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Case of enantiomer impurity identification by normal-phase chiral high-performance liquid chromatography with optical rotation and mass spectrometric detection

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Abstract

An impurity produced in the synthesis of compound **I** is separated and identified as its enantiomer **II** using normal-phase chiral high-performance liquid chromatography (HPLC) with UV absorbance, optical rotation (OR) and mass spectrometric (MS) detection. The results show that the impurity **II** and compound **I** have equal and opposite specific rotations, identical MS spectra and the same MS–MS fragmentation pattern, as required for enantiomers. The procedures presented demonstrate a novel combination of methods for enantiomer identification and characterization that do not require the preparation of individual enantiomer markers or even the racemic mixture, thus reducing the need for additional synthetic work.

Keywords: Enantiomer separation; Optical rotation; Impurity identification; Chiral compounds

1. Introduction

Chiral molecules are currently at the forefront of strategies for the development of safer, more effective, drugs [1–5][1] and other chemical agents [6]. For example, it is estimated that two-thirds of drugs in development are chiral [1–5], a statistic that reflects both the recognition that opposite enantiomers can have quite different pharmacological effects, as well as pressure from increasing regulatory concerns. In addition, the synthesis of single enantiomers is becoming ever more practical due to ongoing advances in stereoselective synthetic procedures and combinatorial chemistry [1–6]. All these factors contribute to the need for new analytical methods to characterize chiral molecules more efficiently, e.g. methods that use smaller amounts of sample, provide data more quickly or eliminate the need for additional synthetic work.

Optical activity measurements are central to the characterization of chiral molecules because this is the only physical property that distinguishes the individual enantiomers. Thus, development of new techniques based on optical activity offer promise to improve the characterization of chiral molecules.

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One approach that has received significant attention is the construction and application of high-sensitivity optical rotation detectors that can be coupled to high-performance liquid chromatography (HPLC) [7–17], flow injection analysis (FIA) [18,19], or most recently, capillary electrophoresis systems [20]. At this time, ''high-sensitivity'' polarimeters can be roughly defined as capable of measuring signals smaller than about 0.1 mdeg, with the most sensitive detectors having limits of detectability near 1–10 μdeg [7–20]. Such systems typically use much smaller amounts of sample (e.g. 1–25 μg) than conventional polarimeters and enable optical rotation measurements to be made on individual components separated chromatographically from complex mixtures. Analogous work has also been described with high-sensitivity circular dichroism (CD) detectors [21–27], but will not be discussed in detail here.

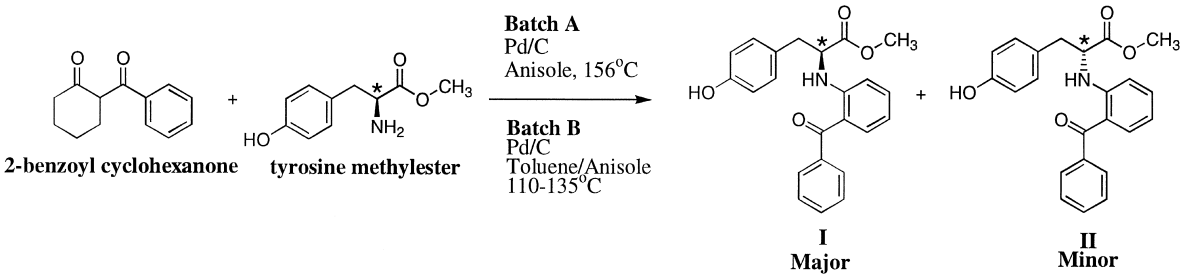
Two applications for OR detectors have received the most attention to date. The most fundamental is to verify the optical activity of the compound of interest and to assign either a positive or negative rotation to the structure. For example, in a seminal paper Yeung and co-workers [7] demonstrated detection of optically active compounds in urine using a laser-based OR detector, but did not attempt any further characterization. Meinard and co-workers [28] used OR–UV ratios to identify diastereomers of deltamethrin produced by photoisomerization and separated by achiral HPLC. Later, Brooks et al. [29] identified the enantiomers of 2,2-dimethyl-1-phenylpropan-1-ol based on the OR–UV peak area ratios obtained in a chiral separation. The second application uses achiral HPLC with UV and OR detection (HPLC–UV–OR) as an alternative to chiral separations for the determination of enantiomeric excess (ee). This is attractive because it reduces testing efforts by enabling achiral chromatographic purity and ee to be measured in a single experiment, eliminating the need to develop a chiral separation method. Procedures for determining ee and specific rotation values from HPLC–UV–OR or FIA–UV–OR data have been described by several groups [30–34].

HPLC–UV–OR can also be used for the identification and characterization of impurities. However, this application has received little attention to date, due primarily to the relatively low sensitivity of most OR detectors and the need to have optically pure standards to facilitate identification [28]. In contrast, HPLC with mass spectrometric detection (HPLC–MS) has become the most powerful tool for the identification of both achiral and chiral impurities [35,36]. Isomeric impurities separated by HPLC are easily flagged by HPLC–MS because they have the same mass as the major component. Individual isomers can then often be distinguished and identified by their fragmentation patterns in an HPLC–MS–MS experiment. Enantiomers, for example, will provide identical MS and MS–MS spectra, whereas diastereomers will typically give different patterns.

Although HPLC–MS is a common technique, it is usually performed using reversed-phase separations with aqueous mobile phases. Normal-phase HPLC–MS with atmospheric pressure ionization (API) sources is still rare (due to the non-polar and flammable nature of the solvents), although the number of examples is increasing. An early study by Hiraoka and Kudaka [37] used HPLC–MS to investigate varying percentages of ethanol in hexane. Recent work with achiral separations includes studies of polyphosphoinositides [38], odapipam and xanomeline [39], difluorophenyl triazoles [40], ethoxylated alcohol surfactants [41], palmitoyl-stearoyl-phosphatidylserine [42] and nonylphenol polyethoxylate surfactants [43]. Examples of MS coupled with normal-phase chiral separations are the enantioselective determination of terazosin in plasma [44], chiral bioanalysis [45] and the determination of omeprazole enantiomers in plasma [46].

The present work focuses on the reactions shown in Scheme 1. Compound **I,** (2*S*)-2-((2-benzoylphenyl)amino)-3-(4-hydroxyphenyl)-propionic acid methyl ester, is a chiral intermediate in the synthesis [47] of a new chemical entity currently in clinical trials. Synthetic scale-up experiments evaluated the preparation of compound **I** via reductive coupling of 2-benzoyl cyclohexanone and tyrosine methylester using Pd/C catalyst in either pure anisole solvent (Batch **I-A**) or a lower boiling toluene–anisole mixture (Batch **I-B**).

Analysis of Batch **I-A** by chiral HPLC with UV absorbance detection revealed a major impurity (see Fig. 1A) that was postulated to be compound **II**, (2*R*)-2-((2-benzoylphenyl)amino)-3-(4-hydroxyphenyl)-propionic acid methyl ester, the enantiomer



Scheme 1.

of compound **I**. However, positive identification was complicated because neither the pure enantiomer compound **II** or the racemic mixture of **I**/**II** were available for comparison at the time these experiments were conducted. Interestingly, Batch **I-B** had only trace levels (see Fig. 2A) of the impurity, presumably because of the lower reflux temperature of the toluene–anisole mixture used to prepare Batch **I-B**. This report presents experiments using normal-phase chiral HPLC–UV–OR and HPLC–MS to identify the impurity in Batch **I-A** as enantiomer **II.**

2. Experimental

2.1. Chemicals

2-benzoyl cyclohexanone was prepared by the method of Denny and Cain [48]. L-Tyrosine methylester was from Aldrich. All other chemicals were American Chemical Society reagent grade or HPLC grade.

2.2. Synthetic methods

Compound **I** was prepared by the reaction shown in Scheme 1 with the following two procedures. Batch **I-A** was prepared by refluxing 2-benzoyl cyclohexanone (92 g, 0.45 mol), L-tyrosine methyl ester (78 g, 0.40 mol) and 10% palladium on activated carbon (17.0 g) for 2 h in 1.0 l anisole (bp=154°C), collecting the resulting water in a Dean-Stark apparatus. The mixture was cooled to 80°C and the Pd/C was filtered, then washed with 50 ml anisole three times. After cooling the mixture to 40°C, 1.0 l hexane was added and the mixture was cooled and stored at −20°C for 48 h. The resulting solid was filtered and washed with 200 ml aliquots of hexane five times to yield 89.0 g crude product **I**. The solid was mixed with 220 ml of methanol and the slurry was refluxed for 30 min. The mixture was cooled to 0°C, the product filtered and washed twice with 50 ml cold (−20°C) methanol, then dried under reduced pressure to yield 67.4 g of Batch **I-A**. A similar procedure was used for Batch **I-B** except the 2-benzoyl cyclohexane (92 g, 0.45 mol), L-tyrosine methyl ester (81 g, 0.42 mol) and 10% Pd/C (17.0 g) were first refluxed in toluene (1.1 l) under an N_2 atmosphere. After 2 h at reflux, anisole (1.1 l) was added and the reaction mixture was heated, removing the distillate (280 ml), until the reaction temperature reached 133 to 135°C, whereupon the mixture was maintained at this temperature for 17 h. The mixture was filtered hot (90°C) through a filter aid pad and the pad was washed with hot ethanol (70°C) until the filtrate was clear (ca. 500 ml). The filtrate was then concentrated under reduced pressure, collecting 700–900 ml of distillate. After cooling the solution to room temperature, heptane was added (1.1 l) over a 20–30 min period and the mixture was chilled in an ice–water bath for 1 h. Crude product **I** was isolated by filtration, washed three times with heptane and dried under reduced pressure at room temperature overnight. Subsequent purification with methanol followed procedures similar to Batch **I-A**. Samples of both batches gave NMR and MS analyses consistent with the desired product [47].

2.3. HPLC Conditions

A Daicel Chiralcel OD (7 μm particles, 250×4.6 mm) column was used with a mobile phase of

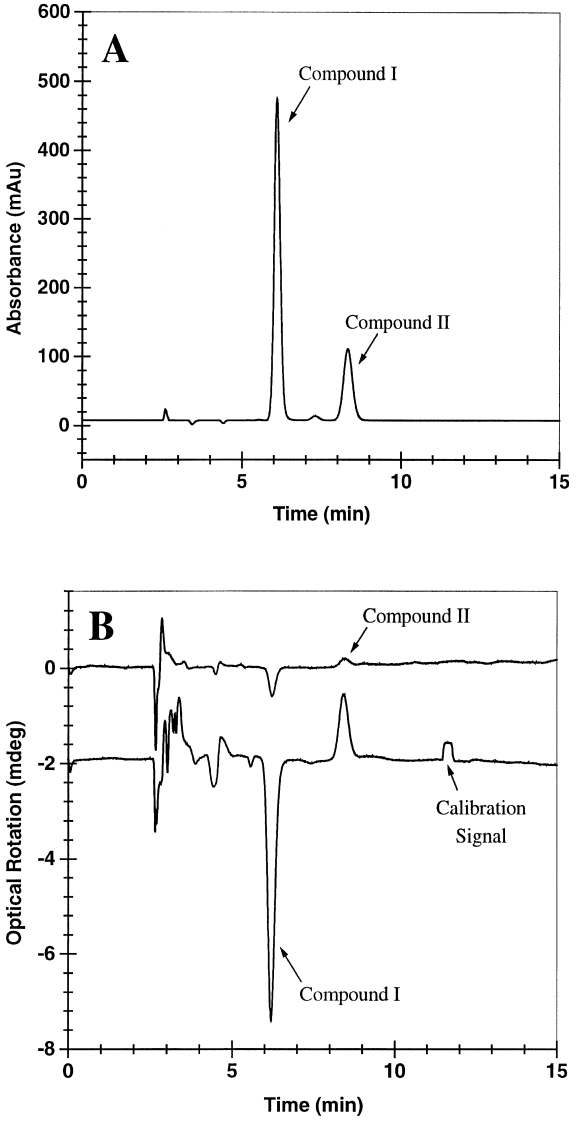


Fig. 1. Normal-phase chiral HPLC–UV–OR chromatogram recorded for injections of 0.96 mg/ml Batch **I-A**: (A) UV absorbance detector response for a 2 μl injection and (B) OR detector responses for a 2 μl (upper trace) and a 20 μl (lower trace) injection. The lower trace is intentionally offset from the origin by −2 mdeg for clarity. The calibration signal shown corresponds to a rotation of 412 μdeg.

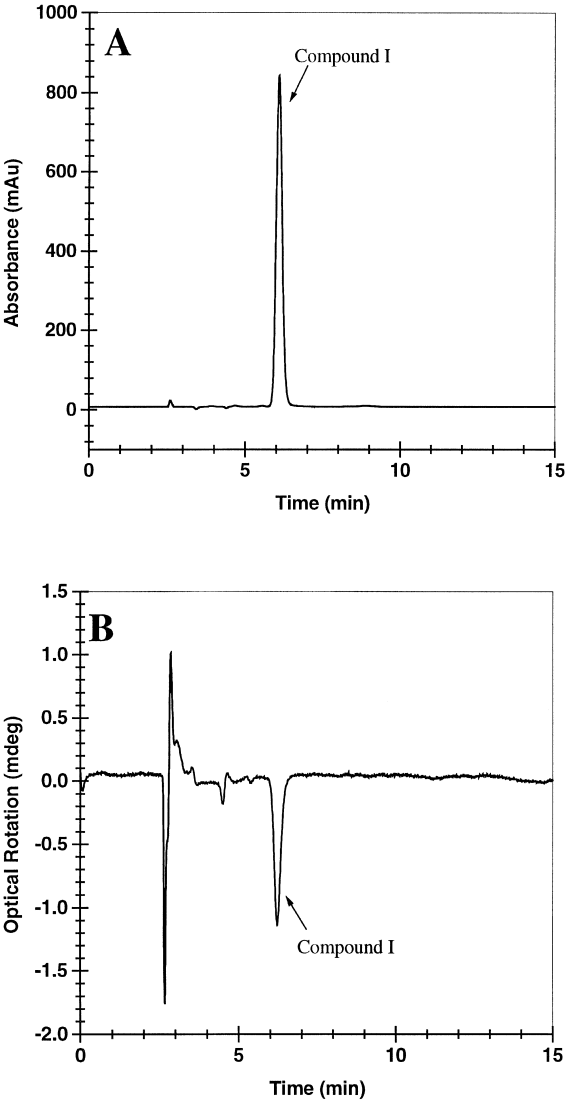


Fig. 2. Normal-phase chiral HPLC–UV–OR chromatogram recorded for a 2 μl injection of 0.91 mg/ml Batch **I-B**: (A) UV absorbance detector response and (B) OR detector response.

23:77:0.1 (v/v/v) anhydrous ethanol–hexane–trifluoroacetic acid at a flow rate of 1.0 ml/min with 230 nm detection and a typical run time of 15 min. The temperature was 24 (±1)°C. Samples were dissolved in the mobile phase. Concentrations and injection volumes are noted in the figures.

2.4. HPLC–UV–OR System

The HPLC system consisted of a Kratos Spectroflow 400 pump, a Hewlett-Packard 1050 autosampler, and an LDC Spectromonitor III UV detector.

The high-sensitivity OR detector used an argon ion laser (Uniphase Model 2011-10SL, 488 nm, 2.0 mW) with an optical bench that was described in detail previously [18]. An integrated chromatography data system (VG Multichrom) digitized the lock-in amplifier output at 3–4 Hz, stored it on a VAX computer and provided data processing routines.

2.5. Specific rotation calculation

Specific rotations (deg ml g^{-1} dm^{-1}) were calculated as shown below:

$$[\alpha]_{488}^{24} = \frac{A_{sam}K_{cal}F}{M_{inj}PL} \quad (1)$$

where A_{sam} is the OR peak area (μV s), F is the flow rate in ml/s, M_{inj} is the sample mass injected in g, P is the purity (% area/area) determined from the UV-detected chromatograms, and L is the flow cell length (0.5 dm). K_{cal} is the instrument response factor (1.17×10^{-9} deg/μV) determined from the detector response to a known optical rotation signal applied via a Faraday rotator as previously described [18].

2.6. HPLC–MS System

The HPLC system consisted of a Waters 610 Solvent System and a Waters 490 Variable Wavelength Detector. The chromatographic conditions were as described above. The outlet of the HPLC system was attached to the inlet of a Finnigan TSQ7000 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source. The high voltages employed in ESI, combined with flammable solvents, can lead to explosions. However, normal-phase HPLC–MS can be safely performed using appropriate procedures, as demonstrated here and in previous work [49]. All of the equipment is inspected before any normal-phase HPLC–MS experiment, especially the tightness of the ionization source, to ensure oxygen excluded. The ESI source was operated at 4.5 kV, which gave a corresponding current of 5.0 μA. The heated capillary was set to 200°C, and the manifold at 80°C. Data were collected in full scan MS mode over the range m/z 100 to 1000 at 2 s/scan. Sheath gas (N_2) was set to 80 p.s.i. and the auxillary gas (N_2) to 20 l/min. Higher than normal sheath and auxillary gas flows were used to ensure positive pressure within the ionization source, reducing the potential for air leaks.

3. Results and discussion

3.1. HPLC–UV–OR data

Fig. 1 shows a chiral HPLC–UV–OR chromatogram recorded for a 0.96 mg/ml solution of Batch **I-A** using the conditions described in the Experimental section. The UV absorbance trace in Fig. 1A shows a major peak (74.0% area/area) for compound **I** at 6.1 min and a minor peak (24.0% area/area) for the impurity at 8.3 min. The relative retention time (RRT) of the impurity peak is 1.37 relative to compound **I**. Careful examination of the UV data also reveals two small additional impurities at 5.5 and 7.3 min. The corresponding OR-detected chromatogram for this injection presented in Fig. 1B (upper trace) shows a negative peak for compound **I** and a positive peak for the 8.3 min impurity, consistent with the behavior expected for enantiomers. Note that the OR peaks are slightly offset from the UV peaks due to the time delay caused by the tubing connecting the two detectors. Fig. 1B also shows a representative OR chromatogram (lower trace) obtained from a 10× larger injection of the same solution of Batch **I-A**. For comparison, the lower trace also shows a 412 μdeg calibration signal, produced by a Faraday rotator, that was used in conjunction with other similar signals to calculate the OR detector response factor. In addition, the larger injection trace shows negative OR peaks for the two impurities observed by UV at 5.5 and 7.3 min, indicating that these compounds cannot be the enantiomer of compound **I**

The UV and OR chromatograms were integrated and the measured peak areas were used to calculate $[\alpha]_{488}^{24}$ values via Eq. (1) for compound **I** and the impurity at 8.3 min. Results from seven injections summarized in Table 1 show that the average specific rotations for compound **I** (-299.4 ± 5.1 deg ml g^{-1} dm^{-1}) and the impurity ($+301.9\pm16.6$ deg

Table 1
Specific rotations for compounds I and II in Batch I-A

Mass injected, μg	$[\alpha]_{488}^{24}$, deg ml g^{-1} dm^{-1}	
	Compound **I**	Impurity (Compound **II**)
9.60	−298.9	333.9
9.60	−302.0	308.7
9.60	−298.2	283.2
19.20	−296.5	288.0
19.20	−297.7	303.1
1.92	−293.3	299.0
1.92	−309.3	297.2
Average	−299.4	301.9
Std. Dev.	5.1	16.6

ml g^{-1} dm^{-1}) are equal and opposite, supporting identification of the impurity as the enantiomer **II**. The results were independent of the mass injected, demonstrating the reproducibility and linearity of the response. The experimental uncertainty is larger than the typical reproducibility of ca. 1% obtained with HPLC autoinjectors because the averages include results from a range of injection volumes and the OR response is typically less reproducible than UV data.

Fig. 2 shows the HPLC–UV–OR chromatogram recorded for Batch **I-B**. The UV absorbance trace (Fig. 2A) shows a major peak (99.2% area) for compound **I** at 6.1 min. Trace impurity peaks (<1% area) were also detected by UV at 5.5, 8.3 and 8.9 min but are not visible in the chromatogram at the scale shown. The OR trace (Fig. 2B) again shows a strong negative peak corresponding to compound **I**, but no detectable positive peak for the 8.3 min impurity. A small negative peak for the impurity at 5.5 min is also apparent, as observed in Batch **I-A**. Quantitative analysis of the UV and OR data from two injections of Batch **I-B** gave the specific rotation results shown in Table 2. The average $[\alpha]_{488}^{24}$ value of -302.4 ± 8.0 deg ml g^{-1} dm^{-1} for compound **I** in Batch **I-B** is identical, within the experimental uncertainty, to the value measured for Batch **I-A**.

Table 2
Specific rotations for compound I in Batch I-B

Mass injected, μg	$[\alpha]_{488}^{24}$, deg ml g^{-1} dm^{-1}
1.82	−308.0
1.82	−296.7
Average	−302.4
Std. Dev.	8.0

The consistent specific rotation values measured for compound **I** in Batch **I-A** and **I-B** demonstrate a key feature of HPLC–UV–OR: the ability to measure specific rotations for individual components of a complex sample, such as Batch **I-A**, via chromatographic separation of the individual compounds. In contrast, conventional polarimetry of Batch **I-A** would give a specific rotation value that reflected the combined optical rotation signals of all components in the mixture. Although such a measurement would reveal the optical activity of Batch **I-A** to be lower than that of Batch **I-B,** due to the addition of negative and positive signals, it would not provide the chromatographic fingerprint (illustrated in Figs. 1 and 2) obtained by HPLC–UV–OR that enables a more complete understanding of the sample.

3.2. HPLC–MS data

HPLC–MS and MS–MS with electrospray ionization were used to confirm that the impurity is compound **II**. Fig. 3 shows HPLC–MS data for the two major components noted in the reconstructed ion chromatogram (RIC) recorded for a 10 μl injection of 0.5 mg/ml Batch **I-A** using the chromatographic conditions described above. The RIC was similar to the chromatogram shown in Fig. 1A, so it is not shown here. Only Batch **I-A** was analyzed by HPLC–MS because Batch **I-B** did not show the impurity of interest. Fig. 3A presents the full scan MS spectrum obtained from the compound **I** peak at 6.4 min in the RIC. The protonated molecular ion peak at m/z 376 is consistent with the compound **I** molecular mass of 375 daltons. Other major ion peaks are apparent at m/z 316, 208, 179, and 137. The full scan MS spectrum for the major impurity peak at 8.4 min in the RIC is shown in Fig. 3B. It is apparent that both full scan spectra are identical which implies that the major impurity is a structural isomer of the desired product, but taken alone does not prove that it is the enantiomer **II**.

HPLC–MS–MS analysis of the molecular ions in Fig. 3 was used to provide final identification of the impurity peak. Enantiomers will give identical MS–MS spectra, whereas other structural isomers are expected to show noticeable differences in their MS–

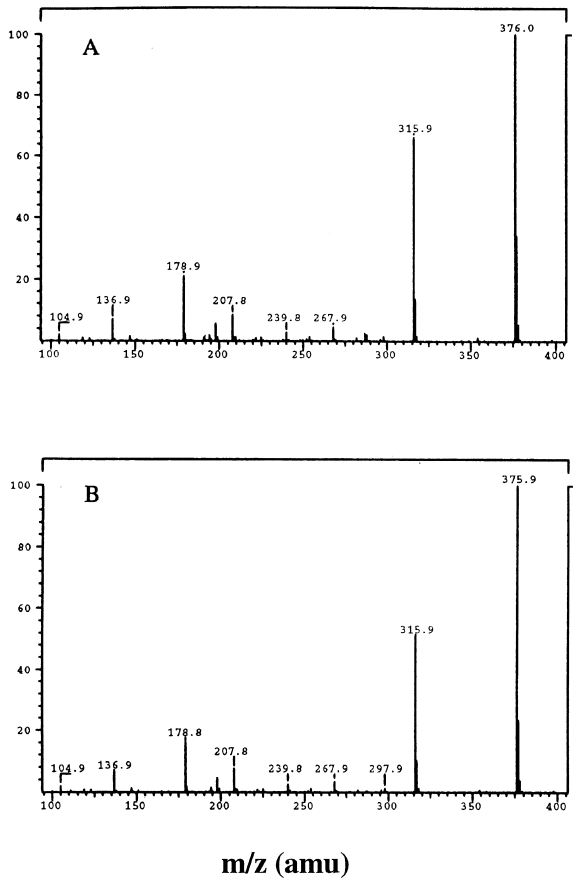


Fig. 3. Full scan mass spectra recorded for (A) the compound **I** peak at 6.4 min and (B) the enantiomer impurity peak at 8.4 min obtained from a 10 μl injection of 0.5 mg/ml Batch **I-A** using conditions as in Fig. 1.

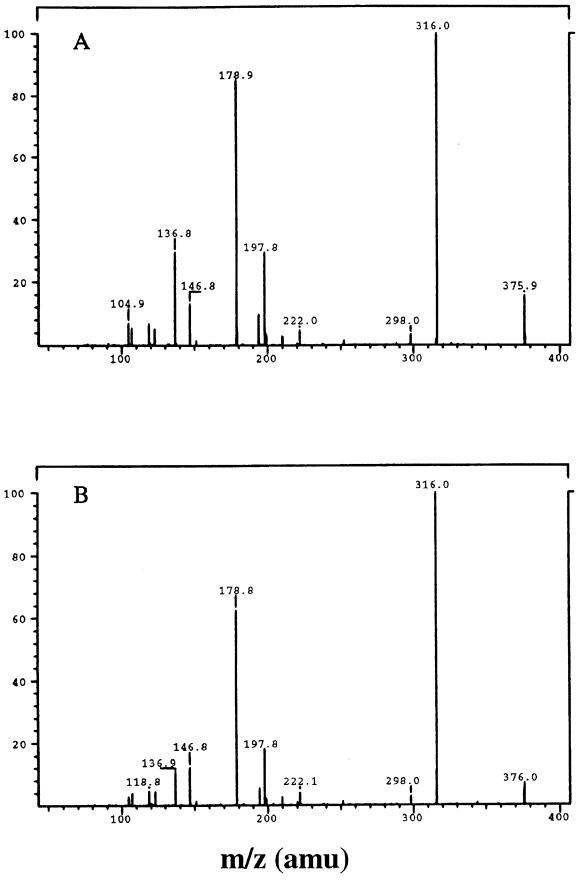


Fig. 4. MS–MS spectra recorded during the experiment in Fig. 3 for (A) the compound **I** protonated molecular ion and (B) the enantiomer impurity protonated molecular ion.

MS fragmentation patterns. Fig. 4A and B show the HPLC–MS–MS data obtained from the protonated molecular ions (m/z 376) observed for compound **I** and the impurity, respectively. The identical MS–MS fragmentation patterns observed for both compounds, with major ions at m/z 316, 198, 179, 147 and 137 gives positive evidence that the impurity is the enantiomer **II**.

4. Conclusions

Chiral HPLC–UV–OR and HPLC–MS experiments show that the impurity in Batch **I-A** and compound **I** have equal and opposite specific rotations, identical masses, and the same MS–MS fragmentation pattern. These data positively identify the impurity as enantiomer **II**. This work illustrates a general method for enantiomer identification and characterization that does not require the preparation of individual enantiomer markers or even the racemic mixture, which eliminates additional synthetic work. It expands upon previous reports because it is the first use of HPLC–UV–OR combined with HPLC–MS for quantitative analysis of chiral impurity data. Although additional confirmation of

structure by NMR [21,50] might be needed in some circumstances (such as for compounds with multiple chiral centers), the type of OR, MS and MS–MS data presented here can provide a reasonable level of confidence in the identification of many chiral impurities, such as compound **II**. Similar experiments could be done using HPLC–UV–CD [21]. On-line CD spectra would provide additional data to confirm structural assignments.

In practice, successful application of the method will be limited by the ability to obtain accurate OR and MS data, which will be a function of the amount of enantiomer impurity present coupled with its specific rotation and ionization behavior. In most cases, it is likely that the OR response will be the limiting factor because of the generally lower sensitivity of OR compared to MS detectors. This report demonstrates successful application of the method for a compound with an enantiomer impurity present at ca. 24% area/area and having a specific rotation of 300 deg ml g^{-1} dm^{-1}. However, the mass-response data in Table 1 indicate that the analysis could also be performed with an enantiomer at the 1–2% area/area level (assuming a similar specific rotation) or lower by appropriate adjustment of the experimental conditions.

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