Review Article

SELECTION OF BUFFERS IN LC-MS/MS: AN OVERVIEW

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ABSTRACT

Liquid chromatography-mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LCMS is frequently used in drug development at many different stages including Peptide Mapping, Glycoprotein Mapping, Natural Products Dereplication, Bioaffinity Screening, In Vivo Drug Screening, Metabolic Stability Screening, Metabolite Identification as well as Impurity Identification. Selecting the proper buffer pH is necessary to reproducible separate ionisable compounds by reverse phase liquid chromatography or LC-MS. Selecting an improper pH for ionizable analytes often leads to asymmetric peaks that are broad, tail, split or shoulder. Sharp and symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, relative standard deviations (RSD) between injection, and reproducible retention times. In the present article, we have concentrated on the aspect when and why buffering is needed in LC-MS, how to choose buffer pH as well as criteria for selecting buffers for ion pair reagents. A properly buffered mobile phase is very important in a successful reverse phase method for optimal peak shape, detection limit, and consistent resistant times. The selection of proper buffer purely depends on analyte's functional group and pKa Values.

Keywords: LC-MS, buffers, relative standard deviations (RSD), reproducible retention times, ion-suppression.

INTRODUCTON

LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. LC-MS is very commonly used in pharmacokinetic studies of pharmaceuticals and is thus the most frequently used technique in the field of bioanalysis. The process is essentially a selection technique, but is in fact more complex. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression, the LC separation can be quite quick. It is common now to have analysis times of 1 minute or less by MS-MS detection, compared to over 10 mins with UV detection[1-3].

LC–MS represents one of the most important tools in the characterization of all organic, inorganic and biological compounds. Buffer solution is a solution which reduces the change of pH upon addition of small amounts of acid or base, or upon dilution.

Buffers in LC-Ms and Need of Buffering

One of the most undesirable processes that can occur during atmospheric pressure mass spectrometric analysis is a nonlinear decrease of ionization by sample or mobile phase. This *ion suppression*, or *ionization suppression*, is an effect whereby the extent of ionization for an analyte is decreased due to competition between analyte and sample matrix components within the atmospheric pressure ion source. Studies have shown ion suppression to be a somewhat proportional effect[4]. The importance of controlling mobile phase pH when analyzing ionizable compounds by reversed phase (RP). HPLC is often

recognized and easily understood, however it is often equally important to control pH when working with field samples of non-ionisable compounds due to the presence of ionisable impurities. Samples containing ionisable compounds are strongly influenced by pH of the mobile phase. In reversed-phase chromatography, mobile phase pH values are usually between 2 and 7.5. Buffers are needed when the analyte is ionisable under reversed phase conditions or the sample solution is outside this pH range. Analytes ionisable under reverse phase conditions often have amine or acid functional groups with pKa's between 1 and 11. A correctly chosen buffer pH will ensure that the ionisable function group is in a single form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring the pH of the injected solution to a less harmful pH.

Careful choice of an appropriate acid or buffer will help ensure success in the LC/MS experiment. Non-volatile aqueous components, whether salts, acids, bases, or buffers, will greatly decrease and even prevent the detection of analyte ions. These non-volatile buffers can also foul ion sources and vacuum regions of mass spectrometers. Non-volatile phosphate or citrate buffers are strongly discouraged for both ionization and practical reasons. Although many instrument manufacturers have developed ion sources rugged enough to tolerate deposition of non-volatile components, frequent cleaning is often necessary. Ion suppression and decreased sensitivity will also be observed when non-volatile buffers are used. In most cases, volatile acids or buffers can be substituted for more traditional HPLC methods. Ammonium acetate or formate buffers can be used with

concentrations ranging from 2 to 50 mM, although a maximum concentration of 10–20 mM is recommended to avoid ion suppression. The acceptable buffer concentration that does not adversely affect ionization has been observed to be highly compound dependent. A useful rule of thumb is to use as low a concentration of buffers as possible to give reasonable chromatographic performance[5].

Criteria for choosing Buffers and concept of volatile and non volatile buffers

Buffers should be chosen such that the pH of the mobile phase falls within the buffer's natural pKa range for maximum buffering capacity.

Buffer capacity outside of these ranges is limited, particularly at lower concentrations. N-methyl morpholine may be used at higher pH ranges and has shown utility in improving sensitivity and chromatographic peak shape for negative ion APCI analysis[6]. Ammonium adducts can be observed in positive ion mode and formate or acetate adducts in negative mode at higher buffer concentrations. Adduct formation is detrimental only in that it can cause greater variability and loss of detection sensitivity for the analyte. These adducts can sometimes be destroyed by increasing the source temperature, voltage, or both. Formic or acetic acid concentrations of 0.1–1% (v/v) are recommended when preparing low pH mobile phases to enhance ionization in electrospray. Trifluoroacetic acid is preferred for protein and peptide separations but should be avoided when negative ion mode is utilized. Ammonium hydroxide, or, in rare cases, triethylamine, are recommended for high pH mobile phases. For basic compounds, 0.1% acid should be mixed with the organic component, whereas water or neutral buffer should be used for neutral or acidic species. The previously discussed buffers can be used as the aqueous component to improve peak shape and resolutions by providing greater control of pH. A direct consequence of the reduced flow rate is the possibility to introduce a steady stream of a non-volatile buffer as a mobile-phase modifier. A micro-particle-beam interface before, and Cap-EI later, demonstrated that the combination of a reduced flow rate and electron ionization enhanced the ruggedness and flexibility to tolerate chemically "aggressive" mobile phases[7].

One of the most challenging applications in LC–MS is to enhance the tolerance toward the non-volatile buffers that are added to the mobile phase to improve chromatographic separation in several applications of biological and environmental interest. As a matter of fact, the presence of non-volatile, ionic species (phosphate and sulphate buffers) in the ESI spray is deleterious. One of the most critical factors in adapting LC methods is the choice of buffer. In volatile buffers interfere with good MS performance. For the best long-term performance, it is highly recommended that the method be modified to use a volatile buffer. Non-volatile species cause salt depositions on the metal surfaces, and could completely block ion transmission. In addition, if the anion and cation pair too strongly with the analyte, then the analyte ions might be prevented from carrying the excess charge on the droplet surface; as a result, the ESI response may be very low. For those reasons, when ESI is interfaced with HPLC, volatile buffers composed of weak acids and bases must replace non-volatile modifiers. Also, strong acids such as trifluoroacetic (TFA), heptafluorobutyric, and hydrochloric acid, which are used as ion-pairing agents, tend to mask the analyte signal in ESI-MS. Electron ionization, used in Direct-EI, is not influenced by the presence of preformed ions in the form of salts or strong acids and bases so that the analyte can be ionized independently. Because of the extremely reduced liquid intake, Direct-EI slowly displaces a negligible salt deposition within the ion chamber as the only sign of a salt presence. After many days of continuous operation, the salt deposition is barely visible in the ion source, and can be removed readily by routine maintenance procedures before any changes in the performance are observed.

The ion suppression/enhancement in LC/ESI/MS strongly depends also on the additives of the mobile phase employed. Additives and buffers in LC mobile phase are primarily employed to achieve reproducible retention time due to their buffering capacity and, secondly, to produce sharper peak shape if used as an ion pairing agent capable of creating neutral species with the target analyte[8].

Additives and Buffers in LC-MS/MS

In LC/ESI/MS the additives and buffer can serve to protonate basic molecules when operating in positive ion mode and vice versa for acidic molecules. Generally in LC/ESI//MS analysis, volatile buffers and additives (i.e. ammonium acetate, formate or hydroxide, formic or acetic acid or trifluoroacetic acid) are preferred to the non-volatile ones (i.e. phosphates, sulphates, borates and citrates). In fact the non-volatile eluent components can be deposited on the ion surface, leading to contamination of the electrodes inside the ion source. This contamination reduces the effective charge density at the electrode surface, emulating a lower voltage for the electrode: a reduction in the signal intensity will be observed over time. However, volatile buffer and additive must be used at a concentration as low as possible, consistently with chromatographic resolution. In fact buffer and additive species compete for surface site on the electrosprayed droplets and an increase in their concentration leads to decrease in the analyte signal: the competition for surface sites of the droplets favours the higher concentration species and, in this case, analyte species become depleted. This effect is compound-dependent: some analytes show only a small decrease of signal while for others the response can be decreased much more. An interesting study on the ion suppression/enhancement due to additives and the

buffer of mobile phase was carried out by Mallet[8] *et al.* APCI (Atmospheric pressure chemical ionization)-

LC–MS is widely used in pharmaceutical industries for performing low-matrix effect bio- determinations of drugs and metabolites in biological fluids. It is more "universal" than ESI (Electrospray ionization) for the analysis of not so polar compounds and can provide better sensitivity[9].

Guidelines for preparing mobile phases

Because slight variations in pH and acid concentration can have a dramatic impact on separation, consistent certain techniques should be employed when preparing mobile phases to ensure good reproducibility. As described in the literature[10], it is generally a good idea to measure an appropriate amount of pure water into a volumetric flask with an accurate amount of salt or acid. The pH of the mobile phase should be adjusted, if required, by adding reagent before diluting to final volume and prior to blending of any organic solvents. For example, blending 25% methanol will raise the apparent, measure pH of the combined mobile phase by about 0.5 pH units. Alternatively, equimolar solutions of different ionic forms of the same buffer (i.e. mono and dibasic phosphate) can be blended to reach the desired pH. When developing a rugged method, it is desirable to select a mobile phase with a final pH at least one pH unit away from any analyte's pK value to cause ionization or suppression of the analytes. There is often some guesswork in this because the effect of type and concentration of organic solvent on either mobile phase pH of solute pK values is not accurately known.

The use of pre-mixed mobile phase (pumping from a single reservoir) is essential to ensure accurate and reproducible mobile phase composition. However, it has become popular to prepare an aqueous buffer and program the instrument to blend organic solvent with aqueous buffer for gradient elution or fast isocratic method development. This practice can result in poor accuracy and incomplete mixing, depending on system maintenance and calibration, magnitude of dwell volume, flow rate and other factors. Isocratic methods that have been developed using instrument blending should be confirmed by premixed mobile phases, and gradient methods should be compared between more than one instrument when possible.

SUMMARY AND RECOMMENDATIONS

Controlling the separation of ionisable compounds can be difficult, and careful attention must be paid to all experimental details in order to accomplish a rugged method. Slight variations in mobile phase preparation can result in pH changes that can have dramatic effects on selectivity, capacity factor (retention factor), peak shape, resolution, and reproducibility. Optimum pH control will usually result in mobile phase containing buffer and acid compositions that will resist change when the sample is introduced and force ionisable analytes into predominantly one form (ionized or neutral) as they enter

the column. Good laboratory practice in preparing mobile phases should be followed to ensure that results can be reproduced within and between laboratories. While instrument solvent blending has become very convenient for fast method development, it is best to evaluate pre-mixed solvent whenever possible to ensure accuracy and equilibration before completing and publishing an HPLC method. This extra step can eliminate the possibility that instrument factors could make separation results difficult for others to reproduce. Mobile phase pH should be selected so that it is at least ± 1.5 pH units from the analyte's pKa. This assures that the analytes are either 100% ionized or 100% non-ionized and should help control run-run reproducibility. At high pH, acidic compounds are ionized and are much more hydrophilic than under ion suppression conditions. These conditions should be selected when fast analysis and low retention are desired. Bio Basic 18 is a good choice under high pH conditions.

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