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Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 889–898

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

'High throughput' solid-phase extraction technology and turbo ionspray LC-MS-MS applied to the determination of haloperidol in human plasma

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Received 9 September 1998; received in revised form 17 December 1998; accepted 1 January 1999

Abstract

A quantitative method for the analysis of haloperidol in human plasma is described. Sample clean-up was performed by means of solid-phase extraction using 3M Empore™ extraction disk plates in the 96-well format, automated with a Canberra Packard pipetting robot. Separation was performed by reversed phase high performance liquid chromatography with turbo ionspray tandem mass spectrometric detection by monitoring the decay of protonated haloperidol of m/z 376 to its fragment at m/z 165, versus the decay of protonated haloperidol-D4 at m/z 380 to its fragment at m/z 169. The validated concentration range was from 0.100 to 50.0 ng ml^{-1}, with an inaccuracy and overall imprecision below 10% at all concentration levels. Validation results on linearity, specificity, precision, accuracy and stability are shown and are found to be adequate. The average sample preparation time for a batch of 96 samples is approximately 50 min. The chromatographic run time is 3 min. A sample throughput of at least 240 samples per day can be achieved.

Keywords: Haloperidol; High throughput sample preparation; 96-Well solid-phase extraction; Robotics; LC-MS-MS

1. Introduction

Haloperidol (Fig. 1) is a potent neuroleptic, which is widely used in the treatment of schizophrenia [1]. Therapeutic plasma concentrations are in the range of 2–12 ng ml^{-1}. In the context of clinical studies to investigate bioequivalence between haloperidol from different formulations or interaction studies to investigate interaction between haloperidol and investigational drugs, which may be co-administered, an assay was developed to determine haloperidol concentrations in human plasma.

Several analytical methods have been reported for the assay of haloperidol in biological fluids including gas chromatography [2–4], radioimmunoassays [5,6], receptor assays [7,8] and high performance liquid chromatography [9–14]. Recently liquid chromatography with mass spectrometric detection (LC-MS) was also reported for

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0731-7085/99/$ - see front matter
PII: S0731-7085(99)00107-7

the analysis of haloperidol in human plasma [15]. Verweij et al. investigated a broad range of drugs with hypnotic, sedative and tranquillising properties, including haloperidol. They state that, using LC-MS-MS, 10–100 times lower detection limits can be achieved for the majority of the compounds compared to methods more commonly applied [16]. The sensitivity, selectivity and the relatively high throughput that can be achieved with LC-MS-MS nowadays makes this technique often the method of choice for the analysis of drugs in biological fluids.

The introduction of LC-MS-MS as a quantitative technique in the field of routine bioanalysis caused a new bottleneck in the total analytical process to appear. The analysis time on the analytical instrument (typically 3–4 min) was no longer the limiting step. Instead, the time spent on sample preparation became the rate-limiting step. Solid-phase extraction (SPE) has become one of the most popular sample preparation techniques for the analysis of drugs in biological fluids and is also used for the determination of haloperidol in human plasma [11,12,15]. SPE is generally characterised by good reproducibility and high recoveries. Additionally, it is less time-consuming and easier to automate compared to the traditional liquid–liquid extraction methods [17].

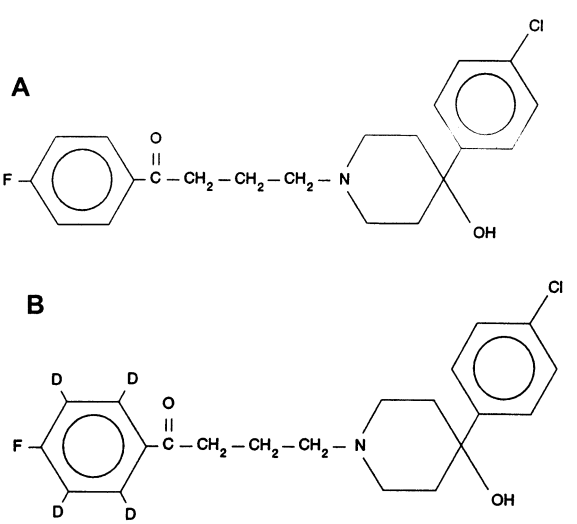

Fig. 1. Structural formulae of haloperidol and haloperidol-D4.

Recently Kaye et al. [18] introduced a novel method for performing SPE more efficiently. They developed techniques for SPE based on 96-well microtiter plates, commonly used in biochemical analysis. The '96-well' SPE technology allows high throughput solid-phase extraction by processing 96 samples in a standard 8×12 microtiter plate format. Sample processing is performed 'off-line' and in 'batch mode' and is therefore ideal for LC-MS-MS and other high throughput applications [19–21].

We have implemented instrumentation for automated 96-well SPE in our laboratory [22]. A bioanalytical procedure for haloperidol on LC-MS-MS was developed and validated using this SPE technology.

2. Experimental

2.1. Chemicals and reagents

Haloperidol and the internal standard haloperidol-D4 were supplied by Sigma (St Louis, MO) and Radian (Austin, TX) respectively. Methanol and ammonium hydroxide were of HPLC grade and supplied by Baker (Philipsburg, NJ). Acetic acid and formic acid were of analytical grade and supplied by Merck (Darmstadt, Germany). Water was purified using a Milli-Ro-10 and a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Solid-phase extraction system

The solid-phase extraction system consisted of a Canberra Packard Multiprobe 104 pipetting robot (Canberra Packard Instruments, Dowers Grove, CT) with 1.0-ml syringes, a vacuum pump (Edwards International, Crawley, UK) and a solid-phase extraction interface (Canberra Packard Benelux). A vacuum control diagram is given in Fig. 2.

Extractions were performed on a 3M vacuum manifold (3M Center, St. Paul, USA) and high performance, octadecyl, standard density, 96-well extraction diskplate (also supplied by 3M). Samples were collected in polypropylene deep well

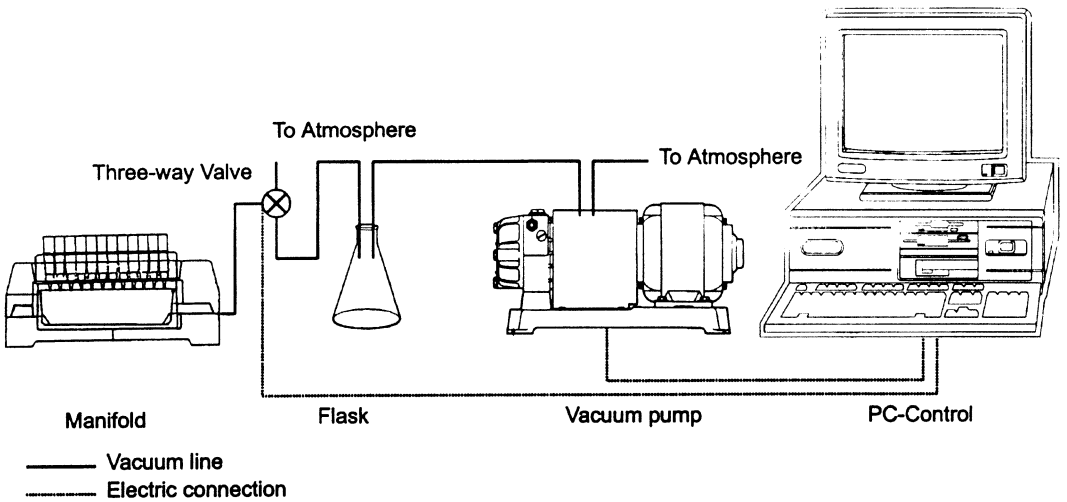

Fig. 2. Vacuum control diagram of the solid-phase extraction system.

collection plates, with a capacity of 1 ml (also 3M). Evaporation of the extracted samples was done with a Zymark Turbo Vap® LV evaporator (Zymark, MA).

2.3. Chromatographic system

A Waters Alliance (Milford, MA) 2690 separations module, delivered the mobile phase consisting of a mixture of methanol and 0.2% formic acid (50:50, v/v) at a flow of 1.0 ml/min (split ratio was 1:3). Injections of 25 μl were made. Samples were stored in the sample compartment at a temperature of 10°C. Reversed-phase high performance liquid chromatography was performed using a Waters 3.5 μm Symmetry C_{18} 100 × 4.6 mm column (Waters, MA) conditioned at a temperature of 40°C.

Detection was performed with a Perkin Elmer Sciex, API 365 triple quadrupole mass spectrometer (PE Sciex, Ontario, Canada) operated in the positive ion mode with a turbo ionspray interface. The probe temperature was set at a temperature of 400°C, the collision energy was 30 eV and the dwell time 500 ms. Data acquisition was achieved using Sample Control software and data analysis was done with MacQuan software version 1.5 (also PE Sciex) on a model 9600/233, Apple Power Macintosh microcomputer.

2.4. Preparation of calibration and validation samples

Stock solutions of haloperidol were prepared by dissolving 10.0 mg of haloperidol in 10.0 ml methanol. Calibration samples and validation samples were prepared by properly diluting the stock solutions of haloperidol with blank, analyte-free, human plasma. For calibration, eight plasma pools were prepared, containing 0.100, 0.200, 0.500, 1.00, 5.00, 10.0, 20.0 and 50.0 ng/ml. For the validation samples, five additional pools were prepared, from an independent stock solution, containing haloperidol concentrations of 0.10, 0.40, 20.0, 45.0 and 100 ng/ml.

2.5. Internal standard solution

A stock solution of haloperidol-D4 was prepared by dissolving 1 ml of haloperidol D-4 standard solution (obtained from the supplier in a concentration of 100 μg/ml) in 10.0 ml of methanol. This stock solution was diluted with water to prepare a working solution with a concentration of 50.0 ng/ml.

2.6. Sample preparation

Within an hour prior to the sample preparation all samples, calibration and validation samples were thawed at room temperature. Subsequently, the samples were homogenized and centrifuged for 10 min at 3200 × *g* prior to loading on the 3M C_{18} extraction disk plates using the modified Canberra Packard pipetting robot. Following conditioning with 250 μl of methanol, 250 μl of water and 250 μl of 0.5% ammonia in water (pH ≈ 10.5), the robot sequentially aspirated 100 μl of internal standard, 100 μl of 2% ammonia in water and 250 μl of plasma sample to be analysed. These aliquots were dispensed to the individual wells of the extraction plate at a high speed providing adequate mixing. After washing with 250 μl of 15% methanol in 0.5% ammonia and 250 μl of water the samples were eluted into a deep well collection plate by two subsequent elution steps with each 500 μl of 0.2% formic acid in methanol. The extracts were transferred to siliconized glass tubes and evaporated to dryness under a gentle stream of nitrogen at a temperature of 45°C. After re-dissolving the residue in 100 μl of mobile phase, 25 μl was injected into the HPLC system. A detailed overview of the sample preparation procedure is given in Table 1.

2.7. Validation experiments

The automated method was validated over the range from 0.100 to 5.00 ng/ml using the validation approach of Wieling et al. [23].

The *linearity* of the method was assessed by plotting the peak area ratios of haloperidol/internal standard versus concentration (weighting factor $1/X^2$) using eight calibration samples in triplicate and performing a goodness of fit and lack of fit test by analysis of variance.

Analysing blank plasma samples from six different healthy individuals were analysed and compared to a standard solution of the pure compounds to assess the *selectivity* of the assay. The *accuracy and precision* at concentration levels of 0.100 (LLQ), 0.400, 20.0 and 45.0 ng/ml were determined by analysing the validation samples in threefold during six analytical runs. The overall, within-run and between-run precision was calculated using one-way ANOVA.

The *recovery* was determined at three concentration levels (0.400, 20.0 and 45.0 ng/ml) by comparing the peak area of the extracted precision and accuracy samples ($n = 6$) with the peak area of non-extracted standard solutions of the corresponding concentration ($n = 6$) in six analytical runs.

For the assessment of the *autosampler stability*, pooled extracts of spiked plasma samples at two concentrations (0.400 and 45.0 ng/ml) were injected every 2 h for a total period of 30 h. During this period the extracts were kept in the sample compartment of the injector (protected from light and at a temperature of 10°C). The peak area

Table 1
Overview of the sample preparation procedure

Step	Process	Reagent	Volume (μl)	Vacuum (s)
1	Conditioning	Methanol	250	3
2		Water	250	12
3		0.5% Ammonia in water	250	12
4	Sample loading	Internal standard	100	180
		2% Ammonia in water	100	
		Plasma sample	250	
5	Washing	15% Methanol in 0.5% ammonia	150	45
6		Water	250	45
7	Elution	0.2% Formic acid in methanol	500	15
8		0.2% Formic acid in methanol	500	20

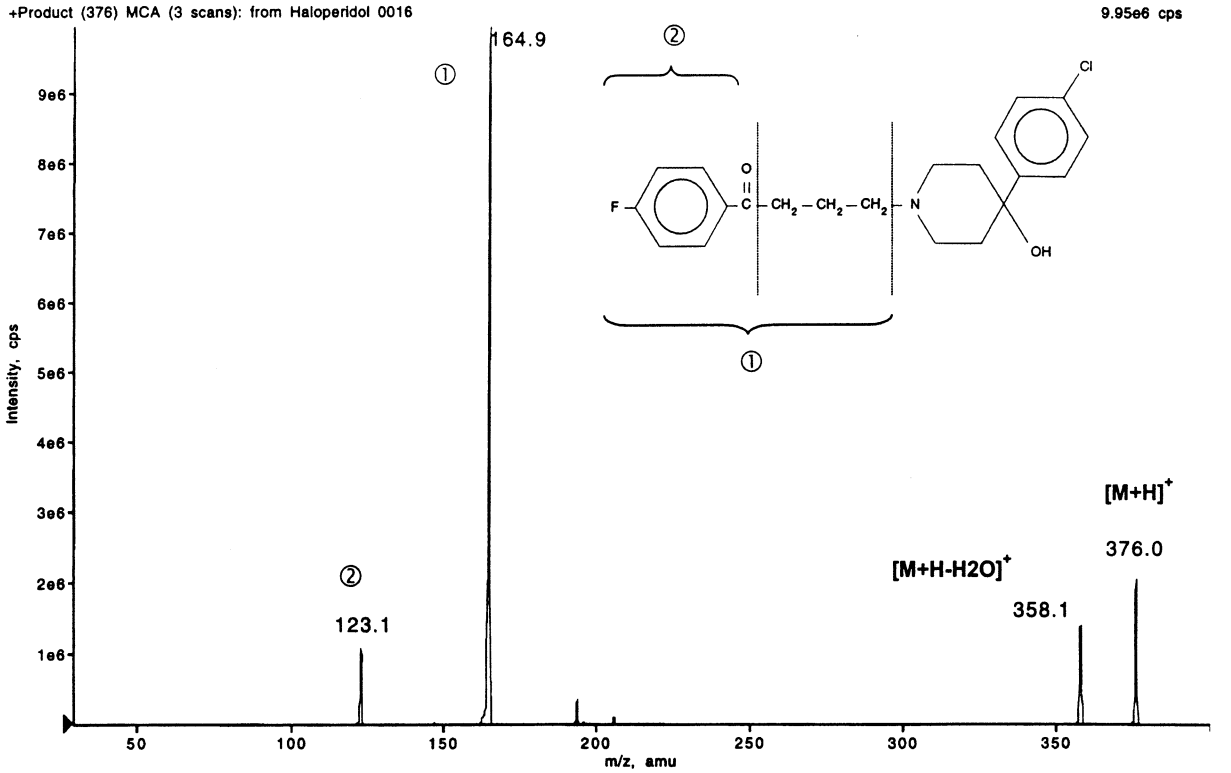

Fig. 3. Mass spectrum of protonated haloperidol and its fragmentation products.

ratios of haloperidol over the internal standard were evaluated using regression analysis.

The stability after repeated freezing and thawing in plasma was investigated during five freezing and thawing cycles at two concentration levels (0.400 and 45.0 ng/ml). After each cycle the concentration of haloperidol was measured.

Dilution of samples of haloperidol was investigated by analysing over-curve control samples; one concentration (100 ng/ml) was diluted five times in sixfold, to obtain a nominal diluted concentration of 20.0 ng/ml in five analytical runs. The data were evaluated comparing the precision and accuracy obtained after analysis of variance with the data from the precision and accuracy experiments at a concentration of 20.0 ng/ml.

3. Results and discussion

3.1. Mass spectrometric conditions

A spectrum of product ions of protonated haloperidol is given in Fig. 3. There are three main fragmentation products observed with m/z values of 123, 165 and 358. Protonated haloperidol exhibits an efficient fragmentation to the product with an m/z of 165. This fragment was used for MRM detection. The mass spectrum (not shown) of the deuterated internal standard exhibits the same fragmentation behavior as haloperidol and gives a fragmentation product with m/z of 169.

In vivo, haloperidol is metabolized to its active metabolite reduced haloperidol (MW is 377). In

theory it is possible that haloperidol-^{37}Cl gives interference with reduced haloperidol-^{35}Cl. However, from Table 2 it is obvious that no interference occurs because the chlorine atom is located in a part of the ion that does not pass through quadrupole Q3. Instead, a reduced group that is located in the part of the ion is passing through Q3 leading to a different m/z for haloperidol and the metabolite. Also haloperidol-D4-^{35}Cl does not interfere with reduced haloperidol-^{37}Cl because the settings for Q3 are different, m/z 169 for the internal standard haloperidol-D4 and m/z 167 for reduced haloperidol.

3.2. Extraction procedure

Haloperidol is a relatively nonpolar compound with a partition coefficient (octanol/buffer pH 7.4) of 20 000. It is almost insoluble in water (1:100 000) and soluble in alcohol (1:50). These characteristics imply that haloperidol can be efficiently isolated using solid-phase extraction on a nonpolar sorbent. The pK_a value of the piperidine group of haloperidol is 8.3, which indicates that it has to be extracted under basic conditions preferably at pH > 10.3. To avoid suppression and clustering in the ionisation process in the interface of the LC-MS it was chosen to use ammonia, as it is volatile and will evaporate in the LC-MS interface.

Table 2
Resolution of haloperidol and related molecular species

Molecular species	Mass	Q1-settings (m/z)	Q3-settings (m/z)
Haloperidol-^{35}Cl	375	376	165
Haloperidol-^{37}Cl	377	(378)	(165)
Haloperidol-D4-^{35}Cl	379	380	169
Haloperidol-D4-^{37}Cl	381	(382)	(169)
Reduced Haloperidol-^{35}Cl	377	(378)	(167)
Reduced Haloperidol-^{37}Cl	379	(380)	(167)

A commonly observed problem in quantitative LC-MS-MS analyses of drugs in biological fluids is formed by the potential matrix effects [24]. The results of quantitative LC-MS-MS analyses can be influenced by ion suppression caused by co-eluting endogenous compounds, especially when a chromatographic system with very short retention times is used. Therefore, the washing procedure has to be optimized in order to remove as much endogenous compounds as possible. For this method, the maximum amount of methanol in 0.5% ammonia was determined which could be used without loss of recovery for haloperidol. Addition of methanol gives an improved selectivity in the sample preparation procedure, because more endogenous compounds will be removed during the washing procedure. Addition of 15% of methanol could be done without losses during the washing procedure. During the final washing step, the SPE column was rinsed with water to remove the ammonia before applying an acid elution solvent.

3.3. Implementation and automation of the method

For implementation of the haloperidol assay we needed approximately 5 days in total. The time needed for development of the method using this systematic approach was 3 days. In our experience the programming and testing of the vacuum settings of the automated system takes approximately 2 days. Time needed for testing and adjustment of these settings depends on the robustness of the SPE procedure.

3.4. Blocking of SPE columns

Blocking is a well-known problem in solid-phase extraction. Individual cartridges or wells become clogged after the addition of matrix. This problem, mainly caused by solid particles in the sample, cannot always be eliminated by thorough centrifugation. Blocking may have a significant effect on the economical advantages of automated analysis because a re-analysis has to be performed in an additional analytical run. This effect is more significant when the number of samples to be analysed in a clinical study is smaller.

To prevent contamination of other wells in an extraction plate the 'liquid sensing' facility of the pipetting robot can be used. With this standard option of the robot, a program can be developed that detects whether any liquid is left in a well after loading of samples and subsequent application of vacuum. If there is liquid left in a well it will be removed and transferred to waste so that wells close to the blocked well are not contaminated during the subsequent washing and elution steps. Identification of blocked wells will be stored in a database file.

3.5. Throughput of the automated method

For a batch of 96 samples (including blank, eight calibration samples and six quality control samples) a sample processing time of about 50 min was required. The chromatographic run time was 3 min. A throughput of at least 240 samples per 24 h can be achieved by one technician.

The method presented is semi-automated and still needs human intervention at different stages in the sample preparation process. The samples have to be placed in the sample racks of the robot system; before elution the deep well plate has to be put into the vacuum manifold and also the prepared samples have to be transferred to vials before injection. The use of XYZ autosamplers and robotic gripper arms may yield applications that are totally unattended and can also further increase the sample throughput.

3.6. Validation results

The *linearity* of the method was established for the concentration range of 0.100–50.0 ng/ml. A test for lack of fit showed that the first-order model ($y = ax + b$), with a weighing factor $1/X^2$, is appropriate for establishing a relationship between concentration and response ($r = 0.999$). The goodness of fit (F-test for regression) is highly significant ($F_{calc} = 1046$, F_{Table} $(\alpha = 0.05) = 4.30$). A slightly significant lack of fit was observed ($F_{calc} = 3.25$, F_{Table} $(\alpha = 0.05) = 2.74$), residuals were rather the result of experimental error (spiking, extraction or integration errors) instead of a consequence of model deviations.

With respect to the *selectivity*, no major interferences (< 0.5 times the response of the LLQ) were found at the retention times of haloperidol or the internal standard (Fig. 4).

For the *lower limit of quantitation*, the lowest concentration of the calibration curve, 0.100 ng/ml was taken. The precision and accuracy at this level were within acceptable limits (C.V. and bias $\leq 20\%$).

A summary of the results of the *precision and accuracy* experiments is given in Table 3. The overall precision (C.V. %) of the method was better than 10% at all concentration levels ($n = 57$). The within-run and between-run imprecision (C.V. %) was better than 8.1 and 13.8%, respectively. The bias varied between -5.6 and $+6.3\%$ at all concentration levels. The results meet the criteria established during the Washington Meeting on Analytical Methods Validation [25].

The *recovery* of haloperidol was consistent over the entire calibration range and was on average 72%. For haloperidol-D4 the recovery was determined on the concentration normally used during routine analysis (plasma concentration of 50 ng/ml) and was found to be 44%. Besides comparable chromatographic and mass spectrometric behaviour one also expects a similar extraction behaviour from a deuterated internal standard. We cannot offer a clear explanation for the discrepancy in the recovery of the internal standard and haloperidol. However, the fact that the recovery of haloperidol-D4 is reproducible and corrects well for changes in the response of haloperidol makes it a good choice as internal standard for this assay.

The results on the *stability* of haloperidol and haloperidol-D4 in the compartment of the autosampler at 10°C show no significant change of the response ratio at the concentration level of 0.400 ng/ml ($+0.1\%$), nor at the concentration of 45.0 ng/ml ($+0.4\%$), indicating that there are no signs of deterioration under these storage conditions. Also, after up to five times freezing and thawing no signs of instability were found. The accuracy for concentration levels of 0.400 and 45.0 ng/ml were found within the acceptance limits of 90.0–110%.

Table 3
Summary of the results of the validation of precision, accuracy and recovery

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Bias (%)	Overall C.V. (%)	Within-run C.V. (%)	Between-run C.V. (%)	Recovery (% ± S.D.)	*n*
0.100	0.106	6.3	9.9	8.0	13.7	ND[a]	12[b]
0.400	0.398	−0.4	4.1	4.5	2.9	75.8 ± 8.3	15
20.0	18.9	−5.6	4.4	4.0	5.4	70.7 ± 8.0	15
45.0	43.2	−4.0	5.0	3.8	7.3	70.8 ± 9.0	15

[a] ND, not determined.
[b] Three observations rejected because of an error in sample preparation.

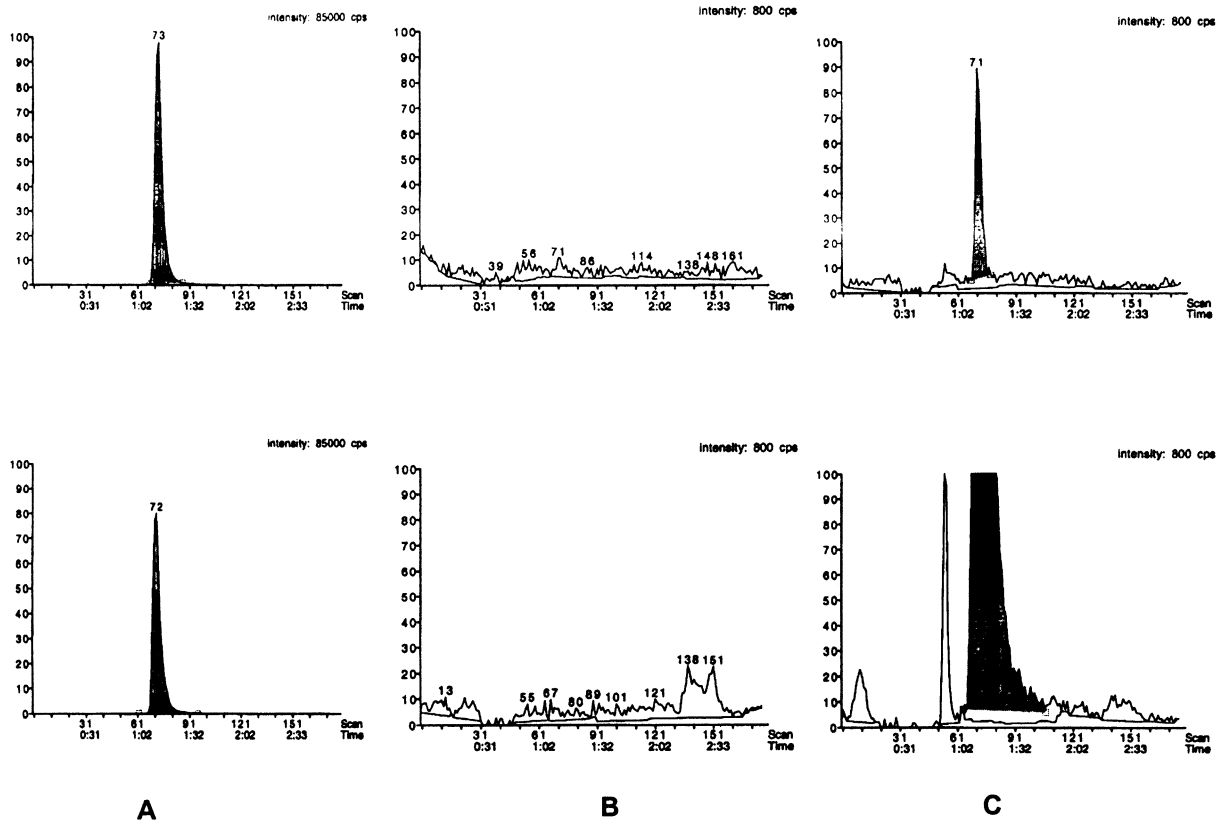

Fig. 4. Representative chromatograms (upper: haloperidol, lower: internal standard); (A) test solution containing the pure compounds; (B) blank plasma sample; (C) spiked plasma sample at the LOQ (0.100 ng/ml); Retention times haloperidol and haloperidol D-4 ≈ 1.1 min.

The results obtained during the *dilution* experiments are comparable with the data obtained during the precision and accuracy experiments. The within-run and between-run precision after dilution were 2.2 and 6.1%, respectively. The bias was −6.4%. The data show that partial volume analysis is allowed in case study sample concentrations above the calibration curve are found or in case there is insufficient amount of sample to perform an assay.

3.7. Application of the method

Considering the number of samples that can be analysed per day and the quality characteristics, the present automated method is suitable for use in routine analysis of plasma samples in clinical studies. The selectivity of LC-MS-MS analysis makes the method suitable for use in drug-interaction studies as well.

4. Conclusions

An automated 'high throughput' sample preparation procedure for haloperidol in plasma using microtiter plate solid-phase extraction technology was implemented successfully in combination with turbo ionspray LC-MS-MS. Validation results indicate that the method shows good accuracy and acceptable precision at all concentration levels.

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