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Quantitative Determination of Cabergoline in Human Plasma Using Liquid Chromatography Combined with Tandem Mass Spectrometry

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A liquid chromatography/mass spectrometry (LC/MS) method using electrospray ionization (ESI) is described for the quantitative determination of cabergoline (N-[3-(dimethylamino)propyl]-N-(ethylamino)-carbonyl-6-(2-propenyl)-ergoline-8β-carboxamide) in human plasma. The method consists of liquid–liquid extraction after addition of deuterated internal standard, and reverse-phase liquid chromatography with electrospray ionization combined with tandem mass spectrometry (MS/MS). Using selected reaction monitoring, the method provides a quantitation limit of 1.86 pg/mL. Calibration curves acquired on five different days showed good linearity ($r > 0.99$) in the range 1.86–124 pg/mL and reproducibility of the slope (% relative standard deviation, RSD = 10.0). The intra-day precision, determined by assaying plasma containing four different concentrations of cabergoline processed in replicate, was found to range from 2.4 to 17.0% (RSD). The inter-day precision, evaluated for the same concentrations, ranged from 7.9 to 10.7% (RSD). The accuracy of the method, expressed as the percent ratio between found to added amount, was 99.1 $\pm$ 10.2% (RSD = 10.3%, n = 78). © **1998 John Wiley & Sons, Ltd.**

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Cabergoline (FCE 21336), N-[3-(dimethylamino)propyl]-N-(ethylamino)carbonyl-6-(2-propenyl)-ergoline-8β-carboxamide (Fig. 1) is a dopamine D2-receptor agonist.[1] Cabergoline is used for the treatment of hyperprolactinemia in humans[2] and has also been shown to be effective in the treatment of Parkinson's disease.[3]

A high performance liquid chromatography (HPLC) method with electrochemical detection (quantitation limits 0.2 and 0.3 ng/mL for plasma and urine, respectively)[4] and a radioimmunoassay method (RIA) (quantitation limits 0.08 and 0.15 ng/mL for plasma and urine, respectively) (E. Pianezzola, unpublished results), were initially developed for the assay of unlabelled cabergoline in biological fluids. These methods permitted the evaluation of urinary kinetics of cabergoline, but the determination of plasma levels of the drug was not possible after administration of single doses of cabergoline lower than 1 mg. (Internal report, unpublished results).

A more sensitive RIA method was subsequently developed to quantify cabergoline in plasma samples.[5] This method made it possible to quantify the drug down to about 12 pg/mL in plasma and 117 pg/mL in urine, and was used for most of the pharmacokinetics studies. The specificity of the method was tested against several cabergoline derivatives synthesized as possible metabolites. The only identified metabolite that cross-reacted with the antibody was a metabolite whose concentration was found to be below the quantitation limit among cabergoline urinary metabolites.[5] After administration of single doses of 0.5, 1 and 1.5 mg of cabergoline to healthy volunteers, maximum cabergoline concentrations in plasma were 33 $\pm$ 12, 40 $\pm$ 8 and 67 $\pm$ 33 pg/mL, respectively, at 2 h using the most sensitive RIA method (S. Pessiani, unpublished results). Due to the very low dosage in humans, it was deemed necessary to develop a more sensitive method for measuring plasma levels of cabergoline.

For quantitative analysis LC/MS, especially with atmospheric pressure ionization, is becoming a powerful technique. In addition, the application of tandem mass spectrometry has improved dramatically both the sensitivity and selectivity of the determinations, compared to those of classical analytical methods. A large number of studies have been performed using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) for the quantitative analyses of drugs in biological fluids[6–10]. Quantitative determination of ergolinic compounds in body fluids was successfully developed using a direct exposure probe and tandem mass spectrometry and gas chromatography combined with MS/MS for concentrations as low as 10 pg/mL[11] and 2 pg/mL,[12] respectively. LC/MS with an electrospray ionization (ESI) interface, in conjunction with tandem mass spectrometry, has been employed recently for analysis of an ergolinic compound in human urine with a quantitation limit of 50 pg/mL[13]. Therefore the combination of HPLC with tandem mass spectrometry through atmospheric pressure ionization was envisaged as a sensitive and specific technique for the measurement of very low levels of cabergoline in human plasma.

A first attempt was made to develop a specific assay by LC/MS/MS with electrospray ionization. A cabergoline analogue was used as internal standard together with a liquid–liquid extraction procedure. The analytical conditions necessary to chromatographically separate cabergoline from the internal standard did not allow a good signal to noise ratio and a good peak shape, and in addition the reproducibility of the data was sometimes affected. Never-

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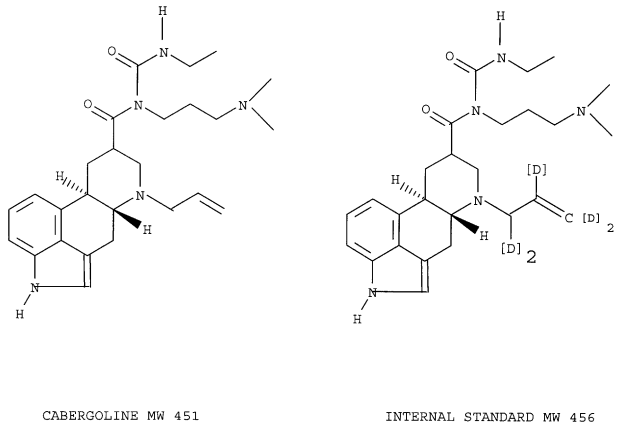


Figure 1. Chemical structure of cabergoline (MW 451) and deuterated internal standard (MW 456).

theless, the method was validated down to 5 pg/mL, with *intra-day* precision ranged from 1.0 to 18.9 (%RSD); *inter-day* precision ranged from 7.3 to 11.7 (%RSD). The method was not considered suitable for routine applications (C. Allievi, unpublished results).

This paper describes a more suitable method which allows the quantitation of cabergoline in plasma at concentration as low as 2 pg/mL, using a deuterated internal standard (FCE 29496) (Fig. 1).

EXPERIMENTAL

Chemicals and reagents

Cabergoline (FCE 21336) and FCE 29496 were synthesized at Pharmacia & Upjohn (Nerviano, Italy). All analytical reagents and solvent were from Farmitalia Carlo Erba (Milan, Italy)

LC/MS/MS equipment

A TSQ 7000 triple-quadrupole instrument (Finnigan-Mat, San Jose, CA, USA), equipped with electrospray ion source and a Digital DEC 3000/300LX Alpha workstation, was used. A Waters 600-MS HPLC pump (Waters-Milford, MA, USA), equipped with a Waters 717 autosampler and a μ-Bondapak C18 column (150 × 3.9mm–10μm particle size), was coupled to the mass spectrometer. The mobile phase was aqueous ammonium formate (10 mM, pH 3) / acetonitrile (70/30 v/v) at a flow rate of 0.5mL/min. The ESI parameters were adjusted to obtain the highest $[M+H]^+$ ion abundance, and were: capillary temperature 270 °C, capillary voltage 10 V, tube lens voltage 65 V, octapole offset 0.8 V, manifold temperature 70 °C, spray voltage 4.5 KV, nitrogen pressure 70 psi.

Quantitative data were obtained by selected reaction monitoring of *m/z* 452 → 381 for cabergoline and *m/z* 457 → 386 for the internal standard, under the following collision-induced dissociation conditions (CID): argon gas pressure 2 m Torr, and collision energy −19 V with a total scan time of 1 s and a peak width of about 2 *m/z* units (at the base of the peak).

Standard solutions and sample preparation

Stock standard solutions of cabergoline and internal standard were prepared separately at a concentration of 1 mg/mL dissolved in HCOOH 0.1M in a 2 mL volumetric flask, previously silanised, giving a nominal concentration of 1000 ng/μL. Three working solutions were prepared every day containing 10, 1 and 0.1 pg/μL of cabergoline and one working solution of 1 pg/μL of internal standard in HCOOH 0.1M.

Five test tubes with 1 mL of human plasma, each containing 21.57 pg of internal standard and 1.86, 4.96, 12.40, 49.60 or 124 pg of cabergoline for calibration curves, plus 16 quality control samples for replicate analyses each containing 21.57 pg of internal standard and 3.10, 8.68, 24.80 or 86.80 pg of cabergoline, were prepared. Five test tubes containing 1 mL of drug free human plasma were also added to the sample stream.

Extraction procedure

To 1 mL of human plasma 0.5 mL of borate buffer at pH 9 (final pH = 8.5–8.6) was added. After vortexing, 2.5 mL of a mixture of methylene chloride/isooctane, 2:3, was added. The samples were shaken for 25 min and then centrifuged at 1500 g at room temperature for 15 min. The organic layer was transferred to a clean tube and 0.2 mL of HCOOH 0.1M was added. After 1 min of vortexing and 15 min of centrifugation at 1500g, the aqueous solution was then transferred to the autosampler vial insert, and 150 μL out of 200 μL were injected into the LC/MS/MS system.

Recovery

The recovery of FCE 21336 was calculated using ^{14}C-labelled cabergoline (specific activity 134.092 μCi/mg). The labelled compound was also used to verify the stability of the product in polypropylene tubes. Three plasma samples containing about 1 μg (309.21 dpm ± 5.87, CV 1.89%, $n = 3$) were submitted for radioactivity determination after the extraction procedure described above.

Calibration graphs

Five calibration graphs were established using blank human plasma spiked with amounts of cabergoline ranging from 1.86 to 124 pg/mL. Each sample, containing also 21.57 pg/mL of internal standard, was submitted to the entire extraction procedure and analysis.

Linearity and limit of quantification

A linear calibration model was applied $A_x/A_{i.s.} = a\ C_x + b$ where A is the peak area, C the concentration, a the slope, b the intercept, x refers to cabergoline and i.s. to the internal standard. Linear regression analysis was applied to the calibration data on five different days, using weighting by $1/y^2$. Comparison between the accuracy obtained for the lowest concentrations, using different weighting regressions, made it possible to select the weight $1/y^2$ as the best method to obtain the lowest relative standard deviation.

The limit of quantification was defined as the minimum concentration of the analyte in plasma giving a signal to noise ratio of at least 3:1 under the assay conditions.

Precision and accuracy

Intra-day and *inter-day* precision were assessed by replicate determinations of cabergoline concentrations in plasma on the same day and on five different days,

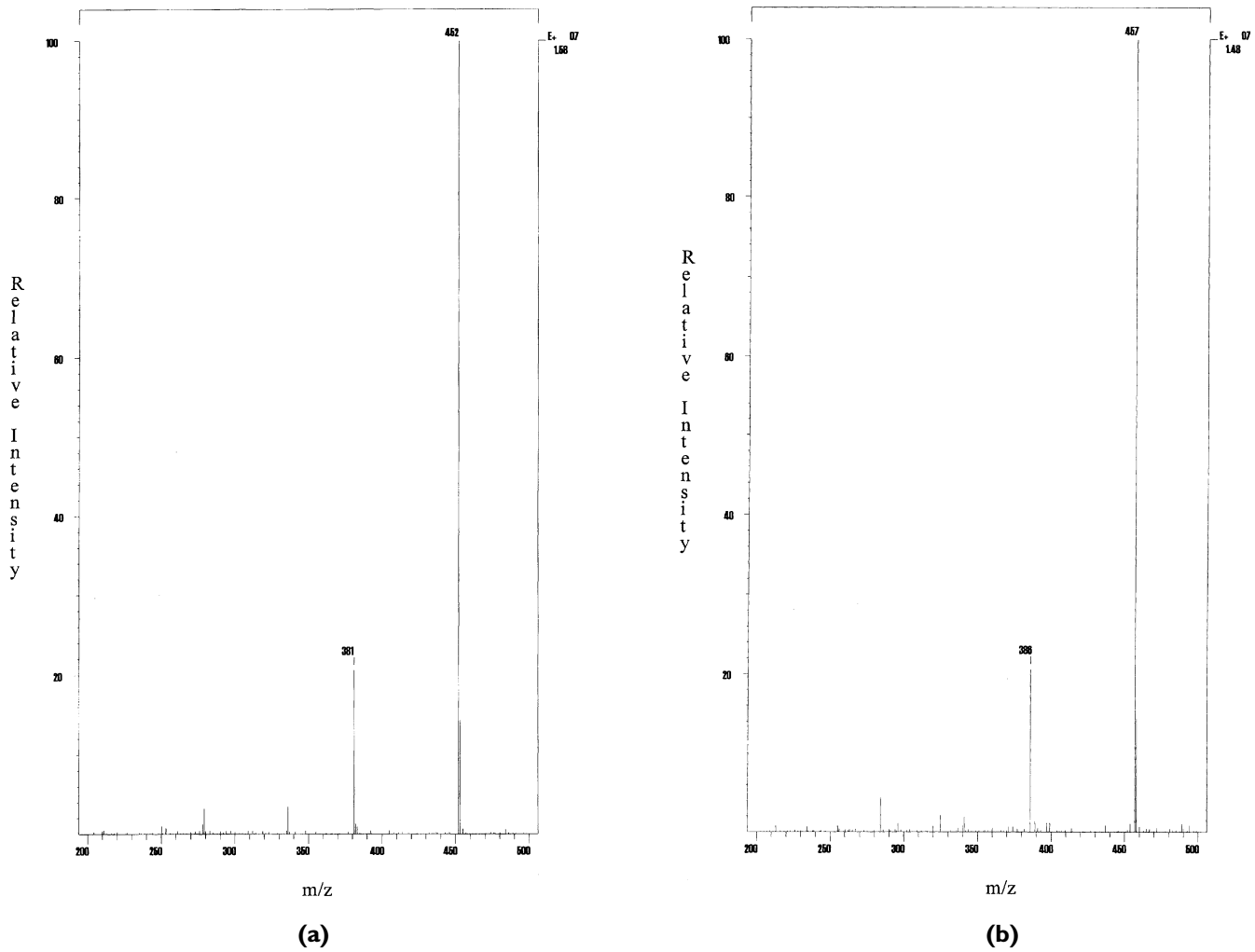


Figure 2. (a) Electrospray mass spectrum of cabergoline, (b) electrospray mass spectrum of internal standard.

respectively. The same samples analysed for the *intra and inter-day* precision were used to calculate the accuracy of the method.

RESULTS

In the ESI mass spectra of FCE 21336 and FCE 29496, the protonated molecular ions $[M+H]^+$ were predominant (Fig. 2(a), 2(b) with low abundance fragmentation observed giving ions of *m/z* 381 and 386, respectively, due to the loss of CH_3-CH_2-N = C = O from the side chain of the urea function. No isotopic contamination of cabergoline was seen in the pentadeuterocabergoline used as internal standard (not shown). No fragment ion of *m/z* 381 was generated during the collision-induced dissociation of the $[M+H]^+$ ion of the internal standard, and no interfering ion of *m/z* 386 was produced during the collision-induced dissociation of the $[M+H]^+$ ion of FCE 21336 standard (not shown). Investigation of the MS/MS behaviour of cabergoline showed that the fragment ion of *m/z* 381 was the most suitable ion for quantitative determination (Fig. 3) by selected reaction monitoring (SRM). The fragment ion of *m/z* 386 was taken, by analogy, as the most adequate ion of pentadeuterocabergoline used as internal standard. The MS/MS fragmentation pathway is briefly described in Scheme 1.

As an example of the results obtained with this method, Fig. 4 shows the chromatograms of blank plasma and spiked plasma samples at concentrations of zero, 1.86 and 124pg/mL of FCE 21336 in the presence of FCE 29496 (21.57 pg/ml). The upper trace of each part of Fig. 4 corresponds to the SRM chromatogram produced by the fragment ion with *m/z* 381, while the lower trace corresponds to the SRM chromatogram produced by the ion of *m/z* 386 from the $[M+H]^+$ ion of the internal standard. No interfering peaks were present in the blank plasma extract.

A small carrier effect occasionally occurred with some of the blank plasma. This problem could be avoided by thorough washing of the autosampler connection lines and loop with 6N nitric acid, followed by rinsing with water and the mobile phase, as well as by replacing the column filters.

In any case this carrier effect, probably due to the very high sensitivity necessary to detect very small amounts of cabergoline, did not seriously affect quantification, as shown by the precision and accuracy data (Table 1).

On each day the calibration curve for FCE 21336 was calculated as described above, and the statistical evaluation showed a good reproducibility of the slope (0.0514 ± 0.0051, %RSD = 10.0, $n = 5$). The correlation coefficient was 0.9951 ± 0.0031 (%RSD = 0.31, $n = 5$). The intercept values submitted to Student's *t*-test were

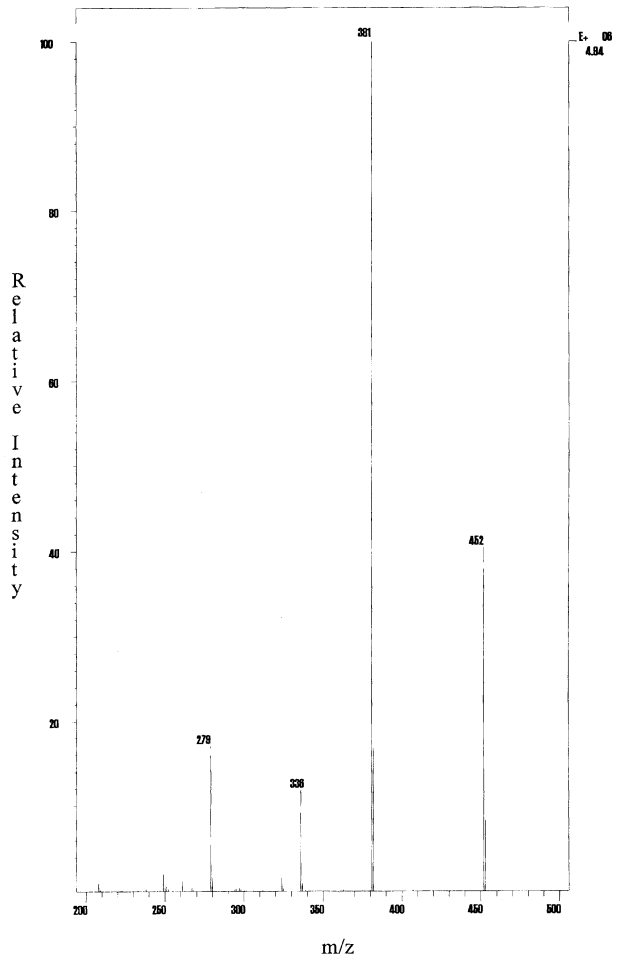


Figure 3. Collision-induced dissociation fragment ion spectrum of the $[M+H]^+$ ion of cabergoline.

not significantly different from zero ($p<0.05$). Figure 5 shows a typical calibration curve.

The extraction yield of FCE 21336, calculated as previously described, was 64.7%. Cabergoline was found to be stable in the polypropylene tubes used for the analyses. Table 1 contains the results of the validation study. These data give the *intra-day* precision and accuracy, the *inter-day* precision and accuracy, and the overall accuracy. The *intra-day* precision, expressed as %RSD of replicate analysis of the concentrations tested, ranged from 2.4 to 17.0. The *inter-day* precision evaluated for the same concentrations ranged from 7.9 to 10.7%.

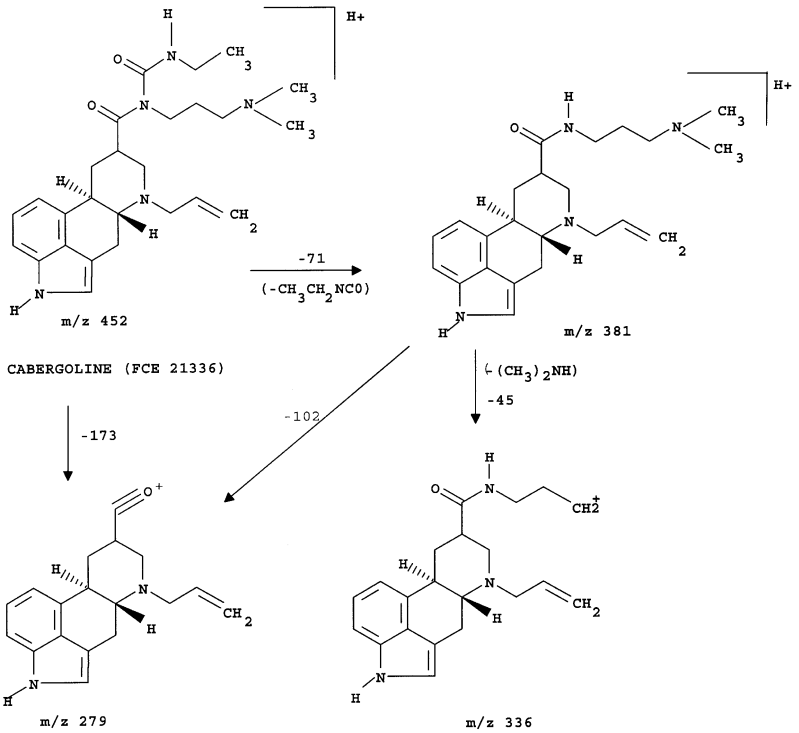
The overall accuracy, expressed as (found/added) amounts, was 99.1 ± 10.2 (%RSD = 10.3).

The limit of quantification was 1.86 pg/mL, corresponding to a mean signal to noise ratio of 18.8 ± 13.1, and is comparable to the sensitivity obtained with similar compounds analysed by GC/MS/MS[11].

DISCUSSION AND CONCLUSIONS

Compared to the first LC/MS/MS method developed, the use of a stable isotope labelled internal standard, along with more suitable analytical conditions, improved the reproducibility of the results as well as the accuracy and precision. In terms of sensitivity, the validated quantitation limit could have been lowered as the signal to noise ratio at 1.86 pg/mL was still very good. However, at lower concentrations the carrier effect might become more important and affect the reliability of the data.

The abundance of $[M+H]^+$ ions for cabergoline was evaluated as a function of the ESI parameters, and CID efficiency for the selected product ion was assessed as a function of CID gas pressure and collision gas energy. CID conditions were adjusted in order to obtain the maximum



Scheme 1. Proposed CID fragmentation pattern of cabergoline.

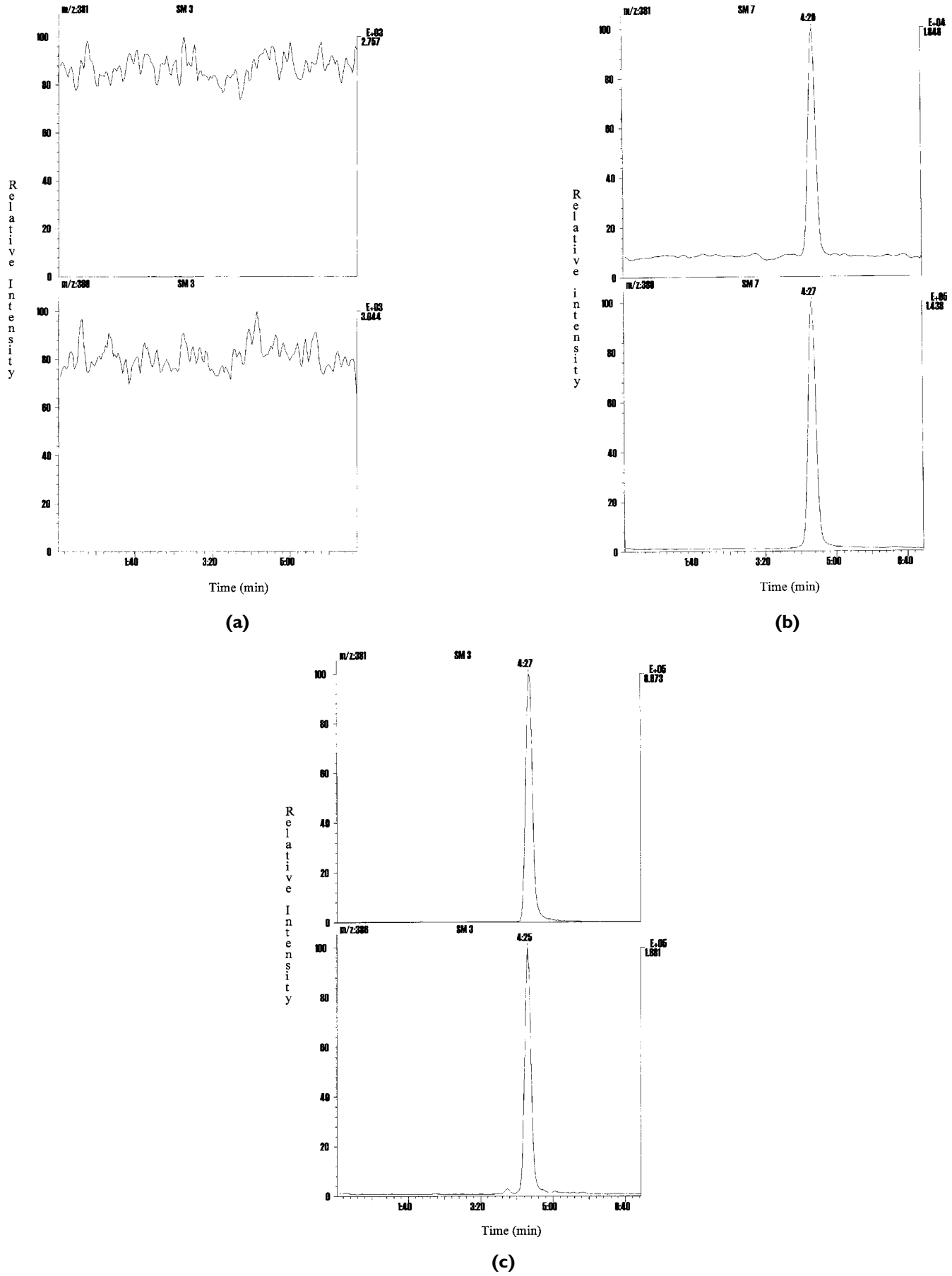


Figure 4. LC/MS/MS selected reaction monitoring chromatogram of: (a) drug-free plasma extract, (b) plasma extract spiked with 1.86 pg/mL of cabergoline (*m/z* 452→381) and 21.57 pg/mL of I.S. (457→386), (c) plasma extract spiked with 124 pg/mL of cabergoline (452→381) and 21.57 pg/mL of I.S (457→386).

Table 1. ***Intra-day and inter-day*** **precision and accuracy of ESI/MS/MS method for the determination of cabergoline in human plasma.**

Day	N° analyses	Spiked amount (pg/mL)	Intra-day precision (%RSD)	Inter-day precision (%RSD)	Accuracy found/added (%)±S.D.
1	5		13.7		
2	4	3.10	17.0		
3	5		4.5		
4	5		5.2		
5	5		2.4	10.2 (n = 24)	97.3 ± 9.9
1	5		10.1		
2	4	8.68	11.8		
3	5		6.5		
4	5		10.8		
5	5		8.4	10.0 (n = 24)	103.6 ± 10.4
1	3		6.7		
2	3	24.8	5.5		
3	3		3.6		
4	3		8.3		
5	3		6.0	7.9 (n = 15)	99.8 ± 7.9
1	3		17.1		
2	3	86.8	9.5		
3	3		2.7		
4	3		4.5		
5	3		7.6	10.7 (n = 15)	94.2 ± 10.1
			overall accuracy 99.1 ± 10.2 (%RSD = 10.3) n = 78		

intensity of the product ion of *m/z* 381. The relatively low collision energy (19 V) was chosen to obtain the highest intensity of the base product ion.

The stability of cabergoline stock solution was evaluated over one month by HPLC determination with ultraviolet detection (λ = 225nm) and found to be very good (99.1% at 15 days and 91% at one month).

The reproducibility of the signal was evaluated for different concentrations of standards (FCE 21336 and internal standard) by repeated injections under various conditions of capillary temperature and scan time. Within the same day good reproducibility of the signal was observed (%RSD ranged from 0.7 to 7.1) for 1000pg and 1pg of cabergoline respectively, n = 5 and %RSD = 14.5 for 100pg of internal standard (n = 35), at a capillary temperature of 270 °C and 1 s scan time. A scan time of 1 s and a capillary temperature of 270 °C were considered the best choices to obtain the maximum intensity of the signal with minimum noise.

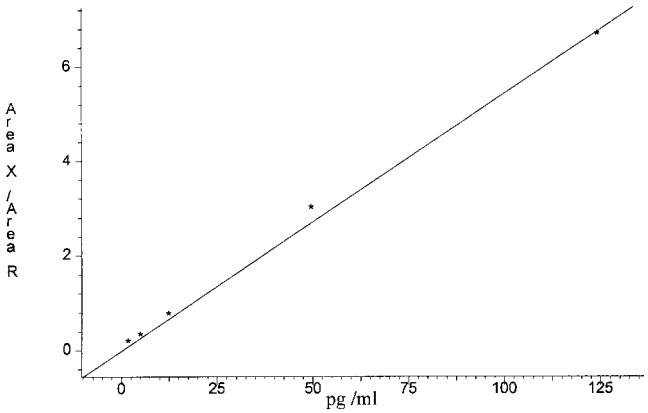


Figure 5. Typical calibration curve of cabergoline in plasma in the range 1.86–124 pg/mL (linear weighted regression $1/y^2$).

Over a few days, a decrease in sensitivity was sometimes observed, probably due to different factors affecting the performance of the instrument. This variation did not affect the detection of the lowest concentration, although in one case a signal to noise ratio of only 3 was observed. The reproducibility of the response in plasma extracts was evaluated during the five days of the validation, comparing the internal standard area within the group of analyses performed on the same day. The %RSD ranged from 17.4 to 30.5 (for 21.57 pg/mL of internal standard). The variability of the response in plasma extracts did not compromise the quantification as the response factor of cabergoline remained unchanged over the whole period of validation (RWR = 1.136 ± 0.117; %RSD = 10.3, n = 25).

Different factors can affect the reproducibility of the response in a range of such low concentrations. For example, cleanliness of the system (autosampler, ESI source, stainless steel capillary, etc.), the position of the fused silica capillary inside the ESI source, the cleanliness and condition of the column filters and all the connection lines, must be considered.

Nevertheless, with a daily fine tuning, the performance of the instrument can be maintained at an acceptable level and allow a large number of plasma samples to be analysed. To date, only methods based on chromatographic separation and mass spectrometry detection can provide the specificity required for the measurement of low levels of drugs in body fluids. In addition, the use of stable isotope labelled internal standards is a great advantage as the requirement for a chromatographic separation can be avoided, and at very low concentrations the identical behaviour shown by the labelled compound provides more reliable data in terms of reproducibility of the response. However, the difficulty in conducting these assays on a routine basis, owing to the very high level of sensitivity that must be maintained, must be considered. A cross-validation study between the LC/MS/MS and the RIA methods will be carried out to validate the suitability of the LC/MS/MS method.

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