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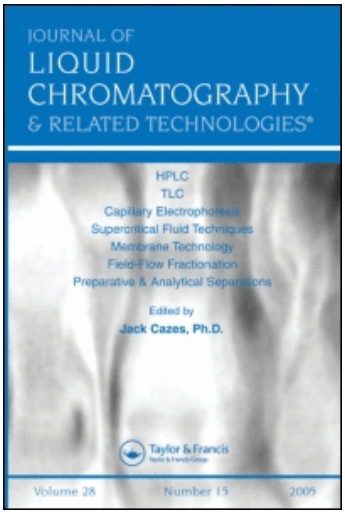

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Liquid Chromatography-Tandem Mass Spectrometric Analysis of Stobadine in Human Plasma

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Abstract: A rapid, sensitive, and specific high performance liquid chromatography procedure for the determination of stobadine in human plasma using electrospray tandem mass spectrometry (MS/MS) detection was developed. Multiple reaction monitoring (MRM) transitions at $202.9 > 160$ (stobadine) and $166 > 103$ (phenylalanine, I.S.) were selected to quantify stobadine by the internal standard method. Linear correlations (r: 0.9971–0.9981) of the calibration curves over the concentration range 20–500 ng mL^{-1} with lowest limit of quantitation (LOQ) of 10 ng mL^{-1} were established. An overall recovery of 94.3% of stobadine from plasma was attained using the SPE technique. The developed tandem mass spectrometry method was proven to be accurate and reproducible as a percent relative error (%RE) ranging from −3.5% to 3.0% and %RSD in the range of 1.4–8.3% were determined. Samples of stobadine in plasma were stable at −20°C for at least 5 weeks. The slope of the calibration curves was 0.00146 ± 0.000087, indicating good stability and the between-day precision and accuracy of the MS/MS method. The validation

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data suggest the utility of the described method in planned clinical studies of stobadine in humans.

Keywords: Stobadine, phenylalanine, human plasma, LC-MS/MS

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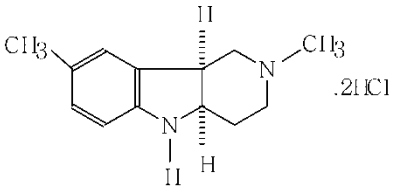
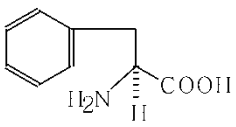
INTRODUCTION

Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,9b-hexahydro-1H-pyrido[4,3b] indole, is a new, potential cardioactive drug with antiarrhythmic and antihypoxic activity, derived from *g*-carboline. It belongs to a group of scavengers of reactive oxygen species and could be a promising drug for treatment of cardiovascular disorders, especially those with neurogenic origin.[1] Consequently, the pharmacokinetics behavior and the drug penetration properties through blood–brain barrier are highly important.



Stobadine



Phenylalanine

Stobadine has a weak chemical functionality that renders drug analysis quite difficult. There is a paucity of literature on the analysis of stobadine. Fluorimetric,[2] TLC,[3] HPLC,[4] and GC[5,6] procedures were described for monitoring of stobadine concentrations in biological samples for in vitro and in vivo pharmacokinetics and metabolic studies. However, the majority of the reported methods applied tedious derivatization reactions and extraction procedures. Recently, tandem mass spectrometry (MS/MS) has been intensively applied in our mass spectrometry laboratories for the analysis of drugs and biomolecules in pharmaceutical products and biological fluids at low concentrations with high degrees of specificity[7–10] for quality control (QC), purity, and clinical studies purposes. This article reports on the application of tandem mass spectrometry for a simple and rapid quantitation procedure of stobadine in human plasma following SPE using phenylalanine as internal standard. Validation of the developed method by measuring the linearity, accuracy, and precision parameters is demonstrated.

EXPERIMENTAL

Materials

Stobadine powder was provided by Prof. L. Benes, Faculty of Pharmacy, Veterinary and Pharmaceutical University, Brno, Czech Republic. DL-Phenylalanine was supplied by Merck, Darmstadt, Germany. SPE cartridges (Waters Oasis®, C18) were purchased from Waters (Waters Corporation, Milford, MA, USA). Water was purified by the Milli-Q System (Millipore Corporation, USA). Other chemicals and reagents were of analytical grade and the solvents were of HPLC grade. Control human plasma samples were kindly donated by the Central Blood Bank, Kuwait. The Mobile phase is composed of acetonitrile/water (50/50 v/v) and 0.025% formic acid and is pumped at flow rate 0.1 mL min^{-1}.

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Instruments

A triple quadrupole tandem mass spectrometer (Quattro LC, Micromass, UK) fitted with a Z-spray ion source was used. The mass spectrometer was operated in positive electrospray ionization mode and was coupled to Waters 2690 Separation Module, Alliance HPLC, and Waters autosampler. System operation and data acquisition were controlled by MassLynx NT 3.5 software. The tuning parameters for MS, MS/MS, and Multiple reaction monitoring (MRM) analyses were optimized by direct infusion of solutions of analytes in the mobile phase to the ionization probe using a Harvard syringe pump at flow rate 10 mL min^{-1}. LC analysis were performed at ambient temperature (25°C) using an Xterra™ MS C18, 2.5 μm (2.1 mm × 30 mm) column (Waters). Volumes of 10 mL of samples were automatically injected into the system. The run-time was ~3 min., injection-to-injection, for all runs.

Standard Solutions

A stock solution of stobadine was prepared by dissolving ~10 mg of the powder in the CH_3CN/H_2O (50/50 v/v) to give a concentration of 1 μg mL^{-1}. Separate aliquots were diluted to 10 mL with the same solvent to give the working solutions of 1 and 10 ng mL^{-1}, respectively. The I.S. working solution was prepared in CH_3CN/H_2O (50/50 v/v) at a concentration 100 ng mL^{-1}. Solutions of stobadine and I.S. were stable for at least 2 weeks, when stored at 4°C.

Human Plasma Extraction Procedure (SPE)

Aliquots of 1 mL of plasma samples (calibration standards and QC samples) were mixed with 20 μL aliquots of the I.S. using the appropriate concentrations

(20 or 200 ng mL^{-1}) for low and high concentration of stobadine, respectively. The plasma samples (1 mL) were loaded onto SPE cartridges (Waters Oasis®, C18) on a vacuum 20-position extraction manifold (Waters). The cartridges were pre-conditioned with methanol followed by water (1 mL each). The loaded cartridges were washed with 50 mM sodium acetate solution (1 mL) and the analytes (stobadine and I.S.) were eluted with 1 mL methanol. The solvent was gently removed at 50°C under N_2 gas and the residue was dissolved in ~100 μL of mobile phase, and 20 μL aliquot was directly injected into mass spectrometer and analyzed using the MRM transitions of 202.9 > 160 and 166 > 103 for stobadine and I.S., respectively. Calibration curves and the found concentrations of stobadine in QC and analyte samples were automatically calculated by the quantify program of MassLynx software using weighted linear regression analysis.

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Method Validation

Linearity

The linearity of the developed LC-MS/MS method was established by measuring the calibration curves over the concentration ranges of stobadine in plasma 20–60 ng mL^{-1} (low) and 50–500 ng mL^{-1} (high). The final concentration of I.S. in plasma was 2 μg mL^{-1}. The spiked plasma samples were extracted and treated as previously described.

Limit of Quantitation

Plasma samples spiked with stobadine at concentration 10 ng mL^{-1} were extracted and analyzed as described above.

Accuracy and Precision

Within-day accuracy and precision of tandem MS method were established at room temperature by analyzing QC samples at low (20 ng mL^{-1}), medium (50 ng mL^{-1}), and high (200 ng mL^{-1}) concentration levels.

Stability Studies

Plasma samples of stobadine at concentrations 50, 100, 20, and 500 ng mL^{-1} were prepared and stored at −20°C. Calibration curves were established on different days for 5 weeks as previously described.

Recovery Studies

QC plasma samples containing stobadine at concentrations 20, 100, and 500 ng mL^{-1} were prepared, extracted, and analyzed as above. Recoveries of stobadine were computed from the calibration curves using linear regression analysis.

RESULTS AND DISCUSSION

Development of MS/MS Method

The tuning parameters for analysis of stobadine and phenylalanine (I.S.) by electrospray MS/MS were examined for optimum detection of the parent and daughter ions. The tuning parameters: capillary voltage, cone voltage, and collision energy were selected as 3.2 kV, 30 V, and 25 eV for stobadine and 3.2 kV, 30 V, and 15 eV for phenylalanine. The source and desolvation temperatures were adjusted at 100°C and 250°C, respectively. A mobile phase consisting of CH_3CN/H_2O in a ratio 1 : 1 and 0.025% formic acid (pH ~4) was appropriate for the analysis of stobadine by positive electrospray ionization. Using the above tuning parameters, the parent ions of stobadine and I.S. were detected at m/z 202.9 and 166, respectively. These molecular mass ions were further used in MS/MS experiments to determine the daughter ions of the analytes, which were detected at m/z 160 and 103, respectively (Figures. 1 and 2). A MRM scan was then selected to determine, specifically, stobadine in the extracted plasma samples. As an MRM scan relates daughter ion to the parent ion, therefore it is possible to detect stobadine and phenylalanine independently in mixtures. MRM transitions at 202.9 > 160 and 166 > 103 (Figure 3) were selected to analyze stobadine and phenylalanine with high degrees of

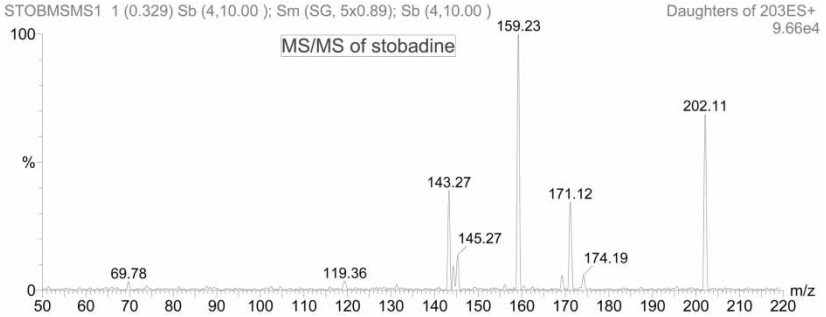

Figure 1. MS/MS of stobadine.

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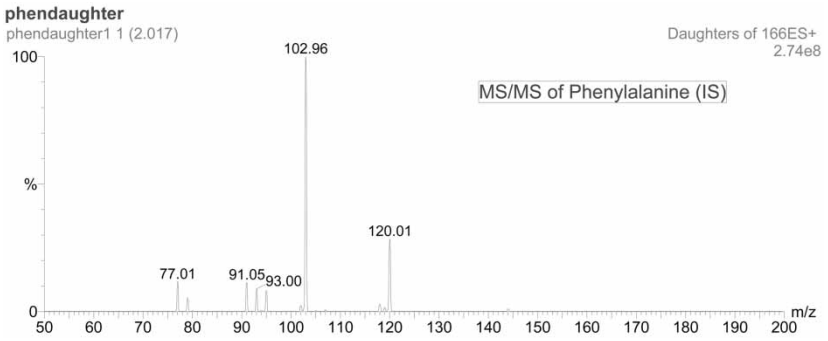

Figure 2. MS/MS of phenylalanine (I.S.).

selectivity. Under these conditions, stobadine and phenylalanine were rapidly detected (<3 min) without need of chromatographic resolution, as their detection was based on fragmentation properties rather than on chromatographic behaviors. Unspiked plasma samples, as blanks, exhibited no interference at the measured MRM transitions of stobadine and phenylalanine, respectively.

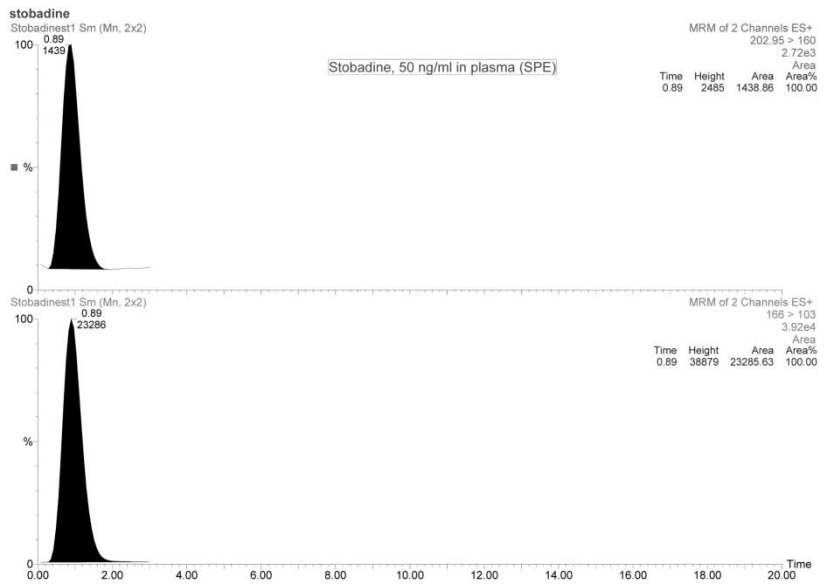

Figure 3. MRM of stobadine (50 ng mL^{-1}) and I.S. (2 μg mL^{-1}) after SPE from human plasma.

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Linearity

Linearity of the developed MS/MS procedure was established over two concentration ranges of stobadine, 20–60 ng mL^{-1} (low) and 50–500 ng mL^{-1} (high), using weighted linear regression analysis. Linearity was indicated by an average slope of 0.002379 (low) and 0.001292 (high) using five duplicate standard curves (Table 1). Figures 4 and 5 show typical calibration curves of stobadine obtained during the validation at two concentration ranges. The limit of quantitation (LOQ) was established as the lowest non-zero human plasma concentration of stobadine that could be accurately and reproducibly quantified. For this validation, the LOQ was found to be 10 ng mL^{-1}. Analysis of plasma samples ($n = 10$) containing stobadine at this concentration level gave an average of 11.47 ng mL^{-1} (Table 1).

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QC samples

The precision and accuracy of the developed tandem MS method were determined by analysis of human plasma QC samples at low, medium, and high concentration levels. Three concentration ranges 20, 50, and 200 ng mL^{-1} were selected for stobadine in plasma. Precision was expressed as percent relative standard deviation %RSD, whereas the accuracy was measured as percent relative error (%RE) from the theoretical values according to the following formula:

$$\text{RE } (\%) = \left[\frac{C_M - C_T}{C_T}\right] \times 100$$

Table 1. Linearity parameters for determination of stobadine in plasma by tandem MS method

Concentration range (ng mL^{-1})	Slope ± SD	RSD (%)	Correlation coefficient ± SD	RSD (%)
Calibration curves[a]				
20–60	0.002379 ± 0.000259	10.9	0.9971 ± 0.00158	0.16
50–500	0.001292 ± 0.000081	6.3	0.9981 ± 0.0017	0.17
LOQ[b]			Calc. Conc. ± SD	RSD (%)
10.0			11.47 ± 1.09	9.50

[a]Five duplicate calibration curves.
[b]$n = 10$.

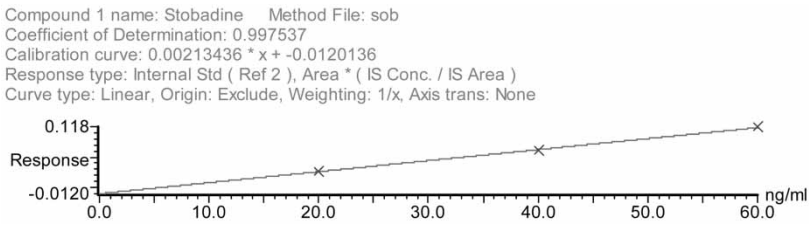

Figure 4. Calibration curve of stobadine in plasma (low concentration).

where C_M, mean determined concentration of QC sample; C_T, theoretical concentration.

Within-day precision and accuracy were evaluated for each QC level. A %RSD ranging from 1.4% to 8.3% and %RE in the range −3.5% to 3.0% indicate good precision and accuracy (Table 2).

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Stability of Stobadine in Plasma

The stability of stobadine stored in human plasma at −20°C was evaluated over 5 weeks by running the calibration curves. The average slope (±SD) was 0.00146 (±0.000087) with %RSD 5.9 (Table 3). The data indicates good stability of stobadine when frozen stored, and proved the between-day precision and accuracy of the tandem MS method.

Specificity

Human plasma samples from different volunteers were spiked, extracted, and analyzed for stobadine and I.S. No peak was found to interfere with the quantitation process using the developed procedure.

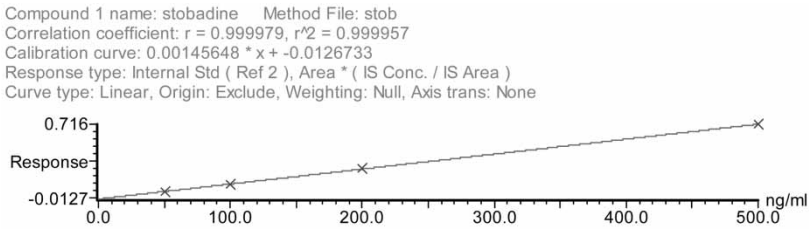

Figure 5. Calibration curve of stobadine in plasma (high concentration).

Table 2. Typical within-day precision and accuracy for determination of stobadine in plasma by tandem MS method

	QCLOW	QCMED	QCHIGH
Add. conc (ng mL^{-1})	20.0	50.0	200.0
Calc. conc. (ng mL^{-1})	19.3	51.5	203.9
$\pm$SD	1.6	3.4	2.8
%RSD	8.3	6.6	1.4
%RE	−3.5	3.0	1.9
n	10	10	10

Note: QC, quality control; RSD, relative standard deviaton; RE, relative error.

Recovery Studies

The SPE procedure of stobadine from plasma was assessed by determining recoveries of stobadine from QC plasma samples at concentrations 20, 100, and 500 ng mL^{-1} using the tandem MS method. The overall recovery for stobadine was 94.3 with a mean %RSD of 8.9% (Table 4). The data indicates the efficacy of SPE extraction using C18 cartridges. Furthermore, the technique is simple and can be used to extract a large number of samples in a short time.

CONCLUSION

In this study, a liquid chromatography procedure was reported for the analysis of stobadine in human plasma using positive electrospray tandem mass spectrometry detection. The analytes were successfully extracted, without ion

Table 3. Stability of the calibration curves of stobadine in plasma at −20°C for 35 days

Day of analysis	Slope of calibration curve[a]
1	0.00147
2	0.00155
7	0.00133
14	0.00145
21	0.00139
28	0.00157
35	0.00149
Mean $\pm$ SD	0.00146 $\pm$ 0.000087
%RSD	5.9

[a]Concentrations selected: 50, 100, 200, 500 ng mL^{-1} in plasma.

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Table 4. Recovery percentages of stobadine from human plasma using tandem MS method

Theor. conc. (ng mL^{-1})	Calc. conc. (ng mL^{-1})	Recovery (%)
20.0		
	18.1	90.5
	16.8	83.8
	17.1	85.5
	18.2	90.9
	15.1	75.1
100.0	97.3	97.3
	98.3	98.3
	108.1	108.1
	104.7	104.7
	91.1	91.1
500.0		
	485.2	97.1
	504.3	100.8
	484.9	96.9
	495.0	99.0
	475.6	95.1
	Overall %recovery	94.3
	$\pm$SD	8.4
	%RSD	8.9
	n	15

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suppression, from human plasma using a SPE technique. The developed LC-MS/MS method was highly sensitive and selective with a LOQ of 10 ng mL^{-1}. Validation data indicates that the current method is accurate and reproducible. The high sample throughput, short-time of analysis, and no necessity of chromatographic separation permit measurement of a large number of samples of stobadine including standards, QC, and analytes during pharmaceutical and clinical studies. Since a limited number of analytical procedures have been published for the determination of stobadine in biological media, the presently described method will be useful for planned pharmacokinetics and metabolic studies.

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