Journal of Chromatographic Science 2013;51:460–467
doi:10.1093/chromsci/bms163 Advance Access publication October 9, 2012

Article

HPLC Analysis of Midodrine and Desglymidodrine in Culture Medium: Evaluation of Static and Shaken Conditions on the Biotransformation by Fungi

Thiago Barth[1], Josefina Aleu[2], Mônica Tallarico Pupo[3], Pierina Sueli Bonato[1]* and Isidro G. Collado[2]*

[1]Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903, Ribeirão Preto-SP, Brazil, [2]Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain, and [3]Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903, Ribeirão Preto-SP, Brazil

*Author to whom correspondence should be addressed. Email: psbonato@hotmail.com; isidro.gonzalez@uca.es

Received 12 July 2012; revised 31 August 2012

A high-performance liquid chromatography (HPLC) method is presented for the simultaneous determination of midodrine and desglymidodrine (DMAE) in Czapek-Dox culture medium, to be used in biotransformation studies by fungi. The HPLC analysis was conducted using a Lichrospher 100 RP18 column, acetonitrile–40 mmol/L formic acid solution (60:40, v/v) as mobile phase, and ultraviolet detection at 290 nm. The sample preparation was conducted by liquid–liquid extraction using ethyl acetate as extractor solvent. The method was linear over the concentration range of 0.4–40.0 μg/mL for midodrine ($r \geq 0.9997$) and DMAE ($r \geq 0.9998$). Within-day and between-day precision and accuracy were evaluated by relative standard deviations (≤ 8.2%) and relative errors (−7.3 to 7.4%), respectively. The validated method was used to assess midodrine biotransformation by the fungi *Papulaspora immersa* Hotson SS13, *Botrytis cinerea* UCA 992 and *Botrytis cinerea* 2100 under static and shaken conditions. Under shaken conditions, the biotransformation of midodrine to DMAE was more efficient for all studied fungi, especially for the fungus *Botrytis cinerea* 2100, which converted 42.2% of midodrine to DMAE.

Introduction

Midodrine is a pro-drug of desglymidodrine (DMAE), developed by the attachment of the amino acid approach glycine to the functional amine of DMAE (Figure 1). The prodrug midodrine is primarily converted into its active drug in the liver and in the systemic circulation by unknown peptidases (1, 2). DMAE is a long acting α-adrenergic agonist that causes elevation of systemic blood pressure, accompanied by a reduction in heart rate (3), and it is therapeutically used for the treatment of orthostatic hypotension (4). Until now, the metabolism of midodrine has not been extensively studied (3), and the production of DMAE by fungi may be used to provide sufficient amounts of the metabolite to further be used in pharmacological, toxicological and analytical tests.

Many researchers have reported the production of drug metabolites using microbial biotransformations (5), and the use of microorganisms for simulating the mammalian metabolism of many molecules of pharmacological importance is well documented (6, 7, 8).

Papulaspora immersa H. H. Hotson (Ascomycetes, Sordariales, Chaetomiaceae) is a familiar soil competitor mold in the culture of mushrooms (9), and it has been recently reported as endophyte in a few plant species such as *Vitex negundo* (10), *Solanum tuberosum* (11), and *Smallanthus sonchifolius* (12). Moreover, this fungus has been highlighted as a producer of phenol-oxidizing and amylase enzymes (13), and was recently used on the biotransformation of albendazole (14). Endophytes are a special kind of fungi that inhabit internal plant tissues without causing visible disease symptoms (15). These fungi are an especially unexplored, or at least underexplored, source for microbial biotransformation (16).

The fungus *Botrytis cinerea* is a fungi imperfecti that belongs to the order Moniliales of the family Moniliaceae. They are geographically widespread, and are polyphagous parasites that cause serious economic losses to commercial crops (17). The biotransformation ability of *Botrytis* species has been summarized, and the hydroxylase and oxidase activities were the more frequently observed reactions (18).

To simultaneously determine midodrine and DMAE in biological matrices (primarily plasma), some methods have been developed based on radioisotope-labeled techniques (19), high-performance liquid chromatography (HPLC) with fluorescence (20, 21, 22) and ultraviolet (UV) detection (23), and capillary electrophoresis (CE) (24). The HPLC method with fluorescence detection described by Posch and Lindner (20) is quite complicated because it requires a column-switching technique (20), and the other HPLC with fluorescence detection methods (21, 22) are not validated. Moreover, the method published by Quaglia *et al.* (23) is enantioselective, not applicable to the intended purpose. More recently, Barth *et al.* (24) described another enantioselective method based on a CE method.

Therefore, the aim of the present study was the development of an HPLC method with UV detection to be used to evaluate the ability of the fungi *Papulaspora immersa* and *Botrytis cinerea* to produce midodrine biotransformation under static and shaken conditions.

Experimental

Chemicals and reagents

The reference substance, midodrine hydrochloride, was purchased from Sigma-Aldrich (St. Louis, MO), whereas DMAE was prepared by acidic hydrolysis following the procedure described by Quaglia *et al.* (4). In this procedure, a solution of midodrine hydrochloride (0.001 mol) in 2 mol/L HCl (10 mL) was refluxed for three hours. After this, the solution was

cooled, alkalinized with 60% NaOH and extracted by ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The crude extract was purified on C18 Sep-Pak cartridges (Waters, Milford, MA) using methanol–water (8:2, v/v) plus 0.5% of triethylamine, as mobile phase. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were used to confirm the structure of DMAE.

Methanol and ethyl acetate were purchased from Carlo Erba (Val de Reuil, France) and acetonitrile was purchased from Merck (Darmstadt, Germany), all of chromatographic grade. Formic acid was purchased from Panreac (Barcelona, Spain). All other chemicals were of analytical grade in the highest purity available. Water was purified with a Milli-Q plus system (Millipore, Bedford, MA).

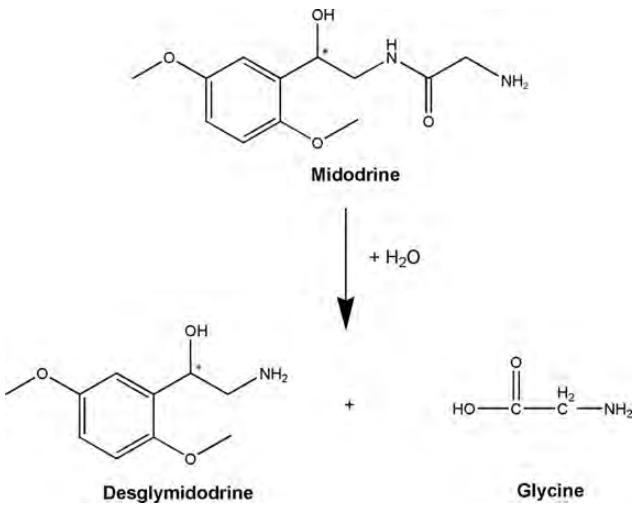

Figure 1. Chemical structures of midodrine and DMAE.

Reference substance solutions

The midodrine and DMAE stock solutions were prepared at the concentration of 1 mg/mL. The working solutions of midodrine and DMAE were 4, 12, 40, 100, 200, 300, and 400 μg/mL. All were prepared in methanol on a free-base basis. The solutions were stored frozen at −20°C and protected from light.

Liquid chromatographic conditions

The liquid chromatographic analyses were conducted using a VWR-Hitachi chromatograph (Darmstadt, Germany), equipped with an L2130 quaternary solvent pump unit and an L2400 UV-Vis detector operating at 290 nm. Injections (25 μL) were performed automatically through an L2200 auto-injector. An EZChrom Elite Client/Server software, version 3.1.6, was used for system control and data acquisition. The analyses of midodrine and DMAE were performed at room temperature (25 ± 2°C) on a Lichrospher 100 RP18 column (250 × 4.6 mm i.d, 5 μm particle size; Merck) using acetonitrile–40 mmol/L formic acid solution (60:40, v/v) as the mobile phase at the flow rate of 1.4 mL/min.

Extraction procedure

Midodrine and DMAE were extracted from the Czapek-Dox culture medium (described in Midodrine biotransformation procedure) by a liquid–liquid extraction (LLE) procedure adapted from Barth *et al.* (24). Aliquots of 0.5 mL Czapek-Dox medium spiked with 50 μL of reference solutions of midodrine and DMAE or samples obtained in the biotransformation process were transferred to 10 mL glass tubes and alkalinized with 50 μL of 10 mol/L sodium hydroxide solution. The samples were mixed by vortex agitation for 20 s. Then, a 4 mL aliquot of extraction solvent, ethyl acetate, was added. The tubes were vortex-mixed for 2 min and then centrifuged at 1,800 g for 10 min. The organic layers (3 mL) were transferred

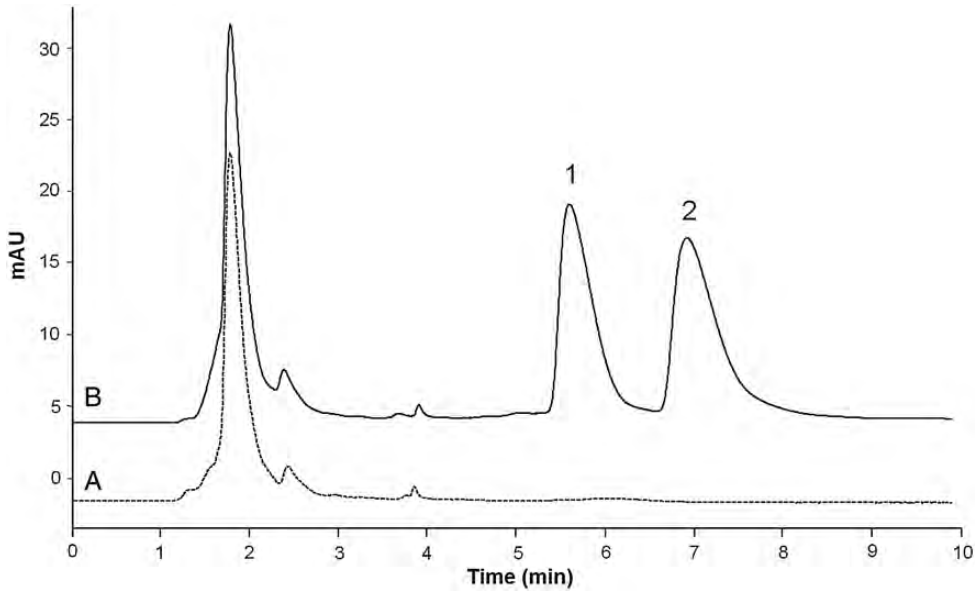

Figure 2. Representative chromatograms (under conditions described in "Liquid Chromatographic Conditions"; all samples extracted by an LLE procedure): blank Czapek-Dox culture medium (A); blank Czapek-Dox culture medium spiked with 10 μg/mL of midodrine and DMAE (B). Peaks: midodrine (1); DMAE (2).

into 10 mL conical glass tubes. The solvent was evaporated to dryness under a stream of compressed air at room temperature. The residues were dissolved in 100 μL of mobile phase and vortex-mixed for 20 s, and then analyzed by HPLC.

Method validation

To determine the extraction recovery, Czapek-Dox medium samples (0.5 mL) were spiked with midodrine and DMAE at the concentrations of 1.2, 20.0 and 30.0 μg/mL ($n = 3$) for each analyte and submitted to the extraction procedure. Another set of samples was prepared by extracting 0.5 mL aliquots of Czapek-Dox medium and then spiking the extract with the same amounts of midodrine and DMAE. The recovery was determined by comparing the peak area of solutions submitted and not submitted to the extraction procedure, and the recovery was expressed as the percentage of the extracted amount.

Calibration curves were obtained by spiking aliquots of Czapek-Dox medium with standard solutions of midodrine and DMAE in the concentration range of 4–400 μg/mL, resulting in concentrations of 0.4–40 μg/mL for each analyte. The linearity of the calibration curves was determined using the correlation coefficient (r) and the F-test for lack-of-fit (FLOF) using a p-value of 0.05. The statistical software MINITAB version 14.1 (State College, PA) was used to perform the statistical calculations.

Table I
Recovery and Linearity of the Method

Analyte	Recovery		Linearity				
	%	RSD (%)	Range (μg/mL)	Linear equation	r	ANOVA Lack-of-fit	
						F-value	p-value
Midodrine	82.0	7.2	0.4–40.0	$y = 126688x - 17947$	0.9997	2.86	0.055
DMAE	83.9	8.6	0.4–40.0	$y = 139789x - 16114$	0.9998	0.25	0.934

Table II
LOQ of the Method

Analytes	Nominal concentration (μg/mL)	Analyzed concentration (μg/mL)*	RE (%)	RSD (%)
Midodrine	0.4	0.439	8.9	5.9
DMAE	0.4	0.443	9.7	5.3

*LOQ, $n = 5$.

Table III
Precision and Accuracy

	Midodrine			DMAE		
Nominal concentration (μg/mL)	1.2	20.0	30.0	1.2	20.0	30.0
Within-day ($n = 5$)						
Concentration (μg/mL)	1.12	20.40	30.98	1.14	19.74	30.05
Precision (RSD, %)	2.4	3.8	3.1	3.6	4.1	2.5
Accuracy (RE, %)	−7.3	1.9	3.2	5.0	1.3	0.2
Between-day ($n = 3$)						
Concentration (μg/mL)	1.12	20.33	31.26	1.12	20.14	29.99
Precision (RSD, %)	4.7	6.7	7.6	3.7	6.9	8.2
Accuracy (E, %)	−6.9	1.6	4.0	7.4	0.7	0.0

The sensitivity of the method was evaluated by determining the limit of quantification (LOQ) (25). The LOQ was defined as the lowest analyte concentration that could be determined with accuracy and precision below 20% (25) over five analytical runs. The LOQ was determined by using Czapek-Dox culture medium (0.5 mL) spiked with 0.4 μg/mL of each analyte (the lowest point in the calibration curve).

The precision and accuracy of the method were evaluated by within-day ($n = 5$) and between-day ($n = 3$) assays using Czapek-Dox medium samples spiked with midodrine and DMAE in the concentrations of 1.2, 20.0 and 30.0 μg/mL of each analyte. The results obtained were expressed as relative standard deviation (RSD, %) and relative error (RE, %).

Freeze-thaw cycle stability, short-term room temperature stability and stability under biotransformation conditions were determined. To perform the freeze–thaw cycle stability, three aliquots ($n = 3$) of samples, prepared in Czapek-Dox culture medium at the concentration of 1.2 and 30.0 μg/mL of midodrine and DMAE, were stored at −20°C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze–thaw cycle was repeated two more times, and then the samples were analyzed after the third cycle. For the determination of short-term room temperature stability, aliquots of samples prepared in Czapek-Dox culture medium at the concentrations specified previously were kept at room temperature (22 ± 2°C) for 12 h and analyzed.

To determine the stability under static biotransformation conditions, an aliquot of 6 mg of midodrine (on a free-base basis) dissolved in 750 μL of sterile water was added to Roux bottles

Table IV
Freeze–Thaw, Short-Term Room Temperature and Autosampler Stability of Midodrine and Desglymidodrine in Czapek-Dox Medium

Analyte	Midodrine		DMAE	
Fresh sample concentration (μg/mL)	1.26	32.05	1.14	33.22
Stability				
Freeze-thaw cycles ($n = 3$)				
Concentration (μg/mL)	1.34	33.59	1.25	33.70
Precision (RSD, %)	2.65	7.70	6.04	6.14
Accuracy (RE, %)	6.49	4.81	9.92	1.44
Short-term ($n = 3$)				
Concentration (μg/mL)	1.35	33.58	1.19	33.92
Precision (RSD, %)	1.22	0.65	2.78	2.88
Accuracy (RE, %)	7.82	4.77	4.60	2.12
Auto-sampler ($n = 3$)				
Concentration (μg/mL)	1.31	33.99	1.12	32.24
Precision (RSD, %)	3.91	0.95	5.00	2.54
Accuracy (RE, %)	4.08	6.07	−1.20	−2.95

Table V
Stability of Midodrine under Biotransformation Conditions

Incubation time (hours)	0	24	48	72	96	120	144	168
Shaken ($n = 3$)								
Concentration (μg/mL)	35.24	34.45	33.18	34.78	33.50	33.78	33.89	32.99
Precision (RSD, %)	5.2	6.3	1.8	10.4	4.9	8.5	9.3	10.4
Accuracy (RE, %)		−2.24	−5.85	−1.31	−4.94	−4.14	−3.83	−6.38
Static ($n = 3$)								
Concentration (μg/mL)	37.34	37.90	37.15	36.25	36.40	35.78	35.96	35.73
Precision (RSD, %)	3.4	7.8	9.7	2.4	10.9	4.7	5.9	9.8
Accuracy (RE, %)		1.50	−0.51	−2.92	−2.52	−4.18	−3.70	−4.31

containing 150 mL of Czapek-Dox medium (40 μg/mL), and submitted to the same conditions used in the biotransformation procedure (described in Midodrine biotransformation procedure). On the other hand, to evaluate the stability under shaken biotransformation conditions, an aliquot of 8 mg of midodrine (on a free-base basis) dissolved in 1,000 μL of sterile water was added to Erlenmeyer flasks containing 200 mL of Czapek-Dox medium (40 μg/mL), and submitted to the same conditions used in the biotransformation procedure (described in Midodrine biotransformation procedure). Daily, during the period of biotransformation (seven days), aliquots of 0.5 mL ($n = 3$) were analyzed.

The stability of the samples placed into the autosampler was evaluated using three aliquots ($n = 3$) of samples prepared in Czapek-Dox culture medium at the concentrations of 1.2 and 30.0 μg/mL of midodrine and DMAE; the samples were extracted and placed into the autosampler at room temperature. The samples were analyzed after 12 h.

The samples of the stability studies were compared with fresh samples at the same concentrations and were considered stable if the deviations (expressed as RE, %) observed for the concentrations from the fresh samples were within $\pm$ 15%.

The selectivity of the method was evaluated by analyzing a sterile Czapek-Dox medium and a sterile Czapek medium added to fungal mycelium.

Midodrine biotransformation procedure

Fungi

The fungus *Papulaspora immersa* Hotson (SS13) used in this experiment was previously isolated as an endophyte from the plant *Smallanthus sonchifolius* (26), and is deposited in the Laboratório de Química de Micro-organismos—FCFRP/USP (Brazil). Moreover, two strains of *Botrytis cinerea* were employed in this experiment. *B. cinerea* (UCA 992) was isolated from grapes from the Domecq vineyard, Jerez de la Frontera, Cádiz, Spain. This *B. cinerea* strain is deposited in the Universidad de Cádiz, Facultad de Ciencias, Mycological Herbarium Collection (UCA, Spain). The other strain of *B. cinerea* (2100) was purchased from the Colección Española de Cultivos Tipo (CECT), Facultad de Biología, Universidad de Valencia, Spain, where this strain is deposited.

P. immersa biotransformation procedure

Three discs of 0.5 cm diameter of potato dextrose agar (PDA) plugs containing the fungal mycelia were aseptically transferred to 9.0 cm diameter Petri dishes containing PDA medium and allowed to grow for seven days at 25°C. Biotransformation was performed using a two-stage fermentation protocol (24, 27). In the first stage (pre-culture), three 0.5 cm uniform discs were cut with a transfer tube and then inoculated in 50 mL Falcon tubes containing 10 mL of pre-fermentative liquid broth (10 g glucose, 5 g tryptone soy broth, 3 g yeast extract, and 10 g malt extract, per liter and with pH adjusted to 6.2 with 0.1 mol/L HCl solution). The Falcon tubes were incubated for four days (96 h) at 25°C on a rotatory shaker (Adolph Kühner, model CH 4127, Birsfelden, Switzerland) operating at 120 rpm.

The second stage (biotransformation) was conducted under static and shaken conditions. Under shaken conditions, the resulting mycelium was transferred to 500 mL Erlenmeyer flasks containing 200 mL of Czapek-Dox medium (50 g glucose, 1 g yeast extract, 2 g $NaNO_3$, 5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 0.01 g $FeSO_4.7H_2O$, per liter) adjusted to a pH range from 6.5 to 7.0 with 0.1 mol/L HCl solution. In sequence, 8 mg of midodrine (on a free-base basis) dissolved in 1,000 μL of sterile water was added to the flask. Control flasks consisted of culture broth (Czapek-Dox) without midodrine and fungus, sterile broth with midodrine and sterile broth with endophytic fungal mycelium. Biotransformation experiments were conducted at 25 °C, with shaking at 120 rpm for 120 h.

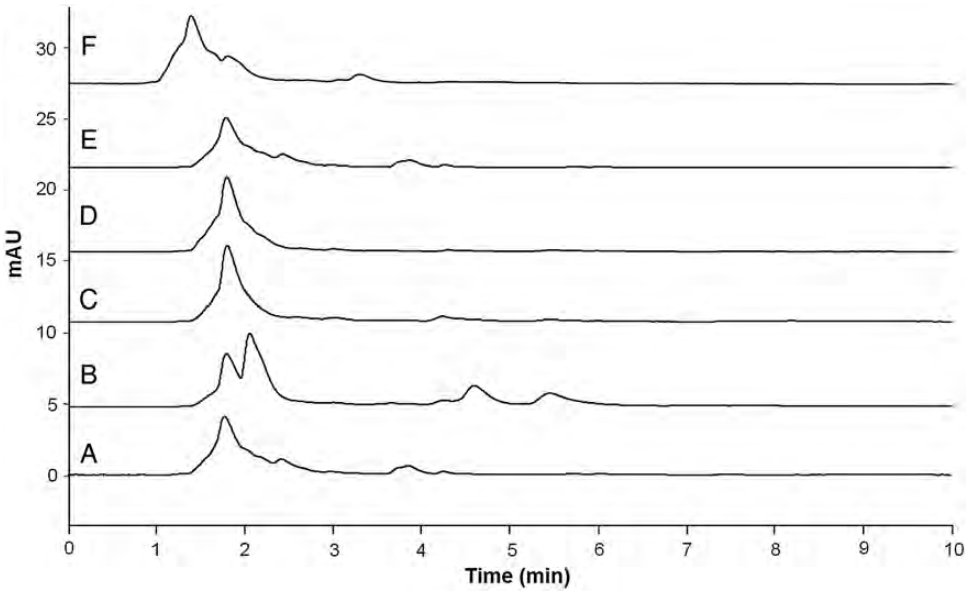

Figure 3. Representative chromatograms of Czapek-Dox medium incubated with: the fungi Botrytis cinerea (2100) under shaken conditions (A); *Botrytis cinerea* (2100) under static conditions (B); *Papulaspora immersa* (SS13) under shaken conditions (C); *Papulaspora immersa* (SS13) under static conditions (D); *Botrytis cinerea* (UCA 992) under shaken conditions (E); *Botrytis cinerea* (UCA 992) under static conditions (F). The chromatographic conditions described in "Liquid Chromatographic Conditions."

Under static conditions the resulting mycelium was transferred into Roux bottles containing 150 mL of Czapek-Dox medium. Furthermore, 6 mg of midodrine (on a free-base basis) dissolved in 750 μL of sterile water were added to the flask. Just as described previously, control bottles were also performed. Biotransformation experiments were conducted at 25°C for 120 h.

Daily aliquots of 3 mL, for both conditions, were collected from the culture flasks, and 0.5 mL were submitted to the extraction procedure and analyzed by HPLC.

B. cinerea strains biotransformation procedure

The biotransformation was conducted under static and shaken conditions. Under the shaken condition, a volume equivalent to 10^7 spores was transferred into 500 mL Erlenmeyer flasks containing 200 mL of Czapek-Dox. After two days' growth, 8 mg of midodrine (free-base basis) dissolved in 1,000 μL of sterile water were added to the flask. Biotransformation experiments were conducted at 25 °C, with shaking at 120 rpm for 168 h.

Under the static condition, a volume equivalent to 10^7 spores was transferred into Roux bottles containing 150 mL of Czapek-Dox medium. Subsequently, 6 mg of midodrine (free-base basis) dissolved in 750 μL of sterile water were added to the flask. Biotransformation experiments were conducted at 25 °C during 168 h. Daily aliquots of 3 mL, for both conditions, were collected from the culture flasks, and 0.5 mL were submitted to the extraction procedure and analyzed by HPLC. As described previously, control flasks were also performed.

The biotransformation kinetic studies were presented as concentration versus collecting interval (hours) profiles. The efficiency of the biotransformation process was calculated (in percentage) based on the amount of DMAE in the culture medium and correlating this amount with the initial amount of midodrine (time 0). Regarding the repeatability of the biotransformation procedure, the experiment was performed in replicate ($n = 2$).

Results and Discussion

The resolution of midodrine and DMAE was conducted on a Lichrospher 100 RP18 column under reverse elution mode. The evaluated mobile phases were prepared using methanol–water and acetonitrile–water mixtures. Formic acid was added to these mobile phases to suppress the ionization of the silanol groups of the silica support, and its interaction with the analytes, reducing the tails of the peaks of midodrine and DMAE that are basic compounds (26). The best separation condition was achieved with a mobile phase consisting of acetonitrile–40 mmol/L formic acid solution (60:40, v/v), at a flow rate of 1.4 mL/min and with detection at 290 nm. Under these conditions, the separation of the compounds was performed in 10 min with enough resolution ($R_s = 1.52$) (Figure 2B).

Method validation

The recoveries of midodrine were approximately 82%, while the DMAE recoveries were approximately of 84%. The RSD (%) for the recoveries of both analytes were lower than 15% (Table I).

Linear regression analyses were performed by plotting the peaks areas of the analytes (y) versus theoretical analyte concentrations (x). The method proved to be linear over the concentration range of 0.4–40 μg/mL for both midodrine and DMAE, with correlation coefficient $r \geq 0.9997$. The linearity of the method was also confirmed by the FLOF test (Table I).

The LOQ using the validated method was 0.4 μg/mL for each analyte. The RSD (%) and RE (%) were lower than 20% (Table II).

The precision and accuracy of the method were evaluated by within and between-day assays. The RSDs and relative errors were lower than 15% (Table III).

The freeze–thaw, short-term room temperature and autosampler stability of midodrine and DMAE in Czapek-Dox medium showed RSDs and relative errors lower than 15%. These results are presented in Table IV. Midodrine was stable for 168 h under the biotransformation conditions (shaken and

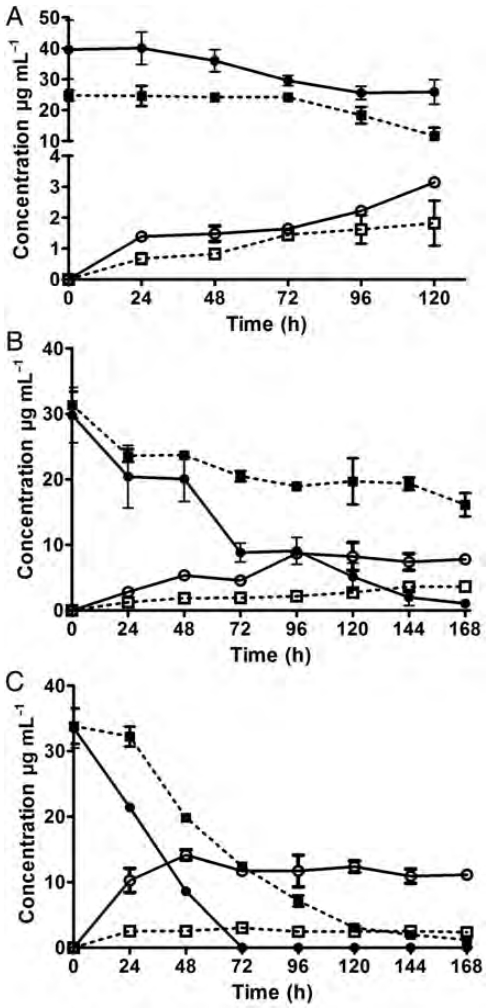

Figure 4. Graphs of concentration time-profiles of midodrine and DMAE in culture medium incubated with: *Papulaspora immersa* Hotson (A); *Botrytis cinerea* UCA 992 (B); *Botrytis cinerea* 2100 (C) under static and shaken conditions. The black circles represent midodrine under shaken conditions; white circles represent DMAE under shaken conditions; black squares represent midodrine under static conditions; white squares represent DMAE under static condition .The bars denote the error of two replicates.

static) (RSD and RE < 15%), and no degradation of midodrine could be observed during this period (Table V).

The studied fungi did not produce any secondary metabolite that presented retention times close to those of midodrine and DMAE (Figure 3).

Biotransformation of midodrine

The midodrine biotransformation was evaluated with three fungi under static and shaken conditions. These different conditions were tested to evaluate the effect of aeration of the cultures and the growth morphology of these fungi. Under static conditions, a weak oxygenation was observed, and under shaken conditions this oxygenation was higher (29). Moreover, under static conditions, the fungi, in general, grew on the culture surface, while under shaken conditions, spherical aggregates called pellets are formed (30).

The biotransformation was monitored for 168 h for the *B. cinerea* strains and 120 h for the fungus *P. immersa*. The concentrations of the analytes were determined based on calibration curves constructed daily. The identification of DMAE was performed by comparing its retention time with that of the reference substance.

The endophytic fungus *P. immersa* Hotson was able to biotransform midodrine in the human active metabolite DMAE (Figure 1). The maximum amount of DMAE was detected in the extracts of the endophytic fungus culture medium after

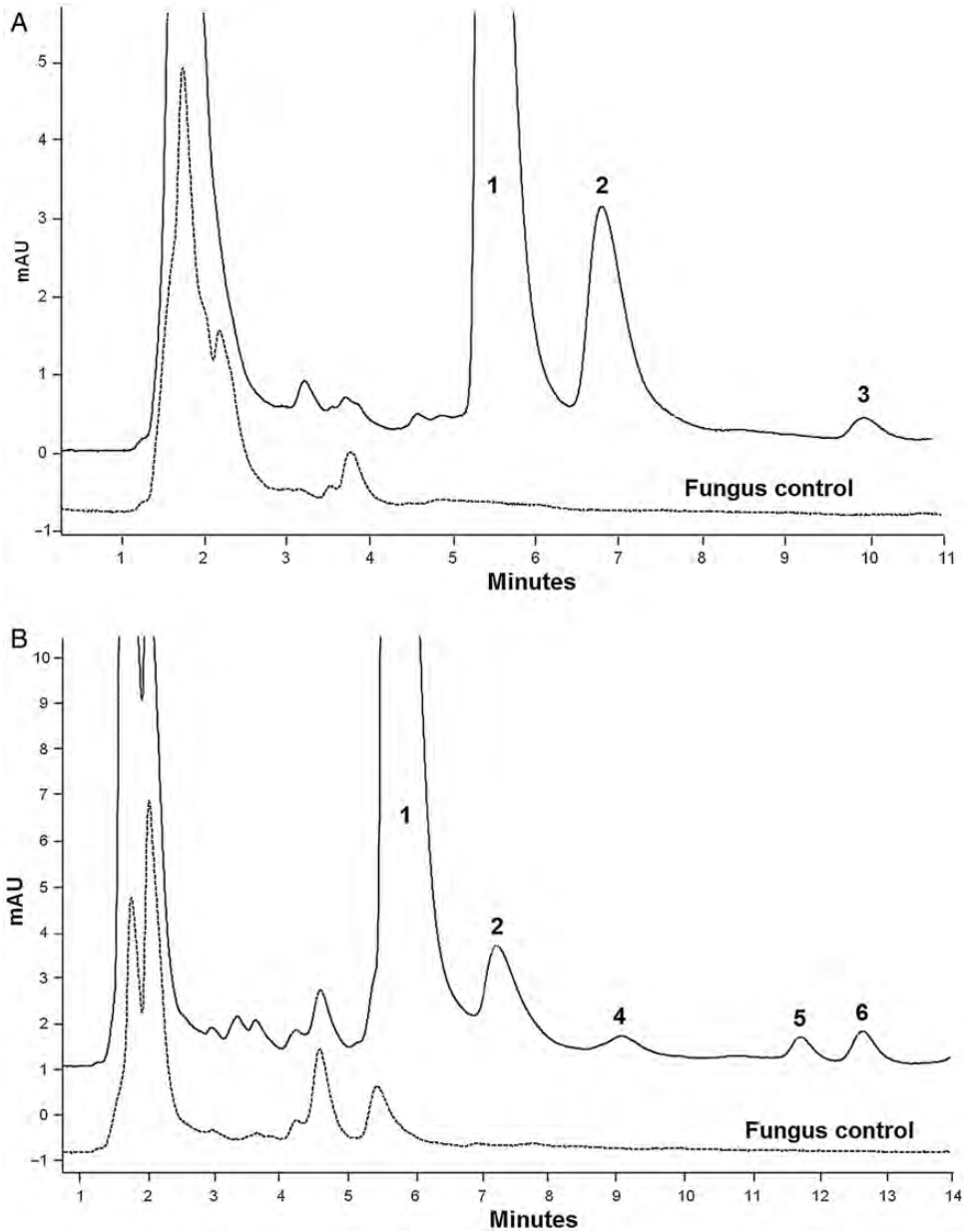

Figure 5. Representative chromatogram of Czapek-Dox medium incubated with the fungus *Papulaspora immersa* Hotson (SS13) and midodrine after 120 h of biotransformation under static conditions (A); representative chromatogram of Czapek-Dox medium incubated with the fungus *Botrytis cinerea* 2100 and midodrine after 48 h of biotransformation under static conditions (B). The chromatographic conditions described in "Liquid Chromatographic Conditions." Peaks: midodrine (1); DMAE (2); unidentified compounds (3, 4 and 5).

120 h of midodrine incubation for both conditions. A slightly higher efficiency (7.9%) under shaken conditions could be observed than under static conditions (7.3%) (Figure 4A). In addition, under static conditions, an additional peak was detected, which can be considered another possible metabolite of midodrine (peak 3, Figure 5A).

The fungus *B. cinerea* has extensively been used to biotransform synthetic (31, 32, 33, 34, 35) and natural products (36, 37) with potential fungistatic and fungicide activities. The conversion of midodrine to DMAE involves an amide hydrolysis reaction, which has already been observed for *B. cinerea* (38).

The strain *B. cinerea* UCA 992 showed better biotransformation results under shaken conditions. The maximal formation of DMAE under static conditions (11.5%) was verified in 168 h of biotransformation (Figure 4B), whereas, under shaken conditions, a higher concentration of DMAE (29.2%) was observed within 96 h of incubation (Figure 4B). Additional peaks at both biotransformation conditions were not observed for *B. cinerea* UCA 992.

B. cinerea 2100 showed higher DMAE formation under shaken conditions (Figure 4C). The maximal amount of formed DMAE under shaken conditions was verified in 48 h of incubation (42.2% of biotransformation). On the other hand, under static conditions, the maximum amount of DMAE was observed in 72 h of incubation (8.9% of biotransformation). Moreover, under static conditions, after 48 h of incubation, three additional peaks were observed. These peaks can also be considered possible midodrine metabolites (peaks 4, 5 and 6, Figure 5B). The unidentified metabolites (peaks 3, Figure 5A; peaks 4, 5 and 6, Figure 5B) were not studied here. The biotransformation under static conditions of midodrine by *P. immersa* Hotson and *B. cinerea* 2100 deserve more studies for the isolation and characterization of these unknown metabolites.

Conclusions

An HPLC–UV method was described for the simultaneous determination of midodrine and DMAE in Czapek-Dox culture medium. The validation parameters were evaluated and met the literature requirements (25). This work presents a simpler method than that published by Posch and Lindner (1989), which uses fluorescence detection and column-switching procedures. This method was successfully employed to study midodrine biotransformation by the endophytic fungus *P. immersa* Hotson and by the phytopatogen strains *B. cinerea* 2100 and *B. cinerea* UCA 992. The biotransformations were conducted under static and shaken conditions. For all studied fungi, the shaken conditions resulted in higher DMAE formation, especially for the fungus *B. cinerea* 2100, which biotranformed 42.2% of midodrine to DMAE.

Acknowledgments

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and for granting research fellowships.

The authors have declared no conflict of interest.

References

1. Mcclellan, K.J., Wiseman, L.R., Wilde, M.I.; Midodrine. A review of its therapeutic use in management of orthostatic hypotension; *Drug and Aging*, (1998); 12: 75–86.
2. Rautio, J., Kumpulainen, H., Heimbach, T., OliyaiI, R., Oh, D., Järvinen, T., *et al.*; Prodrugs: Design and clinical applications; *Nature Reviews Drug Discovery*, (2008); 7: 255–270.
3. Akimoto, M., Iida, I., Itoga, H., Miyata, A., Kawahara, S., Kohno, Y.; The in vitro metabolism of desglymidodrine, an active metabolite of prodrug midodrine by human liver microsomes; *European Journal of Drug Metabolism and Pharmacokinetics*, (2004); 29: 179–186.
4. Quaglia, M. G., Farina, A., Palmery, M., Desideri, N., Donati, E., Bossù, E., *et al.*; Chiral investigation of midodrine, a long-acting α-adrenergic stimulating agent; *Chirality*, (2004); 16: 356–362.
5. Venisetty, V.J., Ciddi, V.; Application of microbial biotransformation for the new drug discovery using natural drugs as substrates; *Current Pharmaceutical Biotechnology*, (2003); 4: 123–140.
6. Smith, R.V., Rosazza, J.P.; Microbial models of mammalian metabolism; *Journal of Pharmaceutical Sciences*, (1975); 64: 1759–1765.
7. Azerad, R.; Microbial models of drug metabolism; *Advances in Biochemical Engineering/Biotechnology*, (1999); 63: 169–218.
8. Asha, S., Vidyavathi, M.; Cunninghamella—A microbial model for drug metabolism studies—A review; *Biotechnology Advances*, (2009); 27: 16–29.
9. Fudo, R., Ando, T., Sato, S., Kameyama, T., Yamanaka, S.; Isolation of isoeugenitin as a fruiting body inducer for *Stimatella aurantiacaI* from a soil fungus *Papulaspora* sp; Bioscience, *Biotechnology and Biochemistry*, (1997); 61: 183–184.
10. Raviraja, N.S., Maria, G.L., Sridhar, K.R.; Antimicrobial evaluation of endophytic fungi inhabitting medicinal plants of the western Ghats of India; *Engineering in Life Sciences*, (2006); 6: 515–520.
11. O'Callaghan, M., Gerard, E.M., Waipara, N.W., Young, S. D., Glare, T.R., Barrell, P.J., *et al.*; Microbial communities of *Solanum tuberosum* and magainin-producing transgenic lines; Plant and *Soil*, (2004); 266: 47–56.
12. Gallo, M.B.C., Cavalcanti, B.C., Barros, F.W.A., Moraes, M.O., Costa-Lotufo, L.V., Pessoa, C., *et al.*; Chemical constituents of *Papulaspora immersa*, an endophyte from *Smallanthus sonchifolius* (Asteraceae), and their cytotoxic activity; *Chemistry & Biodiversity*, (2010); 7: 2941–2950.
13. Adams, P.R.; Mycelial amylase activities of thermophilic species of *rhizomucor, humicola* and *papulaspora*; *Mycopatologia*, (1990); 112: 35–37.
14. Hilário, V.C., Carrão, D.B., Barth, T., Borges, K.B., Furtado, N.A.J.C., Pupo, M.T., *et al.*; Assessment of the stereoselective fungal biotransformation of albendazole and its analysis by HPLC in polar organic mode; *Journal of Pharmaceutical and Biomedical Analysis*, (2012); 61: 100–107.
15. Schulz, B., Boyle, C.; The endophytic continuum; *Mycological Research*, (2005); 6: 661–686.
16. Borges, W.S., Borges, K.B., Bonato, P.S., Said, S., Pupo, M.T.; Endophytic fungi: Natural products, enzymes and biotransformations reactions; *Current Organic Chemistry*, (2009); 13: 1137–1163.
17. Collado, I.G., Sánchez, A.J.M., Hanson, J.R.; Fungal terpene metabolites: biosynthetic relationships and the control of the phytopathogenic fungus *Botrytis cinerea*; *Natural Product Reports*, (2007); 24: 674–686.
18. Aleu, J., Collado, I.G.; Biotransformations by Botrytis species; *Journal of Molecular Catalysis B: Enzymatic*, (2001); 13: 77–93.
19. Kolossa, N., Schutzemberg, W.G., Wiener, H., Krivanek, P.; Plasma level of the prodrug midodrine and its active metabolite in comparison with the α-mimetic action in dogs; *Archives Internationales de Pharmacodynamie et de Therapie*, (1979); 238: 96–104.
20. Posch, W., Lindner, W.; Quantification of midodrine and its active metabolite in plasma using a high performance liquid chromatography column switching technique; *Biomedical Chromatography*, (1989); 3: 153–156.

21. Zachariah, P.K., Bloedow, D.C., Moyer, T.P., Sheps, S.G., Schirger, A., Fealey, R.D.; Pharmacodynamics of midodrine, an antihypotensive agent; *Clinical Pharmacology and Therapeutics*, (1986); 39: 586–591.
22. Lamarre-Cliché, M., Du Souich, P., De Champlain, J., Larochelle, P.; Pharmacokinetic and pharmacodynamics effects of midodrine on blood pressure, the autonomic nervous system, and plasma natriuretic peptides: A prospective, randomized, single-blind, two-period, crossover, placebo-controlled study; *Clinical Therapeutics*, (2008); 30: 1629–1638.
23. Quaglia, M.G., Farina, A., Bossù, E., Cotichini, V.; Human α1-glycoprotein acid as chiral selector in the enantioseparation of midodrine and deglymidodrine racemates by HPLC; *Journal of Pharmaceutical and Biomedical Analysis*, (1998); 18: 171–177.
24. Barth, T., Pupo, M.T., Borges, K.B., Okano, L.T., Bonato, P.S.; Stereoselective determination of midodrine and desglymidodrine in culture medium: Application to a biotransformation study employing endophytic fungi; *Electrophoresis*, (2010); 31: 1521–1528.
25. Food and Drug Administration, Center for Drug Evaluation and Research, Guidance for Industry: Bioanalytical Method Validation, (2001).
26. Gallo, M.B.C., Chagas, F.O., Almeida, M.O., Macedo, C., Cavalcanti, B.C., Barros, F.W.A., *et al.*; Endophytic fungi found in association with *Smallanthus sonchifolius* (Asteraceae) as resourceful producers of cytotoxic bioactive natural products; *Journal of Basic Microbiology*, (2009); 49: 142–151.
27. Borges, K.B., Pupo, M.T., Bonato, P.S.; Enantioselective analysis of propranolol and 4-Hydroxypropranolol by CE with application to biotransformation studies employing endophytic fungi; *Electrophoresis*, (2009); 30: 3910–3917.
28. Mccalley, D.V.; The challenges of the analysis of basic compounds by high performance liquid chromatography: Some possible approaches for improved separations; *Journal of Chromatography A*, (2010); 1217: 858–880.
29. Mansour, H.B., Mosrati, R., Corroler, D., Ghedira, K., Barrilier, D., Chekir, L.; In vitro mutagenicity of acid violet 7 and its degradation products by Pseudomonas putida mt2: Correlation with chemical structures; *Environmental Toxicology and Pharmacology*, (2009); 27: 231–236.
30. Gibbs, P.A., Seviour, R.J., Schmid, F.; Growth of filamentous fungi in submerged culture: Problems and possible solutions; *Critical Reviews in Biotechnology*, (2000); 20: 17–48.
31. Durán, R., Corrales, E., Galán, R.H., Collado, I.G.; Biotransformation of caryophyllene oxide by *Botrytis cinerea*; *Journal of Natural Products*, (1999); 62: 41–44.
32. Durán-Patrón, R., Aleu, J., Galán, R.H., Collado, I.G.; Biotransformation of (4E,8R)-caryophyll-4(5)-en-8-ol by *Botrytis cinerea*; *Journal of Natural Products*, (2000); 63: 44–47.
33. Bustillo, A.J., Aleu, J., Galán, R.H., Collado, I.G.; Biotransformation of the fungistatic compound (R)-(+)-1-(4'-chlorophenyl)propan-1-ol by *Botrytis cinerea*; *Journal of Molecular Catalysis. B: Enzymatic*, (2003); 21: 267–271.
34. Daoubi, M., Deligeorgopoulou, A., Sánchez, A.J.M., Galán, R.H., Hitchcock, P.B., Hanson, J.R., *et al.*; Antifungal activity and biotransformation of diisophorone by *Botrytis cinerea*; *Journal of Agricultural and Food Chemistry*, (2005); 53: 6035–6039.
35. Pinedo-Rivilla, C., Bustillo, A.J., Galán, R.H., Aleu, J., Collado, I.G.; Asymmetric preparation of antifungal 1-(4'-chlorophenyl)-1-cyclopropyl methanol and 1-(4'-chlorophenyl)-2-phenylethanol. Study of the detoxification mechanism by *Botrytis cinerea*; *Journal of Molecular Catalysis. B: Enzymatic*, (2011); 70: 61–66.
36. Aleu, J., Hanson, J.R., Galán, R.H., Collado, I.G.; Biotransformation of the fungistatic sesquiterpenoids patchoulol, ginsenol, cedrol and globulol by *Botrytis cinerea*; *Journal of Molecular Catalysis. B: Enzymatic*, (2001); 11: 329–334.
37. Nuñez, Y.O., Salabarria, I.S., Collado, I.G., Galán, R.H.; The antifungal activity of widdrol and its biotransformation by *Colletotrichum gloeosporioides* (penz.) Penz. & Sacc. and *Botrytis cinerea* Pers. Fr; *Journal of Agricultural and Food Chemistry*, (2006); 54: 7517–7521.
38. Tellier, F., Carlin-Sinclair, A., Fritz, R., Cherton, J.C., Leroux, P.; Activity and metabolism of cyano-oxime derivatives in various strains of *Botrytis cinerea*; *Pesticide Biochemistry and Physiology*, (2004); 78: 151–160.