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Detection of non-UV-absorbing chiral compounds by high-performance liquid chromatography–mass spectrometry

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Abstract

Confirmation of chiral purity is usually carried out via HPLC separation. The mobile phases required to effect good separation, using polar organic phase chromatography on cyclodextrin columns, result in poor detection when the compounds of interest do not have strong UV chromophores. We have investigated the use of HPLC–MS techniques to develop a suitable method of detection for compounds which have UV chromophores absorbing below the UV cut-off wavelength of the mobile phase (~240 nm). This paper describes the development of a robust and reliable, analytical, chiral LC–MS system based on the use of plasmaspray mass spectrometry.

Keywords: Enantiomer separation; Chlorowarfarin; Suprofen

1. Introduction

An increasing number of compounds of therapeutic interest are being developed as single enantiomers. Therefore the need to determine chiral purity is becoming more important within the pharmaceutical industry.

Confirmation of chiral purity is usually carried out via HPLC separation. However, the mobile phases required to effect good separation, using polar organic phase chromatography on cyclodextrin columns, present the chromatographer with a number of problems. The major problem is the HPLC detection of compounds which do not have UV chromophores or have UV chromophores absorbing below the UV cut-off wavelength of the mobile phase (≈240 nm). The use of refractive index and optical rotation detectors is possible when sample size is not an issue. They often lack the sensitivity and specificity required when small sample sizes or low concentrations are encountered in, for example pharmacokinetic studies. To overcome this problem a variety of LC–MS techniques were investigated.

The mobile phase composition is a critical factor in maintaining the enantiomeric selectivity of cyclodextrin columns in the polar organic mode and cannot be compromised to suit the mass spectrometry. A number of mass spectrometer interfaces were available but the options were somewhat restricted as most appeared to have some incompatibility with the mobile phase. The mobile phase proved to be too polar for the particle-beam interface to perform satisfactorily. Electrospray although well suited to the polar conditions showed poor sensitivity in the presence of triethylamine. The chromatography could not be adapted in order to include a suitable buffer for thermospray. In the absence of a buffer the mobile phase inhibited ionisation by

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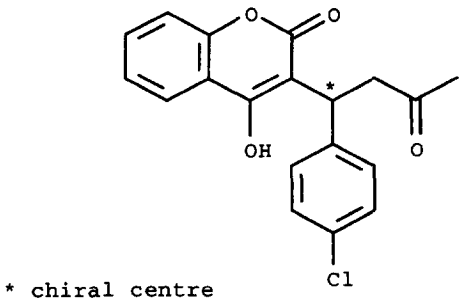

Fig. 1. *p*-Chlorowarfarin.

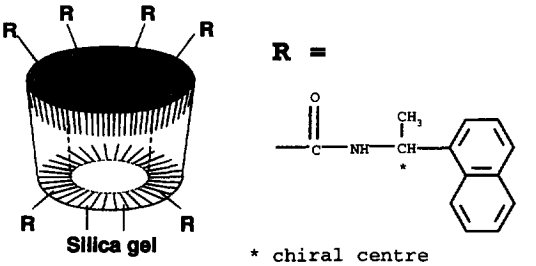

Fig. 3. Beta-cyclodextrin cavity showing the r-naphthylethyl carbamate derivative.

thermospray. Plasmaspray ionisation, sometimes referred to as discharge ionisation [1], which is an option on our thermospray source usually employed to induce fragmentation, proved to be a robust and reliable ionisation method.

The method developed has been used to determine the chiral purity of a number of proprietary compounds. For this reason the initial work has been repeated using two commonly available compounds which serve as good examples of the method described. The first, *p*-chlorowarfarin (Fig. 1), has similar properties to warfarin and is also used as a rodenticide [2].

Since the λ_{max} for *p*-chlorowarfarin is 207 nm (below the 240 nm cut-off for the mobile phase) UV detection is impractical. However, one would anticipate unambiguous mass spectra because it has a relative molecular mass (M_r) of 342 and a distinctive 100:22:35:7 isotope pattern, typical of monochlorinated compounds.

The second compound, suprofen (Fig. 2), is a pharmaceutical compound administered as eye drops to inhibit miosis during eye surgery; oral use of this compound for its analgesic, anti-inflammatory and antipyretic properties ceased due to adverse renal reactions [3].

The λ_{max} for suprofen is 205 nm. This compound has a relative molecular mass of 260 and a 100:16:6 isotope pattern which is somewhat less distinctive than *p*-chlorowarfarin.

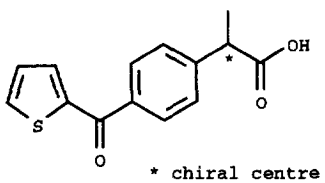

Fig. 2. Suprofen.

2. Background

2.1. Chromatography

The separation technique has been termed the polar organic mode due to the glacial acetic acid and triethylamine in the mobile phase. No water is included. The triethylamine was distilled from potassium hydroxide to ensure quality and dryness of the product.

It has been found [4] that each component of the mobile phase has a role on the final separation. Acetonitrile is the most abundant solvent, usually present at 90% or greater by volume. Methanol essentially controls the retention time, whereby increasing the concentration of methanol, hence increasing the polarity of the eluent, shortens the amount of time the compounds are on the column.

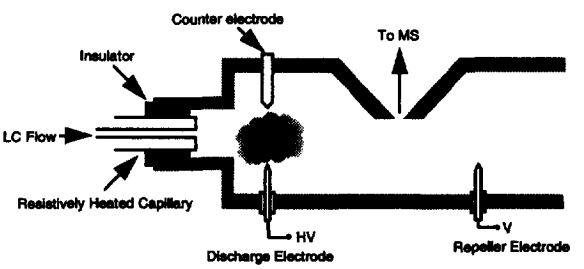

Fig. 4. Schematic diagram of the thermospray ion source. The discharge electrode allows this source to be operated in plasmaspray mode.

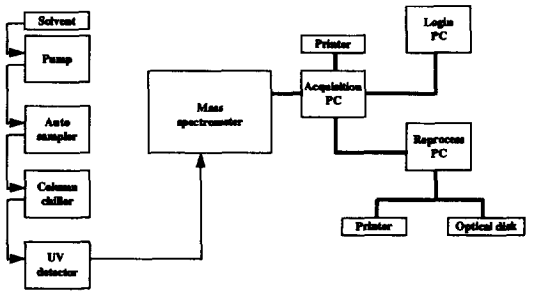

Fig. 5. Block diagram showing the complete system.

The concentration of acid and base also affects retention, the higher the concentration, the earlier the compound elutes.

The enantioselectivity of the separation [5] is governed by the ratio of acetic acid to triethylamine. Both acid and base are required to achieve a chiral separation and the ratio is critical. Temperature also has an effect on resolution. It was found that decreasing the temperature increased the separation in both the examples. The size of the cyclodextrin cavity [6] and the cyclodextrin derivative used is crucial. The two examples required the r-naphthylethyl carbamate β-cyclodextrin as shown in Fig. 3.

The mechanism of retention in the polar organic mode is not yet fully understood, but it does appear to rely on hydrogen bonding. Both *p*-chlorowarfarin and suprofen have a hydrogen donor group and a hydrogen acceptor group on the side chain containing the chiral centre.

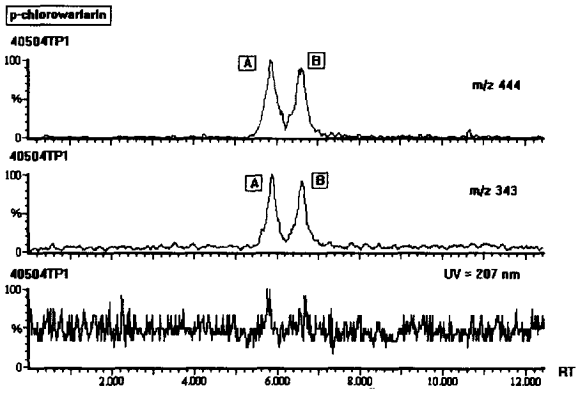

Fig. 6. Mass chromatograms of *m/z* 444 and *m/z* 343 and UV trace at 207 nm for *p*-chlorowarfarin.

2.2. Mass spectrometry

Plasmaspray ionisation is commonly used to induce fragmentation and thereby provide structural information as an aid to sample identification. This complements the molecular mass information obtained by thermospray. The constraints imposed by the mobile phases developed for these chiral separations necessitated the use of plasmaspray as the ionisation technique to obtain useful mass spectral data. The flow-rate required for these methods (0.5 ml/min) is on the lower limit of the normal working range (0.5–1.5 ml/min) of this interface for both plasmaspray and thermospray modes.

The thermospray interface on our mass spectrometer has the facility to be used in plasmaspray mode by the application of a current to the discharge electrode. The electrical discharge in the solvent vapour creates a low current plasma between the discharge and counter electrodes (Fig. 4). The plasma is a region in which positive ions, negative ions, and electrons co-exist. Ionisation in the plasmaspray mode occurs as the sample passes through the plasma. The CI (chemical ionisation) like conditions at high plasma currents present 'harder' ionisation conditions, resulting in fragmentation of the sample molecules. In order to utilise plasmaspray as a 'soft' ionisation rather than fragmentation technique the source must be carefully tuned to minimise the plasma current required to achieve ionisation.

3. Experimental

3.1. Materials

(±)-3-(α-Acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin, referred to as *p*-chlorowarfarin, (±)-suprofen, also known as (α-methyl-*p*-[thienoyl]phenylacetic acid) and triethylamine were purchased from Sigma, Dorset, UK. The acetonitrile and methanol used were HPLC-grade solvents purchased from Romil, Cambridge, UK. The glacial acetic acid and potassium hydroxide were AnalaR grade from BDH, Leics., UK.

The 2000 series r-naphthylethyl carbamate derivative (RN) of the β-cyclodextrin bonded phase col-

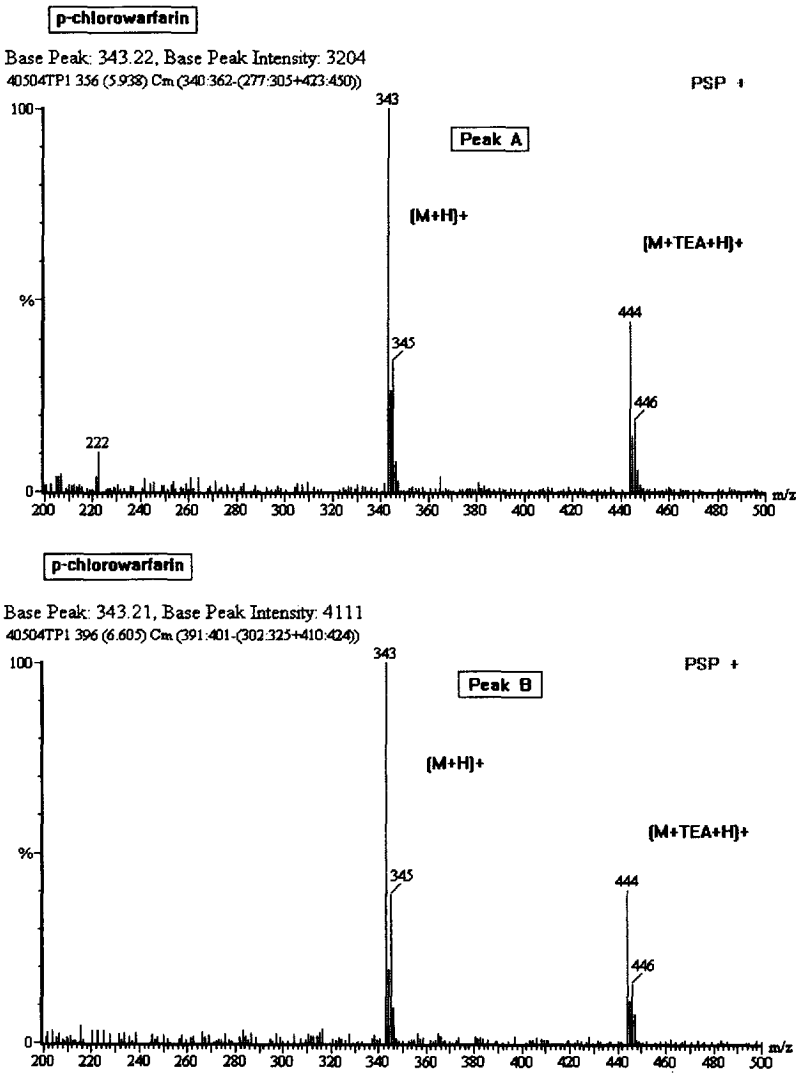

Fig. 7. Mass spectra of both *p*-chlorowarfarin enantiomers (peaks A and B).

umns was used. The column was obtained from BASTechnicol, Cheshire, UK.

3.2. Chromatographic method

3.2.1. Example 1: p-chlorowarfarin

The cyclobond 1 2000 RN column was 25 cm× 0.46 cm internal diameter. The premixed eluent contained acetonitrile–glacial acetic acid–triethylamine in the ratios (99.8:0.1:0.075). A flow-rate of 0.5 ml/min and a column temperature of 0°C was required.

3.2.2. Example 2: suprofen

The cyclobond 1 2000 RN column was 25 cm× 0.46 cm internal diameter. The premixed mobile phase contained acetonitrile–methanol–glacial acetic acid–triethylamine in the ratios (95:5:0.2:0.2). A flow-rate of 0.5 ml/min and a column temperature of 0°C was required.

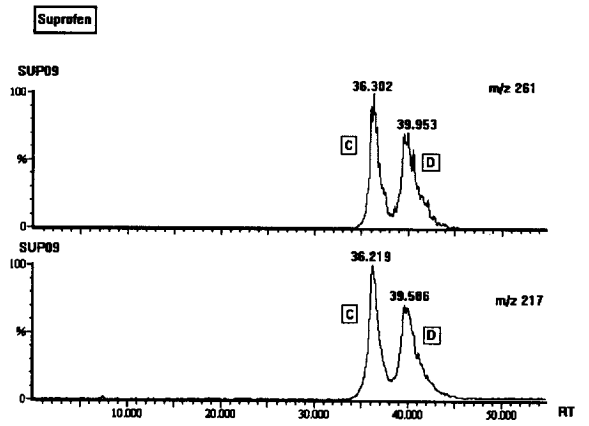

Fig. 8. Mass chromatograms of m/z 261 and m/z 217 for suprofen.

3.3. Mass spectrometry method

3.3.1. Example 1: p-chlorowarfarin

Thermospray interface, positive ion plasmaspray ionisation mode. Mass scan range 150–500 u, scan time 0.9 s and interscan delay of 0.1 s. Plasma electrode current 300 μA and capillary nozzle temperature 220°C.

3.3.2. Example 2: suprofen

Thermospray interface, positive ion plasmaspray ionisation mode. Mass scan range 150–800 u, scan time 0.9 s and interscan delay of 0.1 s. Plasma electrode current 400 μA and capillary nozzle temperature 180°C.

The mass spectrometer was tuned initially on solvent cluster ions e.g. $(CH_3CN)_2H^+$ and $(Et_3N)H^+$ at $m/z=83$ and $m/z=102$ respectively (see section 2.2 for mobile phase details). Fine tuning was then achieved by carrying out loop injections of the sample and optimising on the protonated molecular ions. Once tuned, the system was set up to run in automation mode.

3.4. System description

The complete system is shown in Fig. 5 and consists of the following:

Trio 1000 mass spectrometer, 2–2000 u analyser, fitted with a thermospray interface used in plasmaspray mode. (Fisons Instruments, Wythenshawe, Manchester, UK)

Login computer, 20 MHz 80386, 4.0 MB RAM, 80 MB hard disk. Acquisition and reprocessing computers, 33 MHz 80486, 4.0 MB RAM, 760 MB hard disk (Intel UK, Swindon, Wiltshire, UK). Optical disk, 1GB WORM, Panasonic LF 7010E (Matsushita Electric Industrial, Kadoma, Osaka, Japan). Postscript printer, Oki OL850 (Oki Systems, Slough, UK). Network, Netware Lite Client Version 1.1 (Novell, Hounslow, UK.). Mass spectrometer software, Lab Base V2.14 (Fisons Instruments).

HP1050 HPLC system comprising of quaternary pump, multiwavelength detector and 100 vial auto sampler (Hewlett Packard, Bracknell, Berkshire, UK)HPLC column chiller, model 7950 (Jones Chromatography, Hengoed, Mid Glamorgan, UK)

4. Discussion

4.1. p-Chlorowarfarin

The peaks observed (Fig. 6) correspond to the two *p*-chlorowarfarin enantiomers and have retention times of 5.9 and 6.6 min. A comparison of the UV trace at 207 nm and the mass chromatograms of the $(M+H)^+$ at $m/z=343$ and $(M+TEA+H)^+$ ions at $m/z=444$, shows the much improved sensitivity obtained using the plasmaspray method described.

The mass spectra (Fig. 7), above 200 u, of both enantiomers are dominated by the protonated molecular ion and the protonated triethylamine adduct ions. The presence of these ions allows the M_r of the eluting compounds to be verified with confidence. In this case, the chromatographic necessity to use triethylamine in the mobile phase, which inhibited the use of other LC–MS interfaces, proved to be an advantage with plasmaspray ionisation.

The isotope pattern for both sets of ions closely matches the theoretical 100:22:35:7 ratio expected for *p*-chlorowarfarin, further verifying the identity of the enantiomers.

4.2. Suprofen

The peaks (Fig. 8) corresponding to the two suprofen enantiomers were observed at retention

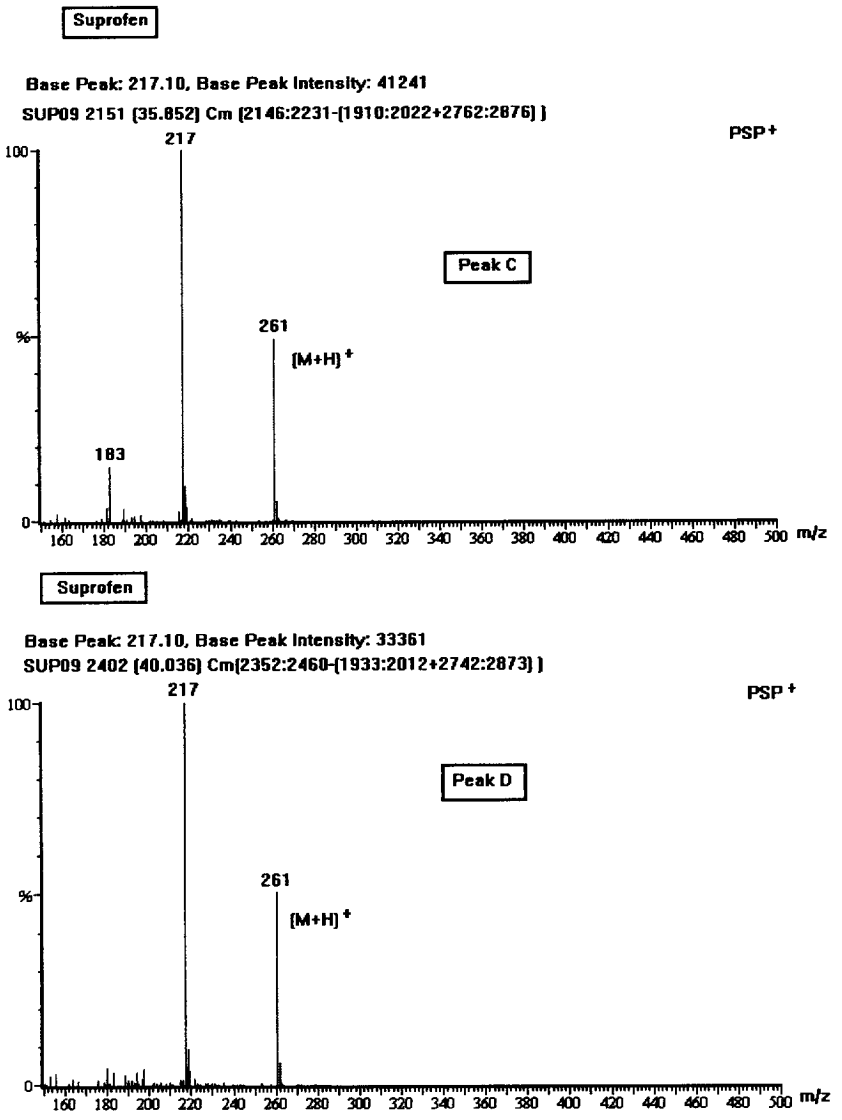

Fig. 9. Mass spectra of both suprofen enantiomers (peaks C and D).

times of 35.9 and 40.0 min. The mass chromatograms of the $(M+H)^+$ at $m/z=261$ and $(M-CO_2+H)^+$ ions at $m/z=217$, also show good sensitivity. The mass spectra (Fig. 9) above 150 u. are dominated by both of these ions. The loss of CO_2 from suprofen must be facile since it gives rise to the base peak in the mass spectrum. Whether this ion at $m/z=217$ is the result of post-ionisation fragmentation or pre-ionisation thermal degradation in the ion source of the mass spectrometer is uncertain.

Although the isotope pattern for suprofen matches the theoretical 100:16:6 ratio, it is similar to other organic compounds of the same relative molecular mass and is of little assistance in identifying the compound. The presence of the $(M-CO_2+H)^+$ ion in the mass spectrum provides an additional charac-

teristic component which further increases the selectivity of this method for suprofen.

5. Conclusion

We have developed an HPLC–MS method for the detection of non-UV-absorbing chiral compounds. While mass spectrometry as a detector for HPLC is common place, we believe that this is the first time it has been successfully coupled to polar organic phase chromatography. The method has been shown to have both greater sensitivity and selectivity than the UV detection for these analyses.

Relative molecular mass data adds to the retention time information but is insufficient to identify a compound with certainty. The combination of M_r, fragmentation and isotope pattern information is a much more powerful data set for compound identification. The availability of such a data set from plasmaspray mass spectrometry provide much greater selectivity than traditional detection methods.

The method has proved robust and reliable in our laboratories. It has been applied to a number of chiral purity determinations of compounds which do not have UV chromophores or where absorbance lies below the effective cut-off wavelength of the mobile phase. This method has the potential to be used in a wide range of polar organic mobile phase chromatography applications such as those described by Armstrong [4,7].

The ability to automate an analysis, is rapidly becoming a benchmark within the pharmaceutical industry [8,9] for a method's robustness and reliability. We are pleased to report that the results described in this paper were achieved in automation using our "Open Access" LC–MS system [10].

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