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Evaluation of the use of LC-MS in supporting stability studies for preclinical study formulations

Emma L. Simmonds [a], W. John Lough [b], Martin R. Gray [a,*]

[a] *Analytical Sciences Department, Sanofi-Synthélabo, Willowburn Avenue, Alnwick, Northumberland NE66 2JH, UK*
[b] *Institute of Pharmacy, Chemistry and Biomedical Sciences, University of Sunderland, Wharncliffe Street, Sunderland SR2 3SD, UK*

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Abstract

Assessing the short-term stability of drug development candidates in one or more formulations to be used during their preclinical evaluation is a routine, but important, task. Typically, this is based on data generated by HPLC with ultraviolet detection of the species of interest and using methodology specifically developed and validated for the purpose. This work describes a feasibility study conducted into the use of HPLC with mass spectroscopic detection for work of this type. Experimental details and the results of trials with three different drugs, each in a different vehicle, are given. It was concluded that, by using mass spectroscopic detection, a well-defined strategy could be used to generate stability data, which offered advantages in terms of specificity, speed and sensitivity over that typically used.

Keywords: Liquid chromatography-mass spectrometry; GLP; Stability studies; Stable isotope labelled internal standards; Preclinical study formulations

1. Introduction

Prior to the initiation of any toxicology study for which Good Laboratory Practice (GLP) compliance [1] is claimed, it is a fundamental requirement that the stability of the test article in the formulation of choice be known. However, this assessment is often performed early in the drug development process, before the stability indicating nature of available assay and impurity methodology, which are generally based on the use of High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection, has been fully established. Additionally, the test article concentration range under evaluation typically extends down to 0.1 mg ml^{-1}, where, depending on the chromophore of the analyte, the determination of degradation products, may not be readily achievable with confidence. Finally, the test article may be presented in a range of different matrices [2], some of which contain ultraviolet absorbing species, leading to further analytical challenges if HPLC-UV based methodology is to be used.

Given the inherent specificity and sensitivity of Liquid Chromatography-Mass Spectrometry (LC-

* Corresponding author. Tel.: +44-1665-60-8556; fax: +44-1665-60-8539.
E-mail address: martin.gray@sanofi-synthelabo.com (M.R. Gray).

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MS), advantages that are routinely exploited for the analysis of biological samples [3–6], it was considered likely that it would prove an attractive alternative to HPLC-UV for the stability assessment of formulations to be used in toxicological investigations. In order to test the feasibility of using LC-MS in this regard, a series of experiments have been performed. In particular it was hoped to assess the extent to which the use of LC-MS would form the basis of a well-defined approach to the analysis of preclinical study formulations, which required the minimum of method development. The adoption of such an approach has been successfully exploited in the bioanalytical field [7–9].

2. Experimental

2.1. Reagents and chemicals

SR121787, SR147778 and SR142801 (Fig. 1) and their stable isotope labelled (SIL) versions ([$^{2}H_4$]-SR121787, [$^{15}N_2$, $^{2}H_6$]-SR147778 and [$^{13}C_6$]-SR142801), which all contained $>99\%$ labelled compound, were supplied by Sanofi–Synthelabo Ltd. HPLC grade acetonitrile was purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK). Purified water was produced in-house by distilling and double deionising the local water supply. Ammonium acetate and formic acid were obtained from Sigma-Aldrich Co. Ltd (Poole, Dorset, UK). Labrasol® was obtained from Alfa Chemicals Gattefosse (Saint-Priest, Cedex, France). Methyl cellulose was purchased from Stancourt Sons & Muir Ltd (Tunbridge Wells, Kent, UK). Hydrochloric acid (1 M) and sodium chloride were purchased from BDH Laboratory Supplies (Poole, Dorset, UK).

2.2. Equipment

HPLC-UV-MS analysis was performed using a Micromass ZMD single quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) coupled to an HP1100 series HPLC system (Agilent Technologies UK Limited, Stockport, UK). The HPLC columns used were a Prodigy ODS (3) (5 μm particle size, 150 × 4.6 mm) purchased from Phenomenex (Macclesfield, Cheshire, UK) for SR121787 analysis and a Waters XTerra™ MS C8 (3.5 μm particle size, 50 × 4.6 mm) purchased form Waters Corporation (Watford, Hertfordshire, UK), for SR147778 and SR142801 analysis. Solvent optimisation was performed using DryLab® chromatography solvent optimisation software, Version 2.05 (LC Resources, Walnut Creek, CA, USA).

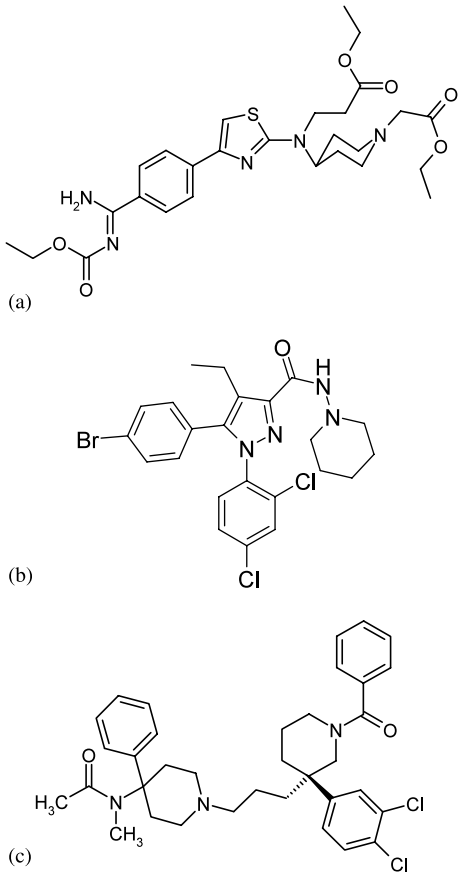

Fig. 1. Structures of SR121787, SR147778 and SR142801. (a) SR121787, (b) SR147778, (c) SR142801.

2.3. Sample preparation and analysis

In each case either previously available chromatographic conditions were adapted for the purposes of this study, or a well defined approach taken to the development of suitable methodology.

In the latter case the HPLC column type and mobile phase components were fixed with appropriate isocratic (for quantification) and gradient (for impurity determination) conditions being developed, with the aid of DryLab®

Samples of each drug candidate in the relevant matrix were prepared and stored as follows:

SR121787 (0.1 mg ml^{-1}) was dissolved in acidified 0.9% saline solution (pH 1.8) and aliquots stored at 25 and 2–8 °C (refrigerated).SR147778 (0.1 mg ml^{-1}) was dissolved in Labrasol®, a bioavailability enhancer which is a well defined mixture of mono-, di- and triglycerides and mono and di-fatty acid esters of polyethylene glycol (PEG) produced from coconut oil and PEG 400. An aliquot of this formulation was stored at ambient laboratory temperature and a second at 60 °C, having first been acidified to a final concentration of 0.1 M hydrochloric acid.SR142801 (1 mg ml^{-1}) was suspended in 0.6% methyl cellulose. One sample was stored at ambient laboratory temperature and a second at 80 °C, having been first acidified to a final concentration of 0.1 M hydrochloric acid.In each case at least one of the storage conditions was designed to cause the relevant drug candidate to significantly degrade over the 7–8 day study period.

Prior to quantification each candidate was diluted with either acetonitrile–water (86:14, v/v) (SR121787) or mobile phase to give a 4 μg ml^{-1} solution, which was then, further diluted 1:1 with the appropriate SIL internal standard to give a final concentration of 2 μg ml^{-1} of each compound. Sample results were determined with respect to a calibration line, established by injecting standards containing 1, 2 or 3 μg ml^{-1} of the analyte and 2 μg ml^{-1} of the relevant SIL.

For the determination of impurities each sample solution was either injected neat (SR121787) or following a 1:1 dilution with mobile phase.

2.4. Chromatographic and mass spectrometric conditions for quantification

Each drug was quantified using the isocratic LC-MS methods described below:

For SR121787 a mobile phase of acetonitrile–10 mM ammonium acetate (pH 6) (90:10, v/v), at a flow rate of 1 ml min^{-1}, split 3:1 in favour of waste prior to the MS, was used. For the remaining two drugs a mobile phase of acetonitrile–water, containing 20 mM formic acid (58:42, v/v for SR147778 and 38:62, v/v for SR142801) at a flow rate of 2 ml min^{-1}, split 20:1 in favour of waste prior to the MS, was used. The column was held at 40 °C in all cases. An injection volume of 10 μl was used for SR121787 and 20 μl for SR147778 and SR142801. Under these conditions the following, approximate, retention times were obtained; 2 min for SR121787 and SR147778 and 1 min for SR142801.

In all cases the mass spectrometer was operated in electrospray mode with positive ion detection. The nitrogen gas flow was 500 l h^{-1} for SR121787 and 400 l h^{-1} for SR147778 and SR142801. Following infusion of drug substance solution into the source a cone voltage of 35 V was found to be suitable in each case. The capillary voltage was 3.5 kV, the source block temperature 120 °C, the desolvation temperature 350 °C and the dwell time 0.05 s. Using the values given in Table 1, single ion monitoring (SIM) data were collected for the respective analytes and SIL's. For SR121787 and its corresponding SIL the $[M+Na]^+$ ions, produced as a consequence of the matrix sodium content, were followed. In the case of SR147778 the most abundant SIL ion ($m/z = 531$) was subject to slight interference from the matrix and accordingly the ion at $m/z = 533$ was used.

2.5. Chromatographic and mass spectrometric conditions for purity assessments

A gradient analysis was performed for each compound to ensure that, as far as possible, any peaks of interest would be eluted. In each case the study samples, the batch of drug substance used to prepare each formulation, at a matching concentration, and a blank solution of the relevant matrix were analysed. The HPLC columns were as described above and were held at 40 °C. Other operating conditions were as follows.

Table 1
Table of ions monitored during stability studies

Compound name	*m/z* Value monitored	Ion monitored	Retention time (min)
SR121787	582	$[M+Na]^+$	2.2
$[^2H_4]$-SR121787	586	$[M+Na]^+$	2.2
SR147778	523	$[M+H]^+$	2.0
$[^{15}N_2,^2H_6]$-SR147778	533	$[M+H]^+$	2.0
SR142801	606	$[M+H]^+$	1.0
$[^{13}C_6]$-SR142801	612	$[M+H]^+$	1.0

For SR121787 a linear gradient was run from 100% A to 100% B (A = 10 mM aqueous ammonium acetate (pH 6.0)–acetonitrile (90:10, v/v), B = 10 mM ammonium acetate (pH 6.0)–acetonitrile (10:90, v/v)) over 30 min, then the solvent composition was held at 100% B for 5 min. The flow rate was 1 ml min^{-1}, with a split of 3:1 in favour of waste prior to the mass spectrometer. An injection volume of 50 μl was used.

For SR147778 a linear gradient was run from 20% B to 55% B over 25 min then 55% B to 100% B over 10 min, then held at 100% B for 5 min (A = acetonitrile–water (10:90, v/v) containing 20 mM formic acid) and (B = acetonitrile–water (90:10 v/v) containing 20 mM formic acid). An injection volume of 20 μl was used.

For SR142801 (with A and B as for SR147778) a linear gradient was run from 100% A to 100% B over 60 min and then the solvent composition held at 100% B for 5 min. In both cases the flow rate was 2 ml min^{-1}, with the flow split 20:1 in favour of waste prior to the mass spectrometer. An injection volume of 10 μl was used.

Under these conditions the approximate retention times were 22 min for SR121787, 19 min for SR147778, and 17.5 min for SR142801.

The mass spectrometer was operated in electrospray mode, with positive ion detection and full scan data from 100 to 1000 Da captured with a scan time of 1.00 s. Different cone voltages were used to ensure that in-source fragmentation could be observed for any significant degradation products (SR121787: 25, 35 and 45 V; SR147778: 35 and 65 V; and SR142801: 25, 35 and 65 V). The capillary voltage was 3.5 kV, the source block temperature was 120 °C and the desolvation temperature 350 °C. The nitrogen gas flow was 500 l h^{-1} for SR121787 and 400 l h^{-1} for SR147778 and SR142801.

2.6. Method validation

Prior to commencing stability assessments, each quantification method was validated in terms of its linearity and accuracy. In the former case the intent was to demonstrate that a linear response extending well beyond the range of interest (80–120% of the nominal drug concentration of 4.0 μg ml^{-1} prior to dilution with the internal standard) was achieved and not to determine the method's lower and upper limits of quantitation. Similarly, accuracy data were only generated within the range of interest, since results, which fell outside this range, would automatically indicate the formulation's unsuitability. These validation procedures are consistent with those published by the International Conference on Harmonisation [10].

2.6.1. Linearity

The linearity of response (drug peak area/internal standard peak area) versus drug concentration was studied over the range 0.4–8 μg ml^{-1} (prior to dilution with the internal standard) for all three compounds. This concentration range corresponded to approximately 10–200% of the nominal analyte concentration in the solution injected. In each case a correlation coefficient of >0.999 and an intercept within $\pm 5\%$ of the 100% value were obtained.

2.6.2. Accuracy

Accuracy was determined as follows; a known amount of drug (0 (blank) 80, 100 or 120% of

nominal) was added to the relevant matrix. Triplicate samples were prepared at each level and taken through the analytical procedure being validated. In each case the mean result was within 98–102% of theoretical and the coefficient of variation of the accuracy results was less than 3%. The blank sample chromatograms shown in Fig. 2 demonstrate that the residual unlabelled material in the SIL continued to be present in trace levels (the highest level detected being 0.1% w/w) and that there were no significant interfering species.

3. Results and discussion

The work involved assessing the stability of three basic drug candidates, each in one of three commonly used preclinical study vehicles; acidified 0.9% saline solution (to be administered intravenously), Labrasol® or methyl cellulose (both to be administered orally). In each case the formulation was stored under relatively mild and relatively stressful conditions, which, in the latter case, were used to demonstrate that sample degradation would be detected using the proposed analytical protocol. The methodology for the analysis of SR121787 was developed from an existing MS compatible method. However, after this a well defined approach to the development of suitable chromatographic methods was used for the other compounds reported here and subsequent work. This comprised three main steps. The first was prediction, via DryLab®, of appropriate mobile phase ratios for the isocratic and gradient methods to be used. This involved using the software to analyse data sets from preliminary gradient trials and subsequent verification of the predicted conditions by experiment. In this manner, it was found that a single HPLC column type (XTerra, 50 mm × 4.6 mm) operated with the same mobile phase constituents (acetonitrile and water both containing 20 mM formic acid) could be used to analyse all the compounds of interest.

The second step concerned application of the rapid isocratic method developed in the first stage to the samples of interest. At suitable intervals the drug content of each formulation was determined by means of the appropriate, internally standardised, single ion response (SIR). In all cases the relevant SIL was used as internal standard as their close structural relationship to the analyte assures that they will be ionised by the same mechanism and will co-elute under the chromatographic conditions of choice.

The final step, conducted at the end of the required sample storage period, involved application of the gradient methodology referred to above. The resultant MS and UV response profiles for each sample of interest, the sample matrix and the drug used to prepare each formulation were used to determine whether or not any degradation products had been formed and if so to tentatively identify them. Where degradants are observed the corresponding UV peak response can be used to estimate their levels relative to the parent compound.

The quantitative and qualitative data obtained for each drug candidate are given in Table 2. In all cases drug degradation was achieved for at least one of the storage conditions used, demonstrating the feasibility of using the approach outlined above to determine the stability of preclinical study formulations rapidly and with confidence. In particular this approach brings advantages in terms of specificity, speed and sensitivity with respect to work for which a non-specific detection system is used each of which is discussed in more detail below.

3.1. Specificity

The strategy described employs fast, isocratic HPLC-MS methods for the determination of sample potency, relying on the detector, rather than chromatographic resolution, to provide their specificity. Whereas co-eluting species are a constant threat to the integrity of HPLC methods that employ UV detection, isobaric species would clearly pose a similar threat to these methods. To encounter such species as degradation products is quite rare but there are exceptions such as cis–trans isomerisation, and, for this reason, it was considered necessary to supplement the quantitative data obtained with qualitative data resulting from application of the appropriate gradient

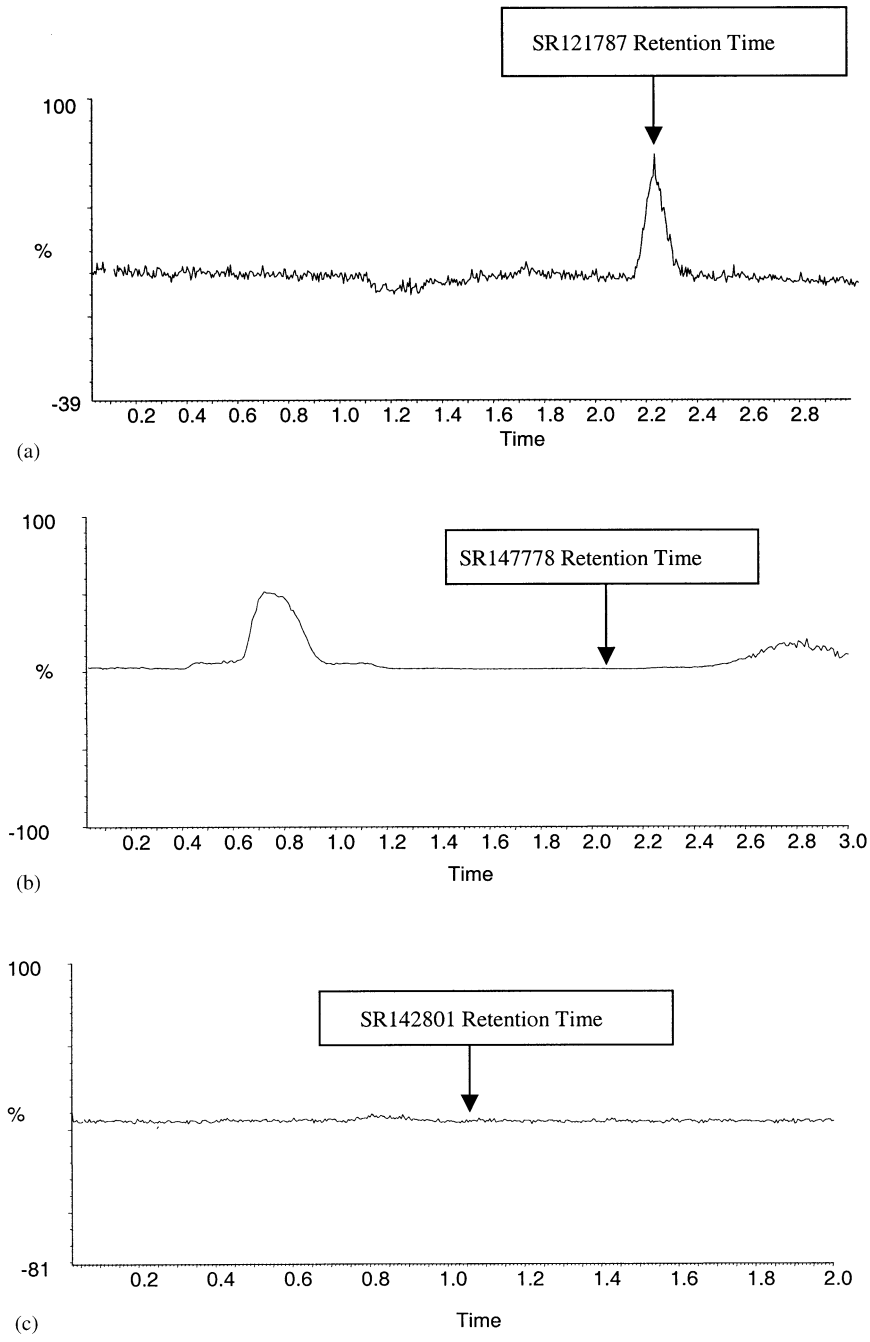

Fig. 2. Blank chromatograms from the accuracy determination for (a) SR121787, (b) SR147778 and (c) SR142801.

methodology. Similarly, it was felt that reliance on qualitative data alone would not provide sufficient assurance regarding product stability. Specifically this would not provide any information on species that were fully retained under the analytical conditions of choice, or where drug was being

Table 2
Stability study results

Drug/matrix	Storage	% of nominal (% relative standard deviation) measured by LC-MS				Impurities found during gradient analysis (following interrogation of the TIC data)
		0 days	2 days	3 days	8 days	
SR121787 in acidified saline	2-8 °C	99.9 (2.3)	90.4 (3.5)	N/A	74.1 (1.1)	After 8 days five new impurities were observed
SR121787 in acidified saline	25 °C	99.9 (2.3)	50.5 (3.0)	N/A	8.3 (2.0)	After 8 days five new impurities were observed
SR147778 in Labrasol®	Ambient	98.9 (2.7)	N/A	97.2 (0.9)	98.1 (2.1)	After 8 days no new impurities were observed
SR147778 in Labrasol®	Acidified	99.1 (1.8)	N/A	89.3 (1.3)	42.2 (2.7)	After 8 days two new impurity peaks were observed
SR142801 in methyl cellulose	Ambient	102.4 (2.4)	100.7 (2.0)	N/A	103.1 (3.3)	After 8 days no new impurities were observed
SR142801 in methyl cellulose	Acidified	96.2 (2.0)	48.6 (1.4)	N/A	14.2 (4.4)	After 8 days three new impurity peaks were observed

lost to mechanisms other than degradation, for example, adsorption to the container walls.

Notwithstanding the above, the results for SR121787 demonstrate that, even when greater than 90% of the drug has been lost through degradation, the remainder could be readily quantified in the presence of co-eluting species. By contrast the apparent SR121787 content of the acidified saline formulation stored for 7 days at 25 °C determined using ultraviolet (270 nm) detection was 50% of the initial value compared with 8.3% by LC-MS. This adequately demonstrates the potential for co-eluting degradants with similar UV spectra to that of the parent compound to adversely affect the data obtained. When this sample was analysed using the gradient method, the main degradant, which elutes, in these conditions, just in front of the SR121787 peak, could be clearly seen (Fig. 3).

As well as proving specific in the presence of co-eluting degradation products, the mass detector can also be used to remove or reduce matrix interference. This was evident for those samples formulated in Labrasol® where this would have made HPLC-UV analysis impractical (Fig. 4).

3.2. Speed

The ability to use rapid, isocratic methods with analyte retention times of less than 3 min, without the need to consider the consequences of co-eluting degradation products, considerably reduces the time required for each analysis, generally reducing it by at least a factor of two. Where this approach is augmented by a well defined strategy to development of the relevant methodology prior to the stability assessment itself, the savings in time and applied resource can be significant. For the work described, mobile phase combinations of acetonitrile, water and formic acid were found to be suitable in all cases, however, subsequent work has suggested that the use of ammonium formate buffer rather than formic acid may be advantageous. Additionally, for drugs that are acidic, the use of mobile phase pH's in excess of seven, for which the XTerra column is well suited, together with operation of the detector in the negative

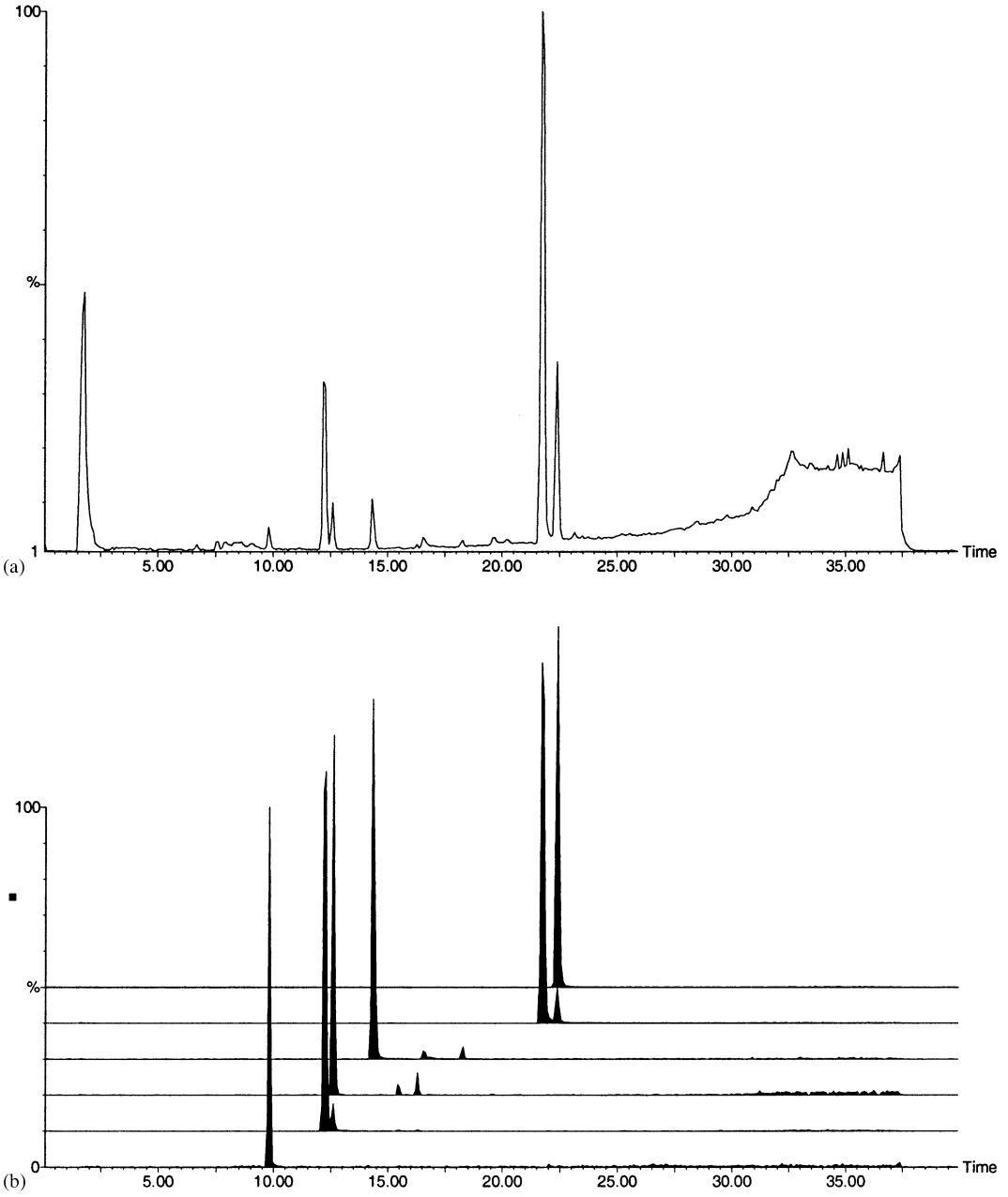

Fig. 3. (a) Total Ion Count (100–1000 Da) following gradient analysis of SR121787 0.1 mg ml^{-1} in saline solution, following storage at 25 °C for 7 days; (b) single ion responses (from top to bottom $m/z = 560$ (parent), $m/z = 561$ (degradant), $m/z = 490$ (degradant), $m/z = 532$ (degradant), $m/z = 533$ (degradant), $m/z = 330$ (degradant)).

ionisation mode, could be envisaged. Not surprisingly, this suggests that a single set of mobile phase components is unlikely to prove suitable for all cases, but a well defined strategy can be envisaged where a few components are rapidly tested according to a pre-determined decision tree.

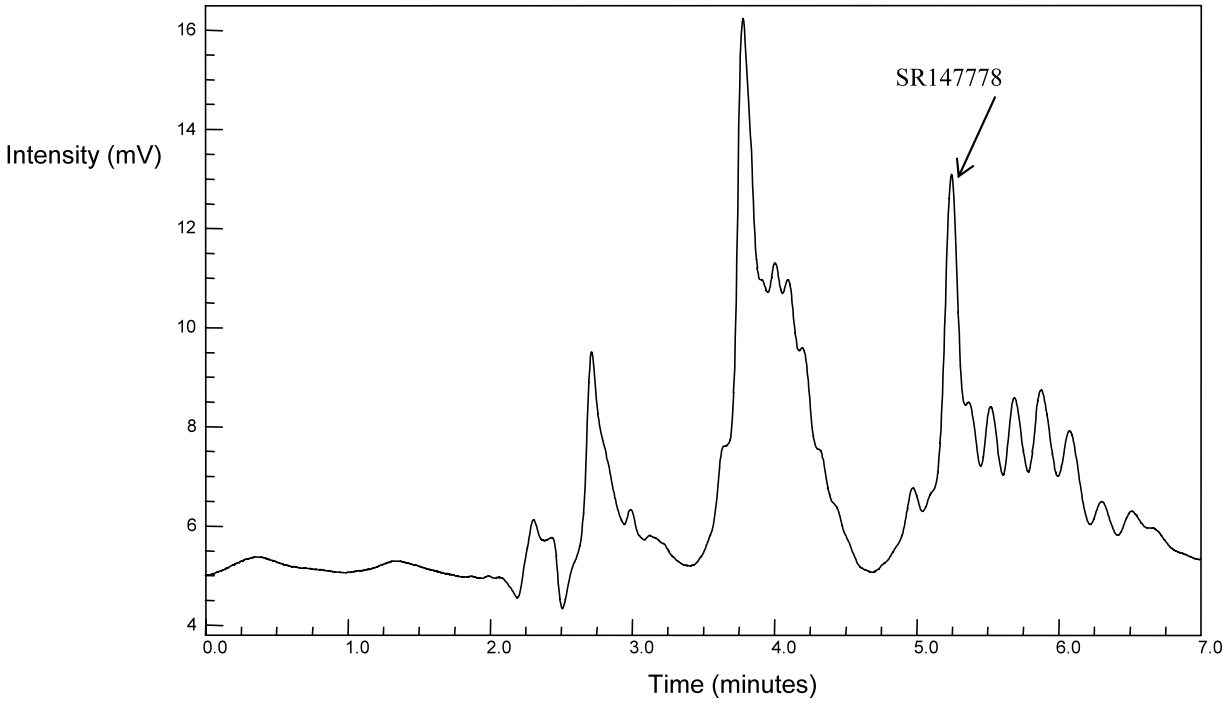

Fig. 4. 0.01 mg ml^{-1} SR147778 in mobile phase containing 0.2 mg ml^{-1} Labrasol by HPLC-UV at a wavelength of 230 nm.

3.3. Sensitivity

In those cases where a drug development candidate has a poor or non-usable chromophore, the advantage of an approach based on mass detection is self evident but, even where this is not an issue, mass detection is likely to be more sensitive than ultraviolet detection. This gives the flexibility to quantify formulation drug contents at relatively low levels without encountering problems relating to poor analyte response or inability to sufficiently dilute the matrix. Several of the samples analysed during this feasibility study contained drugs at 0.1 mg ml^{-1}, which would be sufficiently low for most preclinical study requirements. As such, inclusion of a sample at this concentration, irrespective of the lowest concentration initially requested for study, could obviate the need for repeat stability assessments as the preclinical program advances.

The generally superior sensitivity of the mass detector can also be put to good use during the gradient analysis as it can allow for the easier detection (if not quantification) of degradation products which may not be immediately apparent from examination of the UV response alone due to, for example, their relatively small peak area or their proximity to a gradient artefact, but which nonetheless represent a real change in profile compared with the batch of drug used to make the formulation. In these cases, and due to the unpredictability of mass spectral responses for related species, the level of the impurity, once detected, is best estimated by means of its simultaneously acquired UV response. Naturally, an additional advantage is that the acquisition of full scan mass spectral data for any degradation peaks that are observed may also allow for their identification and, therefore, allow a mode of degradation to be proposed.

3.4. Potential disadvantages

Notwithstanding the above, some disadvantages to the approach described can be foreseen. Firstly, quantitation by mass detection, even with an

internal standard, is less precise than that generally obtained with a modern ultraviolet-based detection system. However, assuming the precision achieved during this work to be broadly representative, appropriate conclusions with respect to formulation stability can still be reached. This is especially true if the quantitative results are, as in the approach described, supported by qualitative data.

A second potential disadvantage relates to the availability of stable isotopically labelled compounds for use as internal standards. It is possible that structurally related compounds could be successfully substituted but, given that stable isotopically labelled compounds are prepared by most pharmaceutical companies as part of their initial and ongoing drug metabolism and pharmacokinetic programs, this seems unlikely to be a major issue.

4. Conclusions

For the representative cases studied, the advantages of using an LC-MS based approach for determination of the short-term stability of preclinical study formulations; those being improved selectivity, sensitivity and speed, with respect to traditional LC-UV based approach have been demonstrated. When used together with a well defined strategy for development of the relevant chromatographic conditions, there is every prospect that this approach will continue to offer significant savings in terms of applied resource, as well as giving additional confidence in the conclusions reached. In this regard, and with minor modifications, it has now been successfully applied to five further compounds.

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