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Direct identification and quantitation of prednisone in the presence of overlapping hydrocortisone by liquid chromatography with electrospray and atmospheric-pressure chemical-ionisation mass spectrometry

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Abstract

The paper describes the application of liquid chromatography interfaced to a triple quadrupole mass spectrometer utilising the multiple reaction monitoring (MRM) mode. The technique was shown to provide detection limits lower than 0.01% for the analysis of prednisone in the presence of hydrocortisone. Prednisone was mixed in concentrations from 0.500 to 0.0005 ppm (corresponding to 1% to 0.001% of the hydrocortisone concentration). These solutions were assayed using MRM observing the product ion transitions of 359.2→147.1 and 359.2→171.2 and was shown to be capable of detecting co-eluting impurities at concentrations of less than 0.001% of the major component. The assay of prednisone was shown to be linear over the range 0.500–0.0005 ppm with a correlation coefficient of 0.999 and a precision of 6.9% at the concentration of 0.005 ppm. The analysis was carried out using both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) as an interface. However, for these compounds APCI provided significantly more sensitive data compared to ESI.

Keywords: Prednisone; Hydrocortisone

1. Introduction

The separation and quantitation of low-level impurities is an ongoing problem with contemporary chemical measurements. The failure to detect these impurities can cause many problems including toxic side effects from pharmaceutical products or costly trade disputes when impurities are only detected when products reach their export markets. The high-performance liquid chromatographic analysis of pharmaceutical products is subject to increasing regulatory controls that specify detailed testing of the analytical method performance. This paper utilises the tandem mass spectrometric capability afforded by the triple quadrupole mass spectrometer to demonstrate that extremely low concentrations of interfering impurities can be detected and quantified as part of a high-performance liquid chromatography (HPLC) selectivity test.

HPLC analysis requires the demonstration of selectivity to ensure that the chromatographic peak is homogeneous and no other interfering compounds co-elute. Many papers have been published on attempts to develop better methods of detecting co-eluting impurities [1–3]. Selectivity is commonly tested for using HPLC coupled with diode array

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detection (DAD) [4–6]. This method has been shown to be capable of detecting co-eluting impurities present at 1% of the compound of interest. However, UV spectra can be similar for different compounds and this substantially reduces the likelihood of detecting compounds that co-elute. The identification and quantitation of low level impurities using the conventional LC–DAD technique, often poses difficult analytical problems as it requires the UV absorption characteristics of the impurity to be slightly different from that of the major component and that some resolution exists between the analytes [3].

The US Food and Drug Administration (FDA) has recently expressed concern that the present detection limit of 1% is insufficient. Pharmaceutical impurities may be considerably more potent than the active ingredient, hence even at 1% of the active concentration impurities could lead to unwanted toxic side effects.

Mass spectrometry (MS) produces complex spectral profiles that are more likely to show differences between individual chemical species than UV spectroscopy. The coupling of HPLC and MS was significantly improved in terms of both sensitivity and the variety of compounds that can be analysed with the development of electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces. ESI and APCI are soft ionisation techniques that allow the MS analysis of large biomolecules such as polypeptides as well as non-volatile or thermally labile molecules under atmospheric pressure. The ESI process involves the use of electrical fields to generate charged droplets and subsequent analyte ions by ion evaporation for MS analysis [7]. APCI involves the conversion of the mobile phase and analyte from the liquid phase to the gas phase and then ionisation of the mobile phase and analyte molecules [8,9].

The coupling of LC with MS permits the analysis of samples that have traditionally been very difficult, such as, polar and/or thermally labile molecules and complex matrices, common in the pharmaceutical field. The coupling of LC to a triple quadrupole MS system, which enables MS–MS experiments to be performed, provides increased selectivity over LC–MS systems. In LC–MS–MS the sample is introduced into a conventional mass analyser. The spectrum provides information on the molecular ion and/or a fragmentation pattern which gives information about species present in the sample. The molecular ion peak pertaining to the compound of interest can then be directed to the second quadrupole. Here this ion is bombarded with nitrogen gas which depending on the pressure (collision energy) causes various degrees of fragmentation. This feature of LC–MS–MS allows the analysis to be extremely selective. By selecting one or two fragments characteristic of the impurity, multiple reaction monitoring (MRM) may be performed where the reactions that convert the $M+$ ion to the fragment ion(s) can be monitored. Thus the detector is able to analyse the impurity even in the presence of an excess of the major component [10,11]. This technique requires the structure of the impurity to be known and is not always possible during a peak purity test [12,13].

A few publications have described the application of LC–MS to the peak purity problem. The work of Lincoln et al. does not attempt to quantify the limits of detection achievable by LC–MS [14] and the work of Bryant et al. shows limits of detection of 0.1% of the active concentration [15].

2. Experimental

2.1. Instrumentation

Experimental work was carried out on a triple quadrupole mass spectrometer (Perkin-Elmer API 365 LC–MS–MS system, Sciex, Concord, Canada). A 20-μl volume of the sample solution was separated on a 150×3.9 mm, 5 μm Novapak C_{18} column at a flow-rate of 1.0 ml min^{-1}, using a 2:1 split prior to LC–MS detection. The mobile phase consisted of acetonitrile–methanol–water (36:4:60) [4]. The LC method was devised to ensure that co-elution would occur for hydrocortisone and its impurity, prednisone.

The potential on the sampling orifice of the instrument was set at +35 V during calibration and was raised to 100 V for the ''orifice-skimmer'' fragmentation. The instrument's m/z scale was calibrated with polypropylene glycol standards under unit m/z resolution and (50% valley definition). For

the optimised APCI-MS conditions, the nebuliser current was set at 2 A, temperature 450°C, orifice voltage 15 V and the ring voltage at 170 V. The MS–MS collision energy was set at 30 V, the Q1 MS ion energy was adjusted to 1 V and the MS–MS ion energy was set at 5 V. The collision gas was nitrogen and was set at a value of 4 units.

2.2. Chemicals

The compounds studied were hydrocortisone (11,17,21-trihydroxypregna-4-ene-3,20-diene) which has a molecular formula of $C_{21}H_{30}O_5$ and a molecular mass of 362.47 and its impurity prednisone (17,21-dihydroxypregna-1,4-diene-3,11,20-trione) which has a molecular formula of $C_{21}H_{26}O_5$ and a molecular mass of 358.44. Both structures are illustrated in Fig. 1.

Prednisone (assay 98%) and the hydrocortisone (assay 98%) were used without further purification (Sigma–Aldrich, Sydney, Australia). It should be noted that the presence of prednisone was detected in hydrocortisone, although this was shown to be less than 0.005% and hence would not interfere with the experimental work described here.

All solvents were of HPLC-grade quality and were used as received (Selby-Biolab, Victoria, Australia). All solutions were made using purified-water (Milli-Q; Millipore).

2.3. Sample preparation

All sample solutions were prepared and diluted with methanol–water (1:1, v/v). Single component sample solutions were made for hydrocortisone and prednisone at a concentration of 100 μg ml^{-1}. The mixtures contained a constant concentration of hydrocortisone (50 μg ml^{-1}) and variable concentrations of prednisone (0.0005–50 μg ml^{-1}).

3. Results and discussion

The aim of this work was to find the most sensitive method for the detection of an impurity which co-elutes with a more concentrated major compound. To achieve this an LC method was developed to allow the impurity prednisone to co-elute with hydrocortisone. It should be noted that prednisone has a similar UV spectrum to hydrocortisone and has previously only been detected at 1% of the hydrocortisone concentration using a DAD method [6]. It should be noted that exact co-elution of the two compounds does not hinder peak purity determination by LC–MS–MS.

Two types of mass spectrometric ionization techniques were available: APCI and ESI. APCI is generally used for samples of low molecular mass, which are weakly polar and apolar [6]. Both hydrocortisone and prednisone are weakly polar and have

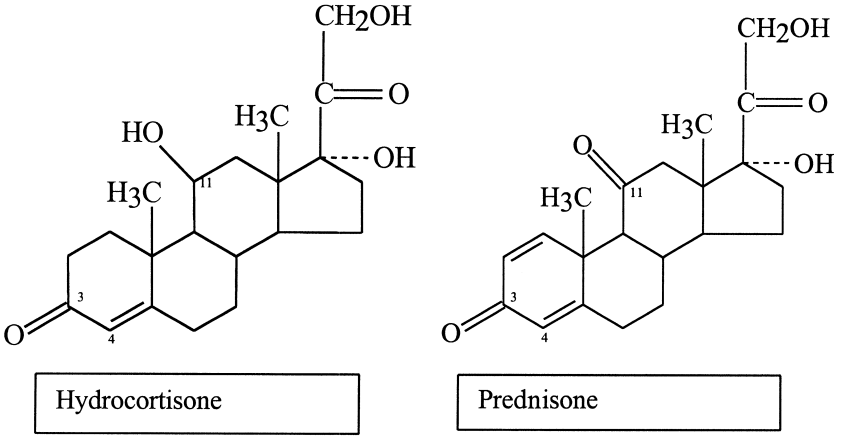

Fig. 1. Chemical structures of the steroids.

relatively low molecular masses. When using APCI the mass spectrum was stable, there was better reproducibility and prednisone could be detected at a lower level than when using ESI. Hence, APCI proved to be more suited in this case.

There were three stages in developing the LC–MS–MS method for the detection of prednisone in hydrocortisone. The first stage involved examining the ion distribution of prednisone using LC–MS. The mass spectra indicated that prednisone fragmented with ease due to the relatively small abundance of the parent ion peak (359 m/z) relative to its daughter ions, which were predominant regardless of orifice voltage and ion spray conditions.

The second stage involved examining the fragmentation pattern of prednisone using LC–MS–MS. The LC–MS–MS conditions were optimised to maximise the fragmentation that allowed more selectivity between the prednisone and hydrocortisone spectra. The optimised APCI-MS conditions are summarised in Section 2.1. Fig. 2 shows the spectrum for prednisone at 50 ppm. From this it can be seen that the major daughter ions from the molecular ion peak of prednisone (359 m/z) were 171 m/z and

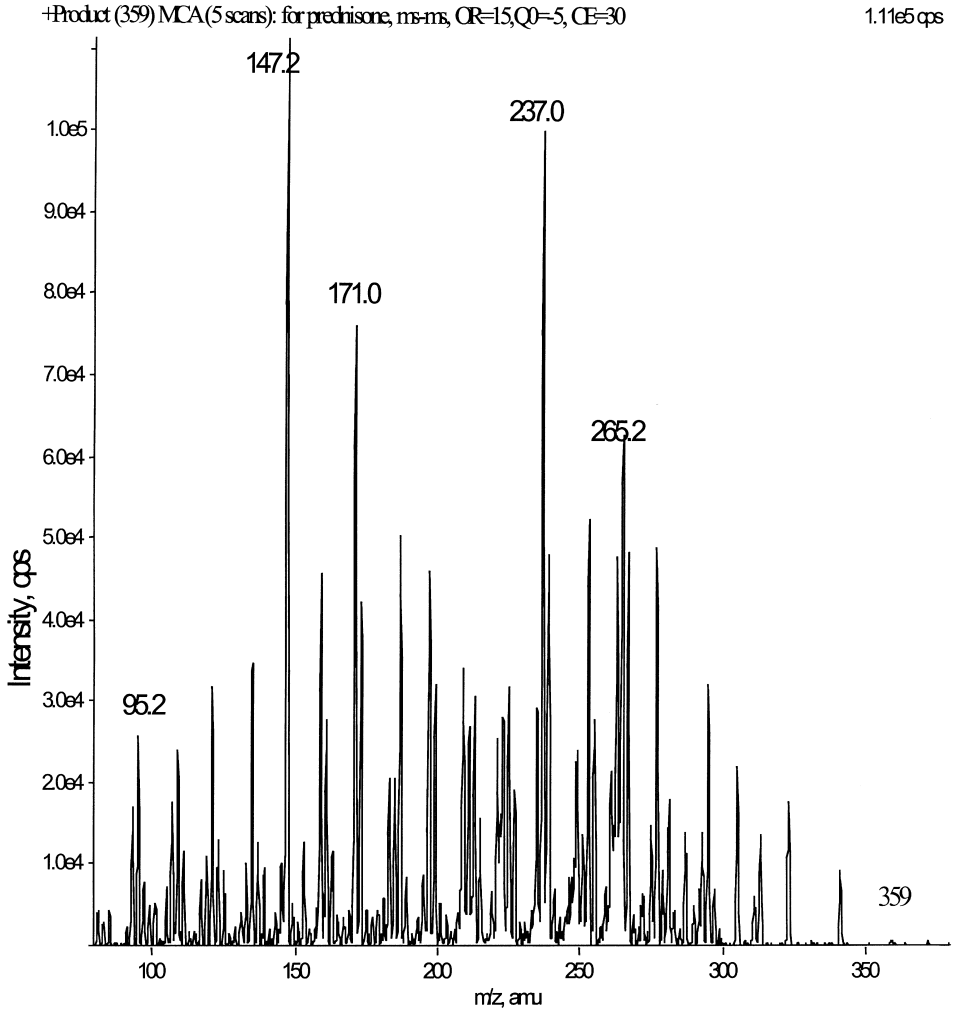

Fig. 2. MS–MS spectra of prednisone at 50 ppm.

147 m/z. A precursor ion scan confirmed that these fragments originated from the 359 m/z molecular ion. The precursor ion scan showed that both fragments were a direct result of fragmentation from the parent ion and were not the result of a recombination. These daughter ions were found to be the most suitable fragments for selective determination of prednisone. Fig. 3 shows the likely structures of these fragments.

To complete the study of LC–MS–MS for peak purity testing, prednisone was assayed using the MRM mode. MRM is used to monitor precursor-to-product ion-transitions and due to its selectivity, is a sensitive technique that can be used for quantitation studies. Further it is known that this technique, unlike SIM, is less likely to produce false positives. Prednisone was analysed by MRM of the product ion-transitions 359.2→147.2 and 359.2→171.0. Fig. 4 shows the chromatographic traces for MRM detection obtained for the lowest limit of detection of prednisone.

A quantitation package, MacQuan, was applied to the data set to obtain a calibration graph. From this the limit of detection was determined to be 2.5 ppb (0.005% of the hydrocortisone concentration). This compared with a limit of detection of 0.5% reported for diode array studies [6]. The assay was linear over the range 0.500 to 0.0005 ppm with a correlation coefficient of 0.999 and a precision of 6.9% at the concentration of 5 ppb.

4. Conclusions

Using LC–MS–MS, the concentration of prednisone was determined in hydrocortisone at a level of 2.5 ppb (0.005%). This work showed that determination of chromatographic peak purity using LC–MS–MS can provide a solution to the problems that accompany LC–DAD and enables impurities to be detected at levels of <0.1%. An assessment of peak purity using LC–MS–MS in MRM mode enables specific compound classification through molecular mass data, structural information through explicit ion fragmentation patterns, high sensitivity, quantitative linearity and high selectivity through specific ion monitoring. Further work is in progress with other compound pairs to determine the ability of LC–MS to identify and quantitate co-eluting impurities.

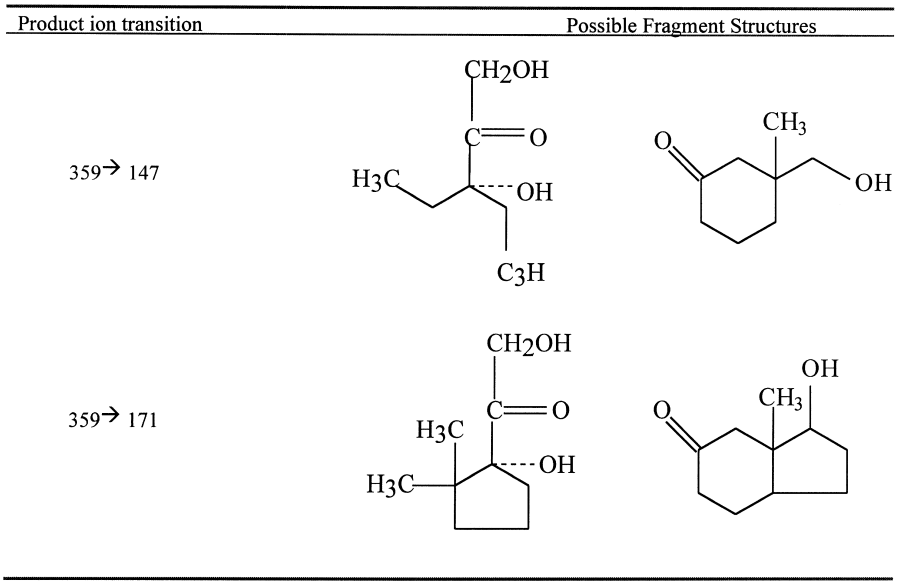

Fig. 3. The chemical structures for the fragmentation of prednisone.

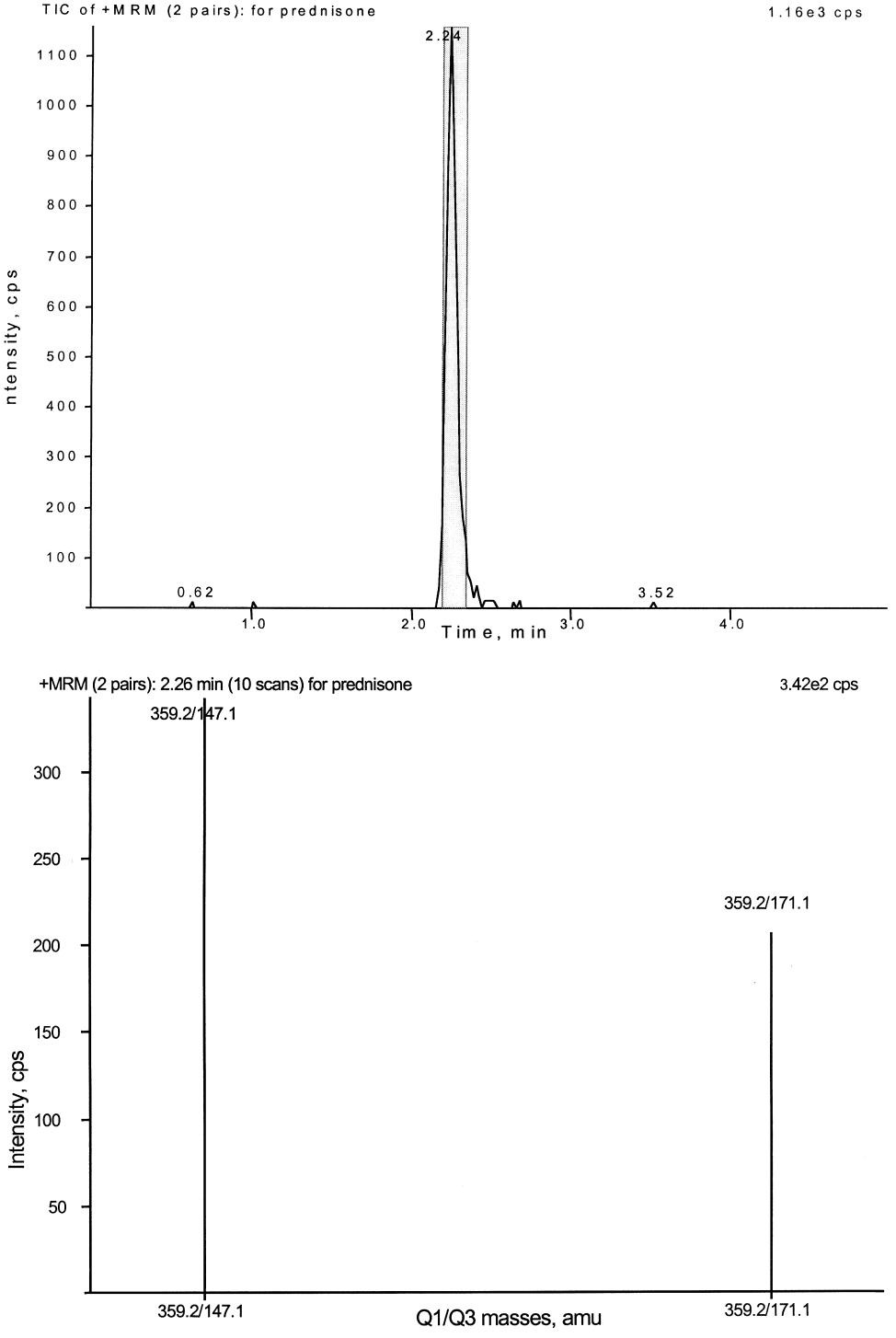

Fig. 4. Chromatographic trace for MRM mode for prednisone at 0.005% of the hydrocortisone concentration.

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