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The Development and Validation of a High Performance Liquid Chromatography (HPLC)/ Tandem Mass Spectrometry Assay for Fenticonazole in Human Plasma and Comparison with an HPLC-UV Method

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A method is described for the determination of fenticonazole in human female plasma. The method utilizes high performance liquid chromatography coupled to atmospheric pressure positive-ion chemical ionization triple quadrupole mass spectrometry. Multiple reaction monitoring is employed for selectivity and sensitivity which enables quantification over the range 0.5–20 ng mL^{-1} with acceptable precision and accuracy. A comparison is made with an existing HPLC-UV assay and the utility of the technology of combined liquid chromatography and tandem mass spectrometry for subnanogram per mL assays is discussed.

Fenticonazole nitrate (**I**) is used as an antifungal in the treatment of vaginal candidiasis. A new product is under development within The Upjohn Company for the treatment of mixed vaginal bacterial and candidiasis infections. The combination cream utilises fenticonazole in conjunction with clindamycin (**II**). Due to the topical route of administration and the relatively low level of systemic absorption, a sensitive assay was required to support further development of this product line.

Existing methods for fenticonazole, based on high performance liquid chromatography,[1] with UV detection, had limits of quantification in the region of 5 ng mL^{-1}. The objective of the current study was to rapidly develop and validate an LC/MS/MS method with a subnanogram per mL limit of quantification in female plasma. This paper briefly reviews the stages in LC/MS assay development, and compares and contrasts the 'chromatographic' assay by HPLC-UV with the 'mass spectrometric' assay by LC/MS/MS.

EXPERIMENTAL

Materials

Fenticonazole nitrate was supplied by Recordati Industria Chimicae Farmaceutica, Milan, Italy, and econazole nitrate by Sigma-Aldrich, Poole, Dorset, UK. Ammonium formate was obtained from Aldrich, Gillingham, UK. Formic acid (Analytical reagent grade), acetonitrile (Far UV, HPLC grade), and methanol (HPLC grade) were obtained from Fisons Scientific Equipment Ltd. (Loughborough, Leics., UK). All water used was produced in-house using a Milli-Q water purification system (Millipore (UK) Ltd., Watford, Herts., UK).

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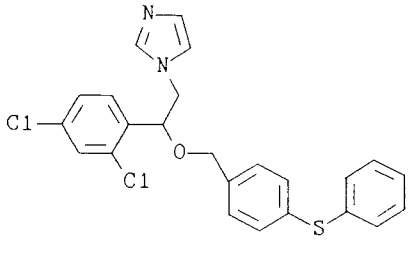
Sample preparation

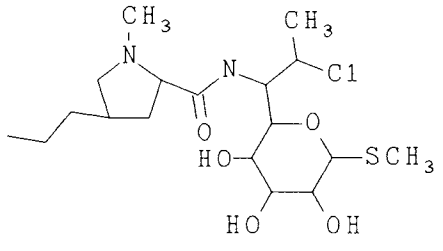
Aliquots (50 µL of clear plasma) were transferred to 1.5 mL polypropylene microtubes, and 100 µL of 2.016 ng mL^{-1} acetonitrile internal standard (econazole) solution was added to each tube and vortex mixed. The samples were centrifuged at 12 000 rpm for 2 min and the supernatant transferred to autosampler vials with low volume inserts using a polypropylene fine-tip pastette.

HPLC using atmospheric pressure chemical ionization (APCI)

HPLC was performed on a Shandon BDS 3 µm C8, 50 × 4.6 mm column (Shandon, Runcorn, Cheshire, UK) with a Brownlee C18, 15 × 3.2 mm guard column (Anachem, Luton, Beds., UK.), maintained at 45 °C. The mobile phase was acetonitrile + 10 mM aqueous ammonium formate (adjusted to pH 3.5 with formic acid) (70 : 30 v/v), at a flow rate of 1 mL min^{-1}. 20 µL of extract was injected using a cycle time of 3.0 min.



I
Fenticonazole



II
Clindamycin

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Table 1. Comparison of experimental conditions

	HPLC-UV	LC/MS/MS
Guard column	Guard Pak μ-BondaPak C18	Brownlee Newguard C18
Column	150 mm × 4.6 mm × 4 μm Phenyl	50 mm × 4.6 mm × 3 μm C8
Flow rate	1.5 mL min^{-1}	1 mL min^{-1}
Mobile phase	76% acetonitrile, 24% (120 mM acetic acid/0.02 mM sodium bisulphate)	70% acetonitrile, 30% (10 mM ammonium formate pH 3.5)
Apparatus	HP 1082 B HPLC Waters 848 autosampler HP3390 integrator	Severn Analytical 6410 pump Waters 715 Ultra WISP PE-SCIEX API III+
Detection	UV absorbtion 254 nm	APCI-MS/MS MRM mode *m/z* 455/199 *m/z* 381/125
Column temperature	40 °C	45 °C
Internal standard	Miconazole	Econazole
Sample aliquot volume	1000 μL	50 μL
Injection volume	80 μL	20 μL
Sample preparation	Multi-step liquid–liquid extraction of basified plasma	Single-step protein precipitation of plasma

The LC/MS/MS analysis was performed using a Perkin Elmer Sciex API III+ triple quadrupole mass spectrometer (PE Sciex, Beaconsfield, UK). The instrument was operated in atmospheric pressure positive-ion chemical ionization (APCI) mode utilizing the heated nebulizer interface. The temperature of the nebulizer probe was set at 500 °C. Curtain, nebulizer and auxiliary gas (nitrogen) flows were 0.8, 0.6 and 2.0 L min^{-1} respectively; the nebulizer gas pressure was 70 psi. Multiple reaction monitoring (MRM) was employed using argon as the collision gas, set to a thickness of approximately 320×10^{12} molecules cm^{-2}, with a collision energy of 35 eV. Parent-to-daughter transitions of *m/z* 455 to *m/z* 199 for fenticonazole, and *m/z* 381 to *m/z* 125 for econazole, the internal standard, and *m/z* 425 to *m/z* 126 for clindamycin were used. The dwell time for each transition was 400 ms. Peak-area ratios for the selected ions were determined automatically using the PE Sciex software package MacQuan. Prior to operation the system is tuned and calibrated in ion spray mode using poly propylene glycol standards. Instrument parameters are optimized for fenticonazole in APCI mode using loop injections of an acetonitrile standard (10 ng mL^{-1}).

Assay validation experiments

Inter-assay variation, accuracy and linearity were assessed from the analysis of three calibration curves, with QC samples, run on three separate occasions. Intra-assay variation, at 0.490, 0.979, 4.896 and 19.584 ng mL^{-1} was determined by analysis of six spiked plasma samples in the same analytical run. The lower limit of quantification (LLOQ), at 0.490ng mL^{-1} was assessed by analysis of six spiked plasma samples in one analytical run.

Stability

The stability of organic (methanol and acetonitrile) standard solutions at four concentrations, stored at −20 °C was assessed by repeat injections over a period of eleven days.

Plasma extract stability

Stability of plasma extracts at room temperature was determined by re-analysis of a standard calibration curve and QC extracts after they had been left in an autosampler at room temperature for 48 h.

24-hour plasma stability

Aliquots (0.2 mL) of the plasma QC samples were left on the bench for 24 h and then analysed using a freshly prepared calibration curve. Samples were prepared in triplicate. The maximum and minimum temperatures over this period were 28 °C and 23 °C respectively.

Freeze/thaw stability

Aliquots (0.2 mL) of QC samples were frozen at −20 °C until solid, removed from the freezer and kept at room temperature until completely thawed. This cycle was repeated a further two times and the samples vortex mixed, extracted and analysed. Samples were prepared in triplicate.

A comparison of the experimental conditions for the HPLC-UV and LC/MS/MS assays are summarized in Table 1.

RESULTS

LC/MS/MS assay validation acceptance criteria were based upon recognized guidelines.[2]

Stability of standard solutions

Aqueous solutions of fenticonazole nitrate were unstable, even at 4 °C, but organic solutions were adequately stable, and were routinely stored at −20 °. Solutions of econazole nitrate showed no evidence of instability during assay validation; methanolic solutions were used for validation, and these proved to be compatible with plasma, given the small volumes of

standard solutions used to prepare calibration and QC samples. Only positive displacement pipettes were used with standard solutions.

Assay Characteristics

Extraction

Extraction efficiency was determined by comparison of the peak areas obtained after injection of a 10.282 ng mL^{-1} methanolic standard and extracts of quality control samples. Extraction efficiencies determined for fenticonazole were 108%, 108% and 109% for the high, medium, and low QC samples respectively. The apparent extraction efficiency is over 100% which probably reflects some evaporation of acetonitrile during sample work-up. This effect, however, appears consistent. The extraction of the internal standard, econazole, is repeatable, the average peak area recorded during validation was $22\,000 \pm 6.1\%$ ($n = 55$).

Linearity and accuracy

The calibration curve was linear over the range of 0.490 ng mL^{-1} to 19.584 ng mL^{-1}. The coefficient of linear regression (r), using a weighting factor of $1/x^2$, was consistently better than 0.99. Back-calculated values for 97% ($n = 36$) of calibrants were within 15% of target for all concentrations.

Repeatability

The inter-assay and intra-assay coefficients of variation (CV) for this assay measured using QC samples were within 10%. The accuracy for all concentrations was within 10% of the target value.

Lower limit of quantification (LLOQ)

A lower limit of quantification (intra-assay CV $< 10\%$) of 0.490 ng mL^{-1} was achieved following extraction of 50 μL of human plasma. This represents approximately 3 pg on column. The accuracy at this level was better than $\pm 10\%$.

Specificity

Reagent blanks and extracts of control plasma routinely gave no peaks interfering with either fenticonazole, or the internal standard econazole. No interfering peaks were found in five control plasma blanks from separate sources. Pooled human (female) plasma used for the calibration curve was interference free, as were other plasma sources investigated during assay development. Clindamycin elutes before fenticonazole or econazole in the HPLC system, and gives a parent ion of m/z 425 and daughter ion of m/z 126. A 1.958 ng mL^{-1} fenticonazole calibration standard spiked with clindamycin at 7 ng mL^{-1} showed no interference (Fig. 4).

The comparative performance statistics for the two assays are listed in Table 2. Key features of the LC/MS/MS assay are the lower limit of quantification (LLOQ) from a much smaller sample size, and a more rapid assay. The inter- and intra-assay precision and accuracy of the two methods are comparable at the 5 ng mL^{-1} level.

DISCUSSION

The chromatogram obtained using HPLC with UV detection for a 5 ng mL^{-1} calibration standard (LLOQ) with miconazole as the internal standard is shown in Fig. 1. The retention time for fenticonazole, peak A, is 12.9 min and the full analysis time is in excess of 15 min. The comparative chromatogram obtained by LC-MS-MS for a 0.5 ng mL^{-1} calibration standard (LLOQ) is shown in Fig. 2. The internal standard used is econazole, the retention time for fenticonazole is 2.25 min and the assay cycle time is 3 min.

The experimental conditions used in the original HPLC-UV assay are described in Table 1. In developing the LC/MS/MS method, four major changes were made:

(i) The internal standard was changed from miconazole to econazole to minimize the effect of the isotopic molecular ion distribution. Econazole and miconazole are closely related analogues (**III**). The tetrachlorinated miconazole has a more complex isotopic distribution than the trichlorinated econazole. The molecular ion region of the APCI spectrum of fenticonazole is illustrated in Fig. 3.

(ii) The extraction procedure was changed from a multiple-step liquid–liquid extraction to a single-step protein precipitation. The sample size was reduced from 1 mL to 50 μL plasma.

Table 2. Comparison of assay performance

	HPLC-UV	LC/MS/MS
Lower limit of quantification	5 ng mL^{-1} (see Fig. 1)	0.5 ng mL^{-1} (see Fig. 2)
Inter-assay precision and accuracy	5.0 ng mL^{-1} ($n = 6$)	4.025 ng mL^{-1} ($n = 9$)
Mean	5.56	4.034
Standard deviation	0.40	0.25
% CV	7.2	6.1
% Accuracy	+11.2	+0.2
Intra-assay precision and accuracy	5 ng mL^{-1}	4.889 ng mL^{-1}
Mean	4.88	4.886
Standard deviation	0.32	0.24
% CV	6.6	4.9
% Accuracy	−2.4	−0.2
Signal:noise ratio at 10 ng mL^{-1} fenticonazole calibration standard	5:1	580:1
Retention time, fenticonazole	12.9 min	2.2 min
Retention time, internal standard	9.9 min	1.6 min
On-column sensitivity at LLOQ	8 ng	3 pg

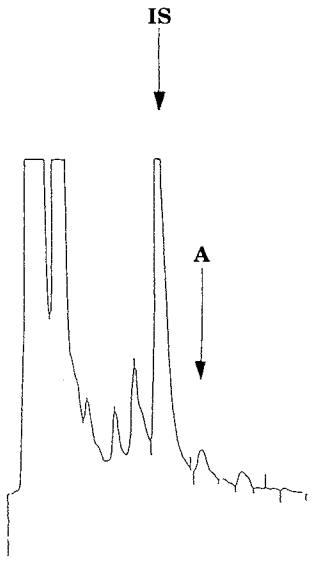
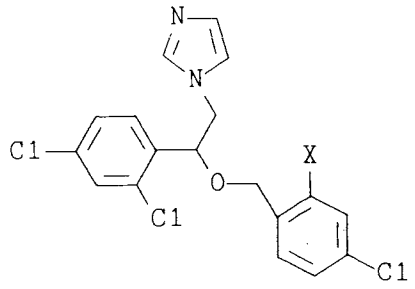


Figure 1. HPLC-UV chromatogram of a 5 ng mL^{-1} fenticonazole (A) calibration standard with miconazole (IS) as the internal standard.

(iii) A volatile acid buffer was used in the mobile phase to assist with ionization.
(iv) A shorter column was utilized to reduce chromatographic run time.

In our laboratories the heated nebulizer (HNI), atmospheric pressure chemical ionization (APCI) interface on the PE Sciex API III + , is generally used in preference to the electrospray (ionspray, ISP) interface for quantitative assays. This is due to its improved sensitivity, robustness and compatibility with LC conditions developed for HPLC-UV. In summary, its ease of use and reliability justify the use of the HNI over the ISP interface.



III
X = H Econazole
X = Cl Miconazole

The majority of LC/APCI-MS/MS assays developed in our laboratories are very similar in terms of guard column, analytical column, flow rate, mobile phase and sample preparation. This facilitates rapid method development for analogous compounds. However, particular attention is paid to sample cleanliness.

Although so-called 'dilute and shoot' urine and plasma assays have been developed, as in this case,

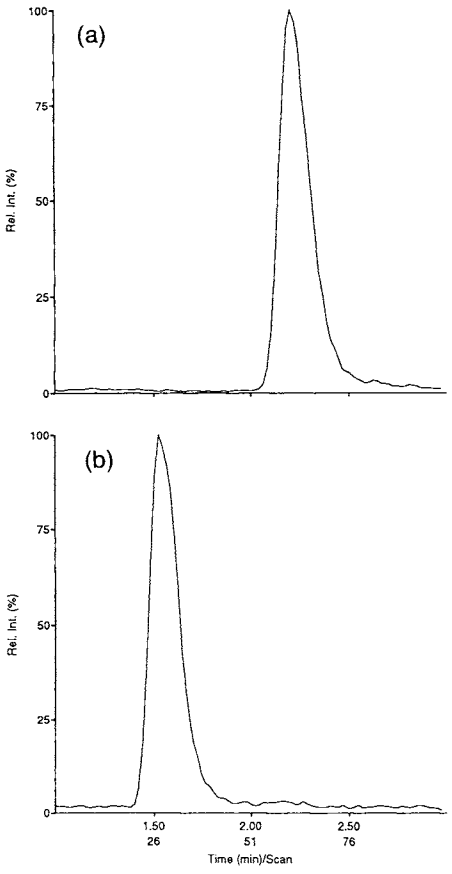


Figure 2. LC/MS/MS mass chromatograms of a 5 ng mL^{-1} calibration standard showing (a) fenticonazole and (b) econazole, the internal standard.

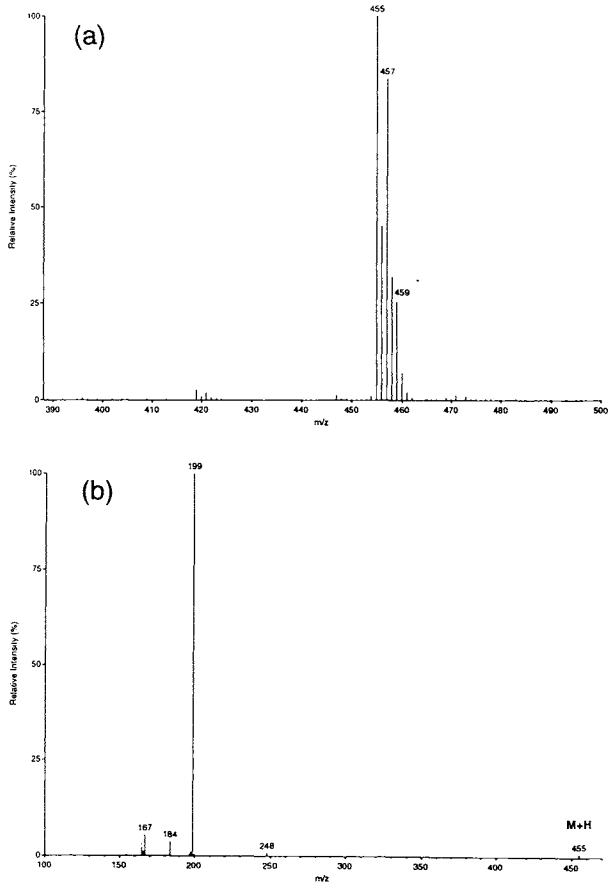


Figure 3. Mass spectra of fenticonazole: (a) APCI spectrum showing the molecular ion region; (b) product-ion spectrum with collision energy of 30 eV.

they are generally insufficiently robust to analyse more than about 80 samples per run. Loss of instrument performance is characterized by a decrease in the signal-to-noise ratio, indicating that cleaning of the APCI interface is required.

In developing the fenticonazole assay, a number of factors affecting assay performance were evaluated and subsequently optimized. These included HPLC mobile phase composition to ensure compatibility with the HNI, efficient ionization and rapid chromatographic elution whilst providing sufficient resolution of the analytes from the solvent front containing potentially interfering endogenous components.

Also evaluated were the MS/MS parameters. It is fortunate that the fenticonazole pseudo-molecular ion ($[M+H]^+$ at m/z 455) is cleaved adjacent to the benzylic carbon to yield almost 100% of a single product ion at m/z 199 (see Fig. 3). The optimization of the MS/MS parameters was therefore limited to ensuring that sufficient collision energy was provided to maximize the formation of the m/z 199 ion. Resolution of the parent ion was maintained at unit mass, despite the presence of the chlorine isotope distribution.

A key factor in the success of this and any high sensitivity assay is the production of a clean blank extract. In this case a simple protein precipitation was sufficient to provide a clean extract, as specificity could be obtained by resolution in the mass domain. The specificity of the assay was confirmed by the analysis of a calibration standard spiked with clindamycin to ensure no interference from the co-administered compound. Figure 4 shows that, even with a run time of 3 min, chromatographic resolution of clindamycin, fenticonazole and econazole has been achieved.

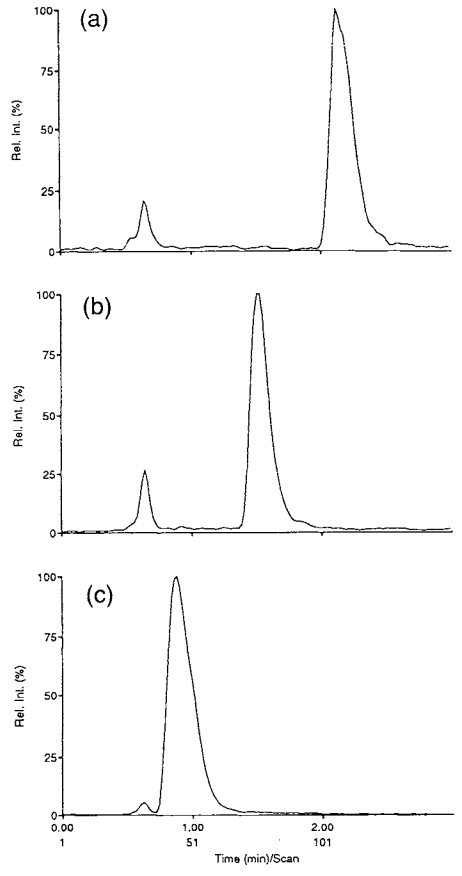


Figure 4. LC/MS/MS mass chromatograms of a 2 ng mL^{-1} calibration standard spiked with clindamycin (equivalent to 7 ng mL^{-1}) illustrating assay specificity: (a) fenticonazole (b) econazole and (c) clindamycin.

GENERAL CONCLUSIONS

Based upon studies conducted in our laboratories, LC/MS/MS has provided a quick and simple method for the analysis of a wide range of compounds, not just those with poor UV chromophores. The utility of this technology has been demonstrated in the support of discovery programmes where rapid method development and sample analysis is required, and also, with large-scale studies where high sample throughput may be the primary consideration.

The introduction of short (3 and 5 cm) HPLC columns with 3 μm packings in a variety of phases allows for very short analytical run times with minimal regard to chromatographic separation, relying on the specificity and selectivity of MS/MS detection to resolve components.

The imminent arrival of relatively low-cost bench-top LC/MS/MS systems coupled to powerful PC-based data systems, (for example, the Finnigan LC-Q ion-trap system offering LC/MS^n), will make this a powerful option available to an increasing number of analysts.

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1. Recordati internal report; July 1991.
2. J. A. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi *et al.*, *J. Pharm. Sci.* **81**, 309 (1992).