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Experimental design for optimization of microwave-assisted extraction of benzodiazepines in human plasma

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Abstract A simple and fast microwave-assisted-extraction (MAE) method has been evaluated as an alternative to solid-phase extraction (SPE) for the determination of six benzodiazepines widely prescribed in European countries (alprazolam, bromazepam, diazepam, lorazepam, lormetazepam and tetrazepam) in human plasma. For MAE optimization a Doehlert experimental design was used with extraction time, temperature and solvent volume as influential parameters. A desirability function was employed in addition to the simultaneous optimization of the MAE conditions. The analysis of variance showed that the solvent volume had a positive influence on the extraction of all the analytes tested, achieving a statistically significant effect. Also, the extraction time had a statistically significant effect on the extraction of four benzodiazepines. The selected MAE conditions—89 °C, 13 min and 8 mL of chloroform/2-propanol (4:1, v/v)—led to recoveries between 89.8±0.3 and 102.1±5.2% for benzodiazepines using a high performance liquid chromatography method coupled with diode-array detection. The comparison of MAE and SPE shows better results for MAE, with a lower number of steps in handling the sample and greater efficiency. The applicability of MAE was successfully tested in 27 plasma samples from benzodiazepine users.

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Introduction

In the short term, benzodiazepines (BZD) are remarkably useful psychoactive drugs, highly effective for disorders such as anxiety, epilepsy and insomnia. However, serious adverse effects can result from long-term regular use in therapeutic doses and from self-prescription or recreational use in excessive doses. The combined disinhibitory effects of alcohol and BZD may also be additive and contribute to aggressive behaviour. Sexual offences and acts of violence (both homicide and suicide) can be related to BZD consumption and can lead to medicolegal complications [1, 2].

The risk of dependence is probably greater with potent, short-acting BZD such as lorazepam and alprazolam. Caution should be exercised in prescribing BZD hypnotics and anxiolytics because they may also induce dependence if used long-term [1]. Quantification in plasma may help to optimize chronic dosing, verify compliance and identify changes in pharmacokinetics [3]. For these reasons, the optimization of reliable, rapid and simple methods for the control of BZD in plasma is required to save time and cost, without losing the sensitivity and reproducibility of the analytical method.

Liquid chromatography has become an important tool for routine determination of BZD owing to its specificity, rapidity and sensitivity [4–8]. Although high-performance liquid chromatography (HPLC) with mass spectrometry detection [5–7] is highly selective, the cost of the instrumentation makes it less suitable for clinical or toxicology laboratories. In contrast, HPLC–UV detection or diode array detection

(DAD) instrumentation is widely available in most analytical laboratories [8–12].

An effective sample pretreatment procedure prior to HPLC separation is often necessary to improve sensitivity. Liquid–liquid extraction [13], solid-phase extraction (SPE) [14–16], online SPE [17] and solid-phase microextraction [18] have usually been successfully employed. Different analytical methods have been effectively developed and established to both identify and quantify these compounds; relevant information on a selected number of BZD-based applications is presented in Table 1. Similar limits of detection (LODs) at the nanogram per millilitre level have been achieved with different detection systems used with gas chromatography or HPLC separation.

Microwave-assisted extraction (MAE) is very suitable for routine analysis, without any additional cleaning step, giving greater efficiency of the method. Furthermore, MAE enables simultaneous extractions producing less waste than other conventional techniques; therefore, it is considered a clean technology (green chemistry). There are a large number of reports concerning application of this technique in environmental sample analysis [34, 35]. MAE has also been used successfully in analysis of medicines, vitamins and drugs of abuse, present in pharmaceutical and biological samples [36, 37].

Factorial designs, which provide a method for simultaneously investigating the effects of multiple variables and multiple responses, have generally been used and preferred to the one factor at a time approach because they detect and estimate any interaction among variables and need fewer experiments to complete the optimization process [38].

Table 1 Methods for the determination of benzodiazepines (*BZD*)

Matrix	Analytes	Pretreatment/extraction	Determination	LOD	Reference
Urine, plasma, serum, meconium	10 BZD	Hydrolysis/SPE Trace-B columns/ethyl acetate/ammonium hydroxide elution	LC-ESI-MS/MS	10 ng mL^{-1}	[3]
Blood	33 BZD	SPE ChemElut cartridges/*t*-butyl methyl ether	LC-APCI-MS	0.1–12.6 ng mL^{-1}	[6]
Plasma	15 BZD	SPE C_1 cartridges/MeOH elution	HPLC-UV	2.6 ng mL^{-1}	[8]
Urine, plasma, saliva	8 BZD	SPE Abselut Nexus cartridges/MeOH/ACN	HPLC-DAD	20–47 ng mL^{-1}	[9]
Urine, plasma	4 BZD	SPE DSC-18 cartridges/MeOH elution	HPLC-UV	100–600 ng mL^{-1}	[19]
Rat plasma	FNZ and metabolites	LLE Toxi-Tubes A/BSTFA derivatization	GC-MS	125 ng mL^{-1}	[20]
Serum, urine	4 BZD	SPE Abselut Nexus and Oasis HLB cartridges/ethyl ether elution	HPLC-UV	1.0 ng mL^{-1}	[21]
Plasma and oral fluid	9 BZD	LLE/diethyl ether	LC-ESI-MS	0.5–1 ng mL^{-1}	[22]
Blood	14 BZD	LLE with ACN/MTBSTFA derivatization	GC-NICI-MS	1–100 ng mL^{-1}	[23]
Urine and blood	FNZ and metabolites	LLE–SPE CECN4 butyl cartridges/ethyl acetate/methanol elution	TFA derivatization-HPLC-DAD/PFPA derivatization-GC-SIM-MS	1–5 ng mL^{-1}	[24]
Hair	14 BZD	LLE/diethyl ether/DCM	LC–ESI-MS/MS	0.5–5 pg mg^{-1}	[25]
Soil	3 BZD	SPE Phenomenex Strata-X/MeOH elution	HPLC-ESI-MS	–	[26]
Hair	9 BZD	SPE Clean Screen columns/ammoniated ethyl acetate elution MISPE/acetic acid in acetonitrile elution	LC-ESI-MS/MS	30–780 pg mg^{-1}	[27]
Urine	11 BZD	SPE extract-clean column C_{18}/MeOH	LC-ESI-MS/MS	30 ng mL^{-1}	[28]
Serum	30 BZD	SPE Oasis MCX cartridges/ACN	LC-ESI-MS	0.1–5 ng mL^{-1}	[29]
Urine and plasma	4 BZD	Automated online SPE Empore™ disks	LC-ESI-MS/MS	0.02–0.15 ng mL^{-1}	[30]
Urine and hair	2 BZD	LLE Toxi-Tubes A/DCM	LC-ESI-MS/MS	0.05–01 ng mL^{-1} 0.5–2 pg mg^{-1}	[31]
Oral fluid	8 BZD	LLE Toxi-Tubes A	LC/MS/MS	0.2–2.1 ng mL^{-1}	[32]
Plasma	6 BZD	SPE Bond Elut Certify cartridges/chloroform/2-propanol elution	HPLC-DAD	7.6–14 ng mL^{-1}	[33]

ACN acetonitrile, *APCI* atmospheric pressure chemical ionization, *BSTFA* *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, *DAD* diode-array detection, *DCM* dicloromethane, *ESI* electrospray injection, *FNZ* flunitrazepam, *GC* gas chromatography, *HPLC* high-performance liquid chromatography, *LC* liquid chromatography, *LLE* liquid–liquid extraction *MISPE* molecularly imprinted solid-phase extraction, *MS* mass spectrometry, *MTBSTFA* *N*-methyl-*N*-(trimethylsilyl)-*N*,*O*-bis(trimethylsilyl) trifluoroacetamide, *NICI* negative ion chemical ionization, *PFPA* pentafluoropropionic anhydride, *SIM* selected ion monitoring, *SPE* solid-phase extraction, *TFA* trifluoroacetic acid, *UV* UV detection

The aim of this study was to determine the applicability of MAE as an alternative to established procedures, such as SPE, for the simultaneous extraction of six frequently prescribed BZD in human plasma—alprazolam, bromazepam, diazepam, lorazepam, lormetazepam and tetrazepam—with the therapeutic and toxic levels shown in Table 2. The development of a feasible and reliable HPLC-DAD method based on microwave extraction for BZD in plasma at concentrations lower than therapeutic levels was performed and validated using spiked samples. The method was applied to the analysis of real plasma samples from BZD users.

Experimental

Chemicals

Standards of the BZD studied were obtained from Cerilliant (Round Rock, TX, USA). Gradient-grade acetonitrile, chloroform, 2-propanol and methanol were purchased from Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q water system from Millipore (Le Mont-sur-Lausane, Switzerland). Individual stock solutions containing 1 mg mL^{-1} of each drug were prepared in methanol. Working-strength solutions containing the six BZD were made by successive dilutions of the stock solutions to obtain final concentrations within the range 0.06–2 μg mL^{-1} in plasma. All solutions were stored frozen. Bond Elut Certify cartridges were obtained from Varian® (Walnut Creek, CA, USA).

Instrumentation

The microwave extraction device was an ETHOS PLUS MPR300/12 S from Milestone® (Agrigento, Italy) equipped with a solvent detector. The device was able to extract 12 samples simultaneously in PTFE-lined extraction closed vessels under the same conditions (temperature and pressure), with simultaneous magnetic stirring of the sample and solvent. An on-board pressure control system was installed for monitoring and controlling the pressure and conditions inside the extraction vessels. This oven allows a maximum power of 1,000 W and the power changes to reach and maintain the temperature selected.

Analyses were carried out with a Waters® (Milford, MA, USA) 2695 chromatograph connected to a model 996 photodiode array detector, also from Waters®. Data were processed with the Millennium 32® software program. Samples were injected into an XTerra® RP8 (250 mm× 4.6-mm inner diameter, 5-μm particle size) stainless steel column from Waters®.

To ensure optimal peak resolution, and hence the efficient separation of the analytes in a reasonably short time (28 min), elution was done with a mobile phase consisting of a mixture of acetonitrile (solvent A) and 0.02 M phosphate buffer pH 7.5 (solvent B), at a flow of 0.8 mL/min, in gradient mode: 0–12 min, 35% solvent A and 65% solvent B; 14–30 min, 40% solvent A and 60% solvent B; 35 min, 35% solvent A and 65% solvent B. The diode-array detector allowed the wavelength range from 200 to 400 nm to be scanned to obtain three-dimensional (wavelength x absorbance x time) chromatograms, although we decided to work at 230 nm, a wavelength very near the absorption maxima of the six BZD, which permits a good simultaneous chromatographic response to be obtained.

Table 2 Therapeutic and toxic levels of BZD

BZD	Therapeutic levels (μg/mL)	Toxic levels (μg/mL/
APZ	0.025–0.10	0.10-0.40
BRZ	0.11–0.17	–
DZP	0.10–1.00	>1.5
LRZ	0.05–0.24	0.30-0.60
LRMZ	0.006–0.016	–
TRZ	0.49–0.63	–

Data from [39]

APZ alprazolam, *BRZ* bromazepam, *DZP* diazepam, *LRZ* lorazepam, LRMZ lormetazepam, *TRZ* tetrazepam

Plasma samples

Drug-free plasma from the Galician Transfusion Centre (Spain) was used for the preparation of calibration standards. Plasma samples were obtained from patients poisoned with BZD and stored at 4 °C, unless the analysis was delayed, in which case the samples were frozen. All studies were conducted in accordance with the World Medical Association's "Ethical principles for medical research involving human subjects" [40].

MAE procedure

The plasma was centrifuged in a MiniSpin Plus centrifuge (Eppendorf®) for 10 min at 14,000 rpm to eliminate compounds that could be coextracted, causing a matrix effect. The MAE conditions were optimized for the extraction of BZD from human plasma as discuss in "Results and discussion". The final optimized procedure was as follows. Borate buffer (2 mL, pH 9.0) was added to 1 mL of plasma containing the analytes and the samples were extracted at 89 °C for 13 min, using 8 mL of chloroform/2-propanol (4:1, v/v). After cooling, the extraction vessels were opened and the extracts were centrifuged for 10 min at 4,000 rpm to separate the organic phase from the aqueous one. The supernatant was collected and concentrated to dryness under a nitrogen stream at 40 °C.

The extracts were redissolved in 200 μL of the mobile phase, yielding a concentration 5 times higher than that originally present in plasma. After ultracentrifugation for 5 min at 14,000 rpm, 20 μL of the supernatant was injected into the chromatograph.

SPE procedure

The method used for the SPE has been robustly validated and a full description of the procedure has been reported elsewhere [33]. In brief, 500 μL of borate buffer pH 9.0 was added to 500 μL of the sample containing the BZD. The drugs were separated from plasma with Manifold (Waters®) and Bond Elut Certify cartridges, which were conditioned with 2 mL of methanol and 2 mL of phosphate buffer pH 6.0. The sample was added and washed with 3 mL of water/methanol (95:5, v/v) and 3 mL of 0.3 M NH_4OH. The samples were then vacuum-dried for 5 min, being eluted with 3 mL of chloroform/2-propanol (4:1, v/v). The extracts obtained were evaporated under a nitrogen stream at 40 °C and reconstituted in 200 μL of the mobile phase, yielding a concentration 2.5 times higher than that originally present in plasma. Finally, 20 μL was injected into the chromatograph.

Validation procedure

The procedure was validated in terms of linearity, repeatability (intraday precision), reproducibility (interday precision) and recovery. In view of the therapeutic and toxic concentrations, calibration curves were generated by spiking blank plasma. The calibration curve was established at six concentrations in the range 60–2,000 ng mL^{-1}. Precision was characterized in terms of the relative standard deviation (RSD) by analysing sets of five spiked human plasma samples at three different concentrations (0.06, 0.4, and 2 μg mL^{-1}). Recovery of the sample was determined by n=5 analyses of samples spiked at three concentrations (0.06, 0.4 and 2 μg mL^{-1}).

Results and discussion

Preliminary experiments

To evaluate the solvent effect on the extraction of BZD from human plasma, studies were performed using different solvents and mixtures commonly employed in traditional extraction of drugs from biological matrices: (1) *n*-hexane; (2) dichloromethane; (3) ethyl acetate; (4) chloroform; (5) chloroform/methanol (1:1, v/v); (6) chloroform/2-propanol (1:4, v/v, 1:1, v/v and 4:1, v/v). For MAE, the solvent used as an extractant requires good permittivity to absorb the microwave energy and transform it into thermal energy. The aqueous matrix of plasma locally superheats the analytes and promotes their release into the surrounding medium. Moreover, the high temperatures reached by microwave heating substantially reduce both the extraction time and the required solvent volume [41]. In all cases 1 mL of blank plasma was spiked with 1 μg of all BZD studied. MAE was performed using the following general conditions: 10 mL of solvent and 6 min at 80 °C. Figure 1 shows the effect of solvent or solvent mixtures on the extraction of the target analytes from spiked samples. Hexane and dichloromethane gave worse results than the other solvents. Hexane is not capable of extracting alprazolam, bromazepam and lorazepam, whereas bromazepam is not extracted when dichloromethane is used. Chloroform/2-propanol (1:1, v/v and 1:4, v/v) mixtures provided extraction yields lower than those found for chloroform for the majority of the compounds studied. Chloroform/2-propanol (4:1, v/v) provided the best extraction results for all the BZD and was chosen as the extractant.

Plasma samples were mixed with a volume of borate buffer pH 9.0 to ensure that BZD were in a nonionized form and they could easily switch to the organic phase. Additions of 1, 2 or 4 mL of buffer to 1 mL of plasma were evaluated using the general MAE conditions described above: 10 mL of chloroform/2-propanol (4:1, v/v), 6 min and 80 °C. The 1:2 ratio mixture gave the best extraction responses for all BZD.

Optimization of the MAE procedure

Temperature, extraction time and extracting solvent volume were simultaneously optimized through a uniform shell (Doehlert) design comprising 12 experiments and three central points [38]. In the experimental domain, five different temperatures (60, 75, 90, 105 and 120 °C), seven times (2, 4, 5, 7, 9, 10 and 12 min) and three solvent volumes (4, 7 and 10 mL) were tested. The design was performed randomly to avoid the influence of external conditions. For each BZD a quadratic polynomial model was considered:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j,$$

where x_i is the coded value of the factors studied (extraction temperature, time and solvent volume) and y is the response (peak area) obtained for each BZD. The b values are the estimated polynomial coefficients: b_0 is the intercept term, b_i coefficients represent the main effect for each variable, b_{ij} coefficients in the quadratic terms are responsible for the curvature effects and $b_{ij(i\neq j)}$ coefficients describe the interaction effects. Experimental design generation and all statistical analyses were carried out using the Nemrod© W

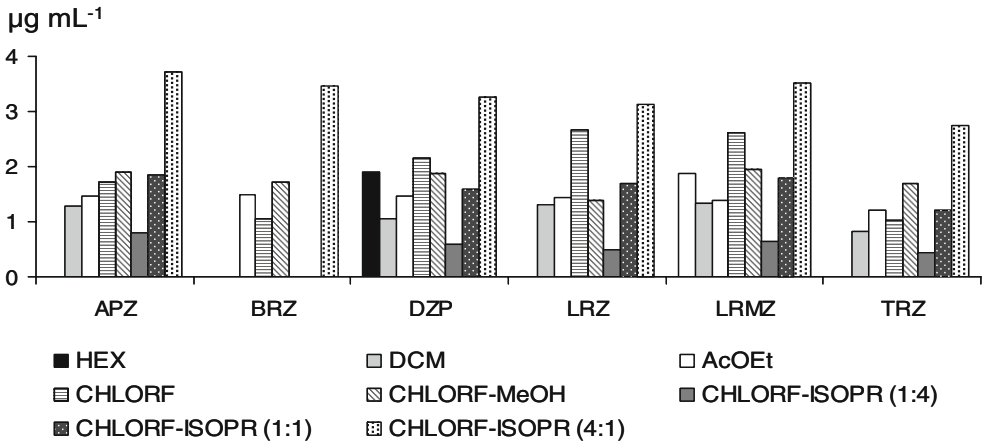

Fig. 1 Concentrations obtained with different extraction solvents tested. *APZ* alprazolam, *BRZ* bromazepam, *DZP* diazepam, *LRZ* lorazepam, *LRMZ* lormetazepam, *TRZ* tetrazepam, *HEX* *n*-hexane, *DCM* dichloromethane, *CHLORF* chloroform, *ISOPR* 2-propanol

statistical package [42]. According to the preliminary study, chloroform/2-propanol (4:1, v/v) and plasma/borate buffer (ratio 1:2) were two factors that were fixed. The MAE efficiency was evaluated through the BZD peak area obtained in each experiment. The estimates of the coefficients for the models were calculated by least-squares linear regression and these models were validated by analysis of variance (ANOVA). The constant term and the coefficients of the main effects (extraction time and solvent volume) were highly significant. The solvent volume was the most important factor, since it was statistically significant and positive for all BZD. The extraction time was also statistically significant for four BZD, with a positive effect for three of them, but with a negative effect for lorazepam. Some interactions and quadratic terms were statistically significant, presenting different effects (data not shown). Three-dimensional response surface plots show the effect of two variables on a given response, at a constant value of another variable (centre of the experimental domain). Figure 2 presents, as an example, some response surfaces developed by the model for two BZD. The best responses were obtained at a medium-high level of temperature and a high level of time for bromazepam (Fig. 2a) and alprazolam. However, lormetazepam, diazepam and tetrazepam show a different behaviour and better responses were acquired at a high level of time but a low level of temperature. For lorazepam, a higher response was obtained when temperature and time were at opposite levels. Also, better responses were achieved at a low level of time and a high level of solvent volume for all BZD. However, a dual behaviour was observed for alprazolam (Fig. 2b), lormetazepam and diazepam; in these cases, a high level of time and any solvent volume level also provided high responses. To find the optimal simultaneous conditions, multicriteria decision-making strategies using desirability function optimization were applied without additional experimentation. These desirability functions were built as partial Derringer functions for each BZD response by Nemrod© W [42]. The responses were transformed into a dimensionless desirability (d_i) scale which ranged from d=0 for a completely undesirable response to d=1 for a fully desired response. In a second step, a global desirability function (D), which represents the global quality of the common optimum, was calculated by combining single desirability functions, usually as the geometric mean. Further details can be found elsewhere [38]. The maximum D obtained was 1.0 for 89 °C, 13 min and 8 mL of chloroform/2-propanol (4:1, v/v) mixture (Fig. 3). Under these conditions, the predicted values of d_i were 1.0 for all drugs.

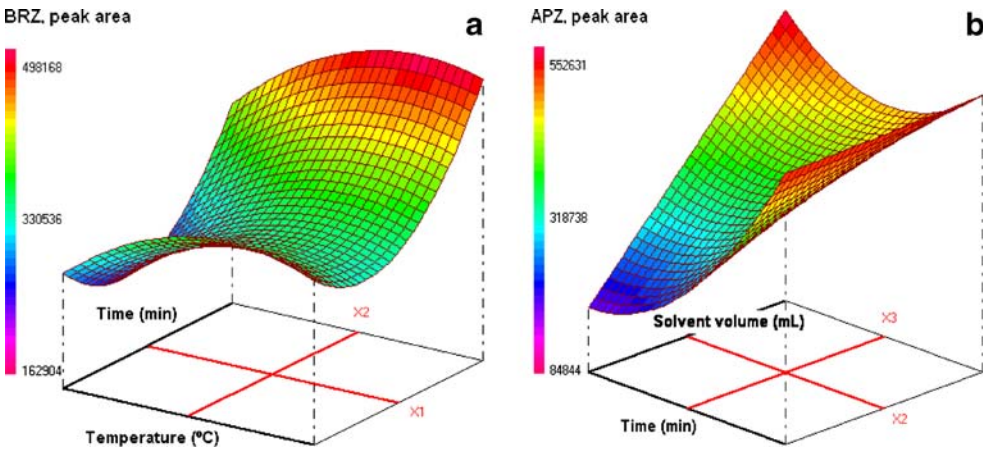

Fig. 2 Estimated response surface as a function of extraction time and temperature for BRZ (**a**) and as a function of solvent volume and time for APZ (**b**) obtained in microwave-assisted-extraction (MAE) optimization using a Doehlert design

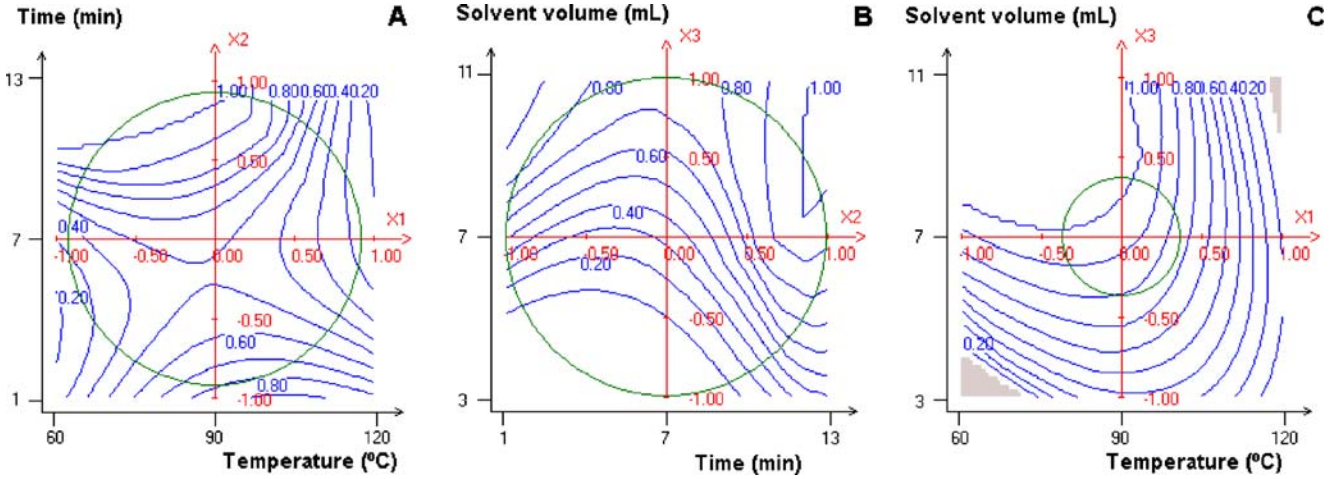

Fig. 3 Two-dimensional response surfaces of global desirability obtained in MAE optimization using a Doehlert design

Performance of the MAE-HPLC-DAD method

The proposed analytical method was validated according to the guiding principles of FDA [43] and ICH [44]. The analytes were identified from their retention times (viz. 7.66 min for bromazepam, 10.52 min for alprazolam, 13.70 min for lorazepam, 17.85 min for lormetazepam, 20.53 min for diazepam and 26.44 min for tetrazepam) and absorption spectra. Endogenous components in the biological matrix did not interfere with the analytes of interest (Fig. 4, chromatogram C) and, therefore, the proposed method was considered sufficiently selective for the determination of BZD in plasma samples.

The linearity was satisfactory for all compounds in the concentration range studied, with regression coefficients oscillating between 0.9997 and 0.9999. Table 3 shows the

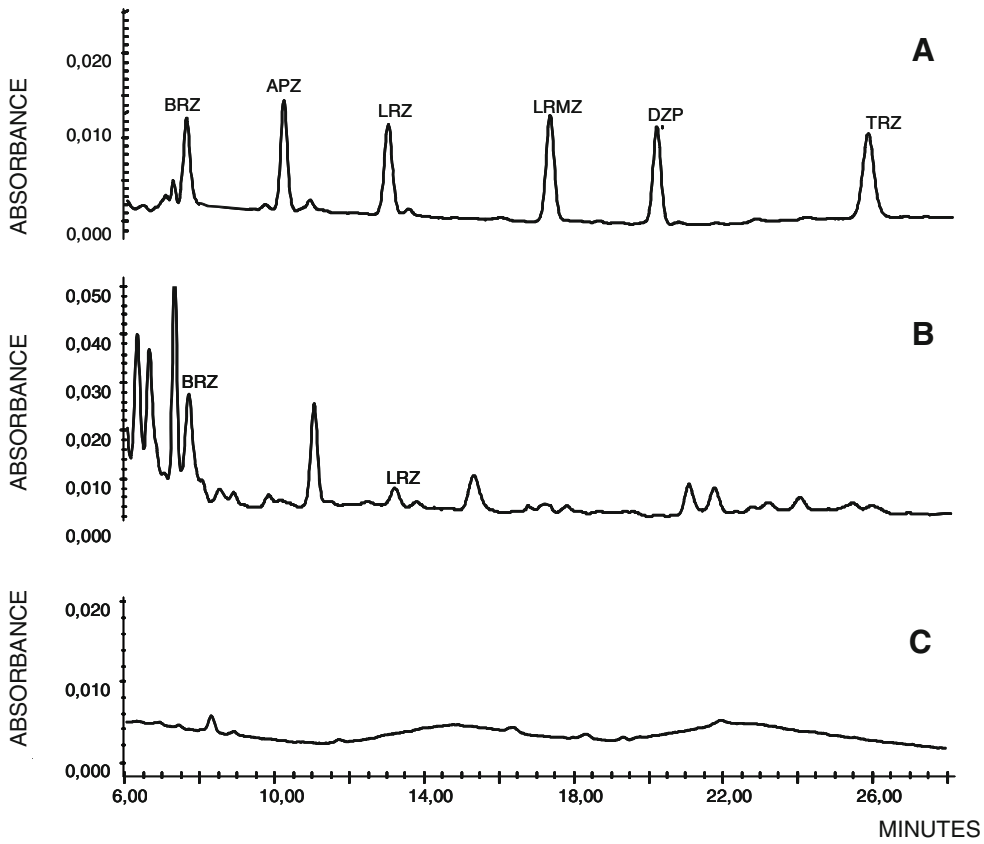

Fig. 4 Chromatograms resulting from a standard of 2 μg mL^{-1} (*a*), a real case (containing BRZ and LRZ) (*b*) and a blank plasma (*c*)

Table 3 Comparative analytical characteristics of the method for the determination of BZD in human plasma by SPE/microwave-assisted extraction (*MAE*)–HPLC-DAD

BZD	SPE				MAE			
	Slope (10^5 counts mLng^{-1})	*r* coefficient	LOD (ngmL^{-1})	LOQ (ngmL^{-1})	Slope (10^5 counts mLng^{-1})	*r* coefficient	LOD (ngmL^{-1})	LOQ (ngmL^{-1})
ALP	1.42	0.9995	8.8	28.7	1.01	0.9998	7.4	24.7
BRZ	1.10	0.9994	14.0	46.7	0.98	0.9998	10.4	34.7
DZP	1.70	0.9999	10.0	33.3	1.10	0.9999	6.2	20.7
LRZ	2.22	0.9995	12.0	40.0	1.04	0.9999	10.4	34.7
LRMZ	2.25	0.9999	7.6	25.3	1.11	0.9997	7.2	24.0
TRZ	1.47	0.9998	10.0	33.3	1.28	0.9998	12.6	42.0

analytical characteristics of the method, calculated with SPE and MAE plasma extracts analysed by HPLC-DAD. The LOD was evaluated as the lowest concentration giving a chromatographic signal-to-noise ratio of 3, and the limit of quantification (LOQ) was evaluated as the lowest concentration giving a chromatographic signal-to-noise ratio of 10 [45]. The LODs achieved ranged from 6.2 to 12.6 ng mL^{-1} and the LOQs obtained ranged from 20.7 to 42.0 ng mL^{-1}. Table 4 shows the recovery and precision (intraday and interday) for all BZD. The intraday precision ranged from 0.3 to 4.3% RSD and the interday precision ranged from 0.3 to 6.0% RSD. The mean recoveries ranged between 89.8±0.3 and 102.1±5.2%.

Comparison between MAE and SPE

To test the statistical significance of SPE or MAE in calibration curves, ANOVA was applied to the calibration slopes obtained with both extraction procedures using the Statgraphics Centurion XV statistical package (Manugistics, Rockville, MD, USA). The results of the test showed that the variation in the calibration slopes of MAE or SPE was significant at the 95% confidence interval (F=10.39 and p=0.0234). Thus, SPE has a slightly higher sensitivity of calibration than MAE and only bromazepam and tetrazepam show similar calibration slope values for both extraction procedures. The data in Tables 3 and 4 show the relative

Table 4 Recovery and precision for analysis of BZD in fortified human plasma for SPE and MAE procedures

BZD	Parameters	SPE (μgmL^{-1}, n=5)			MAE (μgmL^{-1}, n=5)		
		0.06	0.4	2	0.06	0.4	2
APZ	Recovery (%)	83.3±2.8	87.9±5.9	93.6±0.8	102.1±5.2	98.7±3.4	97.3±0.8
	Intraday RSD (%)	2.9	3.5	2.2	2.7	2.4	0.6
	Interday RSD (%)	3.4	6.8	0.9	5.1	3.5	0.8
BRZ	Recovery (%)	65.0±1.3	81.0±3.7	89.0±3.5	100.8±6.0	99.7±3.8	98.2±1.7
	Intraday RSD (%)	1.1	2.4	1.2	3.8	1.3	0.5
	Interday RSD (%)	2.0	4.6	0.4	6.0	3.9	1.8
DZP	Recovery (%)	99.7±4.7	90.1±5.2	94.3±0.7	100.0±1.8	94.3±2.0	97.3±0.3
	Intraday RSD (%)	4.8	5.5	3.3	3.9	2.1	1.0
	Interday RSD (%)	4.8	5.8	0.8	1.8	2.1	0.4
LRZ	Recovery (%)	76.6±3.0	84.9±5.2	87.2±1.4	98.0±5.0	97.3±3.4	95.8±0.4
	Intraday RSD (%)	1.6	5.3	2.6	1.8	3.3	0.3
	Interday RSD (%)	4.0	6.1	1.6	5.2	3.5	0.4
LRMZ	Recovery (%)	99.7±3.7	93.4±6.0	94.3±0.3	98.4±5.3	100.5±4.2	98.4±0.3
	Intraday RSD (%)	5.0	5.1	2.1	1.4	2.9	0.7
	Interday RSD (%)	3.8	6.5	0.8	5.4	4.2	0.3
TRZ	Recovery (%)	76.9±3.5	82.1±4.1	89.3±0.8	99.0±2.7	90.5±3.7	89.8±0.3
	Intraday RSD (%)	4.9	3.3	2.1	2.3	4.3	1.3
	Interday RSD (%)	4.6	5.0	0.9	2.8	4.1	0.3

RSD relative standard deviation

effectiveness of both extraction procedures applied. All BZD except tetrazepam had lower LODs and LOQs for the MAE process. The precision was lower than 7% in all cases, but MAE slightly improved the RSD for the intraday and interday precision.

Two-way ANOVA was used to evaluate differences between recovery experiments (n=5) of three factors: A, extraction procedures (MAE and SPE); B, six BZD analysed; C, three concentrations (0.06, 0.4 and 2 μg mL^{-1}). The significant differences of recovery means were compared by least-significant-difference methods at a confidence level of 95%. No significant differences were found (p>0.05) between the BZD analysed (p=0.5622), the concentrations (p = 0.7059) and their interactions. No difference was observed between the recoveries obtained when one or another extraction procedure was applied to the BZD studied: interaction AB (p = 0.5884). There was no significant difference between the recoveries obtained when one or another extraction procedure was applied at different concentrations: interaction AC (p=0.5043). Finally, the extraction of BZD at different concentrations did not present any difference between the recoveries obtained: interaction BC (p=0.9968). However, significant differences were found (p<0.05) between the SPE and MAE recoveries (F=7.01 and p=0.0111). The multiple range tests confirmed these results and showed that the recovery means obtained with SPE form a homogeneous group, significantly different from the MAE recovery means group, because the recoveries obtained with MAE were higher than those obtained when classic SPE was applied. Also, SPE gave higher data dispersion (from 65 to 103.7%) than MAE (from 89.8 to 105.3%). According to the results of the ANOVA, the multiple range tests showed that the recovery means obtained at the three concentrations studied form a homogeneous group of means. Similarly the recovery means obtained for all BZD also form a homogenous group of means.

Occurrence of BZD in human plasma samples

The optimized MAE and the established SPE processes were applied to ten plasma samples from BZD users and then the samples were measured by HPLC-DAD.

The paired $t$test was applied to compare the mean values from the two sample preparation procedures. The test is based on the paired differences between these two measurement values. The usual null hypothesis (H_0) is that the difference in the mean values is zero [46]. The t statistic is 2.239 for the 95% confidence interval and the p=0.0610. So H_0 is not rejected at α=0.05 and statistical analysis confirmed that MAE and SPE do not provide significantly different results for the extraction of BZD. On the basis of this result, the optimized MAE-HPLC-DAD method is valid for routine toxicological analysis of plasma samples and it was applied to 17 plasma samples from patients undergoing therapy with BZD. In three of the 27 cases processed using MAE, two different BZD were found (bromazepam and lorazepam, diazepam and lorazepam, and tetrazepam and lorazepam). Moreover, BZD found in 12 cases shown toxic levels, whereas 18 cases showed therapeutic levels. The presence of lorazepam was demonstrated in 11 cases (range 41.6–783.8 ng mL^{-1}; mean level 224.9 ng mL^{-1}), followed by bromazepam in nine cases (range 61.6–414.4 ng mL^{-1}; mean level 201 ng mL^{-1}), alprazolam in seven cases (range 48.8–222 ng mL^{-1}; mean level 118.5 ng mL^{-1}), tetrazepam in two cases (range 73.8–457.6 ng mL^{-1}; mean level 265.7 ng mL^{-1}) and diazepam in one case (level 241.2 ng mL^{-1}). Figure 4 shows the chromatograms of a standard solution containing the six BZD at 2 μg mL^{-1}, a real case (containing bromazepan and lorazepam) and a blank plasma.

Conclusions

MAE of human plasma samples is a powerful, fast and high-throughput extraction method. Regarding the total time of analysis, which nowadays is becoming one of the most important factors, MAE is faster than SPE, because 12 simultaneous extractions can be performed in a microwave oven. A simple, rapid and validated MAE-HPLC-DAD method was optimized for sensitive and appropriate determination of BZD in human plasma at therapeutic and toxic levels. The suitability of the method for the routine measurement of BZD in a toxicology laboratory was demonstrated. Furthermore, it can be assumed that the MAE method developed could be applicable to other BZD too.

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