# Comparison of LC detection methods in the investigation of non-UV detectable organic impurities in a drug substance

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## Abstract

HPLC Analysis with different detection methods was shown to be essential in the separation and identification of unknown organic impurities in a drug substance. The impurities were found to exhibit very weak or no response to standard ultraviolet (UV) absorption detection. LC-MS, LC-NMR, indirect, refractive index and evaporative light-scattering detection were used to quantify and identify the impurities in this specific case. The drug substance studied was found to be an ideal analyte for demonstrating the advantages and limitations of several chromatographic detection systems for impurity profile analysis. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Detection; Drug substance; Impurities; LC-MS; LC-NMR; Refractive index; Evaporative light scattering; Indirect detection

## 1. Introduction

For many years chromatographers have used ultraviolet absorption detection (UV) as the means of detecting components in liquid chromatographic separations [1]. UV is the most popular form of detection and the most universally applicable in pharmaceutical analysis. Indirect detection has also been used, but to a limited extent [2,3]. Several other common detection methods are employed such as fluorescence [4,5], electrochemical [6–8], refractive index (RI) [9,10] and more recently, the evaporative light scattering detector is being used routinely [11–17]. In addition, the use of mass spectrometry [18–21], NMR spectroscopy [22–24] and also LC-NMR-MS for on-line detection has started to revolutionise peak identification [25]. Other LC detectors such as those based on circular dichroism [26] and surface plasmon resonance principles [27] are also being developed.

Controlling impurities in drug substance is an essential part of pharmaceutical development. The presence of impurities in a drug substance may have significant impact on toxicology studies and drug safety. An impurity level of only 0.1% is deemed significant from a regulatory standpoint and often identification is necessary. Chromato-

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graphic impurity profiling is used to monitor the level of organic impurities in drug substances. The data obtained from impurity analyses are used to help the understanding of chemical reactions and processes, as well as to ensure conformance to specification. Typically, the impurities are structurally related to the drug substance and require very efficient chromatographic systems to attain adequate separation and detection of all impurities. HPLC is the preferred technique for these analyses, usually with UV detection. However, impurities containing weak or no chromophores can remain undetected.

Here we describe the detection of components that have poor UV absorbing characteristics using LC-MS, LC-NMR, indirect, evaporative light scattering (ELS) and RI for the identification and quantitation of organic impurities in a drug substance. An assessment of the suitability of the detection methods is made with respect to sensitivity, detector response and applicability to general impurity analysis.

## 2. Experimental

### 2.1. Synthesis of SKF-99085

#### 2.1.1. HPLC

The synthesis of SKF-99085 is given in a separate report [28]. The HPLC system was comprised of a Waters 600S controller, 616 pump, 486 detector and a 717 Plus autosampler (Waters, Watford, UK). Purified water and HPLC grade solvents (Fisher, Loughborough, UK) were used (See figures for conditions). Refractive index detection was performed with a Model 7515-A detector (ERMA CR, Tokyo, Japan) and evaporative light scattering detection with a Sedex 55 detector (SEDERE, Alfortville, France). For ELS detection, the nitrogen gas was set at ca 2.0 bar and the evaporation temperature to 50°C. Columns used were Nova-Pak $C_{18}$, 15 cm × 3.9 mm i.d., 4 μm (Waters, Watford, UK) and Kromasil $C_{18}$, 25 cm × 4.6 mm i.d., 5 μm (Hichrom, Reading, UK). All chemicals used were of analytical grade (Aldrich, Poole, UK).

#### 2.1.2. LC-MS

LC-MS was performed using a Hewlett–Packard HP1090 Series II chromatograph (Hewlett–Packard, Stockport, UK) with either a Kromasil $C_{18}$, 25 cm × 4.6 mm i.d., 5 μm column (Hichrom, Reading, UK) at ambient temperature or a Nova-Pak $C_{18}$, 15 cm × 3.9 mm I.D., 4 μm (Waters, Watford, UK) at 40°C. The solvents employed were Solvent A: 5 mM $NH_4OAc$ and Solvent B: acetonitrile (see figures for LC conditions). The eluent flow was split 10:1 prior to introduction to the mass spectrometer so that 100–150 μl min$^{-1}$ flowed into the IonSpray source. UV detection was performed by the HP1090 diode array detector from 200 to 500 nm. Mass spectrometry was performed on a Sciex API-III + (Sciex, Toronto, Canada) triple quadrupole mass spectrometer, using nebulizer assisted electrospray (IonSpray) as the ionization technique. Argon was used as the collision gas. Zero grade air (BOC, Crawley, UK) 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 Da in steps of 0.2 Da, each with a dwell time of 0.5 ms.

#### 2.1.3. LC-NMR

LC-NMR was performed using a 500 MHz JEOL Alpha FT-NMR spectrometer (JEOL, Welwyn Garden City, UK) fitted with a dedicated proton probe. Non-deuterated solvents were used for the mobile phase and solvent suppression of the water and acetonitrile signals was achieved using DANTE and pre-saturation respectively. On-flow data were recorded with 16 transients as approximately 30 s slices through the chromatogram. Stopped flow data were recorded with acquisition times of 30–60 min. HPLC was carried out using a Gilson pump set at 0.5 ml min$^{-1}$ with a Nova-Pak $C_{18}$, 15 cm × 3.9 mm i.d., 4 μm column (Waters, Watford, UK) and an acetonitrile–water (70:30 v/v) mobile phase at ambient temperature. A 10 mg ml$^{-1}$ sample concentration with a 50 μl injection volume was used (500 μg sample loading). Peaks were tracked using UV set at 200 nm prior to transfer via narrow-bore tubing into the NMR probe. A proton spectrum of SKF-99085 was recorded in a mixed solvent sys-
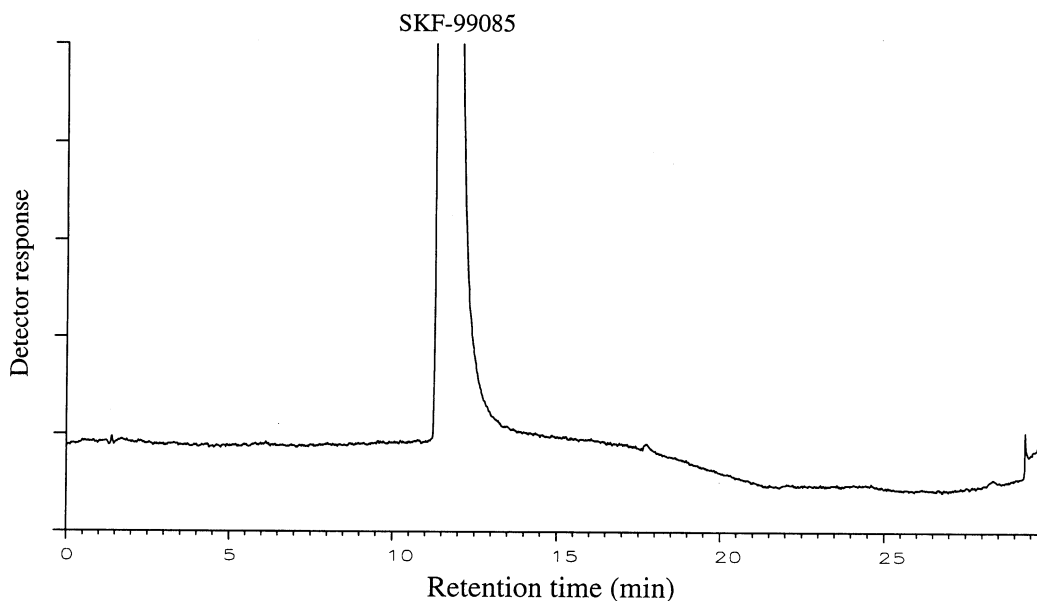
Fig. 1. HPLC impurity profile of SKF-99085 sample using $UV_{280}$ detection with gradient elution. Conditions: Kromasil $C_{18}$ (25 cm × 4.6 mm i.d., 5 μm) at 40°C, elution with acetonitrile–water (80:20 v/v for 12 min), then to 100% acetonitrile (over 5 min), then held (18 min), 1.0 ml $min^{-1}$, 20 μl injection, concentration 5 mg $ml^{-1}$.

tem of acetonitrile–$d_3$:$D_2O$ (70:30 v/v) and the chemical shift of the *t*-butyl group measured to be used as a reference signal ($\delta$ 1.39 ppm).

### 2.1.4. GC

A Varian model 3400 gas chromatograph (Varian, Crawley, UK) was used with a HP-1 methyl silicone 2.65 μm film, 10 m × 0.53 mm i.d. column (Hewlett–Packard, Stockport, UK) and FID detection. The carrier gas was helium. The sample diluent was dichloromethane.

### 2.1.5. Preparative-LC

Preparative-LC was performed on a Septec chromatograph (EM Industries, Wakefield, USA) using UV detection at 200 nm. Work was performed with a Kromasil $C_8$, 25 cm × 5.1 cm i.d., 10 μm column (Modcol Corporation, St. Louis, USA). Scouting and scale-up experiments were carried out on an analytical column packed with identical packing to the preparative column. Mobile phases consisting of acetonitrile–water were used for elution. Mass overload conditions were used with a 10 ml injection volume. Owing to the

low separation factor ($\alpha$) between some impurities it was necessary to recycle heart-cut peaks. Fractions were analysed using HPLC with refractive index detection. Impurities were isolated from the LC fractions by evaporation under reduced pressure to remove acetonitrile followed by freeze drying to give the isolated compounds.

## 3. Results and discussion

### 3.1. Drug substance analysis

#### 3.1.1. HPLC

Fig. 1 shows the HPLC impurity profile of SKF-99085 drug substance using UV detection at an absorption maximum (280 nm) which indicates that the sample is highly pure with minimal related organic impurities by peak area ratio ( > 99.9%). HPLC assay of the sample gave 96.6% w/w relative to a reference material. The discrepancy between the assay and impurity profile result prompted further investigation to determine the cause of the low assay.
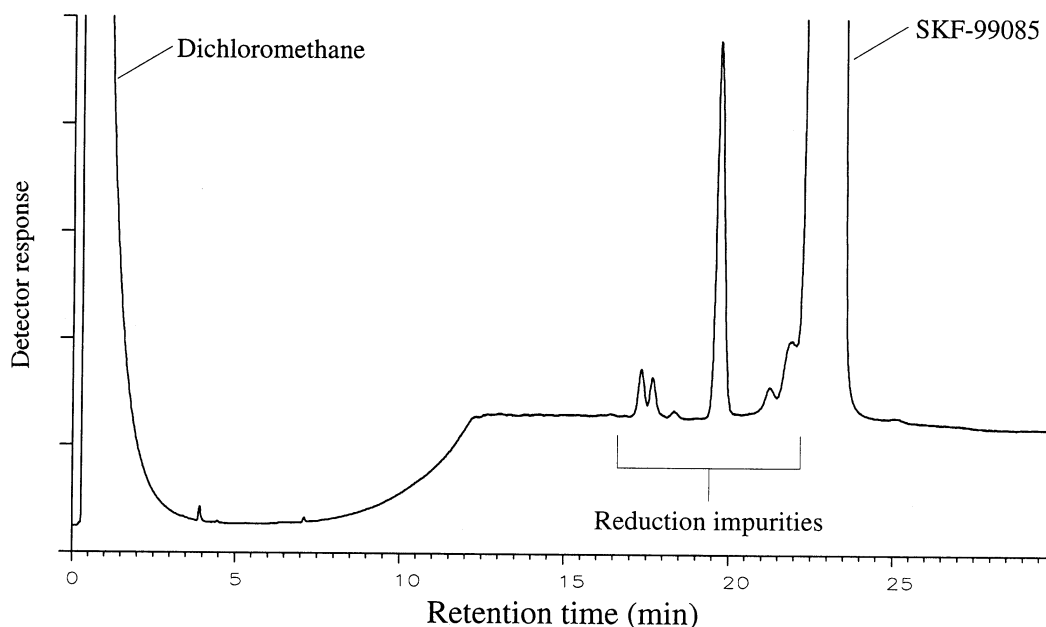
Fig. 2. GC chromatogram of SKF-99085 sample. Conditions: HP-1 methyl silicone (10 m × 0.53 mm i.d., 2.65 μm film), 110–230°C at 10°C min$^{-1}$, Helium at 5.0 ml min$^{-1}$, 1.0 μl injection at 250°C with a 2:1 split ratio, concentration 20 mg ml$^{-1}$, FID at 280°C.

### 3.1.2. GC

A GC impurity screening method was used to analyse the sample. The GC chromatogram shows the presence of several impurity components (Fig. 2). The peak area ratio for SKF-99085 itself by GC was 96.0% which was similar to the HPLC assay value. GC-MS data indicated that the impurities were related to SKF-99085 and were the by-products produced from the conversion of SKF-99086 to SKF-99085 (Scheme 1).

The by-products were impurities formed during the hydrogenation step of the reaction. In this case, the impurities were derived from the reduction of the phenolic ring giving a range of alcohols, some of which tautomerized to cyclohexanone compounds.

The impurities had either no, or very poor, UV response and would not have been detected in the original HPLC impurity analysis. A sample of the reaction mother liquors was found to be enriched with these impurities and this sample (except where stated) was used to study the performance of the LC detection systems.

### 3.2. Identification of organic impurities

### 3.2.1. LC-MS

LC-MS was performed on the mother liquor sample in order to determine the LC elution of the impurities under reversed-phase conditions. LC-MS-MS was carried out on individual impurities in order to obtain structural information and assign tentative structures. The ionisation mode used was IonSpray™ (pneumatically assisted electrospray) which provided protonated molecules for mass analysis. The total ion chromatogram (TIC) obtained from the mother liquor sample under similar chromatographic conditions as in Fig. 1 is shown in Fig. 3 and it can be seen that a number of non-UV absorbing components were observed. In this experiment the UV detector was set at 220 nm, which enabled tracking of the SKF-99085 component; component J could also be detected. Table 1 shows the structural assignments of the major peaks based on LC-MS work and subsequent LC-NMR and preparative-LC work (see following sections). Using collisional activation of the protonated molecules of the
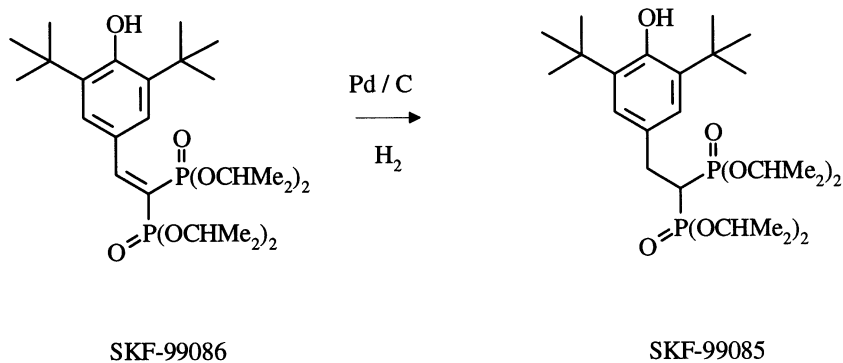
Scheme 1.

impurities with argon gas, fragmentation was induced and tandem mass spectrometry (MS-MS) was used to generate MS-MS spectra. A typical MS-MS spectrum of one of the impurities is shown in Fig. 4. The MS-MS spectra of the reduction impurities was used, in part, for the structural elucidation of the reduction impurities.

Mass spectrometry is a highly specific and sensitive detector for LC applications where UV detection is problematic. LC-MS has been used to perform quantitative work in some cases [29], however, it is expensive and complex.

### 3.2.2. LC-NMR

LC-NMR was used to obtain more structural information on the impurities. In particular, isomeric impurities (C/D/E and G/H/I) were of interest and it was hoped that on-line NMR detection would distinguish the isomers and avoid the need to isolate specimens of the impurities. To accommodate NMR detection, the LC method was optimised to provide enhanced separation at higher column loadings in order to increase the sensitivity of the LC-NMR experiment. LC-NMR was performed on the mother liquor sample and the four largest components were detected in the on-flow mode and subsequently analysed in the stopped-flow mode. Owing to limited sensitivity, only impurities E, G and H could be detected. The data obtained on these components were instructive when interpreted with the complementary LC-MS data. For Impurity E, a single *t*-butyl signal and no evidence for an olefinic or

aromatic proton resonance was consistent with the symmetrical structure listed in Table 1. All signals were visible for the second component, SKF-99085, which was used as the control experiment. The spectrum for Impurity G contained two *t*-butyl signals which suggested that the six-membered ring was not symmetrical and was more consistent with an endocyclic double bond rather than an exocyclic carbonyl group. However, no corresponding olefinic proton was evident to support this structure. Subsequent NMR data obtained on an authentic sample of G (see Section 3.2.3) were consistent with the structure having an exocyclic carbonyl. Non-equivalent *t*-butyl signals are explained by the structures given in Table 1. The spectrum of Impurity H, an isomer of G, contained a single *t*-butyl signal consistent with a symmetrical structure. The absence of an olefinic proton signal was consistent with the proposed cyclohexanone. In this particular case LC-NMR was able to provide supporting data for the structures of the isomers but the data were not good enough to define structures.

### 3.2.3. Preparative-LC

LC-NMR could not unequivocally assign structures to the isomeric impurities, so preparative-LC was used to isolate small quantities of the major components from the mother liquor sample. Impurities E, G, H, J and M were isolated. NMR spectroscopy ($^1$H and $^{13}$C) was used to ascertain structures for the impurities and to corroborate the on-line data (Table 1).
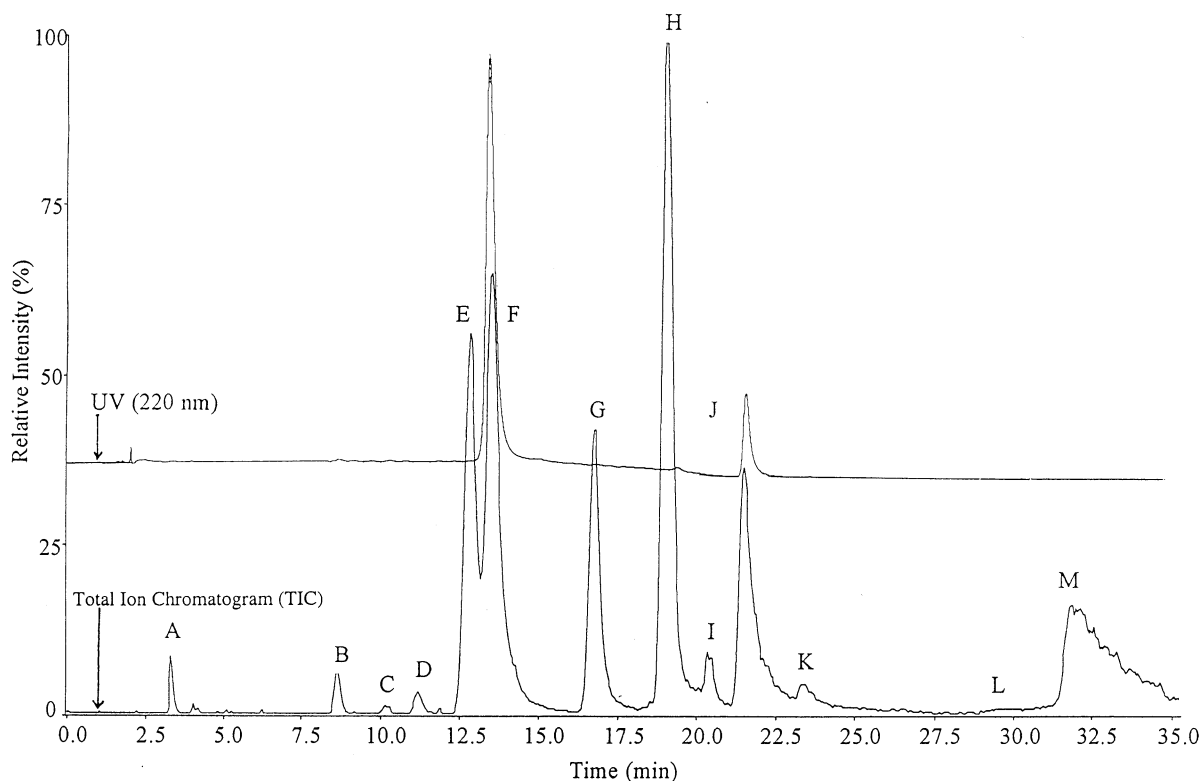
Fig. 3. LC-MS Total ion chromatogram (range 300–700 Da) and $UV_{220}$ chromatogram of the mother liquor sample using gradient elution. Main peak is off-scale. Conditions: Kromasil $C_{18}$ (25 cm × 4.6 mm i.d., 5 μm) at ambient, elution with acetonitrile–5 mM ammonium acetate (80:20 v/v for 12 min) then to 99% acetonitrile (over 5 min) then held (23 min), 1.0 ml $min^{-1}$ split 1:10, 20 μl injection, concentration 1 mg $ml^{-1}$.

### 3.3. Quantitation of organic impurities

#### 3.3.1. Mass Spectrometry

The relative response factors for the major components were investigated by electrospray ionisation (ESI) to assess LC-MS as a suitable quantitative technique. Solutions of known concentrations of the isolated components E, G, H, J and M and SKF-99085 (Peak F) in mobile phase (ca.10 μg $ml^{-1}$) were individually analysed by LC-MS using an isocratic LC method. The mother liquor sample analysed with the isocratic LC method is shown in Fig. 5(a). The response factors relative to SKF-99085 were calculated by measuring the peak areas of the six components obtained from the six TICs. The results are shown in Table 2 and it can be seen that the response factors of the reduction impurities are similar to SKF-99085.

This is not surprising, because the ionization (by protonation) occurs on the phosphorus atoms of the impurities and the proton affinity of the phosphorus atoms should not be affected greatly by the different compositions of the six-membered ring substituents. The similarity of responses in this case makes LC-MS attractive with regard to quantitative characterisation of general samples.

The detection limits (DLs) of the six isolated components were also determined by LC-MS using the same method. They were calculated as three times the noise and the results are listed in Table 3. The DLs were calculated from both TICs and from extracted ion chromatograms (XICs) of the protonated molecules and are given for each of the six components. It can be seen that the use of XICs greatly increases the DLs, due to the fact that most of the noise is removed from the data.

Table 1
Structural assignments of the peaks based on LC-MS data and other techniques where indicated

| Component | A[a] | B | C | D | E[a] | F (SKF-99085) | G[a] |
|---|---|---|---|---|---|---|---|
| Structure |  |  |  |  |  |  |  |
| Mol. formula | $C_{13}H_{30}O_6P_2$ | $C_{28}H_{56}O_7P_2$ | $C_{28}H_{58}O_7P_2$ | $C_{28}H_{58}O_7P_2$ | $C_{28}H_{58}O_7P_2$ | $C_{28}H_{52}O_7P_2$ | $C_{28}H_{56}O_7P_2$ |
| Mol. weight | 344 | 566 | 568 | 568 | 568 | 562 | 566 |
| Technique | LC-MS | LC-MS | LC-MS | LC-MS | LC-MS, LC-NMR ^{1}H NMR, ^{13}C NMR | LC-MS | LC-MS, LC-NMR ^{1}H NMR, ^{13}C NMR |

| Component | H[a] | I | J[a] | K[b] | L[b] | M[a] |
|---|---|---|---|---|---|---|
| Structure |  |  |  |  |  |  |
| Mol. formula | $C_{28}H_{56}O_7P_2$ | $C_{28}H_{56}O_7P_2$ | $C_{28}H_{52}O_6P_2$ | $C_{28}H_{56}O_6P_2$ | $C_{28}H_{56}O_6P_2$ | $C_{28}H_{58}O_6P_2$ |
| Mol. weight | 566 | 566 | 546 | 550 | 550 | 552 |
| Technique | LC-MS, LC-NMR ^{1}H NMR, ^{13}C NMR | LC-MS | LC-MS ^{1}H NMR, ^{13}C NMR | LC-MS | LC-MS | LC-MS, ^{1}H NMR |

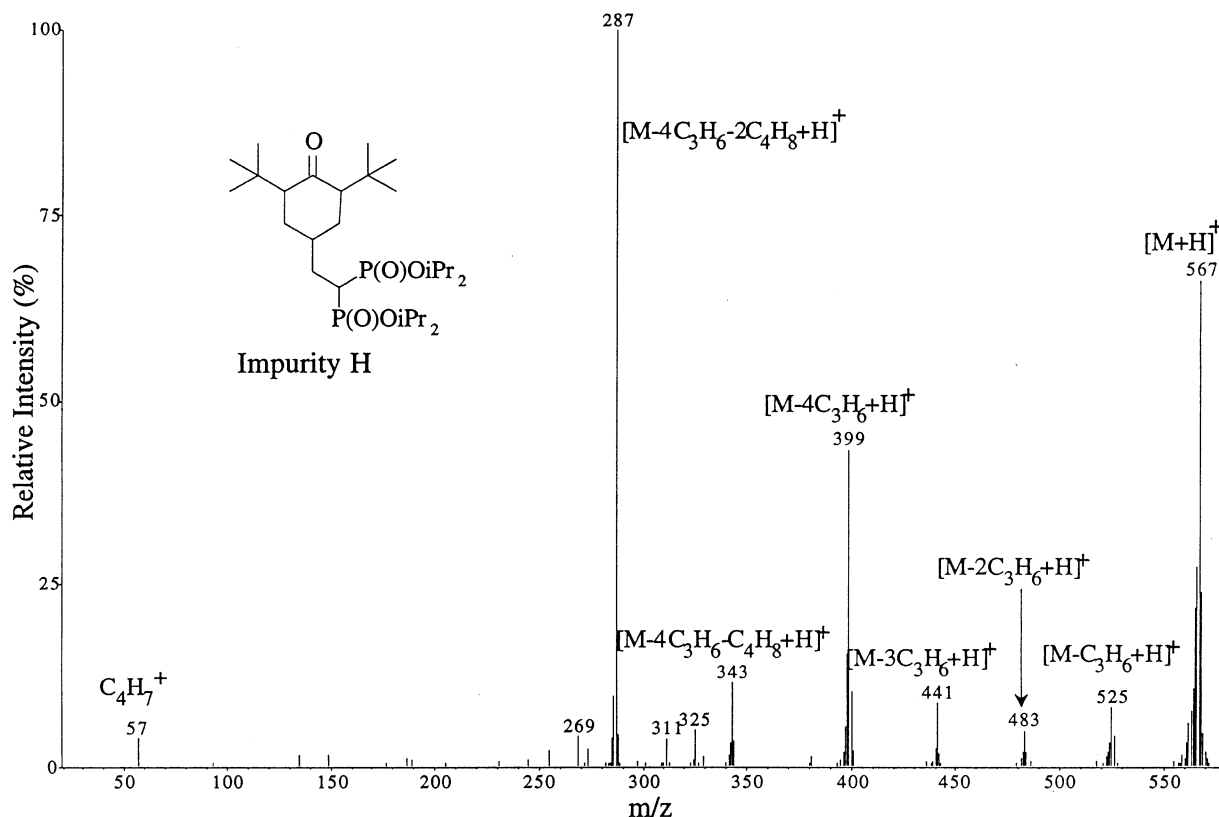[a] Isolated by preparative-LC    [b] Position of double bond not confirmed

R = 
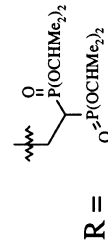
Fig. 4. Electrospray LC-MS-MS spectrum of Impurity H.

### 3.3.2. Refractive index detection

RI detection was used to detect all the major components in the mother liquor sample using isocratic LC conditions (Fig. 5(b)).

To assess detection sensitivity, DLs were determined on authentic impurity samples, in the absence of SKF-99085, at ca 20–280 µg ml$^{-1}$ and were compared with MS and ELS values (Table 3). The DLs using ELS are considerably lower (at least ten times) than when using RI. However, MS detection is far superior to ELS.

RI response factors were similar for these impurities (Table 2) and close to unity compared with SKF-99085 and consequently advantageous for impurity profile analysis based on peak area measurements.

### 3.3.3. Evaporative light scattering detection

Evaporative light scattering detection was directly compared with MS and RI under the same chromatographic conditions (Fig. 5(c)). All the sample components could be detected (except component A) with good sensitivity. It is likely that component A was not detected because of its high volatility.

A separation of SKF-99085 from its reduction impurities was performed using ELS with gradient elution (see RI detection) with a view to improving detection limits of the late-eluting impurities and developing a routine method. Fig. 6 shows a series of separations showing SKF-99085 in the presence of spiked impurities at three concentration levels (SKF-99085 overloads the detector with this detection sensitivity). The detection limits ($3 \times$ noise) for the impurities were found to be between 5 and 10 mg ml$^{-1}$, equivalent to ca 0.1–0.2% w/w with respect to SKF-99085. The detection limit data in Table 3 show that good sensitivity was achieved typical of that required for low level impurity analysis.
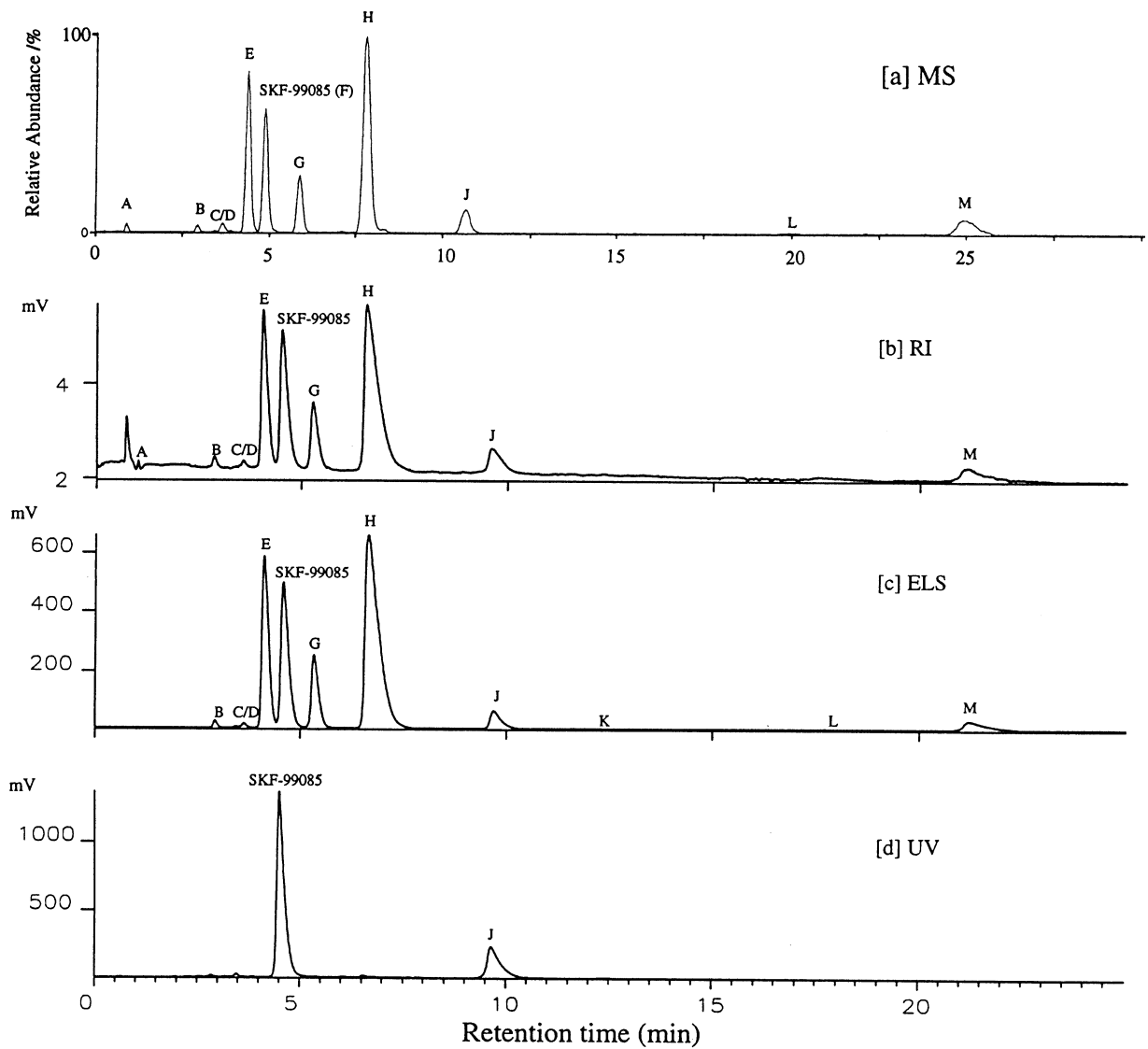
Fig. 5. Comparison of: (a) MS; (b) RI; (c) ELS; and (d) UV$_{220}$; detection using isocratic LC elution of the mother liquor sample. Conditions: Nova-Pak C$_{18}$ (15 cm × 3.9 mm i.d., 4 μm) at 40°C, elution with acetonitrile–water (70:30 v/v), 1.5 ml min$^{-1}$, 20 μl injection, concentration for MS 10 μg ml$^{-1}$, concentration for RI, ELS and UV220 10 mg ml$^{-1}$.

Table 2
Comparison of MS, RI and ELS response factors for SKF-99085 and its reduction impurities

| Detector | Response factor | | | | | |
| | Impurity E | SKF-99085 | Impurity G | Impurity H | Impurity J | Impurity M |
| --- | --- | --- | --- | --- | --- | --- |
| MS[a] | 1.19 | 1.00 | 1.20 | 0.98 | 0.90 | 0.90 |
| RI[a] | 1.15 | 1.00 | 1.23 | 1.22 | 1.15 | 1.37 |
| ELS[a,b] | 0.88 and 1.02 | 1.00 | 1.18 and 1.10 | 1.25 and 1.20 | 2.4 and 2.3 | 4.2 and 5.0 |

[a] Under isocratic elution conditions.
[b] Measured at two different concentrations (0.025 and 0.05 mg ml$^{-1}$).

Table 3

Comparison of MS, RI and ELS detection limits (DLs) for SKF-99085 reduction impurities

| Detector | Detection limit ($\mu g\ ml^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Impurity E | Impurity G | Impurity H | Impurity J | Impurity M |
| MS[a,b] | 0.6 | 0.7 | 0.8 | 0.7 | 2.0 |
| MS[a,c] | 0.04 | 0.02 | 0.06 | 0.06 | 0.04 |
| RI[a] | 20 | 30 | 40 | 110 | 280 |
| ELS[a] | 1.4 | 1.8 | 1.8 | 3.3 | 13 |
| ELS[d] | 5 | 10 | 7 | 6 | 4 |

[a] Isocratic elution (in the absence of SKF-99085) using a 20 μl injection volume.
[b] DLs obtained from total ion chromatograms.
[c] DLs obtained from extracted ion chromatograms.
[d] Gradient elution (in the presence of SKF-99085 at 5 mg ml$^{-1}$) using a 5 μl injection volume.

Hopia and Ollilainen compared ELS with RI detection for some lipids and found that detector sensitivity was comparable; response factors were more variable for RI than ELS [14]. In a comparative study of low level detection of impurities in ursodeoxycholic acid by Roda et al., RI was slightly superior compared with ELS [16]. In addition, RI detection was preferred for quantitation purposes because of its wide linear dynamic range.

The ELS detector response was determined for the reduction impurities (using authentic impurity samples) and was compared with SKF-99085. The detector response is non-linear and follows an exponential curve which makes a direct comparison of response data difficult; the calibration curves were found to be broadly similar for these components (Fig. 7). As an illustration, response factors for ELS (at two levels) are compared to MS and RI in Table 2.

### 3.3.4. Comparison of quantitative data

A comparison of the quantitative data obtained on the mother liquor sample with MS, RI and ELS detection is presented in Table 4. Reasonable agreement with respect to area percent measurements (%Peak Area Ratio or %PAR) was found for the different detectors for this complex sample. For all detection systems the largest component (H) was on-scale.

For drug substance analysis low level impurities are generally related to the drug substance itself using %PAR measurements. Response factors (if known) are used to adjust peak areas for poorly responsive impurities. Sometimes, external impurity standards are used (if available) to determine the % w/w assay for an individual impurity, especially if it is of toxicological importance. Often the working range of the detector is insufficient to allow the drug substance (typically very pure > 98.0% w/w) to be on-scale while detecting low level impurities ($\leq 0.2\%$ w/w) in the same chromatographic analysis. The method of 'high-low chromatography' reported by Inman and Tenbarge can be used to overcome this problem by analysing two sample solutions (one concentrated and one dilute) and relating peak areas [30]. This is a simple operation when using linear responsive detectors (e.g. UV and RI) but is more complex for the ELS. However, peak area data can be linearised using logarithmic regression (log peak area vs log analyte concentration). To determine impurity levels with MS detection, %PAR measurements can be made by comparing the extracted ion chromatograms of the drug substance and the impurity of interest. The 'detector range' is sufficiently wide to enable 0.1 %PAR impurities to be determined.

### 3.3.5. Indirect detection

In this example indirect detection was used to see whether it was possible to screen drug substance for weakly or non-chromophoric components. Indirect detection has been theoretically
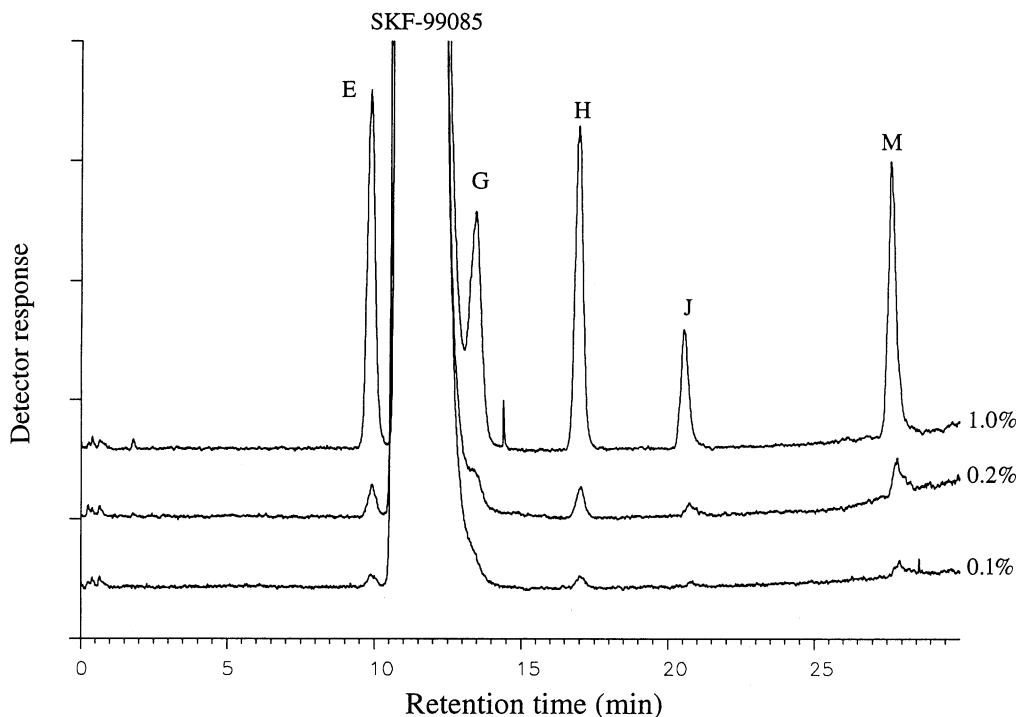
Fig. 6. Separation of SKF-99085 from spiked impurities (E, G, H, J and M at about the 0.1, 0.2 and 1.0% w/w level) using ELS detection. Main peak is off-scale. Conditions: Nova-Pak $C_{18}$ (15 cm × 3.9 mm i.d., 4 μm) at 40°C, elution with acetonitrile–water (60:40 v/v for 10 min) then to acetonitrile–water (90:10 v/v over 20 min), 1.5 ml $min^{-1}$, 5 μl injection, SKF-99085 concentration 5 mg $ml^{-1}$.

described [31,32] and has been used in applications with isocratic chromatographic systems [33,34]. For this separation isocratic LC conditions were used to separate the major components of the mother liquor sample. Several compounds were tested including benzophenone, fluoranthene and 2,4-dihydroxybenzoic acid for suitability as probe molecules. With these compounds it was found that residual UV absorption from some of the mother liquor sample components swamped the small indirect response even at long wavelengths. The best results were obtained with quinizarin (25 mg $l^{-1}$ in mobile phase) which has a strong absorption at long wavelength (480 nm). Under these conditions, it was possible to detect some of the sample components (Fig. 8(a)). RI detection was used in series to monitor the separation and was capable of detecting all the sample components (Fig. 8(b)).

Indirect detection for this example was found to be ten times less sensitive than RI (based on peak height of Impurity H). Researchers have commented that for indirect detection in non-ionic systems, better detection sensitivities are obtained on samples having few components and sensitivities are much better for ionic analytes in ionic chromatographic systems [32,33].

### 3.3.6. Chromatographic system stability

To carry out low level impurity analysis, the chromatographic and detection systems must be stable. Background blank artifacts must be minimised so that low level impurities can be easily distinguished. Both indirect detection and RI suffer from minor perturbations caused by pressure and temperature fluctuations, e.g. Fig. 8(a) shows baseline instability typical of pressure pulsations. Fig. 5(b) shows the detector signal drift typical of RI detection. ELS stability is superior in that it
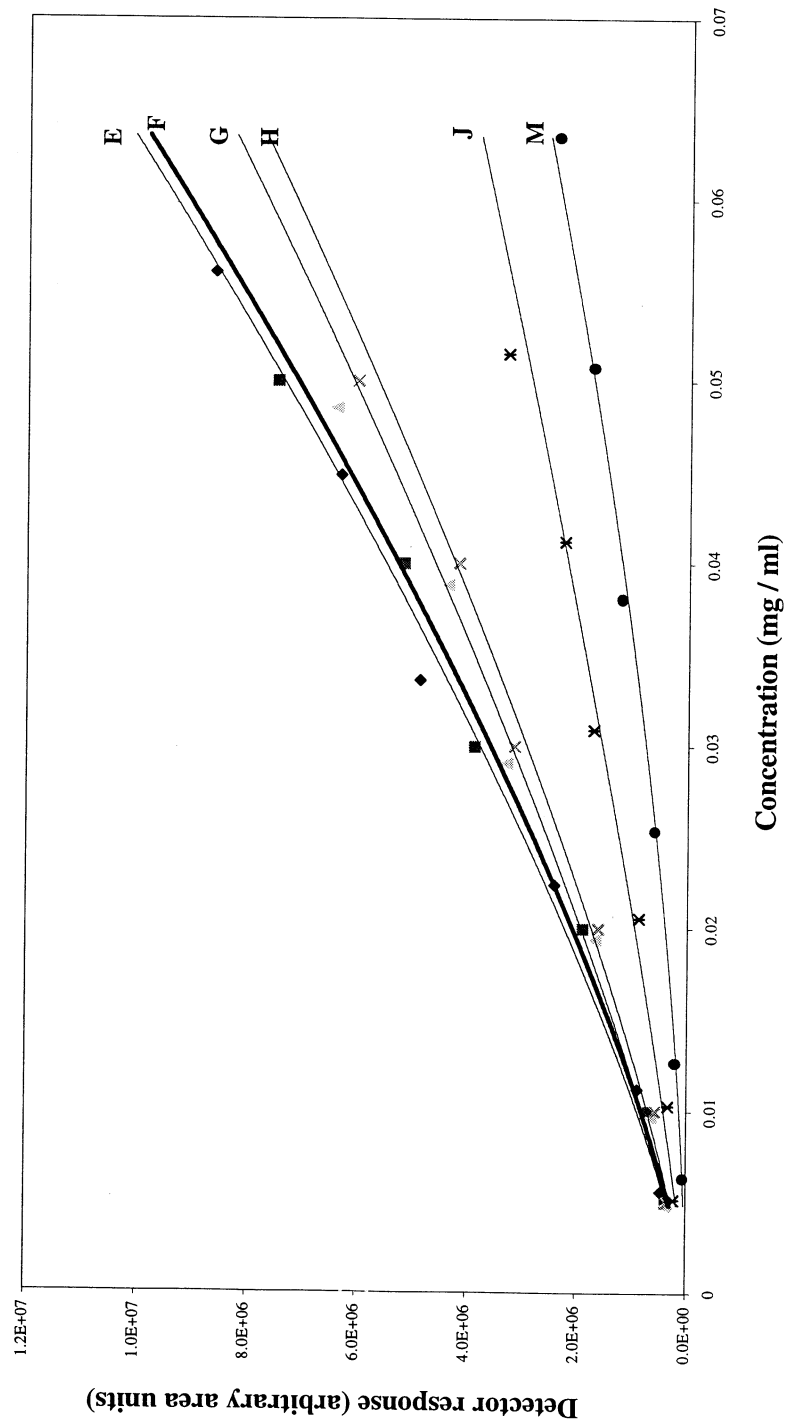
Fig. 7. ELS calibration curves comparing detector response of SKF-99085 with its reduction impurities.

Table 4
Comparison of %PAR data for the major sample components of the mother liquor sample using MS, RI and ELS detection

| Detector | % PAR | | | | | |
|---|---|---|---|---|---|---|
| | Component E | SKF-99085 | Component G | Component H | Component J | Component M |
| MS[a] | 20.3 | 17.0 | 8.5 | 38.0 | 4.6 | 9.2 |
| RI | 27.2 | 23.8 | 11.4 | 28.6 | 4.1 | 2.1 |
| ELS | 27.6 | 23.3 | 11.7 | 31.0 | 2.8 | 1.4 |

[a] Calculated from the total ion chromatogram. Chromatographic conditions are the same as Fig. 5.

does not suffer from baseline disturbances caused by solvent contaminants and system peaks. The baseline quality observed in Fig. 6 is typical.

## 4. Conclusion

LC-MS, LC-NMR, ELS, RI and indirect detection were used to identify and characterise the reduction impurities in SKF-99085. Table 5 illustrates the utility of the detection methods used.

MS and RI could detect all the mother liquor sample components. ELS detection was capable of detecting all sample components except component A. Indirect detection was quite poor. Excellent structural data were obtained from LC-MS experiments. Supporting structural assignments were made from LC-NMR data, however the analysis could not unequivocally distinguish isomeric impurities (C/D/E and G/H/I) due to sensitivity limitations.

ELS detection using gradient elution was found to be the most appropriate chromatographic system for the routine quantitative determination of non-UV low level impurities for this class of drug and for screening mother liquor samples. It was possible to monitor for non-UV absorbing impurities having a wide polarity range with sensitivity typical of that required for impurity analysis. The response factors obtained from calibration curves were broadly similar for the impurities compared with the drug substance making quantitation easier with respect to peak area measurements. However, impurity responses by MS and RI were much closer and thus would be preferred for quantitative work though the high-low chromatography approach needs to be adopted in all

cases because of the limited ranges of the detectors. The RI detector has good range but because of its inherent insensitivity (see detection limits) it is easy to exceed the capacity of the column and compromise chromatographic performance rather than overload the detector-ELS is preferred over RI in this case. MS, RI and ELS were used for the quantitative assessment of sample compositions, e.g. mother liquors, because of the similarity of detector responses for the sample components the results gave acceptable agreement. The detection limits are low for ELS and MS detection with MS being the most sensitive of the detection methods.

The set up of the ELS is simple (nebuliser gas flow and evaporation temperature can be adjusted to enhance signal-to-noise ratio) and quick. No additional precautions need be taken compared with RI, which is inherently problematical, or indirect detection, which can be too involved for general analysis. High performance LC-MS-MS instrumentation is still expensive although benchtop single stage LC-MS systems are now becoming user-friendly, cheaper and widespread.

In this study the ELS detector was found to be a useful means of detection of poor UV responding solutes on a routine basis. RI or preferably LC-MS is needed as a complementary technique to be able to fully characterise samples at the early method development stage. However, each detection method has drawbacks. For example, ELS detection is only possible for solutes that are less volatile than the mobile phase, LC-MS detection requires that a solute be ionised (for electrospray) and the sensitivity of RI detection is low. For this case, there was no single ideal detection system for impurity analysis. Table 6 summarises

Table 5
Comparison of detector utility

|  | MS | NMR | RI | ELS | UV | Indirect |
|---|---|---|---|---|---|---|
| Component detectability (number of components detected in mother liquor sample, Total = 13) | All | 4 | All | 12 | 2 | 3 |
| Quantitation of impurities | Possible | N/A | Yes | Yes | No | No |
| Impurity response factors (area basis) | Similar | N/A | Similar | Broadly similar[a] | N/A | N/A |
| % Range relative to SKF-99085 | 90–120% |  | 115  −137% | 88–500% |  |  |
| Detection sensitivity | Very high | Low | Low | High | High | Very low |
| Chromatographic system stability | Good | Good | Fair | Good | Good | Poor |

N/A, not applicable.
[a] The actual response factor is dependent on solute concentration.

Table 6
General comparison of the advantages and limitations for MS, RI, ELS and UV detectors

|  | MS (Electrospray) | RI | ELS | UV |
| --- | --- | --- | --- | --- |
| Ease of use/set up | Complex (tuning can be necessary) | Complex/lengthy equilibration | Relatively simple/gas flow and temperature adjustment required | Simple |
| Gradient elution | Yes | No | Yes | Yes |
| Incompatible mobile phases | Involatile buffers (>20 mM) | None | Involatile buffers | High absorptivity mobile phases |
| Non detectable analytes | Non-ionizable analytes | Analytes having same RI response as mobile phase | Analytes less volatile than the mobile phase | Non-chromophoric analytes. Analytes with chromophores <UV cut-off |
| Detector baseline stability | Stable but noisy | Drift and pressure effects | Stable | Stable |
| Detector baseline interferences | Chemical background noise from airborne contaminants | Incompatible injection solvents | None | Solvent artifacts with gradient elution. Solvent fronts |
| Detector response | Linear | Linear | Non-linear | Linear |
| Repeatability/precision | — | [16] | [16] | — |
| Detector range (orders of magnitude) | 2–3[a] | 3 | 2 ([35,36]) | 3 |
| Ease of quantitation/calibration | Simple | Simple | Involved ([35,36]) | Simple |

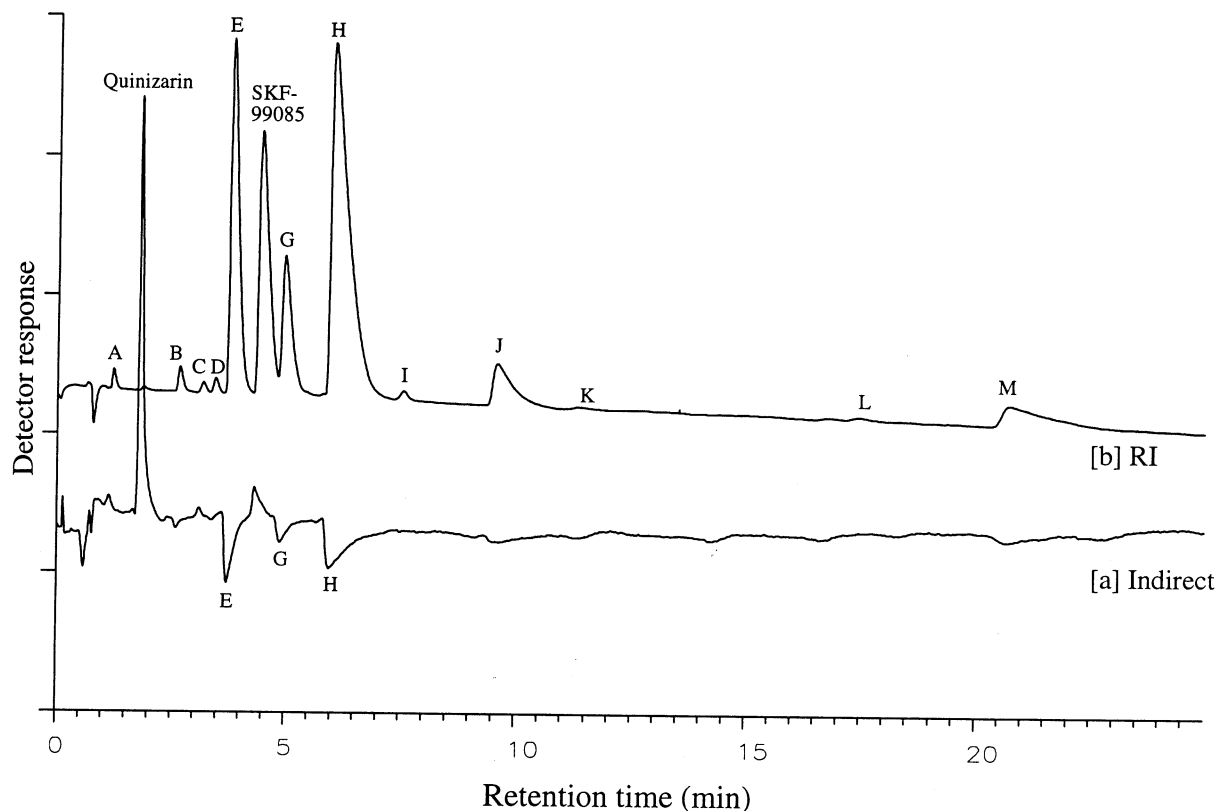[a] Specific to instrument and acquisition mode, e.g. full scan single ion monitoring.

Fig. 8. Comparison of (a) indirect and (b) RI detection using isocratic LC elution of the mother liquor sample. Conditions: Nova-Pak $C_{18}$ (15 cm × 3.9 mm i.d., 4 μm) at 40°C, elution with acetonitrile–water (70:30 v/v containing quinizarin at 25 mg $1^{-1}$), 1.5 ml $min^{-1}$, 20 μl injection. Indirect detection at 480 nm, concentration 10 mg $ml^{-1.}$

the advantages and limitations of the detection systems (including UV for comparison) applied to general drug substance impurity analysis.

## References

[1] P.R. Fielden, J. Chromatogr. Sci. 30 (1992) 45–52.
[2] J. Crommen, J. Pharm. Biomed. Anal. 1 (1983) 549–555.
[3] J. Crommen, P. Herné, J. Pharm. Biomed. Anal. 2 (1984) 241–253.
[4] P. Camilleri, P. Thorpe, J. Chromatogr. 519 (1990) 387–390.
[5] C. Leung, W. Leung, J. Chromatogr. 479 (1989) 361–367.
[6] R. George, M. Masters, Chromatogr. Anal. Feb 41 (1996) 9–11.
[7] J. Nair, J. Chromatogr. 671 (1994) 367–374.
[8] R. Srinivas, L. Igwemezie, Biomed. Chromatogr. 6 (1992) 163–167.
[9] G. Iwinski, D.R. Jenke, J. Chromatogr. 392 (1987) 397–405.
[10] E.J. Benjamin, D.L. Conley, Int. J. Pharm. 13 (1983) 205–217.
[11] M. Lafosse, C. Elfakir, L. Morin-Allory, M. Dreux, J. High Res. Chromatogr. 15 (1992) 312–318.
[12] P.A. Asmus, J.B. Landis, J. Chromatogr. 316 (1984) 461–472.
[13] D.S. Ridley, J.A. Peterson, J. Liq. Chromatogr. 18 (1995) 3035–3048.

[14] A.I. Hopia, V.M. Ollilainen, J. Liq. Chromatogr. 16 (1993) 2469–2482.

[15] M.K. Park, J.H. Park, S.B. Han, Y.G. Shen, I.H. Park, J. Chromatogr. 736 (1996) 77–81.

[16] A. Roda, R. Gatti, V. Cavrini, C. Cerrè, P. Simoni, J. Pharm. Biomed. Anal. 11 (1993) 751–760.

[17] M.D. Lantz, D.S. Risley, J.A. Peterson, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 1409–1422.

[18] S. Pleasance, J.F. Anacleto, M.K. Bailey, D.H. North, J. Am. Soc. Mass Spectrom. 3 (1992) 378–397.

[19] H. Iwabuchi, E. Kitazawa, N. Kobayishi, H. Watanabe, M. Kanai, K. Nakamura, Biol. Mass Spectrom. 23 (1994) 540–546.

[20] A.L. Yergey, C.G. Edwards, I.A.S. Lewis, M.L. Vestal, Liquid Chromatography/Mass Spectrometry Techniques and Applications, Plenum, New York, 1990.

[21] W. Neiseen, J. van der Greef, Liquid Chromatography-Mass Spectrometry, Marcel Dekker, New York, 1994.

[22] M. Spraul, M. Hofman, P. Dvortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem. 65 (1993) 327–330.

[23] J.K. Roberts, R.J. Smith, J. Chromatogr. 677 (1994) 385–389.

[24] M. Spraul, M. Hoffman, P. Dvortsak, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 11 (1993) 1009–1015.

[25] R.M. Holt, M.J. Newman, F.S. Pullen, D.S. Richards, A.G. Swanson, J. Mass Spectrom. 32 (1997) 64–70.

[26] O. Zerbinati, R. Aigotti, P.G. Daniele, J. Chromatogr. 671 (1994) 281–285.

[27] G. Cepria, J.R. Castillo, J. Chromatogr. 759 (1997) 27–35.

[28] European Patent No. 339237, 1989.

[29] B. Kaye, M.W.H. Clark, N.J. Cussans, P.V. Macrae, D.A. Stopher, Biol. Mass Spectrom. 21 (1992) 585–589.

[30] E.L. Inman, H.J. Tenbarge, J. Chromatogr. Sci. 26 (1988) 89–94.

[31] J. Crommen, G. Schill, D. Westerlund, L. Hackzell, Chromatographia 24 (1987) 252–260.

[32] J. Crommen, G. Schill, P. Herné, Chromatographia 25 (1988) 397–403.

[33] G. Schill, E. Arvidsson, J. Chromatogr. 492 (1989) 299–318.

[34] G. Schill, J. Crommen, Trends Anal. Chem. 6 (1987) 111–116.

[35] C.E. Kibbey, Mol. Divers. 1 (1995) 247–258.

[36] V.L. Cebolla, L. Membrado, J. Vela, A.C. Ferrando, J. Chromatogr. Sci. 35 (1997) 141–150.

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