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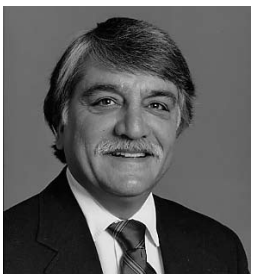
PICKERING
LABORATORIES

Mike Gottschalk
Tel: 1 800 654 3330
Email: mgottschalk@pickeringlabs.com

Michael Pickering
Tel: 1 800 654 3330
Email: support@pickering.com
Web: www.pickeringlabs.com

An Overview of Post Column Derivatization Methods from a Pharmaceutical Applications Perspective

By Michael Pickering, Ph.D.



Dr. Michael Pickering is President of Pickering Laboratories Incorporated (Mountain View, California), founded in 1980. Pickering Laboratories was established to supply the liquid chromatography (LC) market with application-specific chemistry and hardware, with an emphasis on post-column technology. He has held research and development positions with Durrum Instruments (now Dionex Corp., Sunnyvale, California) and Spectra Physics (San Jose, California). In 1974 he earned a PhD in chemistry from the Oregon Center for Science and Technology, now the Oregon Graduate Institute. His research area was synthetic and medicinal chemistry. He received an MS in chemistry from San Francisco State University in 1970, and a BS in Chemistry from St Mary's College of California in 1964.

Introduction

High performance liquid chromatography (HPLC) is the technique of choice for separating analytes pursuant to their detection, identification, quantification and/or purification. Most organic substances, and certainly the majority of pharmaceuticals, are polar, nonvolatile or thermally labile molecules that are amenable to HPLC separation. Moreover, the availability of a large variety of LC column media, particle sizes and near-infinite gradation of mobile phase types and polarities greatly empowers the discriminatory capabilities and ease of adaptation of HPLC. Once separated, analytes of interest must be registered by detecting a characteristic physical property such as the absorption of light, emission of fluorescence, mass, etc. The detected signal, in conjunction with a reproducible retention time for a chromatographic method, is usually sufficient to identify and (with an appropriate set of calibration standards) quantify each analyte of interest. Other things being equal, factors that increase analyte signal strength or the ability to discriminate the signal from background noise or other interferences will lower the limit of detection and quantitation. This is a highly desirable objective for analytical methods used in pharmaceutical applications where analytes of interest are generally active and often monitored at low physiological concentrations. Since much of the effort in chromatographic sample preparation is focused on isolating analytes from sample constituents that may confound detection, techniques that bolster analyte signal strength and selectivity often permit simpler and more efficient sample preparation, with the time saved translated into increased throughput.

Postcolumn Derivatization: Organic Synthesis on the Fly

One approach that has proven eminently suitable for enhancing LC analyte discrimination and detection is postcolumn derivatization (PCD). Given the ubiquity of the signal, ease of use and relatively low-cost of the detection hardware; UV and visible light absorption together with fluorescent emission are typically the detection methods of choice for high-volume and/or routine HPLC analysis. It is these signals, for the most part, that PCD methods are employed to strengthen. While most substances show some absorption in the UV/VIS region of the electromagnetic spectrum, the response is often weak and easily confounded by coeluting substances. PCD overcomes this debility by utilizing synthetic methods to convert chromatographically partitioned analytes to derivatives with chromophores that produce intense absorption or fluorescence peaks, usually shifted to longer wavelength regions of the spectrum to reduce the likelihood of interferences. (Fig.1). The conversion takes place in-line as each analyte exits the column and travels to the detector flowcell. Because the derivative is formed postcolumn, the chromatographic separation is preserved, that is, providing the PCD transport system and reaction chemistry are properly designed and executed with that objective in mind (Fig. 2).

Accordingly, PCD system development is guided by a set of criteria designed to optimize analyte reactivity and enhance signal strength without compromising chromatographic efficiency or reproducibility.
To maximize signal sensitivity and selectivity for both detection and quantitation purposes PCD reagents should contribute as little as possible to the background against which the signal is measured. This rule must be employed with increasing rigor as analyte concentration approaches the method's limit of detection wherein background noise increasingly obscures an ever-weaker signal. Another important PCD developmental criterion requires that reactions and products remain in solution. The formation of a precipitate, with its potential for blocking capillary tubes, bursting reactors and/or fouling detector flowcells, will, along with the formation of other optically interfering phenomena such as emulsions or dispersions, diminish detector response by blocking or scattering the signal. Comparable problems arise when incompletely mixed liquids, differing in constitution or temperature, create localized inhomogeneities that vary in refractive index. Most modern detector flowcells are designed to compensate for these perturbations, but it is far better to avoid the problem by ensuring that solution composition and temperature are uniform throughout before reaching the detector. Other measures that can help reduce signal distortion include the use of pumps that maintain a constant flow rate with high precision. Heating the solution in a PCD reactor, or conversely post-reaction cooling, can result in the evolution of gas bubbles, yet another potential source of optical interference. This tendency can usually be suppressed by applying a modest backpressure on the detector flowcell.

Postcolumn or Precolumn Detection

Both postcolumn and precolumn derivatization methods focus on improving analyte detection through signal enhancement*. Precolumn derivatization may be seen as less demanding since analyte conversion is complete before chromatography is initiated and therefore will not impact post-column band spreading. However, this potential benefit is often gained at the expense of complex and time-consuming sample preparation. Moreover, since the derivatization process is administered to all analytes simultaneously, there is the possibility that conversion will not be uniform and that the relative concentration of analyte derivatives will differ from their precursors in the untreated sample. If this were to occur it could be problematic for quantitation. PCD, by contrast, derivatizes each analyte in isolation, so that given sufficient excess and activity of the reagent, conversion is uniform across the suite of analytes. While it is true that PCD derivatization must be carefully configured and controlled so as to maintain chromatographic resolution, sample preparation is relatively simple compared with precolumn methods, and the in-line nature of the process also facilitates automation. These are methodological characteristics sought after for the many types of high volume analytical applications required across the pharmaceutical value chain, from drug screening through clinical trials, product QC, patient diagnostics and therapeutic monitoring.

HPLC/PCD Integration

Two essential considerations for PCD/HPLC integration are that the PCD reaction system be compatible with the HPLC mobile phase and that neither the reagent addition and subsequent reaction nor the configuration of the PCD apparatus introduce flow irregularities that can cause band spreading or compromise retention time reproducibility. Typical rules of thumb include minimizing the length of the flow path between the column exit and the detector and reducing the derivatization time, if necessary, usually by raising the temperature. Maintenance of precise flow control is as important in PCD reagent addition as it is for the chromatographic separation. PCD pumps should be rugged and utilize components with inert internal surfaces that will contact chemically aggressive reagents.

In many instances, providers of postcolumn and precolumn methods will claim some degree of superiority for their specific approach and/or analytical system. The apparent contradiction of competing claims is resolved when it is understood that these claims reference different analytical situations and that derivatization is integrated with sample preparation, chromatography and detection systems tweaked for optimal performance.

Figure 1 – Spectra of an Amino Acid Derivative with Ninhydrin

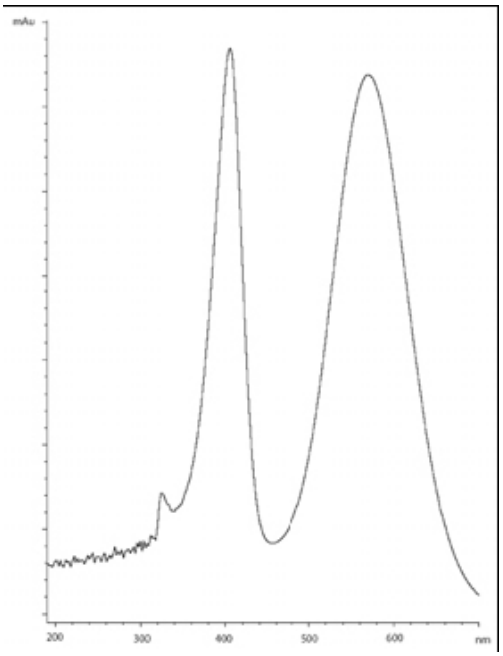
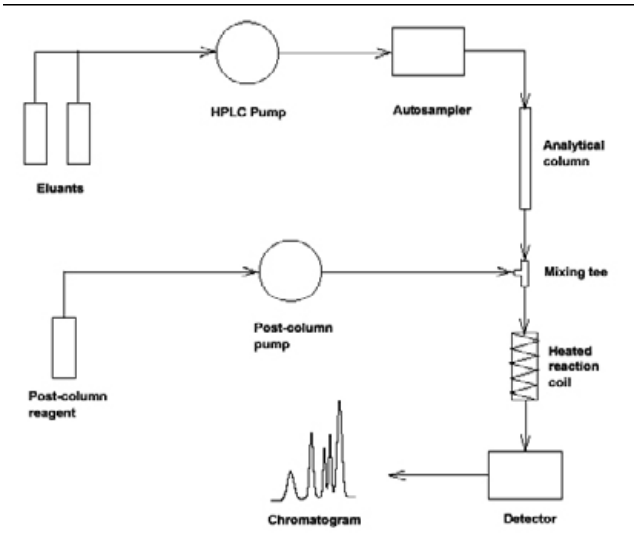


Figure 2 – Post-Column Derivatisation Applies the Techniques of Organic Synthesis Within a Flow-through Reactor that Constitutes the Short Path Between Column Exit and Detector Flowcell



Reactants and conditions must be chosen so that conversion to the desired product(s) takes place rapidly (usually < 1 minute) and reproducibly. Given these constraints, the PCD system must ensure good mixing and take advantage of both favorable reaction kinetics as well as thermodynamics. If the conversion is not sufficiently rapid, it may be necessary raise the temperature, incorporate a catalyst or in some other way accelerate the rate of derivatization. It should be noted that completeness of reaction, while a necessity for quantitation, is not always a required result. In many qualitative applications it may be sufficient that each analyte derivative be formed in abundance great enough to generate a detectable signal.

PCD Method Development and Implementation

Transferring a reaction from a static laboratory vessel to a dynamic flow-through PCD system can require significant modification of conditions and components to achieve the desired result within chromatographic constraints. Therefore, successful PCD development requires familiarity with the tools and techniques of organic synthesis, including molecular structure/activity relationships and solvent effects as well as liquid chromatography expertise. These skills should be bolstered with an ongoing literature-scanning program to identify prospective synthetic methods that could be employed in current and future PCD applications.

The challenge of designing PCD methods for specific groups of pharmaceuticals is simplified somewhat by the fact that molecules developed for a particular therapeutic purpose usually share similar structures and functional groups. These can be selectively targeted for derivatization. Once the optimal synthetic approaches have been designated, (depending on the chromatographic method selected, nature of the sample matrix, analyte concentrations, presence of metabolites, etc.), a more directed literature search may be instituted. Other resources that can be deployed to aid in PCD method development includes privately contracted custom synthesis and the hiring of academic or other expert organic synthesis consultants.

Notwithstanding the ingenuity exhibited in applying diverse organic reaction systems in the development of PCD methods for pharmaceutical analysis (see Table 1), it appears that a small subset of robust derivatization systems have been repeatedly recruited for use with analytes that share common functionalities. One example of this evolution originates with PCD methods for the detection and quantification of amino acids (1), which have been subsequently adapted for other amino functional analytes. The first reagent that gained widespread usage in amino acid analysis was ninhydrin. This compound reacts with the amino acid's primary amino group to generate intensely colored purple derivatives, λ_{max} = 570 nm ($\varepsilon = 2x10^4$). For detection and/or quantification purposes, the underivatized analyte's weak UV absorption at 220 nm (carboxylate moiety, ε = 30-50) is replaced by an intense absorption peak, 4-5 orders of magnitude greater, and shifted to a wavelength region fairly free of interferences. Imino acids, such as proline (secondary amino group) also react with ninhydrin, forming yellow derivatives, λ_{max} = 440 nm, that are also suitable for detection and quantification purposes. Thus, the method has application across the entire suite of amino acid analytes (Fig. 1).

More recently, a reagent system based on o-phthalaldehyde (OPA) was introduced. OPA and alkyl mercaptans react with the primary amino group of amino acids under basic conditions to form highly fluorescent 1-alkyl-2-alkylthio-substituted isoindoles (λem = 465). (Reactivity of secondary amines toward OPA may be increased by addition of an appropriate oxidant prior to OPA addition.) The detection limit for OPA derivatives is approximately an order of magnitude lower than for the corresponding ninhydrin derivatives. (0.1 and 1 nanomoles, respectively), a significant improvement in method sensitivity. Unlike the reaction conditions required to derivatize amino acids with ninhydrin, the OPA reaction does not require heating and shows a desirably low response to ammonia (stable baseline).

Yet, ninhydrin-based methods have remained popular in many applications. One reason may be the relative instability and therefore transient nature of the isoindole product compared with the corresponding ninhydrin derivative. Another may be the quenching exhibited when OPA forms large molecular derivatives, either because multifunctional amino acids such as cysteine can add more than one OPA moiety or the analyte itself is a large molecule as is the case with OPA derivatization of peptides.. Despite these limitations, which are being addressed by ongoing development*†, amino acid analysis is benefited by access to a second PCD method that offers increased sensitivity. The sensitivity of OPA-based PCD methods for amino acids has led to its adaptation to other amino functional analytes, a number of which are pharmaceuticals. Examples include the measurement of bivalirudin (a blood clotting inhibitor) in human plasma and urine (4), an assay for ranitidine (a histamine receptor antagonist) and its metabolites in biological fluid (5) and the determination of Zn-Bacitracin (a topical antibiotic) in adulterated animal feed (6). The rough developmental chronology outlined here illustrates how familiarity with current analytical techniques and organic synthetic methods provides a knowledge base from which new PCD methods can be evolved. Table 1 outlines a series of examples demonstrating the diversity of PCD applications in pharmaceutical analysis.

**Replacement of the original 2-mercaptoethanol with another mercaptan, N,N-dimethyl-2-mercaptoethylamine, produces a more stable isoindole derivative without sacrificing fluorescence intensity (2).*

†The loss of fluorescence that occurs when multiple moieties of OPA add to a multiaminofunctional analyte, such as cysteine, is attributed to internal (concentration) quenching, whereby the folding of the adduct brings the fluorescent moieties into close proximity. There is also a case to be made that the hydrophobic environment around the conjugated fluorescent system, created by the folded conformation, is insufficiently polar to stabilize the highly charged excited state from which fluorescence occurs. The validity of this reasoning is supported by the fact that quenched fluorescence is restored by the addition of a surfactant to solutions of the cysteine derivative of OPA. The presence of the surface active agent shifts the energy balance in favor of an extended conformation in which the fluorescent moieties are no longer in close proximity and gain access to a polar aqueous environment that helps stabilize the transient charged species. Similar reasoning explains why derivatives formed by substituting naphthalene-2,3-dicarboxaldehyde for OPA are not quenched (3). Here, the fluorescent moiety is both rigid and large enough to sterically hinder folding of the adduct thereby preventing quenching effects from coming into play.

Conclusion

From the perspective of developing and deploying PCD methods, there is often no distinction between pharmaceuticals and other targeted analytes save that biological matrices can be somewhat more complex and target analytes may need to be measured at fairly low concentrations. However, it is precisely these considerations that make the sensitivity and selectivity enhancement available through PCD so desirable in pharmaceutical analysis. In addition, as an in-line procedure, PCD can be easily incorporated in end-to-end automated LC analytical systems with demonstrated capacity for delivering the kind of highly reliable high throughput analytical results required in pharmaceutical applications.

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Table 1 – Application of Organic Synthesis to the Post Column Determination of Pharmaceutical Analytes.

Therapeutic Application	Analytes of Interest	Detection Limits	Ref
Treatment of ulcers	Assay of nizatidine, ranitidine, and famotidine, in pharmaceutical preparations.	low ppm	7
Treatment of infection; a broad spectrum antimicrobial,	Detection and quantification of ciprofloxacin, and its known metabolites in urine, serum/plasma, bile, faeces and tissue	low ppb to low ppt	8
Treatment of symptoms for congestive heart failure.	Assay for digoxin in serum, in the presence of its metabolites and endogenous compounds such as steroids	high ppt to low ppb	9
Analgesic	Determination of morphine in plasma.	low to med ppb	10
NSAID--COX-2 inhibitor	Determination of rofecoxib (Vioxx) in plasma.	sub to med ppb	11
Antineoplastic used in treating certain forms of cancer, severe psoriasis, and adult rheumatoid arthritis.	Determination of methotrexate and metabolites 7-hydroxy-methotrexate and 2,4-diamino-N10-methylpteroic acid in biological fluids.	sub to low ppb	12
Phenothiazine tranquillizers.	Determination of demoxepam and other (fenergan, largactilevopromazine and nedaltran).	med to high ppt	13
Metastatic colon cancer drug.	Determination of oxiplatin in serum.	low ppm to med ppb	14
Diuretic	Determination of a synthetic octapeptide vasopressin antagonist (diuretic) in human plasma.	med ppt to ppb	15
Anticlotting drug for stroke prevention.	Quantification of a thrombin inhibitor in plasma and urine.	low ppb	16
Treatment of acute schizophrenia and other mental illnesses	Assay of cis-(Z)-clopenthixol (zuclopenthixol) in urine and plasma.	med ppt	17
Treatment of advanced breast cancer.	Determination of tamoxifen and its desmethylated and hydroxylated metabolites in human plasma.	med ppt	18
Treatment of anxiety.	Determination of oxazepam in urine.	med to low ppb	19
NSAID.	Determination of indomethacin in plasma and urine.	low ppb	20
Antimicrobial used in animal feed (pigs).	Determination of analysis of chlortetracycline.	med ppb	21
Cholesterol lowering.	Determination of pravastatin sodium	low ppt	22
Treatment of infertility.	Determination of clomiphene in plasma.	N/A	23
Oral antibiotic	Determination of ampicillin in plasma and urine.	N/A	24
A broad spectrum antibiotic for treating tuberculosis and MAC—an HIV+ opportunistic infection	Quantification of cycloserine and its prodrug acetylacetonylcycloserine in plasma and urine.	N/A	25
Antimicrobial for treating tuberculosis and HIV+ opportunistic infections.	Determination of ethambutol in serum.	low ppb	26
Anticholinergic for treating incntinence.	Determination of trospium and its metabolite in biological material.	low ppt	27