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Determination of the acylcoenzyme A cholesterol acyltransferase inhibitor 447C88 in plasma using gas chromatography–mass spectrometry and liquid chromatography atmospheric pressure chemical ionisation tandem mass spectrometry

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Abstract

Two mass spectrometry-based methods are described for the determination of 447C88 (I), a novel inhibitor of acylcoenzyme A cholesterol acyltransferase (ACAT), in rat, dog and human plasma. The first method uses gas chromatography–mass spectrometry (GC–MS) with electron ionisation and selected-ion monitoring. The method employs solid-phase extraction of I from plasma and requires alkylation of I using iodoethane. The second method uses liquid chromatography–tandem mass spectrometry (LC–MS–MS) with atmospheric-pressure chemical-ionisation and selected-reaction monitoring. The LC–MS–MS method uses a simplified version of the extraction procedure used for GC–MS and does not require derivatisation of I. While both methods provide the necessary limit of quantitation of 0.5 ng/ml in human, dog and rat plasma with the required precision and accuracy, the LC–MS–MS assay offers increased sensitivity, selectivity and speed over the GC–MS assay. This allows a same day turn round of results for in excess of 100 samples, including sample preparation and data acquisition and processing.

1. Introduction

Current evidence indicates that a raised level of plasma cholesterol is associated with an increased risk of atherosclerosis with increased morbidity and mortality [1]. While dietary modification will continue to be first-line therapy in cases of hyperlipidaemia, clinical trials with first generation cholesterol-lowering drugs such as the bile acid sequesterant cholestamine have demonstrated an important reduction in coronary events in patients receiving long-term therapy [2]. More recently, potent cholesterol lowering drugs have been developed which are competitive inhibitors of the synthesis of mevalonic acid by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [1,3]. The novel compound 447C88, *N*-heptyl-*N'*-(2,4 difluoro-6-{2-[-

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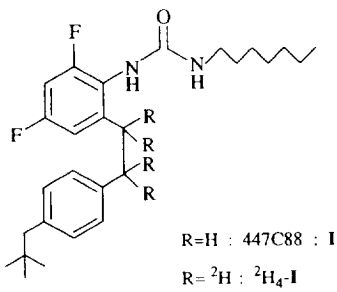

Fig. 1. Structure of the ACAT inhibitor I and its deuterated analogue.

4-(2,2 dimethylpropyl) phenyl] ethyl} phenyl) urea (I, Fig. 1), is a member of a new class of potential hypolipidaemic agents–the acyl-coenzyme A cholesterol acyltransferase or ACAT inhibitors.

ACAT is an integral membrane bound enzyme found in several tissues including intestine and liver. Although most cholesterol enters the gut lumen esterified with fatty acids, de-esterification is essential to the absorption of cholesterol by the gastro-intestinal mucosa. Once in the mucosal cell, cholesterol is transported into the lymph and thence to the blood stream only after re-esterification. The process of esterification within the gastro-intestinal mucosa is thought to be mediated by the enzyme ACAT. The highest activity of this enzyme is seen in rough endoplasmic reticulum where it catalyses the transfer of long-chain fatty acyl residue from acyl-CoA to the β-hydroxyl group of cholesterol to form a cholesteryl ester. Blocking the formation of such esters by the inhibition of ACAT within the gastro-intestinal tract is associated with reduced cholesterol absorption in animal models and may lead to reduced absorption in man and thus a lowering of plasma cholesterol.

Although I has been developed as a non-systemic ACAT inhibitor, it has been observed in plasma following high doses due to limited absorbtion through the gut mucosa. For this reason and to support intravenous administration during pre-clinical toxicological studies, there was a requirement for a sensitive assay for the determination of I in plasma. Early method development work focused on liquid chromatography with either UV or fluorescence detection. Unfortunately, neither approach was able to provide the required selectivity and/or sensitivity. In this paper we describe two mass spectrometry-based methods for the sensitive determination of I from human, dog or rat plasma, using a deuterated analogue as internal standard.

2. Experimental

2.1. Chemicals

Both I and its deuterated analogue [$^{2}H_4$]I were synthesised at the Wellcome Research Laboratories (Beckenham, UK) and stock solutions of these were prepared in methanol. HPLC-grade methanol was obtained from Rathburn (Walkerburn, UK). Acetonitrile 190 (far-UV) was purchased from Romil Chemicals (Loughborough, UK). GPR grade guanidinium chloride (guanidine hydrochloride), potassium *tert.*-butoxide, *n*-pentane (AnalaR), dimethylsulphoxide (AnalaR), water (HiperSolv) and calcium hydride were all obtained from BDH (Poole, UK). Iododethane (99%) and 3-methyl-1-butanol were purchased from Aldrich (Gillingham, UK). A 0.1% (w/v) solution of potassium *tert.*-butoxide solution was prepared in DMSO dried over CaH_2. Control rat and dog plasma were supplied by Charles River (Margate, UK) and Churchill Applied Biotechnology (Huntingdon, UK) respectively, while human plasma was obtained from Occupational Health (Wellcome Research Labs).

2.2. Extraction and derivatisation of I for analysis by GC–MS

All plasma samples were centrifuged at 2000 *g* for 10 min to remove precipitated solids prior to analysis. To each plasma sample (500 μl) was added 15 μl of a 3.5 μg/ml solution of internal standard ([$^{2}H_4$]I) to give a concentration of approximately 100 ng/ml. After mixing and standing for 30 min at room temperature the samples were diluted with an equal volume of

saturated guanidinium chloride solution and vortex-mixed.

Using a Speed Mate 30 solid-phase extraction manifold (Applied Separations), 100 mg of phenyl Bond-Elut (Anachem, Luton, UK) were conditioned with methanol (2 ml) followed by water (2 ml). Plasma samples were applied to the cartridges and slowly drawn through under vacuum. The cartridges were then washed sequentially with water (2 × 1 ml) and 50% aqueous acetonitrile (0.5 ml) before I was eluted from the cartridges with acetonitrile (0.5 ml). The extracts were transferred to 2-ml screw-top autosampler vials (Chromocol, London, UK) and taken to dryness under a stream of nitrogen at 40°C. The residues were dissolved in 250 μl of dry potassium *tert.*-butoxide solution with gentle agitation, immediately followed by the addition of 30 μl of iodoethane. This reaction can be vigorous and should be performed in a fume hood with appropriate care. The vials were capped and left to stand for 30 min at room temperature. The reactions were quenched by the addition of 200 μl of water, followed by 1 ml of *n*-pentane. The vials were recapped, shaken vigorously for 1 min and left to stand until the two phases had separated. The *n*-pentane layer was transferred to autosampler vials (Chromocol) and taken to dryness under nitrogen at 40°C. The residues were re-dissolved in 25 μl of 3-methyl-1-butanol by vortex-mixing thoroughly and the vials capped ready for GC–MS analysis.

2.3. Extraction of I for analysis by LC–MS–MS

Extraction of plasma samples for quantitation by LC–MS–MS used the same solid-phase extraction procedure as described above except that only 300 μl of acetonitrile was used to elute I. This extract was then used directly for LC–MS–MS analysis without preconcentration, derivatisation or any additional clean-up step. For increased sensitivity, 0.5 ml of acetonitrile was used to elute I, but this was taken to dryness and reconstituted in 100 μl of mobile phase (see below).

2.4. Gas chromatography–mass spectrometry

All quantitative GC–MS experiments were performed on a Hewlett-Packard Model HP5890 gas chromatograph coupled to a HP5970B mass-selective detector (MSD). A HP5970C Chem-Station running version 3.1 software was used for instrument control and data acquisition and processing. A HP7673A automatic liquid sampler was used to inject sample extracts (2 μl) into a split/splitless injection port at 280°C, with a splitless time of 0.7 min. Separations were achieved on a 15 m × 0.25 mm I.D., 0.25 μm film-thickness methyl silicone capillary column (Hewlett-Packard) fitted with a 0.5 m × 0.53 mm I.D. uncoated retention gap. Helium was used as carrier gas at a head pressure of 25 kPa and a total flow-rate of approximately 30 ml/min. The oven temperature, after an initial hold at 100°C for 1 min, was ramped to 265°C at 25°C/min, held for 4 min, and finally ramped at 50°C/min to 290°C. The MSD transfer line was maintained at 290°C.

Positive-ion (70 eV) electron ionisation (EI) was used for all GC–MS experiments with the ion source maintained at 200°C. Selected-ion monitoring was used for quantitative GC–MS analyses, monitoring the molecular ions of both the mono- and diethyl derivatives of I (*m/z* 472 and 500) and its deuterated analogue (*m/z* 476 and 504), respectively, with a dwell time of 100 ms/ion.

2.5. Liquid chromatography–tandem mass spectrometry

A Hewlett-Packard Model HP1090A liquid chromatograph equipped with a variable-volume injector, autosampler and ternary DR5 solvent delivery system was used for all LC–MS experiments. A 15-cm ODS column (2.1 or 4.6 mm I.D.) was used in conjunction with an aqueous acetonitrile gradient to evaluate various LC–MS interfaces. A Hewlett-Packard MS Engine was used for EI and NICI (methane) particle-beam experiments and a Finnigan-MAT TSQ-70 mass spectrometer was used for thermospray LC–MS. A PE-SCIEX API-III triple quadrupole instru-

ment equipped with an API source and a heated nebuliser interface was used for quantitatve LC–MS–MS.

For the determination of I in plasma, separations were achieved on a 3 cm × 4.6 mm I.D column packed with 3 μm Spherisorb ODS stationary phase (Jones Chromatography, Wales, UK) fitted with a pre-column frit filter (10 μm). A mobile phase of 80% aqueous acetonitrile was used at a flow-rate of 1.0 ml/min. The column was operated at a temperature of 40°C resulting in a typical retention time of 1.4 min for I and an overall run time of 2.0 min. An injection volume of 20 μl was used. The API source was operated in the negative-ion atmospheric-pressure chemical-ionisation (APCI) mode. Medical grade air at an operating pressure of 5 bar was used as the nebulising gas with an overall flow-rate of approximately 1.6 l/min. High purity nitrogen was used as the curtain gas at a flow-rate of 1.0 l/min. The heated nebuliser interface was maintained at 370°C. Selected-reaction monitoring (SRM) was used for quantitative LC–MS–MS analyses, with a dwell time of 200 ms/transition. A collision energy of approximately 40 eV was used, with argon as the target gas at an indicated thickness of $3.8 \cdot 10^{14}$ atoms cm^{-2}.

2.6. Preparation of calibration curve

A range of calibration standards were prepared in duplicate by spiking control plasma with appropriate volumes of I standard solutions to achieve the required concentration. For GC–MS calibration standards were prepared at 0.4, 1, 2, 3, 10, 20, 50, 80 100, 200 and 300 ng/ml, while for the LC–MS–MS assay the range was 0.5–100 ng/ml. Quality control samples were prepared by spiking batches of control plasma at 1, 10 and 50 ng/ml for GC–MS and 2, 20 and 80 ng/ml for LC–MS–MS analysis. These standards and quality controls were then processed in exactly the same manner as all other test samples.

Calibration curves were constructed by plotting peak-area ratios of I and internal standard against the concentration of I. A weighted (x^{-1}) linear regression line was fitted over the concentration range for both assays. Drug concentrations in unknown and quality control samples were subsequently interpolated from this line.

3. Results and discussion

3.1. Gas chromatography–mass spectrometry

Several gas chromatography–mass spectrometry (GC–MS) methods have been reported for the determination of the HMG-CoA reductase inhibitors simvastatin [3,4], lovastatin [3,5] and pravastatin [3,6] in plasma. Underivatised I was not readily amenable to analysis by GC–MS, due to thermal degradation resulting in poor chromatography and extensive fragmentation under electron ionisation (EI). A similar problem has been reported for substituted urea herbicides [7]. Several derivatisation reagents were therefore investigated. Derivatisation with heptafluorobutyric anhydride gave a good peak shape for the acyl derivative of the corresponding aniline, but no derivative of the intact molecule. Alkylation of the urea group using iodomethane in the presence of potassium *tert.*-butoxide proved more successful, with the production of dimethylated I in high yield which was readily separated from solvents and reagents by back extraction into *n*-pentane. Initially, this procedure appeared suitable for the application to the analysis of I in plasma extracts. However, monitoring the molecular ion (m/z 472) it was concluded that reliable quantitation at low levels (< 10 ng/ml) could not be achieved due to the presence of co-eluting peaks derived from endogenous plasma components. Substitution of iodomethane with iodoethane resulted in the formation of the corresponding mono- and diethyl derivatives (see later). Although of the same mass as the dimethyl derivative, monoethyl-I was well separated from derivatised co-extractives allowing quantitation at lower levels.

Both liquid–liquid and solid-phase extraction of I from plasma were investigated. With untreated plasma the recovery of I using either approach was low (approximately 30% for rat plasma) and was believed to result from the

interaction of the analyte with plasma proteins. The addition of an equal volume of saturated guanidinium chloride reduce the degree of protein binding and resulted in a marked increase in the extraction efficiency ($>90\%$ in rat, dog and human plasma). Solid-phase was eventually selected over liquid–liquid extraction for further development because of the speed and ease of use when analysing large numbers of samples and since it offered greater potential for automation. Of the numerous stationary phases examined, a phenyl bonded phase was selected for the final methodology on the basis of recovery. The solid-phase extraction procedure was optimised as described and the extract was then taken to dryness under a stream of nitrogen prior to derivatisation.

The residues were dissolved in dry potassium *tert.*-butoxide solution, immediately followed by the careful addition of iodoethane. A reaction time of 30 min at room temperature was found to be optimal after which the reaction was quenched by the addition of water. The relative proportions of the mono- and diethyl- derivatives were found to be independent of reaction time and the reagent concentration. The derivatives were separated from the base and DMSO by back-extracting the reaction mixture into *n*-pentane. The organic phase was then removed and again taken to dryness. Reconstitution into the relatively involatile 3-methyl-1-butanol allowed a higher initial oven temperature to be used, reducing the equilibration time and thus the time between injections. A capillary GC method was developed (temperature program etc) based on a methyl silicone column. A retention gap was introduced to improve the ruggedness of the method and extend column life.

The total-ion current (TIC) trace resulting from the full-scan GC–MS analysis of a derivatised extract of dog plasma spiked with I (800 ng/ml) is shown in Fig. 2A. The mono- and diethyl derivatives of I are both observed in the TIC at approximately 11.0 and 11.5 min, respectively. The EI mass spectrum of monoethyl-I derivative is presented in Fig. 3A and while extensive fragmentation is evident, the molecular ion is clearly observed at m/z 472. The corresponding spectrum of the diethyl derivative is shown in Fig. 3B and as expected the molecular ion is found 28 Da higher at m/z 500. The spectra appear similar, both having a base peak at m/z 170 and exhibiting characteristic fragment ions at m/z 457 and 485 and m/z 387 and 415 corresponding to fragmentation along the *n*-heptyl side chain $[M-CH_3]^{+\cdot}$ and $[M-C_6H_{13}]^{+\cdot}$, respectively. The ion at m/z 358 in both spectra is assigned to the additional loss of $HNCH_2$ and $C_2H_5NCH_2$, respectively. A deuterated analogue of I (Fig. 1) was synthesized for use as an internal standard. The corresponding EI mass spectrum of the monoethyl derivative of $[^2H_4]$I obtained by GC–MS is shown in Fig. 3C. The molecular ion is found 4 Da higher at m/z 476 and similar mass shifts are observed for the fragment ions discussed above. Similar shifts were observed in the spectrum of the diethyl-$[^2H_4]$I derivative (not shown).

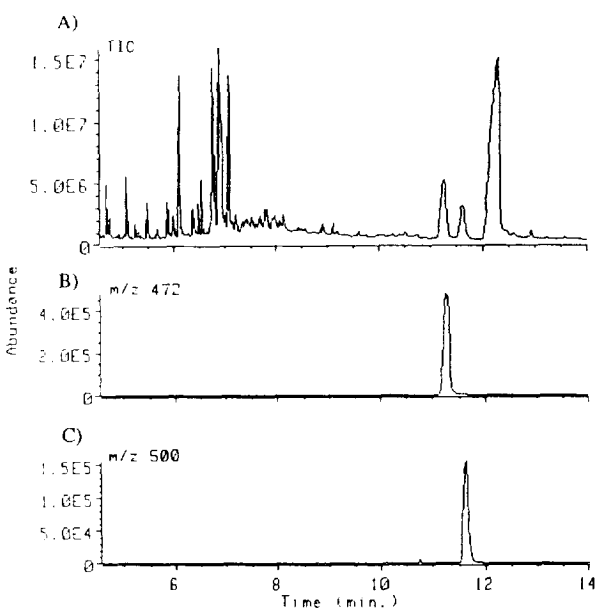

Fig. 2. (A) GC–MS-TIC of dog plasma extract spiked with I (800 ng/ml) and extracted ion chromatograms of (B) mono- and (C) diethyl derivatives of I. Conditions: 15 m × 0.25 mm I.D., 0.25 μm film thickness methyl silicone capillary column (Hewlett-Packard), temperature ramped from 100°C to 265°C at 25°C/min, held for 4 min, and finally ramped at 50°C/min to 290°C.

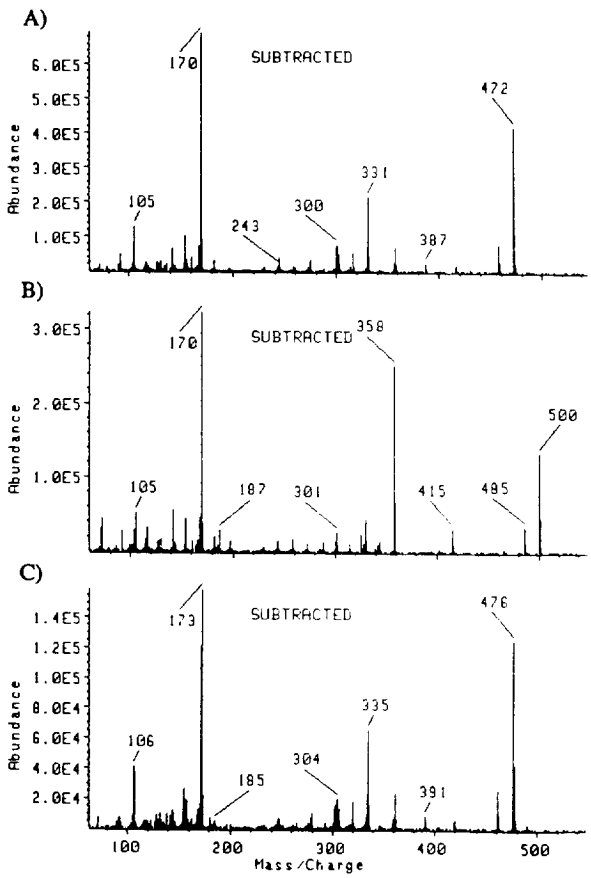

Fig. 3. EI spectra of (A) mono- and (B) diethyl derivatives of I and (C) monoethyl derivative of $[^2H_4]I$. Conditions as for Fig. 2.

The extracted ion chromatograms of the molecular ions of both derivatives are presented in Figs. 2B and 2C, respectively. It can be seen that under the GC conditions employed, the two derivatives are well resolved from each other and from the many endogenous peaks eluting early in the TIC (eliminated by comparison with corresponding blank plasma extracts). Interestingly, particularly in light of the potential therapeutic use of I, the large 'fronting' peak eluting just after the two derivatives is due to cholesterol.

The development work described above, resulted in a viable GC–MS method for the determination of I in plasma. The method was validated in rat and dog plasma and subsequently used to support the preclinical development. The response was linear over the range 0.4–500 ng/ml, although the assay was actually validated over a more limited range (1–300 ng/ml). Precision and accuracy data obtained for dog plasma is presented in Table 1. Typical SIM traces of the ethyl derivative of I obtained for 1 ng/ml and 50 ng/ml standard extracts together with a blank extract from dog plasma are presented in Figs. 4A–C, respectively. The corresponding trace for the $[^2H_4]I$ internal standard (100 ng/ml) is presented for comparison (Fig. 4D), and it can be seen that the deuterated analogue elutes slightly before the unlabeled derivative. Although the molecular ions of both the mono- and diethyl derivatives of I and $[^2H_4]I$ are monitored during the assay, only the more abundant monoethyl derivatives were used for quantitative purposes, with the diethyl derivatives simply providing an extremely useful secondary check to the analyst.

While the GC–MS method is reliable and provides the necessary limit of quantitation, the main drawbacks of the method are that it is labour intensive and slow. The derivatisation, back extraction and two concentration steps are time consuming and a typical batch of 60 extracts would take a technician a whole day to process prior to analysis. These steps do not lend themselves well to automation and the high volume of consumables (reaction vials, etc) demanded by such a procedure is also increasingly a concern. Although the retention time of derivatised I is only around 12 min, the cycle time between injections was normally 30 min due to late eluting peaks and column equilibration. A typical batch would therefore take in excess of 30 h to analyse, which combined with data processing resulted in an overall turn around of three days for the production of final results. During the design period of the Phase I clinical trial to investigate the tolerance and pharmacokinetics of I in man, it was realised that the GC–MS assay would not be able to provide the fast turn around requested by the clinicians for a dose

Table 1
Comparison of accuracy and precision data for GC–MS and LC–MS–MS assays for I

Method	Nominal concentration (ng/ml)	n	Mean concentration (ng/ml)	Bias (%)	C.V. (%)
GC–MS	0.4	6	0.5	19.0	9.1
	1.0	6	1.0	2.3	4.7
	2.0	6	1.6	−17.9	10.5
	5.0	6	5.0	−0.1	6.0
	10.9	6	10.3	2.7	3.5
	20.0	6	18.1	−9.7	5.4
	50.0	5	52.6	5.1	3.8
	100.0	5	99.4	−0.6	4.3
LC–MS–MS	0.5	6	0.5	−2.3	12.6
	1.0	6	1.0	−2.6	6.6
	3.0	6	2.8	−5.8	2.7
	5.0	6	5.1	1.2	7.5
	10.0	6	10.5	4.8	4.8
	20.0	6	20.9	4.4	4.8
	50.0	5	51.8	3.6	6.0
	100.0	5	96.7	−3.3	6.0

$$\%\,\text{Bias} = \frac{\text{mean concentration} - \text{nominal concentration}}{\text{nominal concentration}} \cdot 100.$$

escalation study. An alternative mass spectrometry based method was therefore sought.

3.2. Liquid chromatography–tandem mass spectrometry

Particle-beam (PB) and thermospray (TSP) interfaces were initially evaluated for an LC–MS method. A good positive-ion EI spectrum of underivatised I was obtained via the PB interface (Fig. 5A) with an injection of 250 ng on a 2.1 mm I.D. column using an aqueous acetonitrile mobile phase at 200 μl/min, although as indicated earlier the molecular ion (m/z 444) is too weak to be considered for quantitative purposes. Using identical HPLC conditions, negative-ion chemical-ionisation (NICI) using methane provided an abundant $[M-H]^-$ ion at m/z 443 which would be suitable for selected-ion monitoring. Similarly, an abundant MH^+ ion is observed with little fragmentation in the positive-ion TSP spectrum of I (Fig. 5b). This was obtained with a similar mobile phase used in the PB experiments but with a 4.6 mm I.D. column at a flow-rate of 1 ml/min. Unfortunately, while both these LC–MS interfaces performed well in terms of maintaining chromatographic integrity, it was quickly established that these ionisation techniques would not provide the sensitivity and/or analytical ruggedness for a bioanalytical method capable of replacing the GC–MS assay.

The installation of a triple quadrupole mass spectrometer equipped with an atmospheric-pressure ionisation (API) source, provided an opportunity to develop an equally sensitive assay that could also provide improved selectivity and sample throughput. API-MS has been shown to be an effective and useful detector for HPLC and other chromatographic techniques [8], and several groups have recently demonstrated the versatility of LC–API-MS for the separation and quantitation of biologically active compounds in the nanogram and subnanogram per millilitre range [9–11]. Gas-phase ions are formed in the

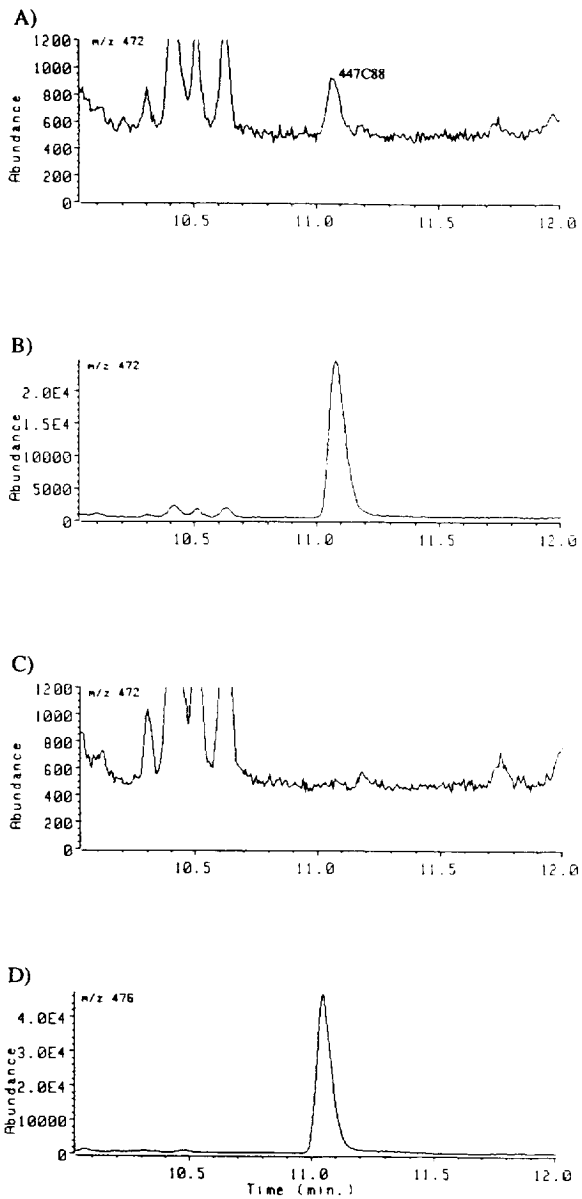

Fig. 4. GC–MS selected-ion monitoring traces (m/z 472) of monoethyl derivative of I of dog plasma extracts containing (A) 1 ng/ml, (B) 50 ng/ml, and (C) 0 ng/ml. (D) Corresponding trace (m/z 476) for [$^2H_4$]I internal standard.

API source by either ion evaporation via an ionspray (ISP) interface, or by atmospheric-pressure chemical ionisation (APCI) using a corona discharge. The latter ionisation mode uses a heated nebuliser interface which allows HPLC mobile phase flow-rates in excess of 1 ml/min to be coupled directly to the API source and it is this approach that has predominantly been used for routine quantitative LC–MS in the pharmaceutical industry [9–11].

ISP and APCI were evaluated for the analysis of I using flow injection analysis of standard solutions into a 50:50 aqueous acetonitrile mobile phase, at 50 and 1000 μl/min, respectively. Both ionisation techniques provided an abundant MH^+ ion at m/z 445 with very little fragmentation under positive-ion conditions and the mass spectrum obtained under APCI is shown in Fig. 6A. Mobile phase cluster ions (e.g. $[MH + MeCN]^+$) observed with both ISP and APCI were reduced by adjustment of the orifice voltage. Although similar sensitivity was observed with both approaches (low ng/ml), the decision was made to proceed with APCI based on its ability to handle higher flow-rates and thus enable 4.6 mm I.D. columns to be used without the need to split.

The negative-ion APCI mass spectrum obtained is shown in Fig. 7A, and is similar to that obtained by PB-NICI with the $[M - H]^-$ ion at m/z 443 as the base peak. In initial LC–MS experiments with plasma extracts using selected-ion monitoring it was found that negative-ion APCI provided greater selectivity than positive ion APCI. Using a 15-cm ODS column it was possible to detect I at 5 ng/ml after the solid-phase extraction stage of the GC–MS assay without pre-concentration of the eluate (500 μl). This alone would result in a 3-fold reduction in the sample preparation time. An isocratic mobile phase of 60% acetonitrile resulted in a retention time of approximately 12 min, similar to that observed for GC–MS. In order to increase the sample throughput and make the LC–MS approach more cost effective, it was necessary to reduce the run time significantly (< 5 min) and this required the introduction of tandem mass spectrometry.

The product ion MS–MS spectra of the MH^+ and $[M - H]^-$ ions of both I and [$^2H_4$]I generated by APCI are presented in Figs. 6B,C and 7B,C, respectively. Comparison of these spectra clearly reveals another advantage of negative-ion

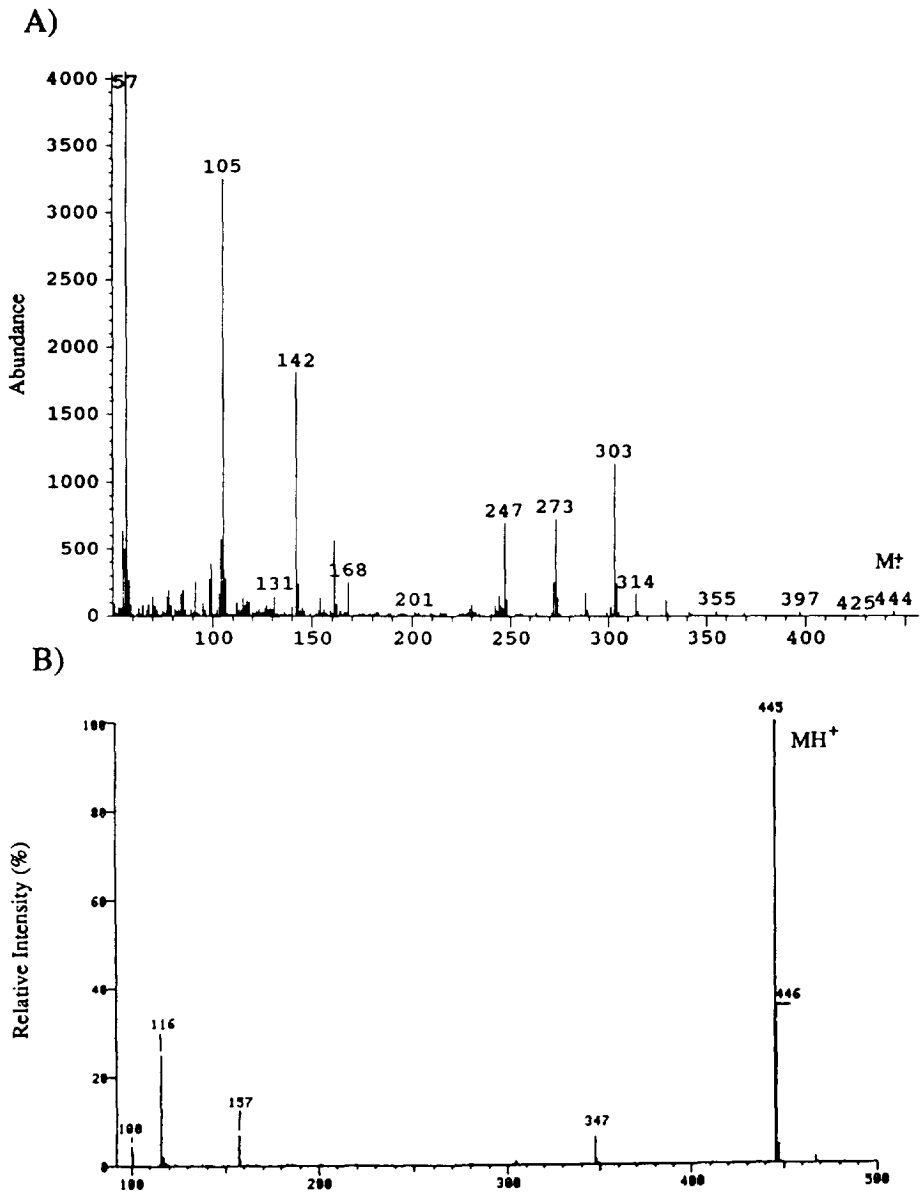

Fig. 5. Positive-ion mass spectra of I obtained by (A) EI particle-beam, and (B) thermospray LC–MS interfaces.

APCI for I; the production of an abundant product ion at m/z 302 (and an equivalent ion at m/z 306 for the deuterated analogue) due to the loss of the n-heptyl urea side chain. Transitions of the respective deprotonated molecules to these product ions were chosen as potential candidates for an LC–MS–MS assay using selected-reaction monitoring (SRM).

Using negative-ion APCI with SRM, it was possible to employ a 3 cm × 4.6 mm I.D. ODS column and increase the acetonitrile content to 80%. Under these conditions the retention time for both analytes was approximately 1.4 min with a total run time for each extract of 2.0 min. The solid-phase extraction procedure used for the GC–MS method was further modified by reduc-

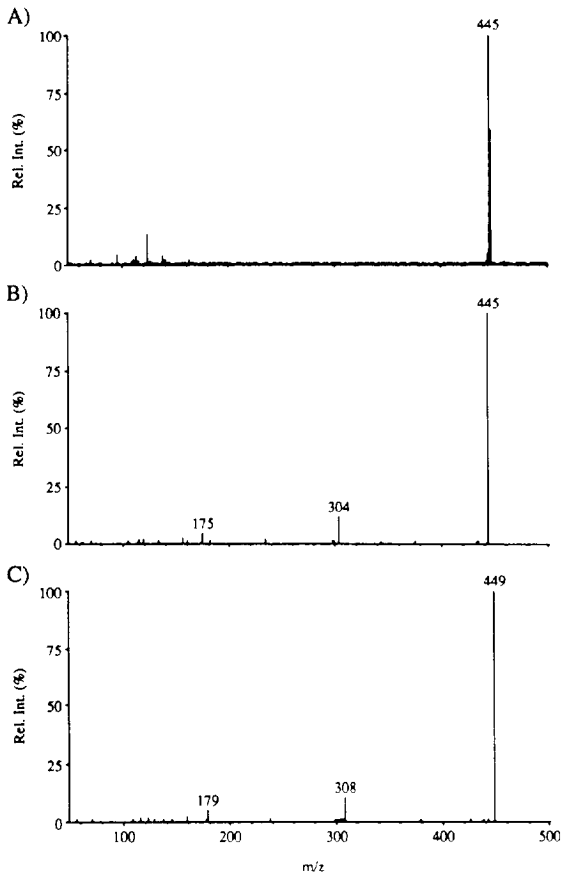

Fig. 6. (A) Positive-ion APCI mass spectrum of I. Product ion MS–MS spectra of MH^+ ions of (B) I (m/z 445), and (C) $[^2H_4]$I (m/z 449). Conditions: 3 cm × 4.6 mm I.D. column packed with 3 μm Spherisorb ODS, aqueous acetonitrile (20:80) at a flow-rate of 1 ml/min, 20-μl injection (5 μg/ml), heated nebuliser interface was maintained at 370°C.

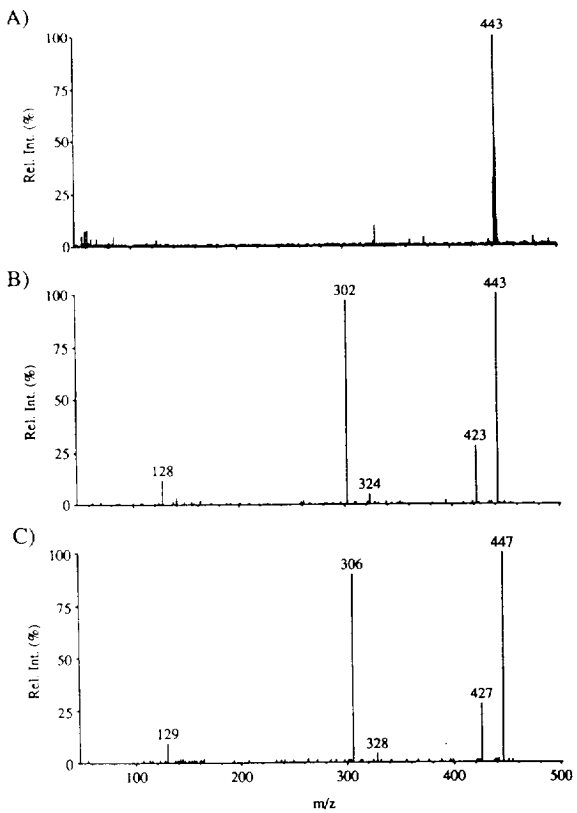

Fig. 7. (A) Negative-ion APCI mass spectrum of I. Product ion MS–MS spectra of $[M-H]^-$ ions of (B) I (m/z 443), and (C) $[^2H_4]$I (m/z 447). Conditions as for Fig. 6.

ing the volume of acetonitrile used to elute I from the cartridge from 500 to 300 μl, of which 20 μl was injected directly onto the HPLC column. This LC–MS–MS method of analysis for I was found to be both sensitive and extremely specific. The SRM response for I at 0.5 ng/ml in human plasma is presented in Fig. 8A, and illustrates the excellent peak shape and signal-to-noise ratio (>50:1) obtained via this approach. As can be expected with both chromatographic and two stages of mass selectivity the trace is clear of any interferences, unlike the earlier GC–MS-SIM chromatograms (Fig. 4). The selectivity of SRM is further demonstrated by the corresponding traces for a blank plasma extract (Fig. 8B). No evidence of interference is observed and the solvent front is only identified by a slight suppression of the noise at approximately 0.4 min. The equivalent SRM trace for the internal standard at approximately 50 ng/ml is also presented (Fig. 8C). The 4 amu difference between the respective precursor and product ions of I and its deuterated analogue, together with unit mass resolution on both quadrupoles ensures no contributions from the internal standard in the analyte channel or vice versa.

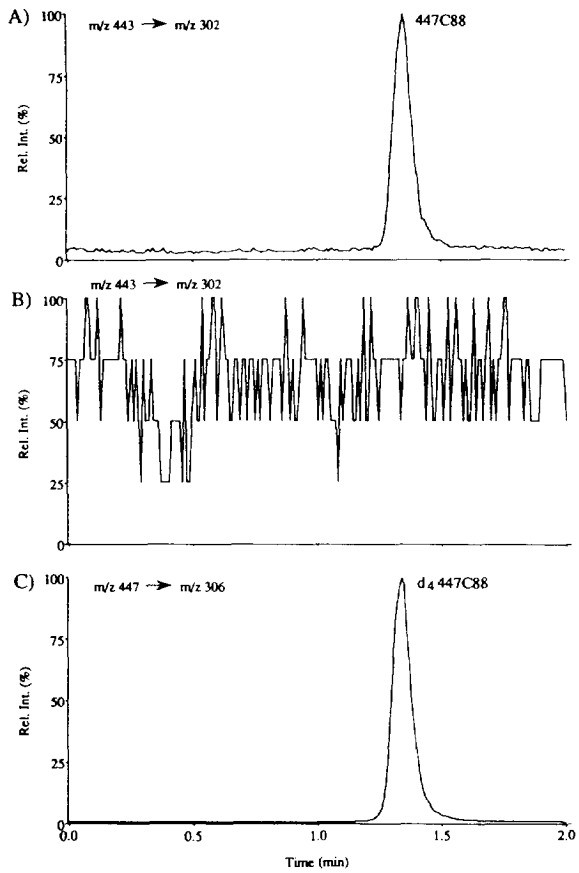

Fig. 8. LC–MS–MS SRM traces (m/z 443 to 302) of I in human plasma extracts at (A) 0.5 ng/ml and (B) 0 ng/ml. (C) Corresponding trace (m/z 447 to 306) for [$^2H_4$]I internal standard. Conditions as for Fig. 6 with an SRM dwell time of 200 ms/transition.

The LC–MS–MS assay was subsequently validated over the range 0.5–100 ng/ml in human, dog and rat plasma. A representative calibration curve generated in human plasma is shown in Fig. 9. Table 1 compares GC–MS and LC–MS–MS validation data for dog plasma. The LC–MS–MS method has proved to be extremely reliable and with the experience with preclinical species it was possible to improve the accuracy and precision of the method for human plasma. Fig. 10 shows a plot of the (low) QC data obtained during support to a Phase I clinical trial to determine the tolerability of I in man. In excess of 1500 samples were analysed in support of this dose escalation study with fast turnaround. It was possible to run 3 individual assays in a 12-h period corresponding to over 240 injections and provided that samples were received in the morning it was possible for results to reach the study director the next day.

4. Conclusions

The sensitivity of APCI and the increased selectivity offered by LC–MS–MS has several advantages over GC–MS for the determination of I. Firstly, the sample preparation time can be dramatically reduced when only the solid-phase extraction step from the GC–MS method is used with no clean-up, derivatisation or concentration steps. Secondly, the run time can be reduced to less than 2 min per injection by using a short HPLC column. As a result the extraction of 80 samples takes approximately 2 h with a further 3 h for their analysis. The other advantage of the LC–MS–MS method is its potential sensitivity. The limit of quantitation for the LC–MS–MS method was originally set at that provided by GC–MS (0.5 ng/ml). However, the LOQ can readily be reduced 10 fold (i.e. to 50 pg/ml) using a combination of increased injection volume and reduced mass resolution. The LC–MS–MS response obtained from I at a concentration of 25 pg/ml in human plasma is shown in Fig. 10.

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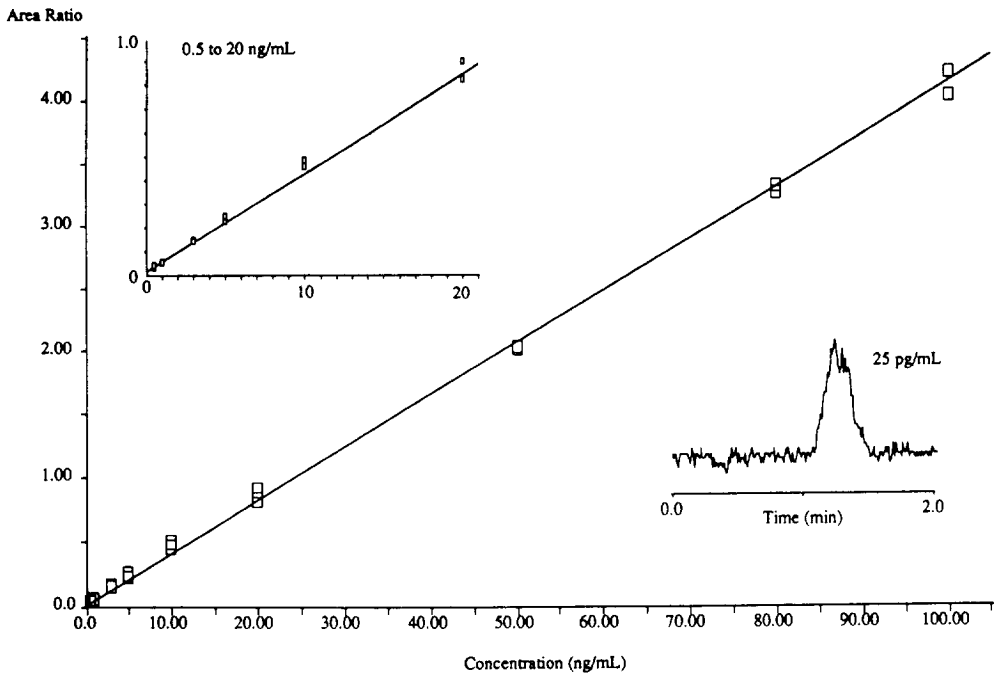

Fig. 9. Representative calibration curve for I in human plasma generated by LC–MS–MS. Insert shows SRM response for 25 pg/ml using modified conditions (see text).

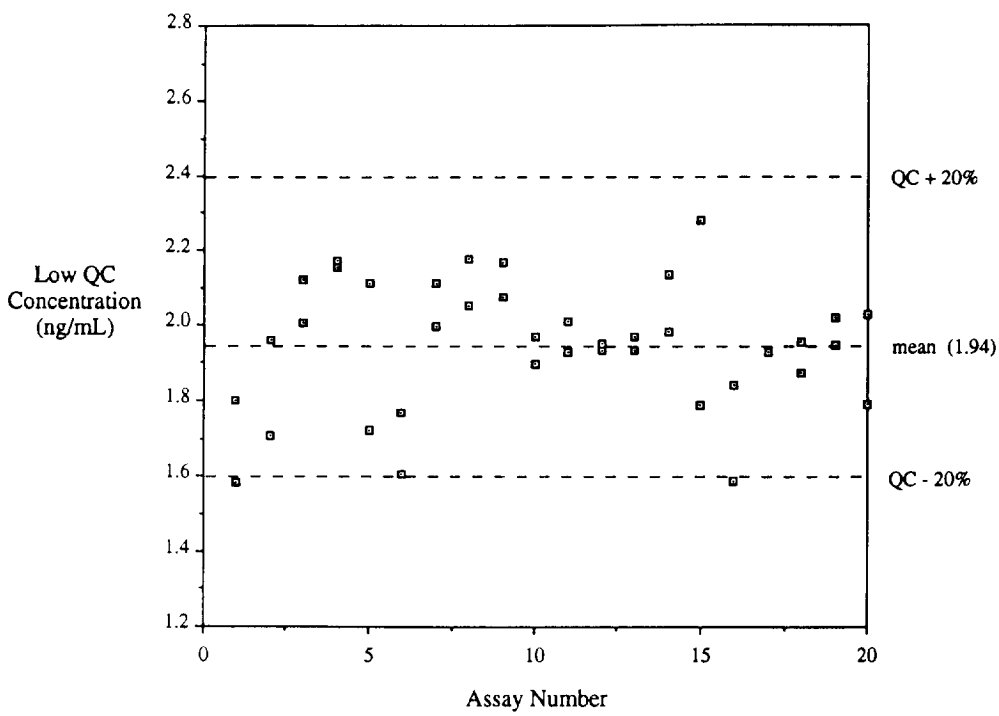

Fig. 10. Plot of measured concentration of low quality control sample (2 ng/ml) from LC–MS–MS method during support to Phase I clinical study with I.

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