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Factors to consider in the development of generic bioanalytical high-performance liquid chromatographic–mass spectrometric methods to support drug discovery

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Abstract

Mass spectrometric detection is gradually replacing ultraviolet (UV) as the method of choice in bioanalysis, especially in the area of early drug discovery where high sensitivity and rapid sample throughput is required and where samples are frequently pooled or ''cocktailed'' prior to dosing or analysis. The change from UV to MS detection requires a significant change in approach since the use of MS poses a number of unexpected problems and limitations which relate to instrument design and the ionisation process. Whilst electrospray ionisation (ESI) allows the analyst to focus on the analyte of interest it is non-selective and blind to background effects which can in certain instances alter the response of the compound of interest, leading to inaccurate data. In addition, when analysing compound mixtures, a number of precautions need to be taken since adduct formation in the MS source, the highly ESI responsive nature of formulating agents and the effect of the isotopic distribution in organic drug molecules can all lead to the production of compromised data. Whilst many of these problems can be minimised or avoided this often results in a complex and inflexible analysis system. Ultimately the analyst has to assess the degree of risk involved and take actions which reflect the use the data.

Keywords: Drugs; HPLC; ESI; Ion-suppression; Mobile phase; Isotopes

1. Introduction

Like most pharmaceutical companies we have been working to generate in vivo and in vitro ADME (absorption–distribution–metabolism–elimination) data earlier in the drug discovery process. To this end we have developed generic high-performance liquid chromatography–mass spectrometry (HPLC–MS) procedures to support the necessary bioanalytical work.

Our methods have been based around a standard reversed-phase (RP) HPLC method with gradient elution and MS detection using a single quadrupole instrument with electrospray ionisation (ESI) and single ion monitoring (SIM). We made this choice over what could be considered the more obvious MS–MS approach for a number of reasons. Firstly the sensitivity and selectivity afforded by single quadrupole MS was for many of our applications more than adequate. More importantly however, single MS instruments could be run successfully using a set of standard conditions without any pre-knowledge of the compound. All our analyses are carried out without the compound having been screened for MS response or the retention charac-

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teristics determined. The only information available is the compound's molecular mass. In contrast, triple quadrupole instruments require the conditions to be optimised for each compound and until very recently this necessitated considerable time consuming operator input.

For the in vivo studies, we have maximised sample throughput and minimised animal usage by employing cocktail dosing, this is also referred to by some workers as *n*-in-one or cassette dosing [1–4]. In our laboratory, typically five test compounds plus a quality control compound are dosed simultaneously to rats and serial blood samples taken.

In most companies the approach outlined above or something very similar will probably have been developed and put in place by bioanalysts/chromatographers. Many of these workers will have had limited or even no experience of MS, probably working in the main with UV detection [4]. Many of the aspects of HPLC–UV analysis which could be considered standard practice are inappropriate in HPLC–MS or need to be modified in some way. Failure to understand these differences can lead to the use of non-optimal methods or at worse the generation of misleading data.

The aim of this paper is to outline some of the constraints imposed by the use of single quadrupole MS instruments in the design of generic HPLC–ESI-MS methods. And to highlight some of the problems that can occur especially where compounds have been cocktail dosed or mixed prior to analysis.

2. Mobile phase considerations

ESI is a relatively non-discriminatory electrochemical process which favours analyte ionisation at low electrolyte (eluent buffer) concentration [5,6]. Optimal performance therefore requires the use of highly base deactivated columns which offer generic applicability, and exhibit good peak shape with weakly buffered eluents. In practice the eluent choice is severely restricted compared to the options available with UV detection. The organic modifier is either methanol or acetonitrile, and volatile buffers must be used to sustain high throughput analysis without source fouling thus minimising instrument downtime. Overall we have found methanol to be the best modifier because it offers a slight improvement in ESI efficiency [6] compared with acetonitrile and it gives better peak shape for basic compounds [7] which represent the largest proportion or drugs and drug candidates. We also favour buffers with near neutral pH (ammonium acetate, ammonium formate) in preference to trifluoroacetic acid (TFA) and formic acid for example, for three main reasons. Firstly the use of a neutral buffer benefits the retention of basic compounds where on-column preconcentration or on-line sample extraction is used. Secondly TFA has a marked suppressive effect on ESI especially in the negative mode but also in positive mode [6]. Finally the use of an eluent at either extreme of pH results in an uneven distribution of compound retention. This can be problematic since we are relying on the separating power of the HPLC to compensate for the relative lack of selectivity of single quadrupole detection.

Having selected the eluent components, their concentration is also critical. Too high a concentration results in suppression of the analyte signal (Fig. 1) whilst too low a concentration results in poor peak shape and efficiency for most basic analytes (Fig. 2) even when using base deactivated columns. This is in marked contrast to UV detection where the eluent buffer concentration would be optimised around the effects on peak shape alone. Obviously in HPLC–MS there has to be a compromise and we have routinely used 50 m*M* ammonium acetate in our eluents; although recent developments in base deactivated phases have al-

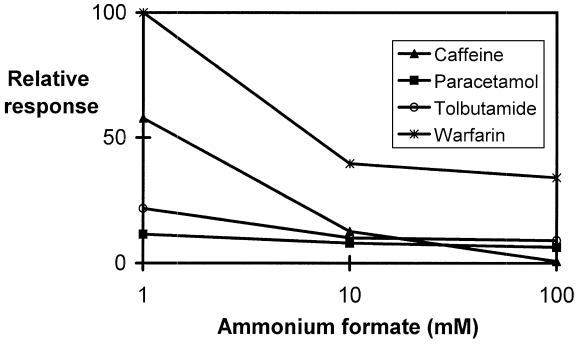

Fig. 1. Variation in relative ESI response for a range of compounds as a function of the eluent ammonium formate concentration. The eluent consisted of methanol–water (20:80).

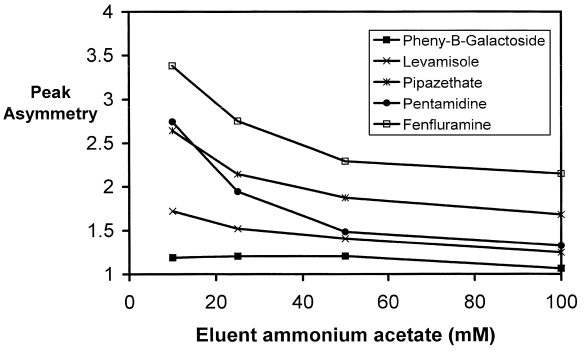

Fig. 2. Variation in peak asymmetry for a range of compounds as a function of the eluent ammonium acetate concentration on a Prodigy ODS3 column. The eluent consisted of methanol–water (35:65).

lowed us to drop this to 10 m*M* without compromising chromatographic performance.

3. Mass spectrometer hardware

Unlike UV detectors where the sample remains in solution and passes through a continuously flushed cell, MS detectors are easily fouled because the sample is desolvated and can form a solid deposit on critical source components. This results in a progressive loss in detection sensitivity which can only be recovered by dismantling and cleaning the contaminated components. Minimal sample must therefore be used for high-throughput applications where instrument down time is especially disruptive. MS detection is also flow-rate dependent and inlet flows must be restricted to achieve high sensitivity. For ESI-MS the flow requirements are particularly low and flows should ideally be kept to $\leq$50 μl/min for most instruments and applications.

These requirements are usually accommodated by reducing the column diameter and injection volume accordingly, or by using a conventional column and a post-column split. Unfortunately both methods have disadvantages that are attributable to poor instrument design.

Narrow bore columns (~1.0 mm I.D.), optimally working at flow-rates of $\leq$50 μl/min are ideally suited to the ESI process. Most of the major manufacturers however are still producing instruments with gradient delay volumes that exceed of 0.5 ml. When used with narrow bore columns this volume causes long delays at the start of the separation which often represents the major proportion of total analysis cycle time. For example when using a 1 mm I.D. column at a flow-rate of 50 μl/min the 0.5 ml delay volume in the pump would increase the analysis time by 10 min; hardly ideal where high throughput analysis and rapid turnaround of data is required. The use of a pre column split would allow the pump to be operated at a higher flow-rate (typically 1 ml/min) thus reducing the delay time whilst providing the appropriate flow-rate for the narrow bore column. This approach is obviously quite wasteful in terms of solvent consumption. In practice we have found 2 mm I.D. columns operated with a post-column split to offer a good compromise in terms of compatibility with both HPLC and MS instrumentation.

The modular design of bench top LC–MS instruments necessitates long transfer lines simply because of the proximity of the LC outlet and MS inlet. When divert valves are incorporated in the system the transfer lines can often extend to 1 m in length. The additional band broadening introduced by this tubing effectively degrades the resolution generated by the column which can be marked when narrower bore columns are used. Losses in resolution can also occur when conventional columns are used because the flow is split post-column to meet ESI requirements. These losses are often underestimated, and can be significant if the splitter is incorrectly positioned and the flow is proportioned by varying tubing length rather than internal diameter. For example, Fig. 3 shows the separation of a two-component mixture acquired on one of our standard LC–MS instruments (Hewlett-Packard 1050-Micromass Platform 2) using a 2.1 mm I.D. column, 0.3 ml/min flow-rate, 50°C column temperature, 0.01 in. (0.254 mm) transfer tubing, and a split ratio of 5:1. The peak resolution was measured with the splitter located in positions A and B as shown in Fig. 4. In each instance the flow split was calibrated by adjusting the length of the waste outlet and measuring outlet flow with the MS in operation. By forming the split in this way one is simply varying the linear flow velocity, consequently the solute bands move more slowly post split leading to increased solute

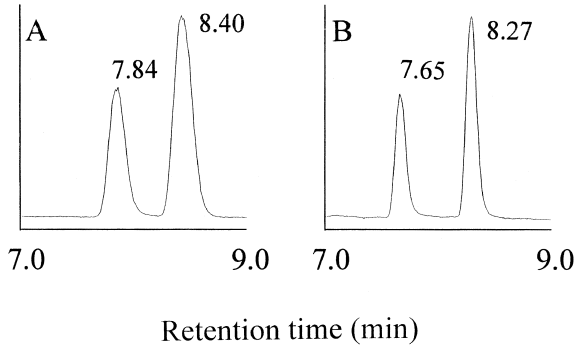

Fig. 3. Chromatograms showing the change in apparent peak efficiency and retention time for two components when the position of the splitter is nearer the switch valve (A) and when the splitter is nearer the MS probe (B).

band broadening as the splitter is distanced further away from the probe.

These effects can of course be eliminated if the split is affected by varying the internal diameter of the split tubing in a manner that generates the same linear flow velocity to the probe and waste outlet. However when we employed this approach using <0.01 in. tubing we observed a higher incidence of probe blockage for some biological applications.

In addition to the lengths of interconnecting tubing the nature of the tubing can also contribute to post-column peak broadening. Like most bioanalytical groups we prefer polyether ether ketone (PEEK) tubing to silica which can often give poor peak shape even when the compounds are not highly acidic or basic [8]. But even with this precaution we have still observed peak distortion or hold up which we attribute to flow path surface interactions. One

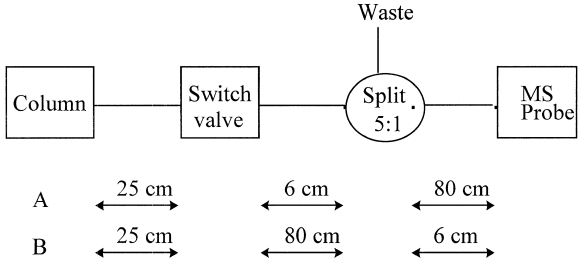

Fig. 4. A schematic of the HPLC–MS set-up showing two configurations where the relative positioning of the splitter with respect to the switch valve and MS probe is varied.

example was noted whilst carrying out simple experiments involving loop injections of caffeine and the β blocker atenolol. In this instance despite simultaneous injection of the two compounds atenolol ''eluted'' before caffeine. Caffeine also showed significant tailing whilst atenolol gave a symmetrical peak.

4. Analytical approach

In our approach we do not pre-screen compounds to check retention time, the preferred ionisation mode or MS response since this would add an unnecessary delay and further complicate our already complex procedures. Our high rate of success relies on the fact that we have an optimised generic gradient system which will successfully chromatograph a wide variety of compounds under one set of conditions. For every compounds assayed we monitor the $(M+H)^+$ and the $(M-H)^-$ ions and occasionally an adduct or the $(M+2H)^{2+}$. (In this terminology M represents the monoisotopic molecular mass of the species, made up of the elements with the lowest isotopic masses, rather than the commonly used average molecular mass). It is important to remember that polyfunctional bases especially where there is a significant distance between the basic centres will often doubly ionise leading to a very poor or a non-existent signal at the $(M+H)^+$ ion but a very strong $(M+2H)^{2+}$ signal which will appear at a mass of half the (monoisotopic mass+2).

When analysing a cocktail of six compounds for example it will be necessary to monitor the full chromatogram for at least, 12 and possibly 18 ions (if adducts are followed). Consequently, it is essential that the MS instrument has a fast scan speed with a short inter scan delay and rapid positive/negative switching so enough data points can be acquired for accurate definition and integration of the HPLC peaks.

Because we typically assay complex samples such as biological fluids a chromatogram for a given mass can contain a number of peaks and the largest may not always correspond to the compound of interest. To identify the analyte peak of interest we simply refer to the chromatograms obtained for our cali-

bration standards which are prepared in the same biological matrix, and look for the peak that changes regularly with concentration. However, not every compound in the mixture will be detected: either because it did not ionise, it formed an ion which we failed to monitor or it was in chemically or enzymically unstable. Occasionally chromatograms from calibration standards will show additional peaks which may actually be more responsive than the compound of interest, thus leading to confusion over which is actually the analyte peak. More often however the compounds may not be detected in test sample chromatograms from in vivo or in vitro experiments for a number of reasons. It is in these circumstances that problems arise and misassignment of a chromatographic peak can occur. These additional peaks may be due to metabolites, adducts, ions made up of heavier isotopes or dose vehicle components, all of which will be discussed in more detail below. Thus, or possibly in spite of the apparent simplicity of the analytical approach, it is necessary to take a number of precautions to prevent compounds from being misidentified especially in those samples where one of the test compounds is absent.

5. Mass conflict

5.1. Isotope effects

ESI spectra of organic compounds contain several ions around the molecular mass region. For example, the positive ESI spectrum for propranolol ($C_{16}H_{21}NO_{2,}$ monoisotopic molecular mass 259) displays a large pseudo molecular ion $(M+H)^+$ at mass 260 as expected. However it also shows ions for a series of heavier molecular species at masses 261, 262 and 263, these represent 18.6, 2.0 and 0.16% of the monoisotopic ion intensity, respectively. These additional ions are due to the presence of heavier isotopes in the molecule, mainly C^{13} in most simple drug compounds. Carbon^{13} is present to a small extent (~1.1%) in all organic molecules. The intensity of these additional ions increases with the total number of carbon atoms in the molecule. For a peptide with a molecular mass of 750 (typical of the upper limit for orally absorbed drugs) then the ion at mass 755, 4 mass units up from the pseudo molecular ion (751) would have an intensity of approximately 2.5% of the pseudo molecular ion. The presence of these ions made up of heavier isotopes effectively result in ''carryover'' where the signal for compound A could be mistaken for compound B. This is crucially important where multiple compounds are being assayed simultaneously and the mass difference between these compounds is relatively small. Thus, to avoid mistaking an isotope ion of one compound for the monoisotopic ion of another compound it is necessary to restrict the masses of compounds that are pooled or combined in one cocktail. Typically we would ensure that there was a mass difference of >4 Da between all compounds in a cocktail.

Despite this simple rule, there are certain instances where mass conflict can still occur and where additional care must be exercised. One such example is with drug compounds containing multiple chlorine atoms. Chlorine contains two major isotopes with masses 35 and 37 having relative distribution of 75 and 25%, respectively. In calculating the mass of chlorinated compounds for MS detection purposes it is important to use the mass of the most abundant isotope. The uneven but significant proportions of these two isotopes leads to great variation in the intensities of the ions resulting from multiply halogenated compounds. Table 1 shows the predicted ion intensities for propranolol with the addition of 1–4 chlorine atoms. The most intense ion for the compounds containing 0–3 chlorine atoms is the monoisotopic $(M+H)^+$ ion. However, with tetra-chloro substitution, the most intense ion is actually the $(M+H)^+ +2$ ion. It is also important to note the relative intensity of the ions up to 6 mass units removed from the $(M+H)^+$ ion which in the case of the tetra-chloro analogue is actually 11% of the most intense ion.

Few drug compounds contain high numbers of chlorine atoms although multiple substitution does crop up during the early drug discovery phase as medicinal chemists explore structure activity relationships. It is important to be aware of these instances since peaks in a chromatogram for a compound with a mass of 506 for example, could actually be due to the higher isotopes of a tetra-chloro compound with a mass of 500.

Table 1
The intensities of the major ions in the ESI spectrum of propranolol and the predicted intensities for its chloro analogues

	Relative intensities (%) for major ions						
	$(M+H)^+$ [a]	$(M+H)^+ +1$	$(M+H)^+ +2$	$(M+H)^+ +3$	$(M+H)^+ +4$	$(M+H)^+ +5$	$(M+H)^+ +6$
Propranolol[b]	100.00	18.57	2.03	0.16	0.14	<0.01	<0.01
+1 Chlorine	100.00	18.57	34.00	6.10	0.66	0.05	<0.01
+2 Chlorines	100.00	18.55	65.98	12.03	11.53	2.00	0.21
+3 Chlorines	100.00	18.54	97.95	17.95	32.63	5.84	3.90
+4 Chlorines	76.97	14.25	100.00	18.35	49.21	8.9	11.03

[a] Monoisotopic peak.
[b] Formula $C_{16}H_{21}NO_2$.

5.2. Metabolite interference

Virtually every compound dosed into a biological system whether in vivo or in vitro has some potential to be metabolised. So when working with cassette or cocktail dosing, it is also necessary to ensure that a metabolite of one compound is not misidentified as another compound in the same cocktail. For example an *N*-methyl aromatic amine with a molecular mass of 400 could be metabolised to the monohydroxy metabolite (416), the dihydroxy metabolite (432) or the *N*-desmethy metabolite (386), or combinations of these which in this case we will ignore. When setting up an assay, we would ensure therefore that this compound was not mixed with other compounds which had the molecular masses of 386, 416 or 432. The reason being that there is a finite chance that one of the metabolites of the *N*-methyl aromatic amine could co-elute with and hence be misidentified as one of the other compounds having the same mass. This would obviously lead to an overestimation in concentration for one of these compounds; or more seriously, this could also lead to false positive result if one of these compounds (molecular mass 386, 416 or 432) were not actually present in the test samples, either because it was not absorbed, was subject to extensive first pass metabolism or was insufficiently responsive to be detected in the assay system.

To fully guarantee the validity of such data the isotope ions of the possible metabolites would also need to be considered. However we would consider this to represent a very small and acceptable risk.

5.3. Adducts

A further problem we have encountered on occasions relates to the formation of adducts in the mass spectrometer. The formation of adducts is obviously condition and compound dependent and tends to be less favoured where the source temperature, cone voltage etc. are set high. However, they are occasionally formed and without appropriate precautions they can easily be mistaken for another compound in the mixture.

Many of the likely adduct forming species are well known, such as ammonium giving an $(M+18)^+$ adduct where the ammonium has obviously come from the ammonium acetate in the HPLC eluent. On occasions we have actually found these adducts to be more intense or giving cleaner chromatograms than the $(M+H)^+$ ions and these have been used in preference for quantification. In certain instances other adducts are formed preferentially over $(M+H)^+$, these include: sodium, $(M+23)^+$; potassium, $(M+39)^+$ and acetate, $(M+59)^-$. In the first two of these, the sodium and potassium, probably result from inorganic materials extracted from the plasma samples. So the nature of the sample itself effects the ionisation in the mass spectrometer. The last of these, M+acetate is again an adduct with an eluent component.

If all the likely adducts were known, and many of them are, then it should be possible to avoid their misidentification by ensuring that compounds are not mixed in cocktails where their masses differ by the mass of the added component. However taken with the ''rules'' outlined above, i.e., no M+16, M−14, M+32, M+4, etc., this would probably preclude the mixing of any compounds to make up a cocktail! This is further aggravated by the fact that that chemists working in a particular therapeutic area will typically make compounds which are simple analogues of one another. Thus they will be producing compounds differing by a methyl (molecular mass

14) or a hydroxyl (molecular mass 16) group, the same mass difference as that between the parent ion and the adduct or metabolite.

The strategy we have adopted to cope with this real problem is to examine and compare both the chromatographic data and the final derived data (e.g., a plasma drug profile) for each compound in a given cocktail. If standards for any two compounds show the same or very similar retention times – we typically employ a retention window of 0.05 min – and they have same peak shapes i.e., the peaks overly, they then potentially one of the peaks could actually be due to an adduct of the other. Unlike metabolites, the adduct is formed post HPLC column so that although it has a different mass to the original compound, it will have the same retention time.

The next stage of the assessment involves consideration of the sample concentration data. If the either of the compounds are not present in the test samples, then the situation can be ignored. If both compounds appear to be present in the test samples and give very similar plasma profiles in terms of shape and absolute concentration, then it is likely that the compound detected with the higher mass is actually an adduct of the one with the lower mass. Thus even though the intensity of the adduct and the $(M+H)^+$ ions may be vastly different they will still produce concentration data that are the same or very similar. We have been able to automatically build such an assessment into our data processing, thus allowing potential adduct problems to be detected whilst not limiting the compounds that can be mixed in a given cocktail.

In selecting compounds to be mixed for analysis we would typically employ the mass rules described above; i.e., all difference >4 Da, and no compound differing by 14, 16, 18, 32 Da, etc. If other metabolic transformations are expected then the appropriate mass difference exclusion rules can be applied. It is a relatively simple matter to produce Visual Basic code within Excel, for example, which will select compounds from a worklist and combine them for dosing or analysis according to a set of predefined rules.

5.4. Dose vehicles

The use of MS detection puts significant constraints on the use of certain dose vehicles, generally those that are polydisperse in nature such as polyethylene glycol 400 (PEG400) and some of the oil-based vehicles, e.g., Cremophor EL.

Virtually all commercial samples of PEG400, which is a widely used dose vehicle with good solvent properties and relatively low toxicity, contain a range of oligomers. Fig. 5 shows a HPLC–MS chromatogram of PEG400 obtained under full scan conditions, it can be clearly seen that it contains several related oligomers. These compounds are non-absorbing (and do not interfere) in the UV but give a very good MS response in both positive and negative ESI. In positive, as well as giving the pseudo molecular ion, $(M+H)^+$, a number of adducts are also observed including $(M+\text{ammonium})^+$ and $(M+\text{sodium})^+$. When used as a dose vehicle there is obviously potential for misidentifying one of these dose vehicle components as a compound of interest or possibly one of these components suppressing the ionisation of a compound of interest.

In one example in our laboratory we were provided with a series of plasma samples from an orally dosed in vivo experiment where unbeknown to the analyst the compound (X) had been administered in a Cremophore EL–dimethyl sulfoxide (DMSO) mixture. In this instance there was a desire to detect not only the parent $(M+H)^+$ ion at mass 458 but also the potential hydroxylated metabolite (M+16), a desmethylated and didesmethylated metabolite (M−14, M−28) and a desmethyl ether glucuronide (M+

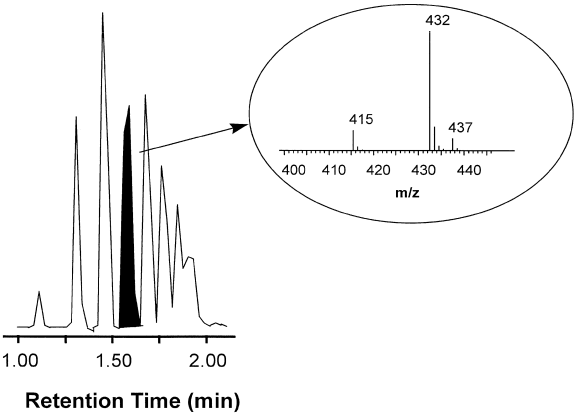

Fig. 5. An HPLC–MS chromatogram for PEG400 showing a cluster of nine peaks associated with the different oligomers. The insert shows the mass spectrum for oligomer with the molecular mass 414. PEGs readily form adducts and a number of ions are observed due to the M+H (415), M+ammonium (432) and M+sodium (437).

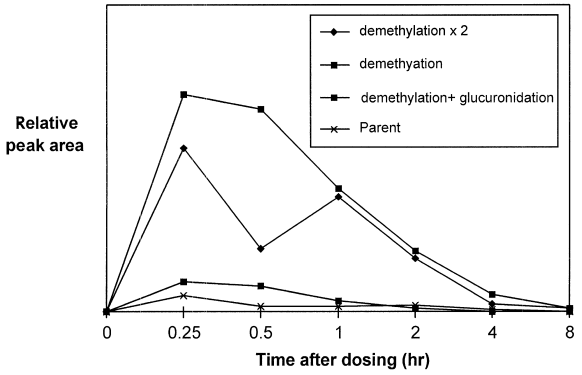

Fig. 6. Relative plasma concentration–time profiles for compound X (molecular mass 458) and three putative metabolites with masses of 444 (loss of methyl), 430 (loss of two methyls) and 620 loss of methyl plus addition of glucuronic acid.

162) metabolite. The analysis showed the presence of the parent compound as well as a large number of ions which corresponded to some of the predicted metabolites. Assuming a response similar to the parent then these putative metabolites were present at realistic concentrations making the results all the more convincing, Fig. 6. Follow up work however, using MS–MS, indicated that these peaks/ions were actually due to components of the dose vehicle (Cremophore EL): polyethoxylated castor oil which is actually a complex and variable mixture containing a wide range of components. Not only do these components respond well under ESI conditions but many of them also have the potential to form adducts $(M+Na)^+$, etc., some of which obviously had the same mass as the expected metabolites. It is important therefore that the analyst has a knowledge of and preferably some control over the design of the live phase of any in vivo experiment to avoid the generation of meaningless data when MS is used.

6. Ion suppression

MS detection with SIM and UV detection differ in one very fundamental fact which makes mass spectrometry a potentially error prone procedure if certain precautions are not taken.

With UV detection for example the selection of an appropriate wavelength such as 210 nm or a combination of two or three different wavelengths makes it possible to detect virtually all compounds, including endogenous components eluting from the HPLC column. Furthermore, if two compounds co-elute then the combined response from these co-eluting materials should represent the sum of their individual responses. This latter point only applies if the linear range of the detector is not exceeded, which in trace analysis, is usually the case.

By contrast in MS detection with SIM the very selectivity of the detector means that you only see what you want to see and potentially you miss an awful lot! More importantly however, in contrast again with UV detection, what you do not see could actually be affecting what you do see in an additive (or subtractive manner). With MS detection, the response of two co-eluting compounds may not be the sum of their individual responses. This latter point comes about since in an electrospray source, there is competition between compounds for the charge and a limit to how much material can be ionised at any one time. Where two compounds co-elute in relatively high concentration and compound A can be ionised more easily than compound B, then the signal from compound B may actually be suppressed and its concentration underestimated [9–11].

This signal suppression can obviously happen where the two components are both of interest, i.e., two test compounds [11], or where one of the components is an endogenous compound [9,10] or a metabolite of another compound. To assess the magnitude of this problem we simulated the first condition by injecting two compounds (minoxidil and clenbuterol) which we knew to co-elute, both as single compounds and as mixtures. We then examined the intensity of the response for a fixed amount of clenbuterol as the concentration of minoxidil was gradually increased. The results of this experiment are shown in Fig. 7.

It can be seen that the relative response for clenbuterol alone (the even numbered injections, plus injection 11), is relatively constant at approximately 100%. However as minoxidil is introduced in increasing amount (injections 1, 3, 5, 7 and 9) there is obviously competition for ionisation and in this case minoxidil wins out and the signal for clenbuterol is reduced by up to 30% at the extreme (injection 9).

Unless you know what is eluting from the HPLC

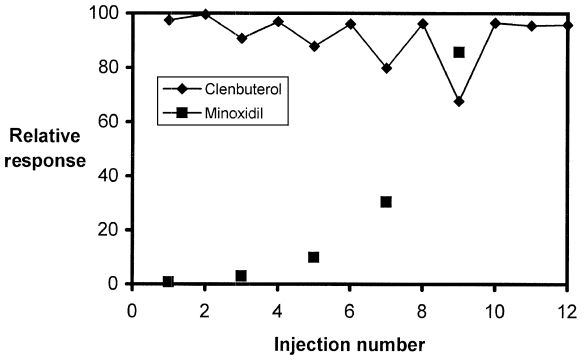

Fig. 7. Relative response for a series of injections of clenbuterol with and without the injection of co-eluting minoxidil. Clenbuterol was injected at a concentration equivalent to 200 ng/ml of plasma and the minoxidil concentrations were varied from 15 ng/ml (injection 1) to 4000 ng/ml (injection 9). Injections 2, 4, 6, 8, and 10–12 were controls which contained clenbuterol alone.

column then it is possible that ion suppression could be occurring throughout the chromatographic run. The more complex the analysis in terms of the nature of the samples, the number of compounds assayed, the simpler the sample clean up and the shorter the chromatographic run time [10,11] then the greater the potential for ion-suppression and the production of inaccurate data. In some circumstances such as in vivo pharmacokinetic analysis for example, the reduction of one concentration point on a plasma profile by 20–30% will probably have little overall effect on the result. However, in permeability experiments or in vitro metabolism experiments, where the conclusion may depend on a single analysis then reduction of response by 20–30% could have a major effect on the experimental outcome and result in some compounds being mis-classified.

7. Linearity of response

ESI-MS has a relatively narrow linear dynamic range: at best it is possible to achieve 3–4-orders of magnitude when operating at low concentration using an optimised compound specific method. Above a certain point a critical ionisation concentration is exceeded and ESI response levels out. This situation is often aggravated when generic conditions are used. For example, in one of our assays we use a fixed set of calibration standards with the average concentration in a chromatographic peak as it enters the MS system of 400 pg/μl (in a 50 m*M* ammonium acetate); even at these relatively low analyte concentrations we observe calibration plots which vary from compound to compound ranging from straight lines to markedly curved plots which require second-order fitting. So, whereas a single calibration point can be used with confidence within the broad linear range of a UV detector and data can be reported in an automated fashion using a standard linear fit; the compound dependence of ESI-MS response requires that several calibration levels must be used and a range of non-linear curve fitting routines investigated to ensure the response curves are accurately defined. Both of these features are clearly major drawbacks for high-throughput applications where fast data analysis and interpretation are paramount. Typically we have found that three standards (including zero and a mid point) are satisfactory when using quadratic fitting.

8. Conclusions

Based on a number of years experience in generating in vivo and in vitro data we believe that a simple approach based on a generic HPLC system with single MS detection using SIM is a highly efficient and effective means of supporting drug discovery. The approach can be applied to a wide range of compound types with a very high rate of success, typically >95%. Apart from the molecular mass of the compounds, no additional information (HPLC retention or MS response data) is required. However, the approach is not fool proof and a full understanding of MS and HPLC fundamentals are required and in particular how these differ from HPLC–UV analysis. Often these differences are counter intuitive. For example the very selectivity of MS detection means that it is more susceptible to interference than UV detection since with UV detection you actually see everything that is eluting from the column.

With an appropriate level of error traps it is possible to guard against many of the potential problems discussed here. How much effort is expended in guaranteeing the quality of the data

depends very much on the analyst, the assays and the use to which the data is being put. In the final analysis it is all about risk management.

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