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The determination of a chlorinated benzofuran pharmaceutical intermediate by HPLC–MS with on-line derivatization

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Abstract

An HPLC–MS method for the analysis of 2-chloromethylbenzofuran, a pharmaceutical intermediate alkylating reagent employed in the preparation of a second generation HIV protease inhibitor, *N*-(2(R)-hydroxy-1(S)-indanyl)-2(R)-phenylmethyl-4(S)-hydroxy-5-(1-(4-(2-benzo[b]furanylmethyl)-2(S)-*N*′-9-*t*-butyl-carboxamido)-pipera zinyl))-pentaneamide, is described. Preliminary analysis of the protease inhibitor by HPLC–MS indicated that the quality of the drug substance was influenced by the composition of the chloromethylbenzofuran. Direct analysis of the chloromethylbenzofuran by LC–MS using atmospheric pressure ionization was unsuccessful, necessitating an alternative approach. The method described incorporated post-column derivatization of the chloromethyl benzofuran using a modification of the drug substance process chemistry yielding a derivative amenable to MS analysis using atmospheric pressure chemical ionization (APCI). This allowed measurement of an impurity in the chloromethylbenzofuran which was incorporated into the protease inhibitor.

Keywords: 2-chloromethylbenzofuran; Protease inhibitor; On-line derivatization; HPLC–MS

1. Introduction

Recently, the introduction of atmospheric pressure ionization (API) for use with mass spectrometry has become very popular in the analysis of complex mixtures. The technique has been particularly strong in the study of compounds of pharmaceutical interest, especially when coupled to a means of separation such as HPLC prior to introduction into the mass spectrometer. This technique has been used for structure elucidation of antibiotics [1,2], determination of degradation products of neuromuscular blocking agents, hypnotics and calcium channel blockers [3,4], identification of synthetic impurities in anti-cancer agents [5] and identification and quantitation of cytostatic and cholesterol lowering drugs and metabolites for pharmacokinetic studies in physiological fluids [6,7].

Notwithstanding these impressive studies, some limitations of API using either electrospray ionization (ESI) or atmospheric pressure chemical

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ionization (APCI) have been noted for the analysis of relatively low molecular weight materials encompassing both polar and nonpolar analytes [8,9]. Sensitivity constraints using ESI may be due to the inability to form ionic species in solution or charge neutralization within the interface region, while constraints observed using APCI can be attributable poor proton transfer to or abstraction from the analyte or analyte volatility.

The usual approach for mass spectrometric measurement of this class of compounds is GC–MS. However, sample volatility, high polarity or thermal lability are limitations and derivatization steps to eliminate or attenuate these restrictions can result in either losses due to sample handling or background due to side reactions or impurities from the derivatizing reagent [10]. An alternative approach using HPLC as a means of separation is the particle beam interface. Despite offering the capability of using either electron impact (EI) or chemical ionization (CI) and library searching [11], disadvantages of the particle beam interface which have been noted include poor sensitivity, non-linear analyte response and extensive instrument optimization [10–12], although a recent report incorporating a capillary-scale nebulizer appears promising for alleviation of some of these limitations [13].

We recently had a need to investigate the composition of a compound of this classification, 2-chloromethyl benzofuran, I. Benzofurans containing pendant groups at the C-2 and C-3 positions which have active halogen sites have been used as intermediates in the preparation of several compounds of pharmaceutical interest such as antihyperglycemics [14], anticoagulants [15], antifungals [16] and cholecystokinin agonists [17]. This compound is one of the materials used for preparation of an oral second generation HIV protease inhibitor, *N*-(2(R)-hydroxy-1(S)-indanyl)-2(R)-phenyl methyl-4(S)-hydroxy-5-(1-(4-(2-benzo[b]furanylmethyl)-2(S)-*N*′-(*t*-butyl-carbox amido)-piperazinyl))-pentaneamide, II, currently being investigated in clinical trials. Our initial HPLC–MS investigations of II using API suggested that an impurity observed in the drug substance was attributable to the quality of I. Further investigations to ascertain the quality of I by direct HPLC-MS analysis using the particle beam interface, ESI or APCI were unsuccessful. We describe an approach for the analysis of I using HPLC with the incorporation of on-line postcolumn derivatization based upon the drug synthetic chemistry. This allowed for MS detection and identification using APCI as an ionization method.

2. Experimental

2.1. Chemicals

Ammonium acetate, ammonium formate, ammonium hydroxide and acetic acid were reagent grade quality (Aldrich, Milwaukee, WI). All solvents were of HPLC grade and were from Fisher Scientific (Bridgewater, NJ). *N*-(2-Hydroxy-1-indanyl)-2-(phenylmethyl)-4-(hydroxy-5-[1-[2-(*N*-*tert*-butylcarbamoyl)piperazinyl]]pentaneamide, III, was supplied by the Process Research Department of Merck Research Laboratories (Rahway, NJ).

2.2. Chromatographic system

HPLC–MS studies were performed using a Hewlett-Packard (Palo Alto, CA) model 1100 pump equipped with a photodiode array detector. The column used for direct analysis of II was a Waters Symmetry C_8, 250 × 3.9 mm (Milford, MA). The mobile phase was 25 mM ammonium acetate adjusted to pH 6.2 with acetic acid and acetonitrile. A gradient from 40 to 80% acetonitrile in 25 min was used at a flow rate of 1 ml min^{-1}. The sample concentration was 1 ml min^{-1} using acetonitrile as the diluent and 10 μg was injected. The column used for I was a YMC J'Sphere H80 C_{18}, 250 × 2.0 mm (Wilmington, NC). The mobile phase was 35% water and 65% acetonitrile at a flow rate of 250 μl min^{-1}. The sample concentration was 2 mg ml^{-1} using acetonitrile as the diluent and 10 μg was injected. UV detection was used at 220 nm for both compounds. For the analysis of I, the

postcolumn derivatizing reagent III was dissolved in a 50:50 mixture of 0.1 M ammonium formate and acetonitrile at a concentration of 0.5 mg ml^{-1}. The ammonium formate portion was pH adjusted using ammonium hydroxide. The solution was introduced after the photodiode array detector using a Waters Model 600MS pump via a ZDV tee. The flow rate was 0.5 ml min^{-1}. The combined HPLC effluent/derivatizing solution was then passed through a 0.015 mm × 25 m reaction coil heated with a Haake water bath (Berlin, Germany) and then into the mass spectrometer. The derivatization scheme is shown in Fig. 1.

2.3. Mass spectrometry measurements

Mass Spectrometry was performed using a Finnigan (San Jose, CA) model 7000 triple stage quadrupole. Both electrospray and APCI in the positive ion mode were employed for the preliminary analysis of II. Electrospray ionization was performed using a spray needle voltage of 4.5 kV and a capillary interface temperature of 250°C. APCI ionization was performed at a corona current of 5 μamps, a vaporizer temperature of 400°C and capillary interface at 150°C. The nitrogen sheath and nebulizing gas was maintained at 70 psi and 30 units (rotameter), respectively. For the analysis of I, APCI ionization was used. Full scan MS measurements for I and II were from 400 to 900 amu at a dwell time of 2 ms. MS/MS and SRM experiments were performed using argon as the collision gas at 2.0 mtorr and a collision energy of −27 eV.

3. Results and discussion

3.1. LC–MS analysis of the HIV protease inhibitor drug substance

During drug development, a key feature is the design of a scaleable, economically feasible, synthetic scheme for the bulk active pharmaceutical to support clinical trials and act as the framework for commercial production. It is not uncommon during this development cycle to have a new unknown organic impurity appear in development samples of drug substances. In order to assess the viability of a potential process, it is incumbent upon the research laboratory to identify the impurity. This is generally carried out to assist in its removal and to ensure a new impurity does not have potentially toxic or unexpected pharmacological effects, which could jeopardize clinical trials. In the synthetic development effort of II, we were required to identify a new impurity in lab samples and establish the source.

One of our approaches for the control of organic impurities in II was an HPLC gradient method. In this method where the retention time of II was 20 min, a new impurity was observed at a retention time of 22.5 min. The level observed was 0.4% area at the wavelength used for the analysis, i.e. 220 nm. This necessitated identification of the impurity and LC–MS and MS/MS was the technique of choice. Since the parent compound II contained a basic piperazine moiety which offered a potential proton sink, API in the positive ion mode was used for the mass spectrometric investigations. Under conditions employing either ESI or APCI ionization, the protonated

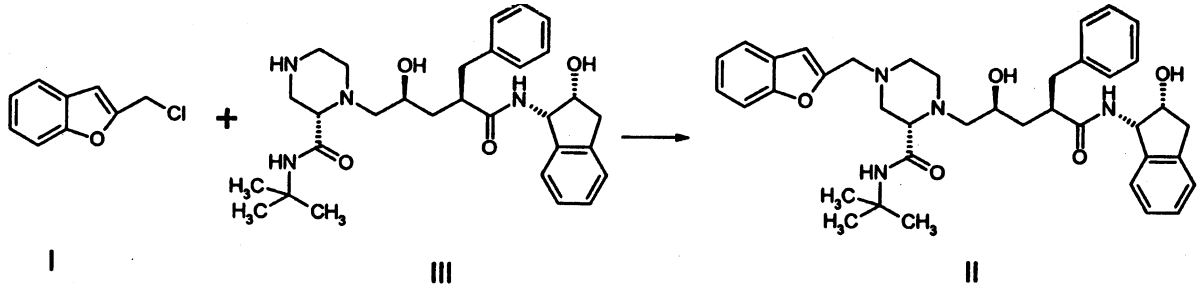

Fig. 1. Derivatization scheme used for the analysis of the chloromethylbenzofuran, I.

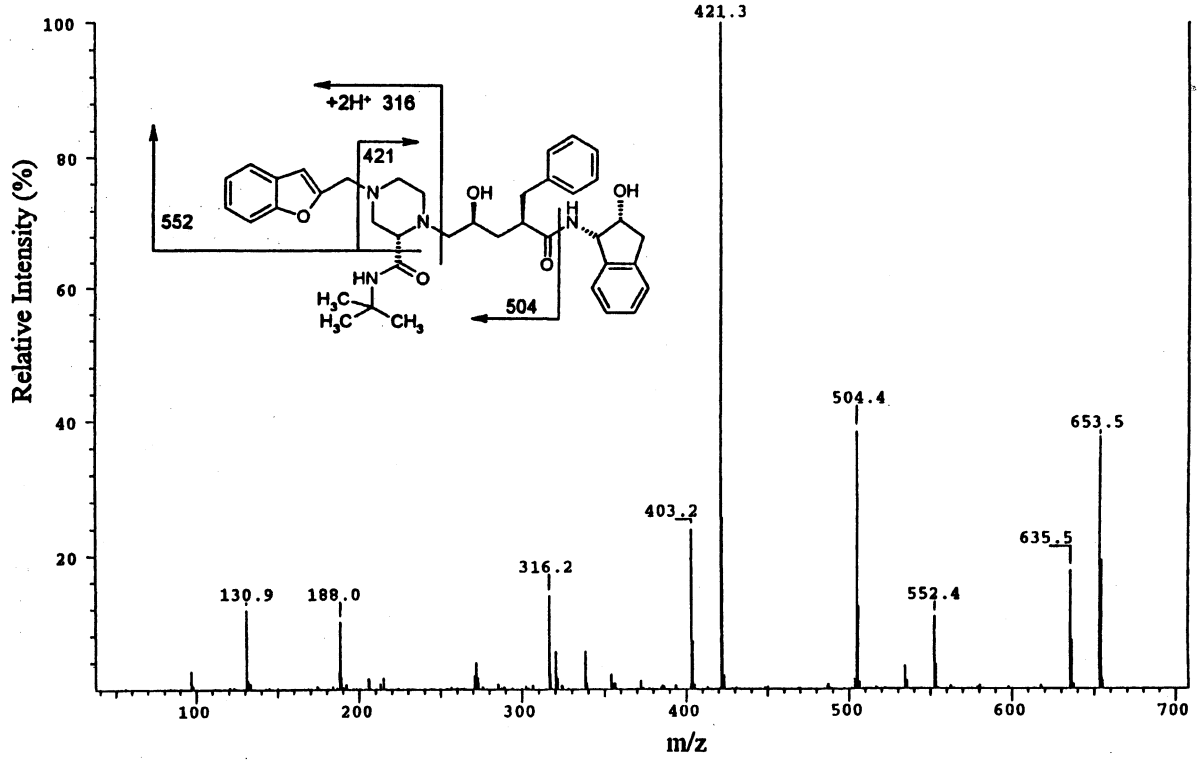

Fig. 2. APCI LC–MS/MS product ion spectrum of the $(M+H)^+$ ion (m/z 653) of II acquired at a collision cell pressure of 2.0 mtorr and energy of -27 eV. The proposed fragmentation pathway is shown in the inset.

molecular ion, $(M+H)^+$, was easily observed in full scan MS mode for both II and the impurity of interest with little fragmentation observed. The results are consistent with the gentle ionization process afforded by API leading to gas phase ions of low internal energy as noted by others [18]. The protonated molecular ions were 653 and 687 amu for II and the impurity, respectively. Although the lack of fragmentation observed for the impurity precluded any structural assignment, the mass differential relative to II, $+34$ amu, and the intensity of the $M+2$ isotope, 40%, suggested chlorine substitution within II.

To elaborate the structure of the impurity, LC–MS/MS experiments were performed. The parent precursor ion, as $(M+H)^+$, was selectively passed through the first quadrupole, Q1, and allowed to enter the collision cell, Q2, followed by mass analyzing the resultant product ions in Q3. The resolution in Q1 was decreased resulting in a 4.5 amu band width at one half band height for the precursor ion, allowing a significant portion of the $M+2$ isotope of the precursor ion to be transferred to the collision cell, facilitating identification of fragments containing chlorine. To discern the fragmentation pathway, II was used as a model compound.

Using a collision gas pressure of 2 mtorr and an applied potential of -27 eV across the collision cell, a 60% decrease in the intensity of the parent ion mass was noted concomitant with the appearance of numerous fragments in the product ion spectra. The product ion spectrum of II, shown in Fig. 2, showed structurally significant ions at m/z 552, 504, 421 and 316. The fragmentation pathway, shown in the inset of the figure, features loss of the *tert*-butylaminocarbonyl (m/z 552), the amino-2-hydroxy indanyl (m/z 504) and combined loss of the *tert*-butylcarbonyl and methylbenzofuryl (m/z 421) moieties. Cleavage at the C-5

position adjacent to the substituted piperazine moiety results in m/z 316. The proposed fragmentation pattern is consistent with previous LC–MS/MS metabolism studies of the first generation protease inhibitor analog, indinavir [19,20] in which methylpyridyl replaces the methylbenzofuryl moiety. The product ion spectrum of the impurity of interest, shown in Fig. 3, indicated chlorine substitution within fragments at m/z 586, 538, 437 and 350 and a lack of chlorine substitution in a fragment common to II, m/z 421. The proposed fragmentation pathway of the impurity, analogous to that of II, was consistent with chlorine substitution within the methylbenzofuryl group. This was deemed plausible since the preparation of this class of compounds can involve the use of a halogen containing alkylating reagent [21,22] which potentially may contain reagent impurities having halogen polysubstitution.

3.2. LC–MS analysis of 2-chlormethylbenzofuran

Direct analysis of I, the source of the impurity in II, by LC and MS using the particle beam interface was hampered by lack of sensitivity. ESI or APCI without derivatization was not pursued since preliminary data obtained by flow injection of I and either positive or negative ion mode MS detection was of no value; no interpretable spectra for I were obtained, even after extensive modification of the MS operational parameters known to have significant effects upon analyte response such as nebulization gas flow rates, heated capillary interface temperature and applied voltage to the capillary interface and tube lens. This result is similar to MS data obtained under API conditions for low molecular weight polar and non polar materials having phenyl groups with alcohol, ether, β-diketone, enol, cyano or alkyl halide substitution patterns [9,23]. An alternative ap-

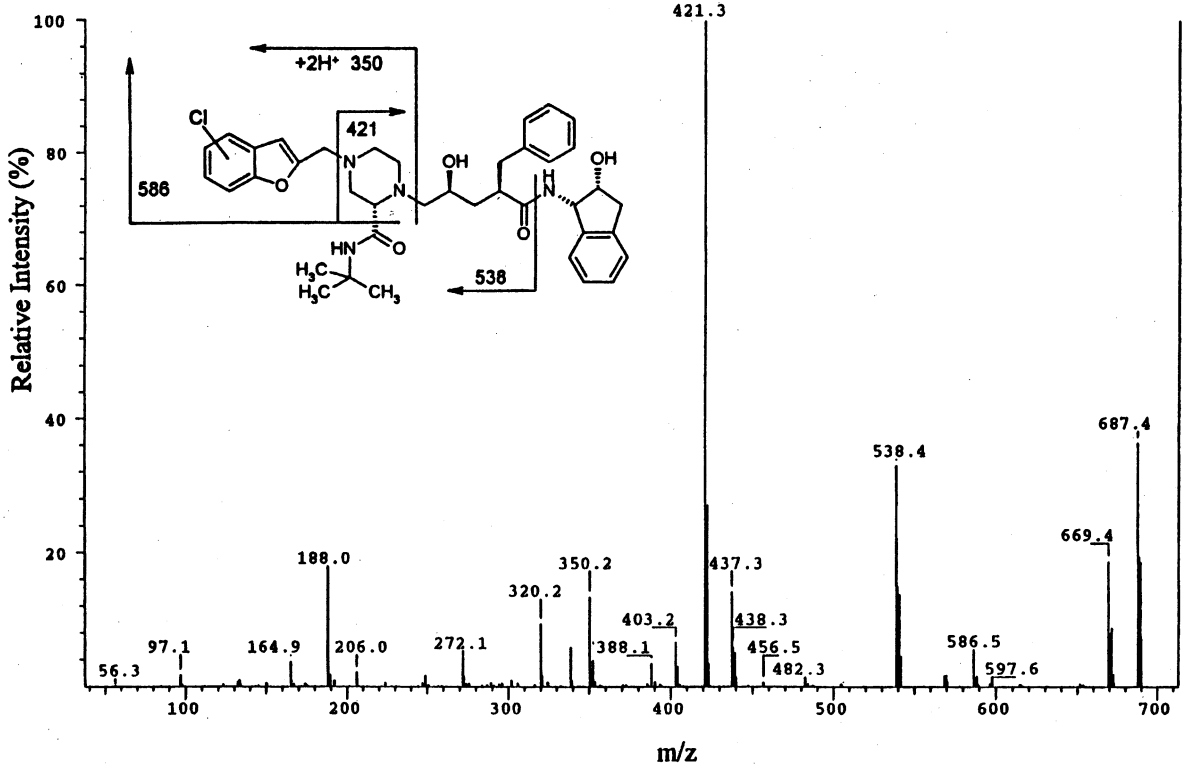

Fig. 3. APCI LC–MS/MS product ion spectrum of the $(M+H)^+$ ion (m/z 687) of the undesired impurity observed in II. The proposed structure of the impurity and fragmentation pathway are shown in the inset.

proach which seemed feasible for the analysis of I was conversion of I to a species having better mass spectral response characteristics under API conditions. Since the performance of II was known, a strategy of converting I to II by reaction with III, mimicking synthetic approaches for formation of this class of compounds [22], was undertaken. The formation of II was accomplished by chromatography of I followed by on-line postcolumn alkylation with III.

The postcolumn alkylating reagent III was prepared in a hydroorganic mixture of acetonitrile and water containing ammonium formate as the base. It was introduced after the UV detector and just prior to a 25-m reaction coil held at elevated temperature and finally the mass spectrometer. In order to increase the residence time in the reaction coil, the chromatography of I was performed using a flow rate of 250 μl min^{-1} and a narrow bore HPLC column. Since suppression of the ion intensity in ESI mass spectrometry by endogenous components of sample matrices and also bases, which are present at high concentration in the alkylation solution, has been reported [24,25], APCI was used as the ionization source for MS measurements. To maximize ion transmission from the atmospheric region to the analyzer region in the mobile phase/derivatization solution environment, the response of the protonated molecular ion of II, m/z 653, was studied as a function of the interface capillary and tube lens voltages. A maximum of 121 and 98 V for the tube lens and capillary, respectively, was found to give highest throughput for this ion. These settings were used throughout the rest of the study.

The HPLC retention time of I with UV detection was 7.9 min and after background subtraction of the derivatizing reagent, m/z 523, showed the expected derivative at m/z 653 amu. Another component having m/z 653 amu was observed for a band at a retention time of 9.3 min, suggesting an isomeric material. The component having the expected m/z of the chlorinated species, m/z 687 amu, was observed at a retention time of 11.3 min. The product ion spectrum of this component was remarkably similar to that of the impurity observed in Fig. 3 for the bulk drug substance, providing confirmatory evidence for the assignment of this band as a chloromethylchlorobenzofuran. The total ion and the mass chromatogram of m/z 687 amu, shown in Fig. 4, illustrates that this is the only component in the sample which gives a derivative consistent with a chloromethylchlorobenzofuran. Note that the retention times of the derivatives observed in the total ion and mass chromatogram differs by ~ 6 mins from those using UV detection; this is due to the residence time in the postcolumn reaction coil. These chromatograms were acquired using a mass range from 530 to 900 amu, thus eliminating background from the derivatizing reagent III.

3.3. Derivatization parameters

The influence of derivatizing reagent concentration, reaction temperature and buffer pH upon the derivative response was investigated. The formation of II by flow injection of I and without chromatography was used as the model, with the MS measurement of the intensity of m/z 653 amu used as the response. No change in response was noted when the concentration of the derivatizing reagent III was varied from 0.1 to 1 mg ml^{-1}. This result suggests that under conditions which can potentially lead to suppression of the MS signal by endogenous matrix components, APCI is a robust means of ionization, consistent with a previous study [24]. The effect of both temperature and pH upon the MS response is shown in Fig. 5. In the temperature range from 50 to 90°C, the response increased by nine-fold. A potential side reaction of alkyl halides at elevated temperature in the presence of base is elimination [26], however no α-hydrogen is available in the case of I and this pathway may be obviated here. The pH of the ammonium formate buffer was studied in the range from 6.0 to the practical limit of 10.5. At pH 6.0, no response was observed, while the response increased 6.2-fold from a pH of 8 to 10.5. This is consistent with an S_N2 type of reaction, in which displacement is enhanced by the basicity of the nucleophile [27].

The yield of the reaction was estimated by flow injection introduction and comparison of the area of the m/z 653 amu ion obtained for I with post column derivatization versus that of solid II. The

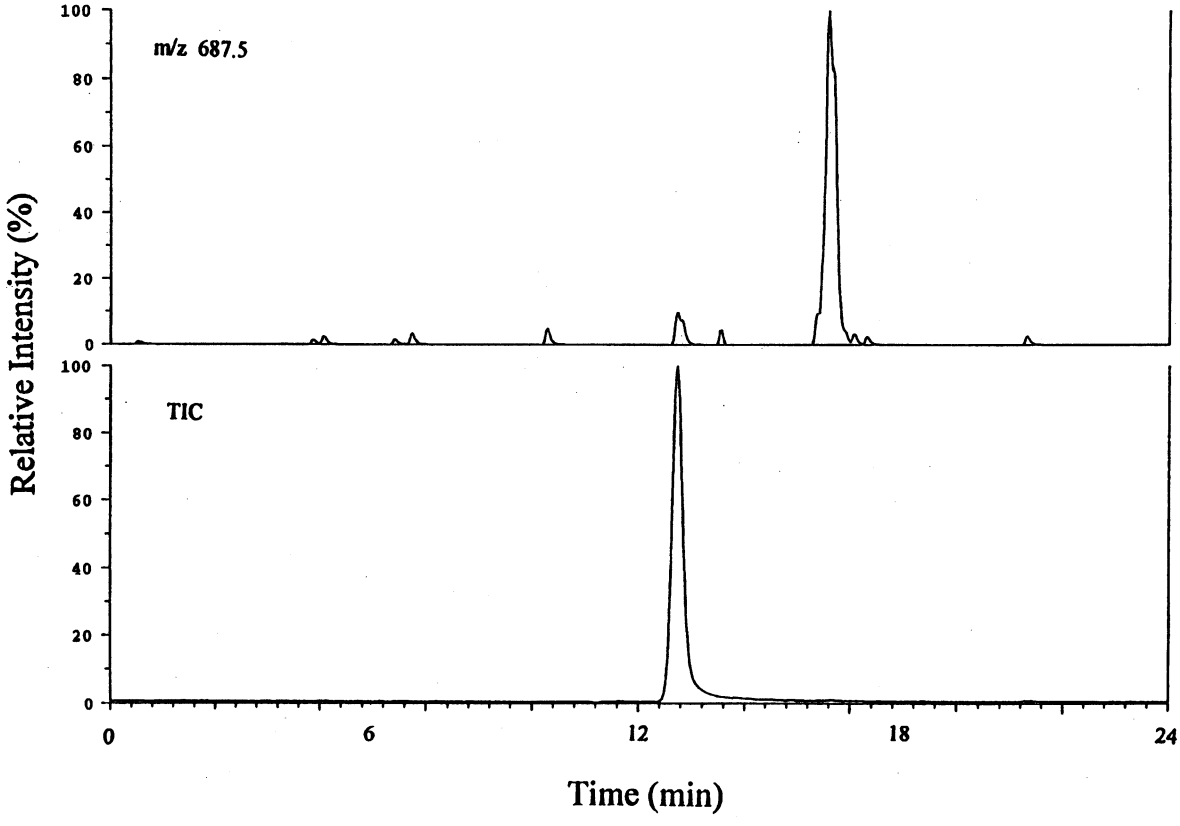

Fig. 4. APCI–LC–MS total ion and mass (m/z 687.5) chromatograms of a sample of the chloromethylbenzofuran, I, after online derivatization.

yield of II obtained in this manner was $\sim 10\%$. Off line derivatization of I with III using DMF as a reaction solvent and triethylamine as the base, simulating the actual process chemistry conditions, gave a reaction yield of 80%. It has been noted that nucleophilic processes are enhanced in dipolar, aprotic solvents compared to protic solvents such as water or alcohol, due to increased solvation of the transition state intermediate in the apolar system relative to the protic one, and hydrogen bonding effects of the nucleophile and protic solvent [27,28]. The use of the aprotic solvent system was not used for MS measurements due to the lower volatility of DMF and triethylamine which can lead to cluster ion adducts of these species within the mass spectrometer resulting in both high chemical noise and decreased ionization [18,29].

3.4. Detection limits

The limit of detection of the chloromethylchlorobenzofuran was obtained in full scan and single reaction monitoring (SRM) modes using a buffer pH of 10.5 and reaction temperature of 90°C. In the full scan mode the limit of detection was made by comparing the area of m/z 683 to that of a known amount of m/z 653 at a S/N of 3 for both ions, assuming an equal MS response, and also when injecting 10 ug of I on column. The limit of detection of the chloromethylchlorobenzofuran impurity in full scan MS mode was found to be 10 ng, or 1 part per thousand relative to I. In the SRM mode, the limit of detection was measured by serial dilution of a standard of I and monitoring the transition from $687 \Rightarrow 421$ m/z. The estimated detection

limit at 3 S/N was 100 pg, or 10 parts per million relative to I.

4. Conclusions

A pharmaceutical intermediate compound having poor intrinsic MS ionization efficiency in API mode, chloromethylbenzofuran, was subjected to postcolumn alkylation with a suitable amine resulting in a derivative having very good MS performance. We suggest this strategy may be generalized for drug substances and intermediate precursors to identify and control impurities by MS provided the synthetic process chemistry can be used or modified to operate under API ionization conditions and the derivative has acceptable MS response. Although the reaction yield was relatively low due to incorporation of reagents which are more compatible with the APCI interface, the selectivity offered by MS allowed low level determination of a chloromethyl chlorobenzofuran impurity which ultimately was undesirably incorporated into the bulk drug substance. On a practical note, we were able to introduce a rather high concentration of derivatizing reagent without adversely influencing the ionization of the derivatives or physically plugging the capillary interface just prior to the quadrupole analyzer region.

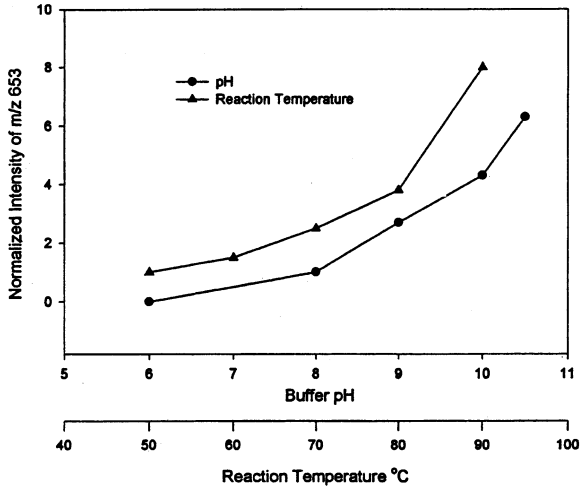

Fig. 5. The effect of temperature and pH upon the normalized yield of the reaction of the chloromethylbenzofuran, I, with the piperazinyl pentaneamide, III, as determined by flow injection and APCI–MS measurement of the ion intensity of the product, II, m/z 653.

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