging technique in derivatization is discussed.

Key words: Tandem mass spectrometry, ultraviolet, pre-column derivatization, liquid chromatography.

INTRODUCTION

Chemical derivatization is routinely employed to improve the chromatographic and/or mass spectrometric characteristics of an analyte. In gas chromatography (GC), derivatization is often required to achieve the necessary volatility. Specific derivatization of analytes can simplify the separation from interferences in the sample during liquid chromatography (LC), for example by reversed-phase (RP) separation or ion-exchange methods after the introduction of a nonpolar group or a charged function, respectively. A permanent charge in a molecule, such as the positive charge of a quaternary ammonium moiety, increases the ionization efficiency and thus the sensitivity in electrospray ionization (ESI) mass spectrometry (MS). Furthermore, by modification with a defined structural element, derivatization often enables the prediction of specific fragmentation reactions in tandem MS (MS/MS), which enhances the specificity of the method (Kretschmer et al., 2011).

Derivatization has long been accepted as an effective modification technique that can improve the overall specificity and sensitivity of trace analyses. The usual methodology involves a solution-based derivatization procedure. Over the past decade, immobilized solid-phase reagents on a polymeric solid support have become increasingly popular for easy conversion of analytes into more detectable species (Falc´o et al., 1997).

Liquid phase separation techniques, including high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), are the most frequently used techniques for determination of organic substances in various matrices. Unfortunately, many substances of interest cannot be detected because they lack the structural properties necessary for the production of signals compatible with common HPLC or CE detectors, such as ultraviolet (UV) absorbance and fluorescence. This problem can be overcome by inducing derivatization reactions that add chromophoric or fluorophoric groups to the investigated molecules (Ku´smierek et al., 2011).

Two forms of derivatization techniques applicable to

chromatographic analysis are recognised. One involves the reaction of the analyte of interest with a reagent and performing some sample clean up before chromatographic separation and detection are carried out. This is the pre-column derivatization procedure. In some instances, the derivatization procedure is carried out after separation of components in procedures referred to as post-column derivatization reactions. Each technique has its relative merits and demerits and challenges which must be addressed before a successful derivatization procedure is conducted.

The application of any pre-column derivatization methodology is fraught with some challenges. Since the reactions are conducted prior to column separation, the selection and optimization of critical response parameters are always there to be tackled. Some of the critical parameters that are apparent are presented in this paper. This review paper chronicles some of the pre-column reactions and reagents that have been adopted in analysis of pharmaceuticals and related compounds and provides some of the requisite critical parameters that must be optimized for a successful procedure. Some of the challenges accompanying adoption of various techniques are also presented.

PRE-COLUMN DERIVATIZATION FOR UV DETECTION

Perhaps, the greatest number of applications of pre-column derivatization reactions is the conversion of drugs to UV-absorbing compounds. In the earliest development of HPLC where single wavelength detectors were operational, it became clearly evident that drug substances have to be converted into species that can absorb at 254 nm. However, with the advent of multi-wavelength detectors and photo-diode array detectors (PDA), conversion of pharmaceuticals to UV-active components are dwindling, except of course for those drugs that lacks sufficient chromophores like the artemisinins. An account of some of previously utilized techniques for UV-derivatization prior to LC separation is given here.

Petsch et al. (2004) described a fully automated pre-column derivatization reaction for the quantitative determination of dithiocarbamates in plasma samples. For complexation, an aliquot of the sample was mixed with an aliquot of a 5 mM Co (II) solution on the autosampler tray. Optimum conditions for the reaction were achieved at a tray temperature of 20°C and a reaction time of 11 min. LC separation was performed on a C_{18} ODS column using a binary gradient consisting of phosphate buffer and methanol. The coloured complex of dithiocarbamate and Co (III) was quantified by UV detection at 330 nm. The assay was applied for the determination of pyrrolidine dithiocarbamate, a potent antiviral compound for the inhibition of human rhinoviruses and influenza viruses, in plasma samples.

Shishan and Mei-Yi (1986) reported a pre-column reaction of LC of qinghaosu and a method for the determination of qinghaosu in animal plasma. Qinghaosu was converted to the UV-absorbing compound Q260 by treating it with 0.16% NaOH at 45°C for 30 min and thereafter acidifying the solution with acetic acid. Optimum conditions of the pre-column reaction were investigated. Plasma samples were extracted with ethyl acetate. After evaporation, qinghaosu in the residue was converted to 0260 by using the precolumn reaction and determined by HPLC. The pre-column derivatization of artemisinin derivatives is particularly a welcome idea as all the artemisinins require one form of activation or the other in order to make them UV-active. Recently, Adegoke et al. (2012) reported the reaction of artemisinin and its three regularly used derivatives (artesunate, dihydroartemisinin and artemether) by pre-column derivative formation with 4-carboxyl-2,6-dinitrobenzene diazonium ion with high sensitivity and good reproducibility.

Conversion of amino acids to UV-absorbing species is an age-long reaction process for detecting amino-acid sequence of proteins. Pre-column derivatization of amino acids with reagents is official in the (European Pharmacopoeia, 2011). Reagents adopted include phenylisothiocyanate for detection at 254 nm and (dimethylamino)azobenzene sulfonyl chloride (DABS-Cl) for visible detection. The latter is particularly useful for the detection of imino acids such as proline. Likewise, a sensitive HPLC method for quantification of sulphydryl and disulfide amino acids in human plasma using ultra violet spectrophotometric detection was developed by Katrusiak et al. (2001). Pre-column derivatization with 5, 5`-dithio-bisnitrobenzoic acid (DTNB) and an optional pre-derivatization reaction with dithiothreitol allowed both quantitative reduction of disulfides for measurement of total amino acid levels and the measurement of the reduced forms. A dynamic range of 500 to 750 mmol/L allowed the major analytes of interest to be quantified in plasma without sample dilution. The assay is a sensitive and precise method for the determination of sulphydryl and disulfide amino acids in plasma and cell extracts. In another procedure, a reproducible method for the routine determination of amino acids in protein hydrolysates using 9-isothiocyanatoacridine as a HPLC pre-column derivatization reagent was reported by Oravec and Podhradský (1995). Derivatization is carried out by adding the isothiocyanate dissolved in dry acetonitrile to a buffered amino acid solution without the following extraction of the excess reagent. The resulting N-(9-acridinylthiocarbamoyl) amino acids were separated by one single isocratic HPLC analysis with the UV detection at 280 nm.

Pre-column conversion of ammonia and a number of aliphatic amines into phenylthiourea or its derivatives by reaction with phenyl isothiocyanate, followed by HPLC,

has been used for their determination in environmental waters. Optimum conversion was found when the reaction was carried out in sodium hydrogen carbonate-carbonate medium at 40°C for 15 min. Well separated peaks were obtained on a C_{18} column with an acetonitrile-water gradient (1 ml min^{-1}) of 30% acetonitrile for an initial 5 min which was increased linearly to 100% over 15 min and then maintained isocratic for 5 min, the acetonitrile ratio finally being returned to 30% in 5 min. The derivatized analytes were subjected to off-line solid phase extraction on C_{18} sorbent. The method was applied to tap, underground, river and aquarium waters (Sahasrabuddhey et al., 1999).

Centella asiatica is a plant belonging to the Umbelliferae family, hydrocotyle order, which has been used for many years in the treatment of venous ulcers and venous hypertension for its activity on connective tissue metabolism and endothelial integrity. Asiaticoside, one of the principle terpenoids in *C. asiatica*, is presumed to be converted to asiatic acid (AA). AA was determined by a novel precolumn derivatization RP-HPLC method with UV-Vis detection for the quantitative determination of total concentration in beagle dog plasma. AA was extracted with *n*-hexane-dichloromethane-2-propanol (20:10:1, v/v/v) from plasma, which had been hydrolyzed by acid and derivatized with *p*-toluidine. Chromatographic separation was achieved on a C_{18} column using gradient elution in a water-methanol system. Detection was set at UV wavelength of 248 nm (Zheng and Wang, 2009). Derivatization of aminoglycosides using 2,4,6-trinitrobenzenesulfonic acid (TNBS) has been widely reported. It was reported first by Benjamin (1973) for the spectrophotometric assay of these antibiotic drugs in serum and other biological fluids. Quantitative HPLC determination of amikacin in pharmaceutical formulations using precolumn derivatization was first described by Gambardella et al. (1985). Gambardella et al. (1985) reported the use of TNBS as a derivatization reagent in a pyridine-water mixed solvent for the pre-column derivatization of several aminoglycosides including amikacin. A modification of the Gambardella method was reported by Lung et al. (1998) by modifying the TBNS and pyridine concentrations adopted.

Sypniewski and Bald (1996) reported an assay that measures the total, reduced, and protein-bound captopril, the orally active antihypertensive drug, in whole human blood and urine. The procedure involves a precolumn derivatization of the drug *via* its sulfhydryl group with l-benzyl-2-chloropyridinium bromide followed by solid-phase extraction and RP-HPLC separation with UV detection at 314 nm. Oxidized and protein-bound captopril is converted to reduced form by the use of triphenylphosphine and derivatized and quantified in the same manner. The proposed method offers the possibility of determining the *in vivo* redox status of captopril in blood of patients orally given a standard dose of at least 12.5 mg of captopril as part of the treatment of hypertensive disease and/or congestive heart failure.

HPLC method has also been developed for the determination of aliphatic thiol drugs, such as N-acetyl-L-cysteine, captopril and mercapto propionylglycine in pharmaceutical formulations. The procedure involves a pre-column derivatization of the thiol drug with ethacrynic acid followed by RP-HPLC separation and UV detection. The conditions for a rapid and selective reaction of the thiols with ethacrynic acid were investigated. The method proved to be suitable for a reliable and selective quality control of commercial dosage forms of the examined thiol drugs (Cavrini et al., 1987).

Pre-column derivatization has also found usefulness in residue determination of pharmaceuticals in food products. Since the concentrations of drugs in such residues are expectedly small, application of a derivatization procedure will be an advantage. Ampicillin has also been determined in milk samples by extraction with trifluoroacetic acid solution followed by concentration on a conditioned C_{18} solid phase extraction column. Acetylation with acetic anhydride in aqueous solution (pH 8.0) was done at ambient temperature for 3 min followed by reaction with 2 M 1,2,4-triazole and 10^{-2} M mercury (II) chloride solution (pH 9.0) at 65°C for 10 min. Separation was accomplished on a C_{18} column with a mobile phase containing phosphate buffer (pH 6.5, 0.1 M), the ion-pairing agent tetrabutylammonium hydrogen sulphate, acetonitrile and methanol. Detection limit for ampicillin in milk was 3 ng mL^{-1} (Verdon and Coueder, 1996). An HPLC method was developed for the determination of isoxazolylpenicillins (oxacillin, cloxacillin and dicloxacillin) residues in milk. This method involves extraction of the penicillins from milk with phosphate buffer pH 8, deproteinization by acidification with sulfuric acid followed by clean-up and concentration on a C_{18} solid-phase extraction column and reaction with 1,2,4-triazole and mercury(II) chloride solution pH 9.0 at 65°C. The derivatized compound is eluted on a C_8 column with a mobile phase containing acetonitrile, methanol and phosphate buffer 8:2:1 (pH 6.5, 0.1 mol L^{-1}) loaded with sodium thiosulfate and ion-pairing tetrabutylammonium hydrogenosulphate. The three penicillins were quantified down to 15 μgL^{-1} in line with the EU criteria of the directive No. 93/256/EEC (Verdon and Coueder, 1998).

An accurate, sensitive and selective RP-HPLC method was developed for the analysis of two halogenated 8-hydroxyquinoline derivatives; clioquinol (CQN) and iodoquinol (IQN) using the complexation ability of the studied compounds with Pd(II) ions. RP chromatography was conducted using a 300 × 3.9 mm i.d. stainless steel column packed with 10 µm Bondclone phenyl at ambient temperature. A solution containing 0.005% w/v of Pd(II)-chloride in a mixture of acetonitrile-methanol-water (3:3:4 v/v/v) of pH 3.7 as a mobile phase pumped at a flow rate of 0.75 ml min^{-1}. UV-detection was performed at 282 and 285 nm for CQN and IQN, respectively. The presence of metronidazole (MNZ) or tolnaftate (TFT) with the studied

drugs does not affect their accurate determination. The results were reportedly satisfactorily, accurate and precise (Rizk et al., 2002).

Sulphonamides have also been determined by HPLC following pre-column derivatization. In one of such procedures, a chromatographic procedure with pre-column derivatization to form the N-(l-naphthyl)ethylenediamine dihydrochloride azo dyes was proposed for the analysis of several sulphonamides (sodium sulphacetamide, sulphadiazine, sulphaguanidine, sulphamerazine, sulphamethizole, sulphamethoxazole, sulphanilamide and sulphathiazole) in pharmaceutical preparations (tablets, pills, capsules, suspensions and drops). The separation is performed with a 0.05 M sodium dodecyl sulphate/2.4% pentanol eluent at pH 7. The pre-column derivatization improved the resolution in the chromatograms and increased the selectivity in the determination of mixtures of sulphonamidcs and in preparations where other drugs were present (Garcia-Alvarez-Coque et al., 1995).

Using 7,7,8,8-tetracyanoquinodimethane (TCNQ) as a new derivatization reagent for HPLC and tin LC (TLC), novel methods were described to detect secondary amine-bearing antidepressants (paroxetine, desipramine, fluoxetine, nortriptyline, maprotiline). The HPLC method is sensitive enough to detect these drugs in plasma at therapeutic levels, whereas the latter has potential to detect them in overdose or forensic cases. The methods are based on purple chromogens formed by the displacement reaction of the drugs with TCNQ. The resulting chromogens are directly separated by either RP-HPLC on a C_{18} column or TLC on silica gel plates. For HPLC, acetonitrile-water (60:40) was used as mobile phase, with detection at 567 nm and separation in 40 min. For TLC, three developing solvent systems were used. By HPLC, 36 $ngmL^{-1}$ spiked plasma concentrations of the drugs gave easily detectable signals whereas by TLC, detection limits varied mostly between 240 and 480 ng mL^{-1}. The HPLC method was applied to real plasma samples (Oztunc et al., 2002).

A pre-column derivatization of the aminoglycosides with a 2,4,6-trinotrobenzenesulphonic acid reagent and UV detection (350 nm) has also been reported. Commercial bulk products and pharmaceutical drug formulations of aminoglycoside antibiotics obtained by fermentation (kanamycin, gentamicin, sisomicin and tobramycin) or by synthesis (amikacin) were analyzed with HPLC on a C_8 RP column (Gambardella et al., 1985).

The procedure for measurement of different forms of four plasma thiols cysteine, cysteinylglycine, glutathione and homocysteine was proposed by Bald et al. (2004). The analytes were derivatized with thiol-specific UV labelling reagent, 2-chloro-1-methylquinolinium tetrafluoroborate, and separated from each other, reagent excess and plasma matrix constituents by RP-HPLC with detection at 355 nm. Oxidized forms are converted to their thiol counterparts by reductive cleavage with sodium borohydride prior to derivatization step. The method was found linear within the physiological and pathological ranges of thiols and is applied for plasma samples donated by apparently healthy volunteers.

The halogens judging from their importance in therapy and biological systems have also been determined through pre-column derivatization. Bromide was determined (1) in the presence of a large excess of chloride and other ions to evaluate its level in sea water, concentrated hydrochloric acid and salts, (2) after selective mineralization of aliphatic bromo-compounds to measure total bromide residues of fumigants such as 1,2-dibromoethane in water and (3) to assess bromate levels in water after removal of any free bromide, and reduction of bromate to bromide. The method involves a pre-column derivatization of bromide to 4-bromoacetanilide by reaction with 2-iodosobenzoic acid and acetanilide, sample clean-up by solid-phase extraction using C_{18} cartridges and HPLC with detection at 240 nm. The method is simple and precise and has a limit of detection of 1 µg/L bromide (Jain et al., 1996). Similarly, derivatization of iodide into 4-iodo-2,6-dimethyiphenol and HPLC of iodophenol with UV detection has been found to be a suitable method for the sensitive detection of Iodide. The pre-column derivatization involved oxidation of iodide with 2- iodosobenzoate at pH 6.4 in the presence of 2,6-dimethyiphenol. The reaction mixture was chromatographed on an octadecylsilane column using a mobile phase of acetonitrile-water, 60:40 (v/v), and detection at 220 nm. Methods have also been evolved for Iodine (pre-column reaction without using 2-iodosobenzoate) and iodate (reduction with ascorbic acid to iodide and its derivatization) and for the analysts of iodide, Iodine and iodate in the presence of each other. Application of the method has been made to the determination of Iodide in natural water, sea water, iodized salt, milk and pharmaceuticals. The relative standard deviation (RSD) was in the range 0.4 to 2.9% (Verma et al., 1992).

PRE-COLUMN DERIVATIZATION FOR FLUORESCENCE DETECTION

Pre-column derivatization for fluorescence detection is also widely applied using a variety of reagents and conditions. The advantages of fluorescence detection over UV detection stems from the highly sensitive nature of fluorescence procedure itself. Since fluorescence quenching is a serious procedural error at high concentrations, many compounds determined by fluorescence spectroscopy are usually done at very low concentration. Coupling fluorescence detection with HPLC has dramatically improved the sensitivity recorded to the extent that detection limits for some techniques are in pico- and femto-mole range. Reviews of some of the reported techniques are later presented.

An HPLC method involving on-line pre-column oxidative cleavage and fluorimetric detection was

developed for the determination of methotrexate in plasma. Plasma samples were subjected to protein precipitation followed by solvent purification and then injection into the chromatographic system. Cerium (IV) trihydroxyhydroperoxide (CTH) was introduced as a packed oxidant before analytical column for the conversion of methotrexate into highly fluorescent 2,4-diaminopteridine derivatives. The oxidative cleavage of methotrexate occurs during the flow of 0.04 M phosphate buffer (pH 3.5) containing the drug through CTH column with a flow-rate of 0.2 mL/min at 40°C. The proposed method is highly sensitive, specific and applicable to biological fluids (Emara et al., 1997). Amino acids have been particularly assayed after pre-column derivatization by HPLC with fluorescence detection. The methods for fluorescence detection is widely reported in the European Pharmacopoeia (2011) using reagents such as 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (with excitation of derivatized components at 250 and emission at 395 nm), *o*-phthalaldehyde in conjunction with a thiol reagent for primary amine groups, 9-fluorenyl methyl chloroformate for both primary and secondary amino acids as well as 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole for primary and secondary amino acids (EP, 2011). In another report, on an RP Hypersil BDS C_{18} (200 mm × 4.6 mm, 5 µm) column, 20 amino acids, which were derivatized using 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl) as pre-column derivatization reagent were separated in conjunction with a gradient elution. Optimum derivatization was obtained by reacting amino acids with BCEC-Cl at room temperature for 5 min in the presence of sodium borate catalyst in acetonitrile solvent. The fluorescence excitation and emission wavelengths were 279 and 380 nm, respectively (Zhao et al., 2008). In another method, a systematic approach to determine the optimal reaction conditions, with respect to accuracy and sensitivity of the quantitative determination of primary amino acids in a single run, using *o*-phthalaldehyde-2-mercaptoethanol derivatization was reported. To this end, an experiment was designed in which the effects of reaction time, concentration of 2-mercaptoethanol and type of solvent were determined simultaneously. The response of all parameters tested was found to be interrelated: the effect of a change in one reaction condition also depended on the other reaction conditions. The reaction conditions determined in this research resulted in accuracy better than 0.25 µM, an average reproducibility of 0.6% and an average sensitivity of 136 fmol (Dorresteijn et al., 1996). In another method, the determination of secondary amino acids is reported. Primary amino acids are removed with *o*-phthaldialdehyde-(OPA) mercaptoethanol, followed by fluorescence labelling of secondary amino acids with 9-fluorenylmethyl-chloroformate (FMOC-Cl). The reactions are run in sequence and are completed in less than 2 min at room temperature. The stable FMOC derivatives are easily separated on short RP-LC columns. The detection limits for hydroxyproline, sarcosine and proline are in the low femto-mole range (Einarsson, 1985). In another procedure, 4,7-Phenanthroline-5,6-dione (phanquinone) was used as a fluorogenic labelling reagent in pre-column derivatization for the quality control of amino acids in pharmaceuticals. The amino acid adducts were efficiently separated by C_{12} RP HPLC using a ternary mixture of triethylamine (TEA) phosphate buffer (pH 2.5, 0.05M)-methanol-tetrahydrofuran (THF) as mobile phase by varying composition gradient elution and detected fluorometrically. The results obtained by the proposed method were compared statistically, by means of the Student's t-test and the variance ratio F-test, with those obtained by a rapid reference method, which involved OPA as pre-column reagent; no significant difference was found. The stronger derivatization conditions (60 ^{O}C, pH 8, 60 min) required for the method with phanquinone are compensated by the major stability of derivatives and by the absence of fluorescent degradation products (Gatti et al., 2004).

A simple procedure is described for the derivatization of primary short- and long-chain alcohols and the HPLC separation and determination of the resulting derivatives. The alcohols were derivatized to their 2-(4-carboxyphenyl)-6-methoxybenzofuran esters with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride as the dehydrating agent. The coupling (esterification) reaction proceeded rapidly and smoothly in the presence of a base catalyst (Haj-Yehia and Benet, 1996).

In another approach, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was used as an alternative to the most common derivatization reagent, OPA, to determine sixteen biogenic amines in wines. OPA and AQC were compared in terms of the range of linearity, the limit of detection and the sensitivity (calibration line slopes) resulting from the application of each of the derivatization reagents. Sample handling is minimal because derivatization is fully automated by means of an injection programme. After derivatization, the derivatives were analyzed by RP-HPLC with gradient elution and fluorimetric detection. All the amines studied eluted in less than 25 min under the optimum conditions established. The method was successfully applied to the determination of the above amines in several types of wines from the Tarragona region (Busto et al., 1996).

A sensitive determination method for melatonin was developed by derivatizing melatonin under alkaline conditions in the presence of hydrogen peroxide. The resultant fluorophore was excited at 247 nm and the emission wavelength was 384 nm. This method was successfully applied to the determination of melatonin in rat pineal gland (Iinuma et al., 1999).

Fluorogenic acid hydrazides are well known to be useful as fluorescence derivatization reagents for carboxylic acids in HPLC with the advantage that the derivatization using such compounds can be readily performed under mild conditions in aqueous solution in

the presence of a coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Ohkura et al., 1994; Yasaka and Tanaka, 1994; Yasaka et al., 1998). A unique acid hydrazide, 2-(5-hydrazinocarbonyl-2-oxazolyl)-5,6-dimethoxybenzothiazole, that is characterized by the benzothiazole structure conjugated to an oxazoline moiety was synthesized, and its applicability as a pre-column derivatization reagent for carboxylic acids in HPLC was examined in view of sensitivity and separability. The sensitivity of the hydrazide for carboxylic acids was determined using lauric acid to be 0.1 pmol (per 10 µL injection volume) at a signal-to-noise ratio of 3 (Saito et al., 1995). The lipid aldehyde 4-hydroxy-2-nonenal (4-HNE) was derivatized with a novel fluorescence labelling reagent, 4-(2-carbazoylpyrrolidin-l-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-ProCZ). The labelling reaction was carried out at 60°C for 10 min in the presence of trichloroacetic acid. The resultant 4-HNE-NBD-ProCZ hydrazone was separated from other aldehyde-NBD-ProCZ derivatives by HPLC with a ternary eluents consisting of water, methanol and acetonitrile (Liu et al., 1996). 6,7-Dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide was found to be a highly sensitive fluorescence derivatization reagent for carboxylic acids in HPLC. The reaction conditions were optimized for various C_5-C_{20} saturated fatty acids. The derivatives were detected spectrofluorimetrically at 447 nm with excitation at 365 nm. The detection limits (signal to noise ratio = 3) for the acids were 3 to 6 fmol for an injection volume of 10 µL. The reagent was also applied to the derivatization of some metabolites of arachidonic acid (Yamaguchi et al., 1990).

A highly sensitive and simple RP-HPLC method for the quantitative determination of free fatty acids in human serum was also presented. The method is based on the direct derivatization of serum fatty acids with 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide. The derivatization reaction proceed in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 37°C (Iwata et al., 1992).

Some carboxylic acids have been derivatized for fluorescence detection. Iwata et al. (1992) found that 2-phenyl-5,6-benzimidazole, which is produced by the reaction between benzaldehyde and 1,2-diamino-4,5-dimethoxybenzene, gives a more intense fluorescence than 6,7-dimethoxy-l-methyl-2(1l1)-quinoxalinone-3-propionylcarboxylic acid hydrazide. Hence, 4-(5,6-dimethoxy-2-benzimidazoyl)benzohydrazide (DMBI-hydrazide) was prepared, which has a novel 2-phenyl-5,6-benzimidazole moiety, as a fluorescence derivatization reagent for carboxylic acids.

An HPLC method for the determination of rofecoxib in human serum was developed by Amini et al. (2005). The method is based on pre-column derivatization of the analyte to a phenanthrene derivative. Rofecoxib and internal standard were extracted from serum using liquid-liquid partition. Upon exposure to UV light, the drug undergoes a photocyclization reaction giving a species with high absorbance which was determined by RP-HPLC.

A highly sensitive and simple method for the determination of prostaglandins (PGs) by HPLC with fluorescence detection is described. PGs are converted to the corresponding fluorescence derivatives by the reaction with 4-(*N*,*N*-dimethylaminosulphonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole (DBD-PZ) in the presence of 2,2′-dipyridyl disulphide and triphenylphosphine in acetonitrile. The reaction is completed at room temperature after 30 min. The DBD derivatives of nine PGs are separated within a single 45 min chromatographic run on a RP ODS column with a linear gradient elution using water and acetonitrile. The detection limits (signal-to-noise ratio of 3) calculated from the standard mixture of PGs (6-keto-$F_{1\alpha}$, $F_{1\alpha}$, $F_{2\alpha}$, E_1, E_2, D_2, limaprost, A_1 and B_1) are in the range 1.7 to 5.0 fmol. The applicability of the proposed procedure is evaluated to the detection of PGs added to rat plasma (Toyo'oka et al., 1992).

A simple and selective method for the multiple residue determination of eight sulphonamides in consumers' milk was also reported. The drugs are sulphisomidine, sulphadiazine, sulphamerazine, sulphadimidine, sulphamonomethoxine, sulphamethoxazole, sulphadimethoxine and sulphaquinoxaline. The milk sample was deproteinized with the same volume of 2 M hydrochloric acid and filtered. A 1-ml volume of the titrate was mixed with 1 ml each of 1.25 M sodium acetate solution and a buffer (pH 3.0) for derivatization with 0.6 ml of 0.02% fluorescamine solution in acetone. An HPLC analysis was carried out on a C_{18} column with a mobile phase of acetonitrile-2% acetic acid (3:5) at 55°C using a fluorescence detector at an excitation wavelength of 405 nm and an emission wavelength of 495 nm. The method was applied to 25 milk samples and all appeared to be free from the drugs (Takeda and Akiyama, 1992).

Aflatoxins have been widely derivatized for sensitive fluorescence detection by HPLC. In a reciew by Kok (1994), various derivatization methods for the fluorimetric detection of aflatoxins after separation by HPLC were reviewed. Optimum conditions for the reactions are discussed. In terms of sensitivity, the three described derivatization schemes give similar results. The methods are compared with respect to experimental convenience, selectivity, reproducibility and suitability for automation.

A new method for determining cycloserine in plasma samples based on derivatization of cycloserine with *p*-benzoquinone was described. The reaction is reported to take place at the same time as the process of plasma deproteinization due to the presence of ethanol as solvent in the solution of the derivatization reagent. Four derivatives are obtained from this reaction. The main derivative is well correlated with the cycloserine

concentration. This method was validated and applied to the determination of cycloserine in blood plasma samples of several healthy volunteers (David et al., 2001).

PRE-COLUMN DERIVATIZATION FOR CHEMILUMINESCENCE DETECTION

In a review by Yamaguchi et al. (2002), the principles for chemiluminescence of luminol-type compounds and their wide and powerful application to the detection system in LC and CE as derivatization reagents were presented. The reagents can be classified into two types, chemiluminescence labelling and chemiluminogenic reagents. The former reagents are highly chemiluminescent themselves and used for tagging their intense chemiluminophores to analytes, whereas the latter are weakly chemiluminescent but generate intense chemiluminescence by reaction with analytes. The LC methods utilizing chemiluminescence derivatizing reactions with luminol-type reagents allow the analytes to be detected at pmol to sub-fmol levels. Furthermore, the chemiluminogenic reactions show high selectivity owing to their selective reaction against the analytes permitting facile and reproducible detection.

Catecholamines occur in various biological materials in extremely small amounts and play physiologically important roles. In order to evaluate physiological investigations, a highly selective and sensitive method for the determination of catecholamines is required. Yakabe et al. (2002) reported a highly sensitive luminol-type chemiluminescence (CL) derivatization reagent for 5-hydroxyindoles, 6-aminomethylphthalhydrazide (6-AMP), and applied the reagent to the HPLC method for the determination of 5-hydroxyindoles (Ishida et al., 1997; Yakabe et al., 2000). The method is based on the derivatization reaction of 5-hydroxyindoles with 6-AMP in the presence of potassium hexacyanoferrate (III) to give highly CL derivatives. The authors also found that 6-AMP reacts with catecholamines under different conditions from those for 5-hydroxyindoles to give the corresponding derivatives, which produce CL by a reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate (III) in an alkaline solution.

Ishida et al. (1995) also reported the development of another reagent for the sensitive determination of amines by HPLC with CL detection following a derivatization reaction. 6-Isothiocyanatobenzo [*g*] phthalazine-1, 4 (2*H*,3*H*)-dione was synthesized as a highly sensitive and selective chemiluminescence derivatization reagent for primary and secondary amines in LC. Di-*n*-butylamine, dibenzylamine and *n*-nonylamine were used as model compounds to optimize the derivatization conditions. The reagent reacts selectively with amines in the presence of TEA to give highly chemiluminescent derivatives which produce CL by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate (III) in alkaline solution. The chemiluminescent derivatives of the three amines can be separated within 12 min by RP-HPLC with isocratic elution, followed by CL detection. In another report, Ishida et al. (1992) gave an account of the utilization of 4, 5-Diaminophthalhydrazide (DPH) as a highly sensitive reagent for the CL derivatization of α-dicarbonyl compounds. The reagent reacts with α-dicarbonyl compounds in dilute HCl in the presence of β-mercaptoethanol to give highly chemiluminescent quinoxaline derivatives which produce CL by reaction with hydrogen peroxide and potassium hexacyanoferrate (III). The DPH derivatives of five α-dicarbonyl compounds were determined.

A sensitive determination method for a non-fluorescent anti-arrhythmic drug, mexiletine, in rat plasma is presented utilizing a HPLC peroxyoxalate chemiluminescence (PO-CL) detection system. After an internal standard (4-methylmexiletine, 4.35 pmol) and 0.1 N sodium hydroxide solutions were added to 5 µL rat plasma, the solution was poured onto an Extrelut 1 column. Both mexiletine and the internal standard were eluted with diethyl ether and then the eluate was evaporated to dryness. The residue was dissolved in 0.2 M borate buffer (pH 8.5) and mixed with dansyl chloride (75 nmol) in acetonitrile. After standing of 90 min at room temperature, 0.5 N HCl was added to the reaction mixture to stop the reaction and a 2/45 aliquot of the mixture was subjected to a HPLC PO-CL detection system using bis(4-nitro-2(3,6,9-trioxadecyloxycarbonyl)phenyl)oxalate (TDPO) and hydrogen peroxide. The method was applied to the measurement of the time courses of plasma mexiletine concentration after oral administration of the drug [25 mg (115.9 µmol)/kg] to rats (Nishitani et al., 1992).

An HPLC assay of methamphetamine (MP) and its related compounds, that is, ephedrine (EP), norephedrine (NE), *p*-hydroxymethamphetamine (*p*-HMP), *p*-hydroxyamphetamine (*p*-HAP) and amphetamine (AP), with peroxyoxalate chemiluminescence detection was developed by using 4-(*N*,*N*-Dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) as a fluorescent labelling reagent. A mixture of hydrogen peroxide and bis[4-nitro-2(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate in acetonitrile was used as a post-column chemiluminogenic reagent. The method was successfully applied to the assay of MP and its metabolites in urine samples from MP addicts (Nakashima et al., 1992).

Ishida et al. (1997) reported the synthesis and application of 6-AMP as a highly sensitive and selective chemiluminescence derivatization reagent for 5-hydroxyindoles in LC. 5-Hydroxytryptophan, serotonin and 5-hydroxyindole-3-acetic acid (5-HIAA) were used as model compounds to optimize the derivatization conditions. The reagent reacts selectively with the indoles in the presence of potassium hexacyanoferrate(III) to give highly chemiluminescent derivatives which produce CL by reaction with hydrogen peroxide in the presence of

potassium hexacyanoferrate(III) in alkaline solution. This same reagent was later applied for the determination of serotonin and its major metabolite, 5-HIAA in human urine (Yakabe et al., 2000).

PRE-COLUMN DERIVATIZATION FOR CHIRAL COMPOUNDS AND ENANTIOMERIC SEPARATIONS

The application of LC to optical resolution has developed in two ways: the direct resolution of enantiomers on a chiral stationary phase, and derivatization with a chiral reagent followed by LC of the diastereomers on conventional columns (Nambara et al., 1978). Majority of the applications of pre-column derivatization have been for enantiomeric amino acids. The reaction usually converts them to diastereomers which can then be readily separated. In one of such procedures, Nambara et al. (1978) synthesized two chiral derivatization reagents; (-)-1,7-dimethyl-7-norbornyl isothiocyanate and (+)-neomenthyl isothiocyanate from their corresponding amines and used them to transform amino acids into the thiourea derivatives which absorb at 243 nm and effectively resolved by normal phase chromatography with cyclohexanne/ethylacetate as mobile phase. Some of the amino acids derivatized and determined were alanine, valine, norvaline, proline, glutamic acid, threonine among others.

In another procedure, Nimura et al. (1980) reported a novel method for RP-HPLC resolution of amino acid enantiomers by the formation of diastereomers using a new chiral reagent, 2,3,4,6-tetra-*o*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). GITC reacts readily with enantiomeric amino acids at room temperature and the reaction mixture can directly be injected into the chromatograph. The derivatives were detected spectrophotometrically at 250 nm. Complete resolutions were observed for all enantiomers examined on a RP column eluted with aqueous methanol. Some of the amino acids analyzed as amino ethyl esters are serine, alanine and tryptophan. However, another procedure utilized non-esterified amino acid intermediates by the formation of diastereomers using two chiral reagents, GITC and 2,3,4-tri-*O*-acetyl-α-D-arabinopyranosyl isothiocyanate (Kinoshita et al., 1981). These compounds react readily with enantiomeric free amino acids at room temperature and the reaction mixture can be injected directly into the chromatograph.

A novel approach to enantiomeric separation was reported by Ôi and Kithara (1984) where N-acetyl-DL-amino acid methyl esters were separated on L-valine bonded onto silica gel columns and chromatographic separations were effectively conducted for alanine, leucine among others.

Another application reported a new and highly sensitive method for the determination of amino acid enantiomers is also presented. Fluorescent diastereoisomers are formed by reaction with OPA and *Boc*-L-cysteine prior to chromatography. The reaction occurs rapidly at ambient temperatures and is performed by an automated pre-column derivatization device integrated into the HPLC system. Separation of the diastereoisomers is carried out by RP chromatography with gradient elution and the derivatives are detected fluorimetrically (Buck and Krumen, 1984).

A novel approach was reported by Bruckner and Strecker (1992). Nucleophilic replacement of one halogen atom (chlorine, fluorine) in the trihalo-*s*-triazines (2,4,6-trihalo-1,3,5-triazines), cyanuric chloride or cyanuric fluoride by reaction with either methanol, 2-naphthol, 1-methoxynaphthalene, or 4-aminoazobenzene furnished UV-absorbing, fluorescent of chromogenic dihalo-*s*-triazines. Substitution of a further halogen atom in these compounds by reaction with L-alanine amide provided chiral monohalo-*s*-triazines. The remaining halogen atom was substituted by reaction with selected D- or L-amino acids to form diastereomeric derivatives which were separated by RP (C_{18}) HPLC using mixtures of water, acetonitrile and trifluoroacetic acid as eluents. Because of its possibilities for selection among a large number of detection groups in combination with various chiral moieties, the approach is considered to be a general method for the design and construction of tailor-made reagents suitable for pre-column derivatization and indirect liquid chromatographic separation of amino acid enantiomer.

In another derivatization procedure developed for converting enantiomeric amines into diastereomers for resolution by HPLC, two chiral reagents, (-)-α-methoxy-α-methyl-1-naphthaleneacetic acid and (-)-α-methoxy-α-methyl-2-naphthaleneacetic acid, were prepared and optically resolved by fractional crystallization of their (+)-α-methylbenzylamine salts. The diastereomeric amides formed from amino acid methyl esters and (-)-α-methoxy-α-methyl-1-naphthaleneacetic acid by the N,N`-dicyclohexylcarbodiimide method were efficiently resolved on a normal-phase column and responded with satisfactory sensitivity in the UV detector (Goto et al., 1978).

Carboxylic acids have also been derivatized for enantiomeric separation. In a report by Goto et al. (1980), two highly sensitive chiral derivatization reagents, D- and L-1-aminoacetyl-4-dimethylaminonaphthalene, were synthesized from 1-dimethylaminonaphthalene. Condensation of carboxylic acids with the chiral reagent was readily effected in the presence of a water-soluble carbodiimide. The diastereoisomeric amides formed from N-acetylamino acid and α-arylpropionic acid enantiomers were efficiently resolved by normal phase chromatography (µPorasil column) with hexane/ethyl acetate or hexane/THF as a mobile phase. With a fluorescence detector (excitation 320 nm. emission 395 nm), the detection limit was 0.1 ng.

A new triflate-type fluorescence chiral derivatizing

reagent, (*S*)-(1)-1-methyl-2-(6, 7-dimethoxy-2, 3-naphthalimido)ethyltrifluoromethanesulfonate was also developed for the determination of the enantiomers of carboxylic acids. By introducing the two methoxy groups on the naphthalimido ring moiety, the red shift in the fluorescence spectrum and a high resolution in reversed-mode separation of the diastereomers of chiral carboxylic acids were achieved (Yasaka et al., 1998).

PRE-COLUMN DERIVATIZATION FOR MASS SPECTROMETRIC DETECTION

LC/ESI-MS/MS is one of the most prominent analytical techniques owing to its inherent selectivity and sensitivity. In LC/ESI-MS/MS, chemical derivatization is often used to enhance the detection sensitivity. Derivatization improves the chromatographic separation, and enhances the mass spectrometric ionization efficiency and MS/MS detectability. In a review by Santa (2011), an overview of the derivatization reagents which have been applied to LC/ESI-MS/MS was presented, focusing on the applications to low molecular weight compounds, such as carboxylic acids, alcohols and amines.

The analysis of free carboxylic acids by ESI-MS is subject to a number of challenges. While positive ionization is the method of choice for the large majority of compounds in biomolecular analysis, free carboxylic acids need to be detected in negative ionization mode, and ionization efficiencies are generally rather low. It is therefore difficult, if not impossible, to achieve simultaneous detection of carboxylic acids together with a large number of different analytes, such as within a whole set of biomarkers. Various derivatization reagents, including diethyl-aminoethyl chloride or bromide, 2-nitrophenylhydrazine, pyridinium compounds, benzofurazan reagents and tris(trimethoxyphenyl) phosphonium compounds have been used to solve this problem and make carboxylic acids compatible with positive ionization ESI-MS (Kretschmer et al., 2011). In order to develop a generic positive ionization ESI LC–MS method for a variety of interesting substance classes, a new derivatization strategy for carboxylic acids was developed (Kretschmer et al., 2011). The carboxylic acid group is labelled with the bromine containing reagent based on carbodiimide chemistry.

The stability of amino acids derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) was investigated using a combination of HPLC, solid-phase extraction, photodiode array spectrophotometric detection, and MS characterization. The degradation of amino acid derivatives, generated using β-mercaptoethanol as a nucleophile, was characterized under a variety of environmental influences, with a focus on understanding the degradation kinetics and identifying the degradation products. The predominant degradation product observed under most reaction conditions was the non-fluorescent lactam form of the originally fluorescent isoindole derivative. MS/MS experiments were used to demonstrate unimolecular degradation of the protonated isoindole in the absence of solvent or atmosphere, suggesting an intramolecular reaction mechanism involving the hydroxyethylthio group (Manica et al., 2003).

A sensitive and reproducible LC–ESI/MS/MS method, which was combined with the pre-column dansyl chloride derivatization to enhance the signal intensity of analytes, was developed by Zhuang et al. (2008) to determine blood 4-dimethylaminophenol (DMAP) concentrations. By using this method, pharmacokinetic studies were conducted in dogs after i.m. and i.v. administrations. It has been proved that this LC–MS/MS combined with pre-column derivatization method can be used as a routine analytical method to provide enhanced measurements for blood DMAP concentrations.

OVERVIEW OF REPORTED METHODS, CHALLENGES AND FUTURE PROSPECTS

This review has made an attempt at chronicling some of the reported pre-column derivatization procedures in literature spanning close to 60 years. The techniques have evolved through the desire of scientists to find solutions to the issues of analyte detectability, selectivity of analytical methodology, sensitivity of determination, wide applicability and convenience of analysis. One key thing that stands out is that as diverse as pharmaceuticals and related compounds are, so diverse are also the reagents that have been developed to analyze them. The reason is not far-fetched, since pre-column derivatization reactions are based on specific functional group interaction, it is expected that the reagents adopted mechanisms of derivatization reactions will equally be diverse. The initial development of pre-column derivatization reactions appears to have focused more on the conversion of drugs and related compounds to UV-active species which can be readily detected in the UV region or in the visible region as contained in the reviewed literatures. As advancement in the design and available detection systems in LC progressed, the derivatization methodologies shifted to the development of methods that can convert drugs to fluorescent species.

These induced fluorophores were characterized by high sensitivity. Of particular interest is the wide range of methods that have developed for the analysis of amino acids. This appeared to be a welcome idea in the mid 20th century since amino acid chemistry was developing and their understanding in biological processes and disease progression will require availability of techniques that can determine these compounds at very low concentrations. In the procedures specified by European Pharmacopoeia, some of the reagents can detect and determine the amino acids by fluorescence in the pico- and femto-molar

range, which is not possible with HPLC-UV methods.

Advancements of techniques have also brought into fore the conversion of drugs and other compounds to chemilumniscent derivatives. This is revealed in the reviewed literature where a variety of reaction conditions and reagents were adopted to effect CL behaviour in the compounds. One clear draw-back in my opinion is the need for multiple reagents and reaction steps to effect chemilumniscent behaviour which of course will require that more attention to be paid to design and application of reagents that can achieve 1- or 2- step conversions of these molecules for ease of detection.

Enantiomeric separations of organic compounds have always posed some difficult analytical challenges to an average scientist engaged in such study. The separation of chiral compounds and enantiomers has gone through processes of selective chiral separation on chiral columns, which is still popular. However, worthy of note is the development of some reagents that can readily convert enantiomers to separate diastereomers which can be separated on conventional RP columns in HPLC and then detected by UV or fluorescence. While this appears cost-effective, the challenge at making available less time-consuming techniques at the expense of acquiring specialised columns must be addressed.

The combination of HPLC-MS/MS as a hyphenated technique has once again advanced the applications of HPLC to the determination of pharmaceuticals and other related compounds. The advantages to be gained are enormous ranging from low detectability, small sample size and amenability to forensic studies, since the sensitivity of MS is combined with the selectivity of HPLC. Apart from high cost, pre-column derivatization for HPLC-tandem MS appears to be highly favoured as the technique of the 21st century and beyond.

The stability of the pre-column derivatization reagent is a primary challenge in these procedures. Since it is expected that the conversion take place readily and accurately, the reagent is expected to be stable all throughout the analysis and a good reagent will also be one that can be applied at varied temperatures and reaction media (such as change in pH, ionic strength, extreme acid or base applications etc). The reagent concentration must be one that can be conveniently prepared without recourse to extreme conditions. It is also expected that the reagent must have what can be described as convenience of application. Thus, a reagent that is too toxic for the analyst or that must be handled with extreme caution will lack wide applicability.

The reaction stoichiometry is one practical challenge in ensuring that a pre-column derivatization procedure is applicable. Most of the reactions are carried out using excess of the reagent in order to ensure a complete conversion of the analyte to the desired derivative. This then leave the analyst with the challenge of removing excess reagents not used up. A good technique thereafter will be the selection of detection system that will be responsive to derivatized products while discriminating between the residual reagent and the compound derivatized. This scenario has been accomplished in most of the literature reviewed as the detection is usually carried out at regions where detector response is optimal for the derivatized species formed. This is one challenging area where application of HPLC-MS/MS is of greater advantage since specific ions related to derivatized components can be monitored without undue interference from the reagents or other starting materials.

The time required for the derivatization procedure to be completed is also a critical factor that must be optimized. A lot of improvements have emerged in this respect. In the older literatures, time for derivatization of up to 1 h were reported, whereas newer reagent applications have dramatically resulted in less time of pre-column processing.

One other challenge in pre-column derivatization is the required work-up procedures for the derivatized components to make them ready for HPLC analysis. It is expected that the work-up time should be as simple and as less-time consuming as it can be possible. Thus, adequate attention must be paid to shortening the work-up procedures to ensure large throughput of samples. The reproducibility and by extension the reliability of the adopted methodology for pre-column reaction is also another challenging choice in pre-column derivatization. It is expected that any method adopted must be one that can be reproduced at other times.

Perhaps, the greatest challenge in adopting a pre-column derivatization reaction is the specificity of the reagent to the analyte of interest. Since pre-column derivatization reactions are carried out in the sample matrix and are potentially susceptible to side reactions and interferences in the reaction sequence, the reagent adopted must be able to selectively determine the component of interest. While this condition is often difficult to attain in solution processes (where most derivatization reactions take place), recent advances have focused on the development of solid-phase microextraction (SPME) of the sample to be analyzed and then eluting them onto HPLC. Stashenko and Martinez (2004) provided an excellent review of SPME and the advantage of low detection limits and selectivity it has afforded.

CONCLUSION

Many wide-ranging techniques have been applied for the pre-column derivatization of pharmaceuticals and related compounds in order to enhance detectability and specificity. The avalanche of development of reagents and techniques will obviously continue as more procedures for detection of separated components from HPLC become available.

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