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Determination of liquid chromatographic peak purity by electrospray ionization mass spectrometry

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Abstract

A technique is described whereby the purity of an HPLC peak can be determined using electrospray liquid chromatography–electrospray ionization mass spectrometry. Electrospray mass spectra acquired across an HPLC peak are summed and examined for co-eluting impurities. The mass spectrometer is set up to produce solely cationised molecules and background noise is minimized so that minor coeluting impurities can be observed down to a level of <0.1% of the major component. This method offers advantages over using diode-array UV detection (LC–DAD) for the determination of HPLC peak purity, namely components with similar UV spectra can be distinguished, the molecular mass of the impurity can be determined and structural data can be obtained by using tandem mass spectrometry (MS–MS). The effectiveness of the technique is demonstrated with drug substances of pharmaceutical interest which have been chromatographed on an HPLC system designed to intentionally co-elute a number of impurity standards with these compounds.

1. Introduction

The use of high-performance liquid chromatography (HPLC) for quantitative and qualitative analysis of pharmaceuticals is now standard. The establishment of the purity of a chromatographic peak is essential for both the validation of chromatographic methods and the determination of analyte purity. This is particularly important when developing methods for assays, impurity profiles, and when analysing stressed samples and samples retained for stability studies. Chromatographic methods applied to the determination of pharmaceutical drug substance should ideally detect as little as 0.1% of an impurity under a chromatographic peak even if the resolution is low and the UV spectra of the analytes are very similar.

Currently a number of methods are employed for the determination of HPLC peak homogeneity [1–10] using multiple-wavelength UV detection which essentially detects the presence of impurities by deviations in the UV spectra of the analyte. Early liquid chromatography–diode-array detection (LC–DAD) methods for peak purity control used very simple univariate analysis based on the calculation of ratiograms [1,6,8]. The presence of coeluting impurities could be determined by comparing the absorbance ratio typically at two selected wavelengths measured across the LC peak. Pure peaks are characterised by having the same absorbance ratio across the peak. An impure peak can be detected by a change in the absorbance ratio over the time

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period where the impurity elutes. The sensitivity of this method strongly depends on the selection of two wavelengths which show the greatest discrimination between the main component and the known impurity. The ratiogram approach is most suitable in a "quality control" environment where the spectrum of any coeluting impurity is well known. In a research environment this method is not so useful as there are often no spectral data available for the impurity and the two most discriminating wavelengths cannot be selected.

Correlating multiple chromatographic peak area data integrated at different wavelengths has allowed more sensitive assessment of peak purity by univariate analysis [6]. More recently, commercial packages have been made available for LC–DAD based on multivariate analyses which are based on the concepts of principal-component analysis (PCA) [3,9,10] and factor analysis (FA) [2–4,7] and which make full use of the data available. These methods can be applied without having to make any assumption about the chromatography and UV spectra and therefore are particularly relevant to the calculation of peak purity. These methods compare entire UV spectra recorded at various points across the LC peak with the spectrum recorded at the apex of the peak, which can reveal details of the retention time as well as the UV spectrum of the coeluting impurity. This incorporates the assumption that the main component should be purest at the apex and, if possible, free of any coeluting impurities at this point. All these methods require the UV absorption characteristics of the impurity to be slightly different from that of the major component and this condition is often not met for impurities closely related to the major component. The other criterion for UV detection of coeluting impurities is that there is a difference in the retention times of the two (or more) overlapping species, i.e. some resolution must exist.

An assessment of chromatographic peak purity of drugs using mass spectrometric data (LC–MS particle beam) has been reported [11] using principal-component analysis but the results are inconclusive. Here we present data describing a technique for assessing peak purity that is sensitive, simple to interpret, and unambiguous. Ionspray is one of the atmospheric-pressure ionization (API) techniques that are rapidly becoming the ionization methods of choice for the mass spectrometric analysis of organic molecules of pharmaceutical interest. The two techniques commonly used, electrospray ionization (ESI; including ionspray) and atmospheric-pressure chemical ionization (APCI), can ionize small molecules with minimal fragmentation and high sensitivity [12–14]. By using nebulizer-assisted electrospray (ionspray) we have shown that peak purity can be verified down to an impurity level of ~0.1%.

2. Experimental

2.1. Chemicals

The compounds in this study, famciclovir (BRL-42810) [15] and its impurities (BRL-43594, BRL-45145, BRL-48428, BRL-48951, BRL-55842), ropinirole (SKF-101468-A) [16], and its impurity SKF-96266-A (all > 99%), were used without further purification. Solutions of famciclovir and ropinirole ($10^{-4}M$) were prepared in acetonitrile–5 mM NH_4OAc(aq; pH 7.0) (80:20). Generally, 2 μl of these solutions was used for an LC–MS analysis so that about 0.4 μg was injected on-column. Solutions of famciclovir ($10^{-4}M$) were prepared in the same eluent containing one of the following: BRL-43594, BRL-45145, BRL-48428, BRL-48951, BRL-55842, at both 10^{-6} M and 10^{-7} M (1% and 0.1% spikes). Likewise solutions of ropinirole (10^{-4} M) were prepared containing SKF-96266-A at 10^{-6} M and 10^{-7} M (1% and 0.1% spikes). Again, 2 μl of these solutions was used for an LC–MS analysis.

2.2. Mass spectrometry

Mass spectrometry was performed on a Sciex API-III (Sciex, Toronto, Canada) triple quadrupole mass spectrometer using nebulizer-as-

sisted electrospray (ionspray) as the ionization technique. Zero grade air (BOC) was used for nebulization and high-purity boil-off nitrogen was used as the curtain gas. Spectra were obtained in positive-ion mode using Q3 by scanning from 100 to 1000 u in steps of 0.2 u, each with a dwell time of 0.5 ms. The amount of analyte injected for LC–MS analysis was such that as abundant a signal as possible of the protonated or ammoniated molecule of the major component was obtained without overloading the detector (~0.4 μg), which allowed for the greatest dynamic range for detection of minor coeluting impurities. Spectra were background subtracted and summed across the main LC peak. Ion chromatograms of peaks above the noise level (which was normally about 0.2% abundance of the protonated molecule of the main component) were obtained to determine whether they were from the background or from some eluting component. MS–MS was employed to determine which peaks (if any) were adduct ions of the main component. CID was performed using argon as the collision gas with a gas thickness of $\sim 4 \cdot 10^{14}$ atoms cm^{-2}.

2.3. HPLC

HPLC was performed using a Hewlett–Packard HP1090 Series II chromatograph with a Kromasil 5 μm C_{18} 250 × 4.6 mm column at ambient temperature. The mobile phase employed was acetonitrile–5 m*M* NH_4OAc(aq; pH 7.0) (80:20), flowing at 1 ml min^{-1}. The eluent flow was split 10:1 prior to introduction to the mass spectrometer so that 100 μl min^{-1} flowed into the ionspray source. UV detection was performed by the HP1090 diode-array detector from 200 to 500 nm. Data analysis was carried out using the ChemStation V. 522 (Pascal Series) software which also performed the peak purity calculations shown in the results. The HPLC method was such that co-elution could be achieved for the drug substances and impurities analysed.

The same chromatographic conditions were also used on a Waters 996 HPLC system. Data analysis was performed on the Millennium™ 2010 Chromatography Manager software system which uses the Spectral Contrast™/Threshold Contrast™ algorithm for peak purity determination.

3. Results and discussion

The LC method was developed so that the drug impurities would co-elute with the drug substance. So that a comparison of peak purity determination by UV diode-array methods could be performed, the LC method was designed to prevent exact coelution of the main component and impurity. It should be noted here that exact coelution of two components is not an obstacle for peak purity determination by LC–MS. It was found that by using a Kromasil $5C_{18}$ column with a mobile phase containing a high organic content, compounds of similar structures could be co-eluted to the extent that no separation could be observed by visual inspection of the main peak. Fig. 1 shows a typical UV chromatogram of famciclovir obtained under these conditions. The structures of the compounds used in this study are shown in Scheme 1.

There is no reason why any number of mass spectrometric ionization techniques could not be used to determine the purity of HPLC peaks but electrospray ionization does offer a number of significant advantages: (a) for a vast majority of drug substances, only cationised molecules are produced, allowing simple spectral interpreta-

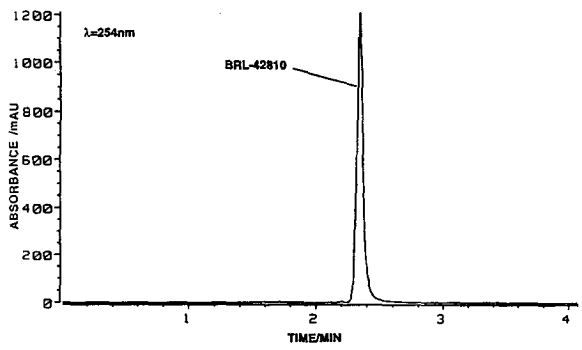
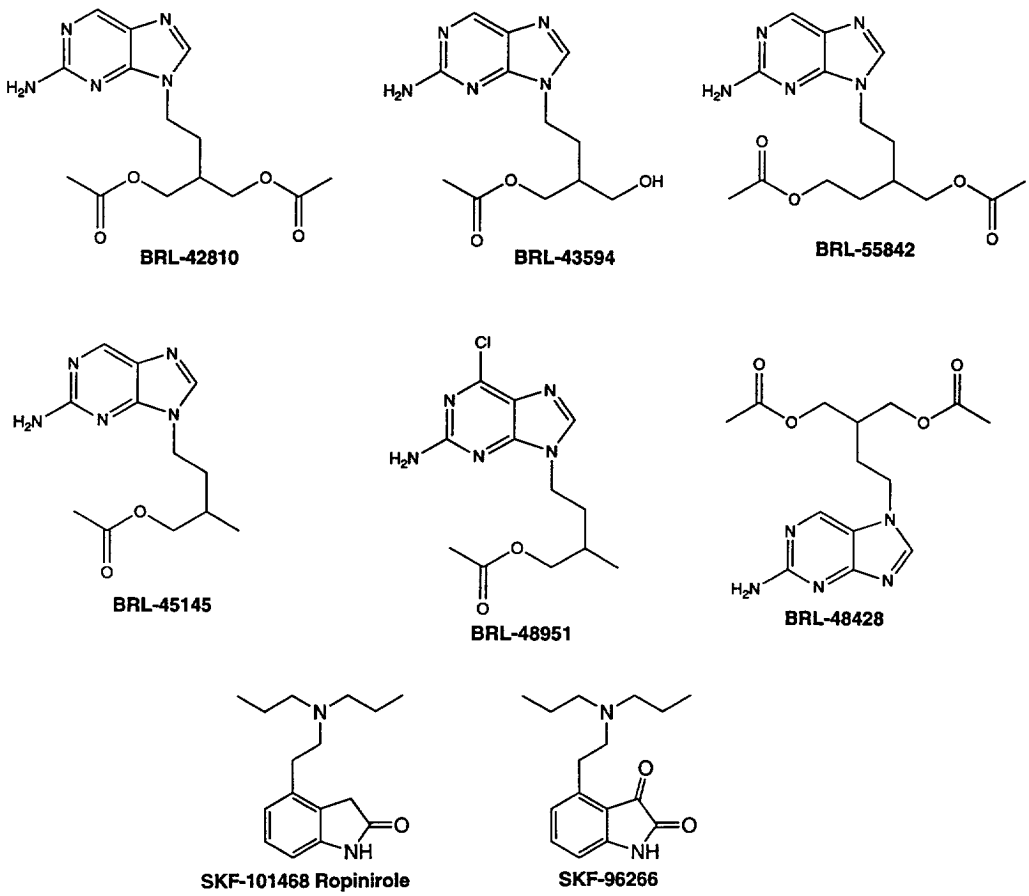


Fig. 1. UV chromatogram of BRL-42810.



Scheme 1. Structures of compounds used in this study.

tion, (b) very little chemical noise is produced [unlike fast atom bombardment (FAB) and thermospray], (c) the technique is very sensitive, and (d) suppression effects are minimised. It should be noted that this technique would probably work as well with atmospheric-pressure chemical ionization (APCI) which yields similar results for the ionization of organic molecules [17,18]. The mass spectrometer was set up such that the base peak (in this study the protonated molecule of the drug substance) was of high abundance but not overloaded. The orifice voltage, which controls the degree of fragmentation within the source region, was set so that fragmentation and background chemical noise were minimised. The spectra acquired across the peak were summed and background subtracted to give a spectrum of almost entirely cationised molecules. A scan time of about 2.2 s was employed which allowed optimal signal-to-noise values to be obtained for minor components. Care was taken in that the region selected for subtracting was entirely free of any eluting components, so as not to subtract any isomeric coeluting impurities. A typical spectrum of chromatographed famciclovir obtained using this method is shown in Fig. 2, and it can be seen that the main component is the protonated molecule of famciclovir. The same spectrum is shown in Fig. 3a with the y-axis magnified by a factor of 16. Other ions are observed such as $[M + Na]^+$ (m/z 344), $[M + K]^+$ (m/z 360), and $[2M + H]^+$ (m/z 643) as well as some fragment ions of famciclovir at m/z 136, 262 and 280.

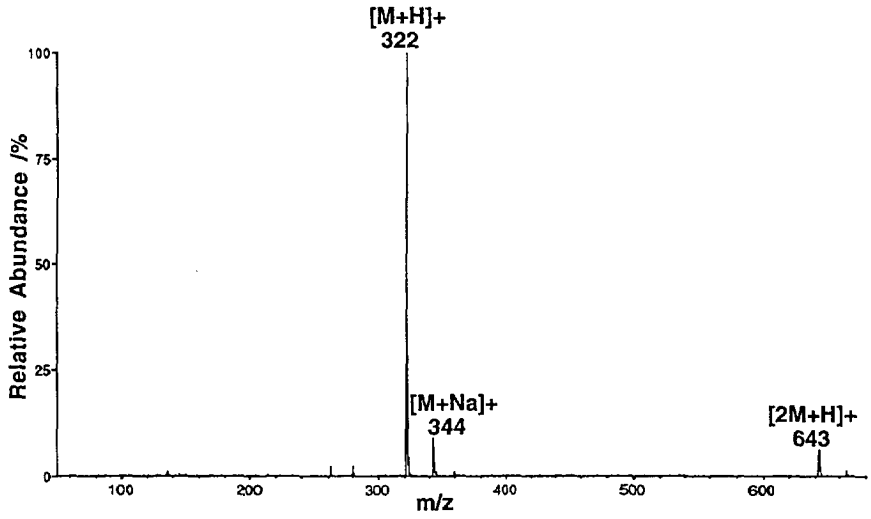


Fig. 2. LC–MS spectrum of BRL-42810.

3.1. High and low orifice voltages

The degree of in source fragmentation can be controlled by varying the so-called orifice voltage. Low voltages tend to preserve non-covalently bound clusters formed in the source with the solvent, modifier and/or analyte, which can lead to artefact peaks which could be mistaken for cationised molecules. High voltages will break up these clusters but will also cause some fragmentation of the analyte compound. The effect of varying the orifice voltage while performing LC–MS on famciclovir can be seen in Fig. 3. At 70 V there are virtually no significant background peaks but a small number of low abundance fragment ions are seen. At a lower orifice voltage (50 V) there is no fragmentation but there are more background ions, and more

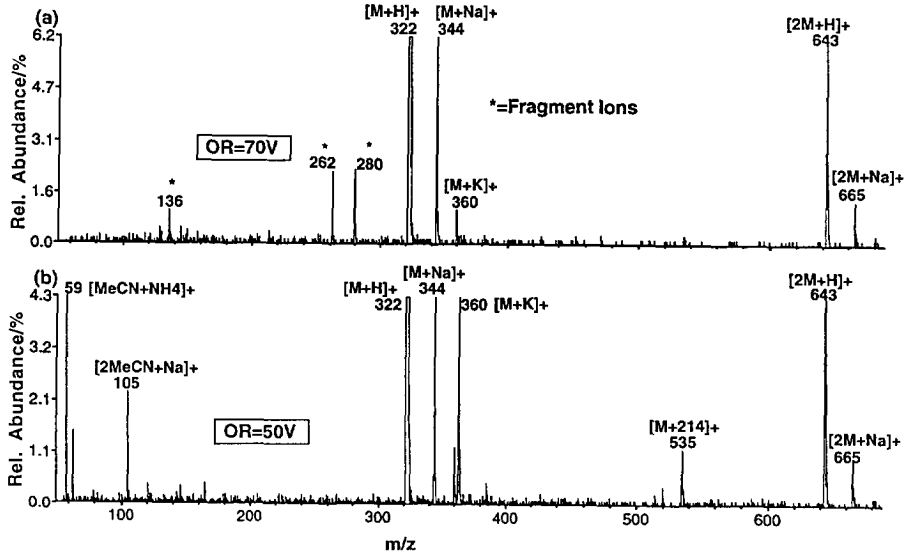


Fig. 3. Electrospray spectra of pure (>99%) BRL-42810 ($M_r = 321$) obtained at (a) high and (b) low orifice voltages.

clustering is observed. In the example shown in Fig. 3, a low orifice voltage analysis can be used to determine whether the small peaks at m/z 136, 262, and 280 are cationised molecules or fragment ions. The fact that they disappear under low orifice voltage conditions indicates that they are fragment ions and in the figure are marked with an asterisk. It is possible that they are background cluster ions which disappear at low orifice voltages due to insufficient "cluster breaking" but it is certain that these peaks would be removed during background subtraction. A high orifice voltage analysis likewise should remove all possible background cluster ions. Ions common to these two analyses are therefore likely to be cationised molecules. Protonated dimer species formed in the source region are almost always observed on our instrument for small molecules, especially when large amounts are analysed. The identity of these source dimers can be verified by MS–MS experiments or by repeating the analysis at an orifice voltage >100 V which will completely break up the source dimers but not the cationised molecules.

The example shown in Fig. 3 is of a pure sample of famciclovir but an example of LC–MS of an impure famciclovir peak is shown in Fig. 4. The famciclovir solution was spiked with 1% BRL-43594 which can be seen as a protonated molecule of m/z 280 at an abundance of 5.2% relative to the protonated molecule of famciclovir. Interestingly, the protonated molecule of this impurity, m/z 280, is isobaric with a fragment ion of famciclovir. The peak observed at m/z 280 in Fig. 4a is therefore a mixture of protonated molecules of BRL-43594 and a fragment ion of famciclovir. By repeating the analysis at an orifice voltage of 50 V, fragmentation of famciclovir can be prevented thereby leaving the ion at m/z 280 to be made up solely of protonated molecules of BRL-43594. This effect of varying the orifice voltage can be seen in Fig. 5 which shows ion chromatograms for m/z 280 for impure famciclovir recorded at high and low orifice voltages. The fragment ion m/z 280 of famciclovir is only observed at the higher orifice voltage (Fig. 5a). The low orifice analysis (Fig. 5b) shows that some of the ions of m/z 280 are from the protonated molecule of the impurity, BRL-43594.

3.2. Versatility of the technique

Samples of famciclovir were spiked with BRL-55842 ($M_r = 335$), BRL-45145 ($M_r = 259$), BRL-48951 ($M_r = 297$), and BRL-48428 ($M_r = 355$) at the 1% level, the impurities being clearly observed by LC–MS. For example, ionspray spec-

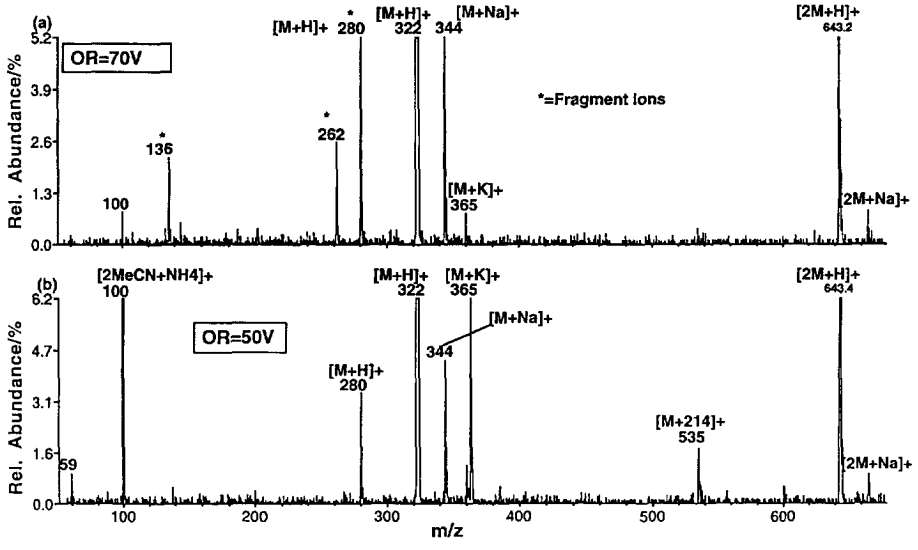


Fig. 4. Electrospray spectra of impure BRL-42810 ($M_r = 321$) spiked with BRL-43594 ($M_r = 279$) at (a) high and (b) low orifice voltages.

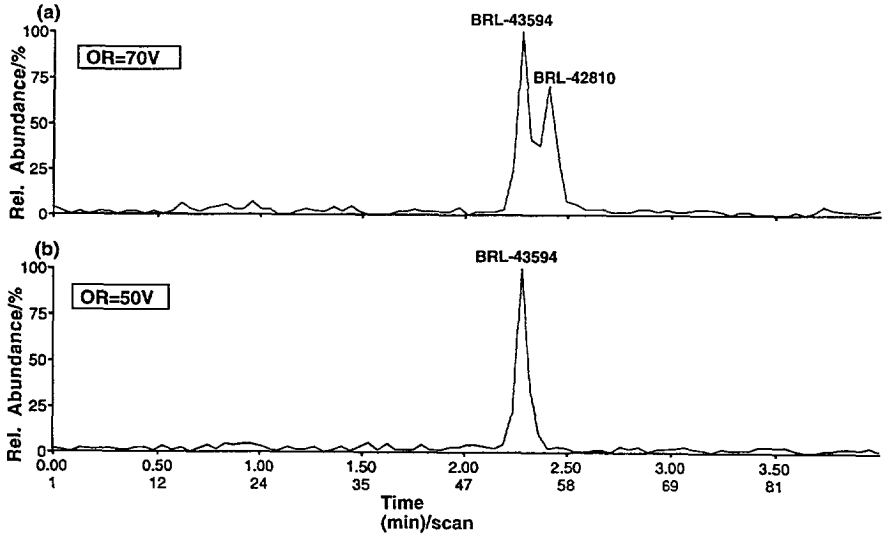


Fig. 5. Ion chromatograms of *m/z* 280 of BRL-42810 spiked with 1% BRL-43594 at (a) high and (b) low orifice voltages.

tra of impure famciclovir LC peaks spiked with 1% BRL-48951 and 1% BRL-48428 are shown in Fig. 6. It should be noted that all these impurities except BRL-48428 have identical UV spectra to the main component famciclovir and would not be detected as coeluting impurities at almost any level by UV methods. An example from a different class of compounds is shown in Fig. 7 in which a sample of ropinirole (SKF-101468-A) spiked with (a) 1% and (b) 0.1% SKF-96266-A indicating the versality of the technique.

3.3. Detection limits

Fig. 7 also demonstrates the sensitivity of the technique. Even at the low level of 0.1%, the protonated molecule of SKF-96266-A is clearly

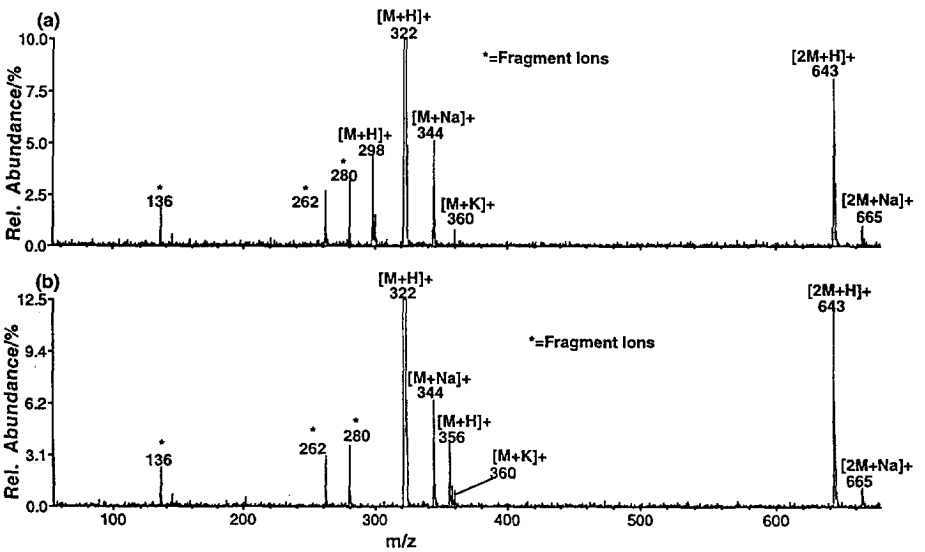


Fig. 6. Electrospray spectra of BRL-42810 ($M_r = 321$) spiked with (a) 1% BRL-48951 ($M_r = 297$) and (b) 1% BRL-48428 ($M_r = 355$).

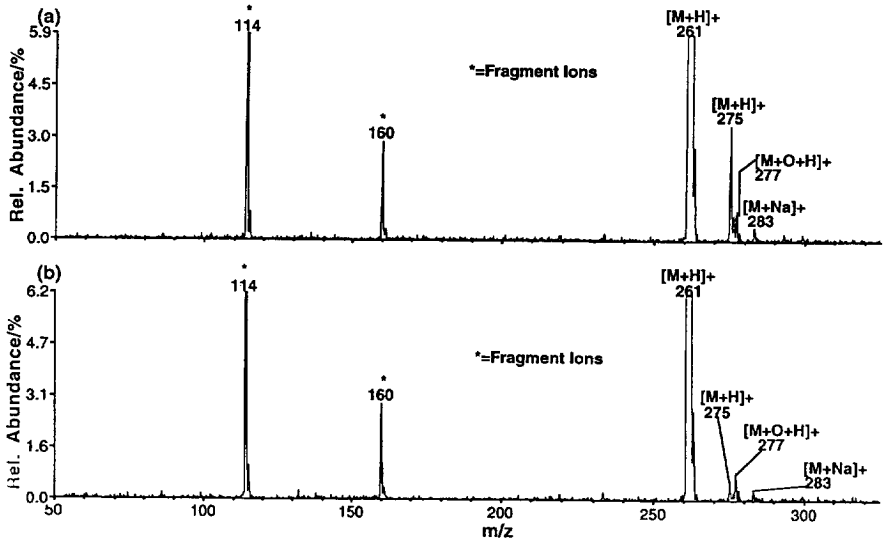


Fig. 7. Electrospray spectra of SKF-101468-A ($M_r = 260$) spiked with (a) 1% SKF-96266-A ($M_r = 274$) and (b) 0.1% SKF-96266-A.

visible at an abundance level of 0.7% relative to the protonated molecule of ropinirole (Fig. 7b). Assuming that organic compounds of the same class might be expected to have similar response factors when analysed by electrospray ionization mass spectrometry, it should be possible to quantitate the approximate level of the coeluting impurity. However, the linear dynamic range of the detector on the Sciex API-III is relatively small in full-scan mode and low level impurities could not be directly quantitated by comparison of relative abundance levels. The fact that the protonated molecule of SKF-96266-A has an abundance level of 0.7% relative to the protonated molecule of ropinirole when spiked at the 0.1% level indicates that the ropinirole cationised molecule response is much smaller than expected. A comparable analysis on an instrument with a large linear dynamic range in full-scan mode should allow an approximate quantitation assuming equal response factors for the two coeluting components.

3.4. Disadvantages

Response factors between different classes of compounds can vary by orders of magnitude with electrospray which can make approximate quantitation unreliable. However, in drug substances, for example, impurities are generally structurally chemically related to the main component giving rise to equivalent response factors. Another problem is that isobaric coeluting species would not be detected although if it is a gross impurity it may be detectable using MS–MS. It should be stressed here that determination of the purity of an HPLC peak is a negative concept. This method, as with diode-array UV detection, will not establish the absolute absence, or otherwise, of coeluting impurities and/or degradation products. It will, however, increase confidence in the establishment of peak purity.

3.5. Comparison with UV methods

There are a number of methods for determining peak purity by UV diode-array detection. As stated earlier, they rely on the impurity (i) having a different UV spectrum to the main analyte and (ii) not coeluting exactly with the main analyte. Fig. 8 shows the results of a peak-purity determination on the sample of ropinirole

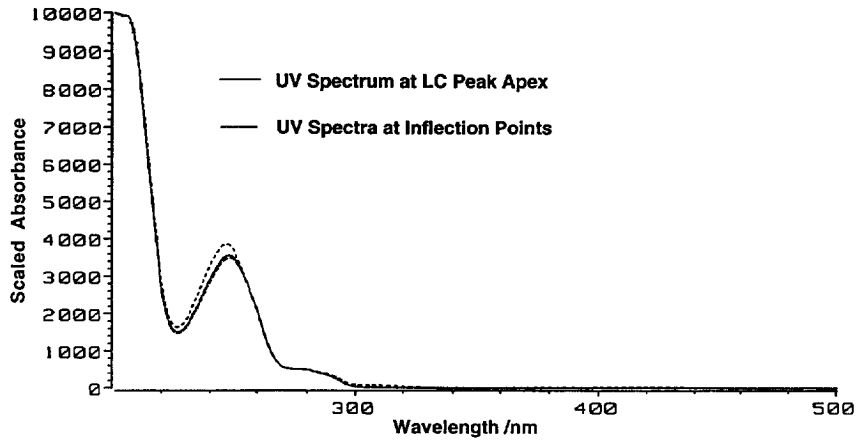


Fig. 8. UV spectra of an impure LC peak (1% SKF-96266-A in SKF-101468-A) recorded using the peak purity function on the HP-1090 ChemStation Data System.

spiked with 1% SKF-96266-A on a Hewlett-Packard 1090 ChemStation (Pascal Series) V. 5.22 data system. This software system allows the investigation of peak purity using two different approaches. The first approach is the use of ratiograms across the peak and is only a qualitative technique while the second approach is based on univariate analysis [19]. Using the latter technique, the software expresses peak purity as a number, with a value of 1000 representing a pure peak through a perfect degree of overlap of the normalized spectra at the upslope, apex and downslope. A low peak-purity value corresponds to poor overlap between the normalized spectra and therefore indicates an impure peak. Because this approach does not require prior knowledge of chromatography or spectra it is most applicable to the early stages of the development of chromatographic methods for the analysis of drug substance where the nature of the coeluting impurities or decomposition products are unknown and therefore, this approach was adopted in this study. In Fig. 8 some spectral differences are seen, but there is no information on the identification and abundance level of the impurity, and the retention time of the impurity can only be said to occur before or after the apex of the main peak. Another problem with this technique is that if the coeluting peak does not elute at the apex or the inflection points then it will not be clearly observed, as is the case shown in Fig. 8.

The other UV-DAD software package evaluated in this study was the Millennium™ 2010 Chromatography Manager system which uses the Spectral Contrast™/Threshold Contrast™ algorithm. This package is used in conjunction with the Waters 996 high-resolution diode-array detector. Differences in spectral shape are quantified by converting spectra to vectors. Differences in vector direction are measured between the spectrum recorded at the LC peak apex and all the other spectra in the LC peak and is denoted as a purity angle. Spectra that have the same shape have vectors that point in the same direction, while spectral differences show up as variations in the spectra vectors. The uncertainty of the spectral difference will strongly decrease with increasing concentration of the impurity and with increasing resolution of the coeluting impurity from the main component. The Threshold Contrast™ algorithm is required to measure the degree of uncertainty of the variation in the spectral vector. Graphically the maximum difference can be accentuated by using the Maximum Impurity algorithm which displays the spectrum at the retention time where the purity angle to noise angle is greatest as calculated by peak purity. An example is shown in Fig. 9 and it can be seen that not only has it determined that the

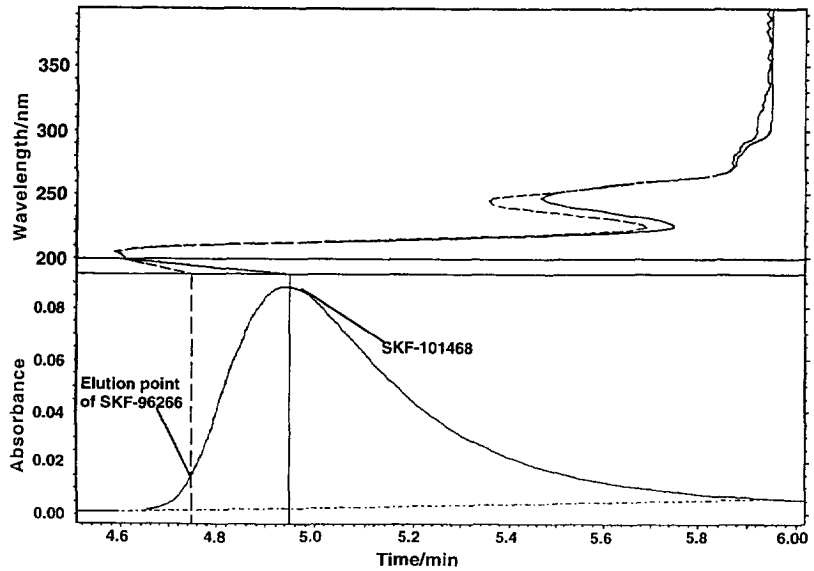


Fig. 9. UV spectra of an impure peak (1% SKF-96266-A in SKF-10468-A) recorded using a Waters 996 System with Millennium™ software. Top: comparison of the spectrum at the apex and the spectrum containing the maximum impurity. Bottom: UV chromatogram.

peak is impure but it has also determined the elution time (4.55 min) for the impurity (SKF-96266). This method was able to detect co-eluting SKF-96266 in this system down to a level of 0.1% of ropinirole. However, for the example of BRL-48428 in famciclovir it was only possible to determine that the main peak was impure down to an impurity level of about 1%, due to the fact that the UV spectra of BRL-42810 and BRL-48428 are similar. The UV spectra for famciclovir along with its impurities BRL-43594, BRL-45145, BRL-48951, BRL-55842 are all identical and UV diode-array detection could not be used to determine HPLC peak purity of spiked famciclovir solutions. It should be noted that peak purity as determined here is a negative concept and not an absolute determination of peak purity. This study will however increase confidence in determining the absence of coeluting impurities in the main component.

4. Conclusion

Electrospray ionization mass spectrometry (ESI-MS) is a versatile, sensitive and rapid technique for the determination of LC peak purity. Coeluting impurities can be rapidly determined down to a level of 0.02% and below. Both the molecular mass and the retention time can be determined of the impurity and unlike UV methods, compounds with identical or similar UV spectra can be distinguished. The sister technique of ESI-MS, atmospheric-pressure chemical ionisation (APCI), would also be suitable for this type of analysis.

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References

[1] J.B. Castledine, A.F. Fell, R. Moden and B. Spellberg, Anal. Proceedings, 29 (1992) 100.
[2] H.R. Heller, D.L. Massart and J.O. de Beer, Anal. Chem., 65 (1993) 471.

[3] H.R. Keller, P. Kiechle, F. Erni, D.L. Massart and J.L. Excoffier, J. Chromatogr., 641 (1993) 1.
[4] H.R. Keller, D.L. Massart, Y.Z. Liang and O.M. Kvalheim, Anal. Chim. Acta, 267 (1992) 63.
[5] H.R. Keller, D.L. Massart, Y.Z. Liang and O.M. Kvalheim, Anal. Chim. Acta, 263 (1992) 29.
[6] J.B. Castledine, A.F. Fell, R. Modin and B. Sellberg, J. Chromatogr., 592 (1992) 27.
[7] H.R. Keller and D.L. Massart, Anal. Chim. Acta, 246 (1991) 379.
[8] F.V. Warren, B.A. Bidlingmeyer and M.F. Delaney, Anal. Chem., 59 (1987) 1897.
[9] Y.Z. Liang, M.D. Hamalainen, O.M. Kvalheim and R. Anderson, J. Chromatogr. A, 662 (1994) 113.
[10] R.W. Andrews and H. Richardson, J. Chromatogr. A, 683 (1994) 3.
[11] D. Lincoln, A.F. Fell, N.H. Anderson and D. England, J. Pharmaceut. Biomed. Anal., 10 (1992) 837.
[12] A.P. Bruins, T.R. Covey and J.D. Henion, Anal. Chem., 59 (1987) 2642.
[13] E.C. Huang, T. Wachs, J.J. Conboy and J.D. Henion, Anal. Chem. 62 (1990) 713A.
[14] S. Pleasance, J.F. Anacleto, M.R. Bailey and D.H. North, J. Am. Soc. Mass Spectrom., 3 (1992) 378.
[15] R.A.V. Hodge, D. Sutton, M.R. Boyd, M.R. Harnden and R.L. Jarvest, Antimicrob. Agents Chemother., 33 (1989) 1765.
[16] R.J. Eden, H. Hughes, D.A.A. Owen and M.S. Wallduck, Br. J. Pharmacol., Proc. Suppl., 91 (1987) 380P.
[17] J.P. Kiplinger, Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL (1994) p. 329.
[18] R.D. Voyksner, J. Keever and R. Straub, Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL (1994) p. 330.
[19] H.P. Sievert and A.C.J.H. Drouen, Diode Array Detection in HPLC, in L. Huber and S.A. George (Editors), Marcel Dekker, New York 1993, pp. 000