

DEVELOPMENT AND VALIDATION OF THE GC METHOD FOR THE QUANTITATIVE DETERMINATION OF SEMI-VOLATILE SOLVENTS IN THE PHARMACEUTICAL SUBSTANCE BOSENTAN

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Keywords: Bosentan (BO), dimethyl sulfoxide (DMSO), ethylene glycol (EG), acetic acid (AA), GC method, validation

Bosentan is an active substance in the orphan drugs used to treat pulmonary artery hypertension (PAH). It is a dual endothelin receptor antagonist (ERA) with the affinity for both receptors of the A and B: endothelin-A (ET_A) and endothelin-B (ET_B). Under normal conditions, ET_A or ET_B receptors cause constrictions of the pulmonary blood vessels. By blocking this interaction, bosentan decreases pulmonary vascular resistance (1).

During the synthesis of bosentan the following solvents were used: ethanol, methanol, acetone, toluene, ethylene glycol, acetic acid, DMSO. Benzene is a potential contaminant of acetone. The acetic acid and ethylene glycol were used in the last stage of the bosentan synthesis. The DMSO was used prior to the last step in the synthesis. According to the International Conference on Harmonization (ICH) guidelines (2), all these residual solvents should be controlled to ensure the safety and quality of the finished product. Ethanol, acetone, methanol, toluene and benzene are determined by static headspace gas chromatography (HS-GC) methods. Volatile organic components (VOCs) are often determined by static headspace gas chromatography. This technique is very robust and combines easy sample preparation with good selectivity and low detection limits (high sensitivity). The HS technique is characterized by low sample matrix interference. The European Pharmacopoeia (Ph. Eur.) (3), describing a general HS procedure for Identification and Control of Residual Solvents in a drug substance, classifies solvents into three classes on the basis of their toxicity level and the degree to

which they can be considered an environmental hazard (4). The AA and DMSO fall into Class 3 with the permissible daily exposure (PDE) of 50 mg/day (2). The EG is classified into Class 2 with the permissible daily exposure (PDE) of 6.2 mg/day (2). According to the Guideline Q3C (R5) 11 (2) the acceptable limits – maximum allowable limits in respect to sample preparation – are as follows: 620 ppm for the ethylene glycol, 5000 ppm for the acetic acid and 5000 ppm for the DMSO.

Headspace technique is not suitable for less volatile solvents because they cannot reach the injector and the column. As a consequence, the determination of high boiling/semi-volatile solvents becomes challenging. Therefore, the described method for the determination of high boiling VOCs like the acetic acid (AA), dimethyl sulfoxide (DMSO) and ethylene glycol (EG) presents an interesting example of dealing with high boiling/semi-volatile solvents.

In the literature there is a lack of descriptions of the methods for the simultaneous determination of the AA, DMSO and EG in bosentan and other substances. However, AA, DMSO and EG were determined separately, with other analytes and in different matrices (5–7). If a sample is not liquid, first, it has to be dissolved in a suitable medium to obtain a clear, homogeneous solution. This implies that residual VOCs have to be determined in the simultaneous presence of the sample matrix and dilution medium.

The development and validation of the gas chromatographic method with direct injection for

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the quantitative determination of residual AA, DMSO and EG in the pharmaceutical active substance bosentan has been described in this work.

EXPERIMENTAL

Chemicals

Chemicals of an analytical grade were used for the validation. The active substance – bosentan – was synthesised in PRI (Pharmaceutical Research Institute, Warsaw, Poland). The 99.7% AA and 99.7% DMSO were provided by Sigma-Aldrich (Steinheim, Germany), the 99.5% EG and acetonitrile were provided by POCH (Gliwice, Poland).

Method optimization

In order to analyze the high boiling VOCs in a short time, so that the method could be implemented as a routine method of analysis, a further optimization was needed. The mixture of three high boiling point (b.p.) VOCs, namely AA (b.p. 118°C), DMSO (b.p. 189°C) and EG (b.p. 198°C), was used as the standard solution in order to generate data. A higher initial temperature and a faster ramp were selected. Thus, the analysis time of the optimized temperature program was reduced to 30 min. Working standard solutions were prepared based on different sample sizes to investigate the effect of the sample size. Different split ratios were also studied. The injector port temperature was considered to be a very important parameter. In order to investigate whether the bosentan decomposition was the reason of an unknown impurity at the retention time of the ethylene glycol, the analyses under different injector temperatures

were performed. The results of these investigations are presented in Figure 1. Stable injections have been achieved as the injector temperature was changed from 220 to 160°C. If the injector temperature is too low, the AA, DMSO and EG in the sample matrix may not vaporize completely before they are transferred into the column. Therefore, lower injector temperatures were not investigated. Complete GC parameters can be found in *Method description*.

Method description

Chromatographic conditions

Chromatographic separations were performed on a DB-WAX column (poly(ethylene glycol) film thickness 0.5 μm , 60 m long, 0.32 mm ID). The experiments were performed on a Shimadzu GC-2010 gas chromatograph (GC) equipped with a Shimadzu AOC-20i autosampler and a flame ionization detector (FID).

GC parameters: inlet heater 160°C, detector 260°C, oven initial temperature 60°C, raised at the rate of 5°C/min to 185°C, then raised at the rate of 30°C/min to 240°C, 3 min at the final temperature. Nitrogen was used as the carrier gas at 100 kPa (constant flow, approximately 3.48 mL/min) and the split flow of 10 mL/min. The air flow rate of 400 mL/min, the hydrogen flow rate of 40 mL/min were used for FID and 1 μL was used for injection.

Preparation of standard and test solutions

All solutions were prepared directly before the analysis.

The acetonitrile (ACN) was used for the standard and test solution preparation as the diluent.

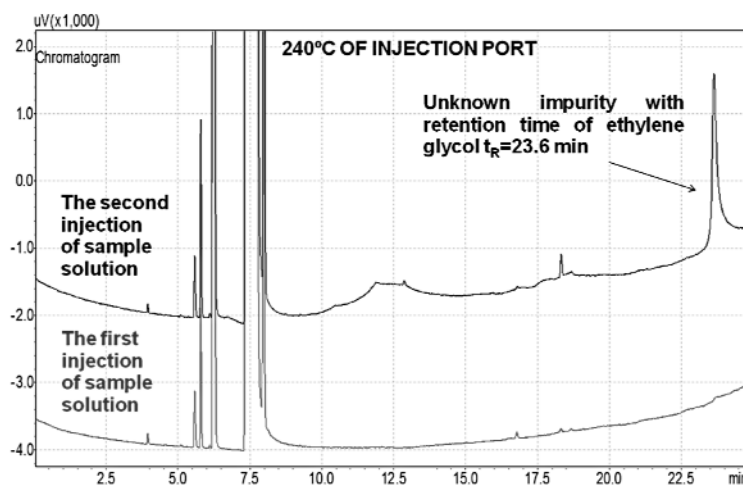


Figure 1. Overlain chromatograms of the first and second injection of the sample solution in temperature 240°C of the inlet heater

Standard solutions were prepared from the standard stock solutions by diluting an appropriate volume in the diluent to reach 100% of the specification limit concentrations – 620 µg/mL of EG, 5000 µg/mL of AA and 5000 µg/mL of DMSO (the SST solution) and to reach 10% of the specification limit concentrations – 62 µg/mL of EG, 500 µg/mL of the AA and 500 µg/mL of DMSO.

Test solutions were prepared by dissolving approximately 100 mg of bosentan in 1.0 mL of diluent.

Additional validation solutions were prepared as follows:

Specificity solution

The specificity solution was prepared by dissolving the appropriate amounts of all solvents from the synthesis route and a potential contaminant in the ACN to reach 100% of the specification limit concentrations: (ethanol (5000 µg/mL), methanol (3000 µg/mL), acetone (5000 µg/mL), toluene (890 µg/mL), EG (620 µg/mL), AA (5000 µg/mL), DMSO (5000 µg/mL), benzene (20 µg/mL).

Reference solutions

Spiked test solutions (Solution I, Solution II, Solution III, Solution IV) were prepared by dissolving 100 mg of bosentan in 1.0 mL of the corresponding standard solutions. Solution I contains 50 µg/mL of AA, 50 µg/mL of DMSO and 6.2 µg/mL of EG, that corresponds to 500 µg/mL of AA, 500 µg/mL of DMSO and 62 µg/mL of EG in the tested substance. Solution II contains 250 µg/mL of AA, 250 µg/mL of DMSO and 31 µg/mL of EG, that corresponds to 2500 µg/mL of AA, 2500 µg/mL of DMSO and 310 µg/mL of EG in the tested substance. Solution III contains 500 µg/mL of AA, 500 µg/mL of DMSO and 62 µg/mL of EG, that corresponds to 5000 µg/mL of AA, 5000 µg/mL of DMSO and 620 µg/mL of EG in the tested substance. Solution IV contains 74.4 µg/mL of AA, 60 µg/mL of DMSO and 6.2 µg/mL of EG, that corresponds to 6000 µg/mL of AA acid, 6000 µg/mL of DMSO and 744 µg/mL of EG in the tested substance.

Chromatographic procedure

For the GC analysis, a portion of each solution was transferred into a crimp-top vial with a fixed insert. The vial was closed with a PTFE/rubber crimp cap. One microliter of the blank (ACN), 1.0 µL of the test solution and six 1.0 µL replicate injections of the standard solution (SST solution) were chromatographed separately. Under the described conditions, the retention time is about 18.1 min,

about 22.7 min and about 23.1 min for AA, DMSO and EG, respectively. The resolution of not less than 1.5 between the determined solvents was set as the system suitability requirement for the system suitability solution. To verify the system precision, six replicate injections of the system suitability solution were injected and the relative standard deviation (RSD) for the area peak of the residual solvents from six injections was calculated. The RSD of not more than 10.0% was set as the system precision acceptance criterium for the determined solvents peak areas. The acetic acid, DMSO and ethylene glycol in the test sample were determined against the mean areas of respective solvents obtained from the replicate injections of the system suitability solution.

Evaluation

In order to establish the validation parameters, the peak area (x), mean peak area (\bar{x}), relative standard deviation (RSD), and confidence interval $\bar{x} \pm \Delta x$ were evaluated.

The recovery was calculated using the following formula (1):

$$\text{Recovery} = \frac{W_{\text{std}} \cdot A_{\text{sol}}}{A_{\text{std}} \cdot W_{\text{sol}}} \times 100\% \quad (1)$$

where: W_{std} – weight of the analytes in mg in 1 mL of the standard solution; W_{sol} – weight of the analytes in mg in 1 mL of the solution I, II, III or IV; A_{std} – peak area of the analytes in the chromatogram of the standard solution; A_{sol} – peak area of the analytes in the chromatogram of the solution I, II, III or IV.

For intermediate precision, the Snedecor F-test was performed using the following formula (2):

$$F = \frac{SD_1^2}{SD_2^2} \quad SD_1 > SD_2 \quad (2)$$

where: SD_1 – standard deviation from the results obtained by the first analyst. SD_2 – standard deviation from the results obtained by the second analyst.

RESULTS AND DISCUSSION

The validation procedure was performed based on the ICH requirement (8). During the validation, the specificity, precision, linearity, accuracy, limits of detection (LODs) and quantitation (LOQs) and robustness were investigated.

Specificity

The specificity of the method was evaluated by injecting the following samples: the blank (ACN –

diluent), test solution, reference solution, specificity solution, solvent solutions (ethanol solution, methanol solution, acetone solution, toluene solution, EG solution, AA solution, DMSO solution, benzene solution).

The peak area of the analytes in the chromatogram of the reference solution (spiked test solution) was greater than the corresponding peak area in the chromatogram of the test solution. The reten-

tion times of the analytes in the chromatogram of the reference solution correspond with the retention times of the analytes in the chromatogram of the solvent injected separately. Spiking the sample with the analyte did not cause the peak to split. In the chromatogram of the blank there were no peaks with the retention time of the analytes. All peaks in the chromatogram of the specificity solution are separated, $R_s \geq 1.5$ between the determined solvents (Table 1).

Table 1. Specificity results of the acetic acid, DMSO and ethylene glycol determination.

Compound name	Specificity solution		Solvent solutions injected separately
	Retention time [min]	R_s	Retention time [min]
Acetone	4.97	-	4.98
Methanol	5.62	5.21	5.62
Ethanol	6.03	3.70	6.03
Benzene	6.22	1.39	6.21
ACN	7.23	5.51	7.27
Imp. of ACN	7.60	2.43	7.62
Toluene	7.82	2.11	7.83
Acetic acid	18.13	93.09	18.01
DMSO	22.74	46.19	22.73
Ethylene glycol	23.14	3.84	23.14

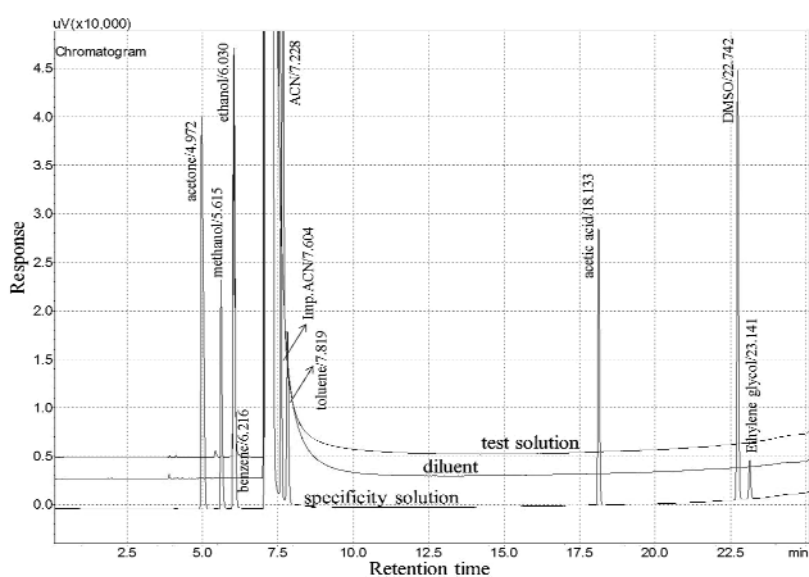


Figure 2. Overlain chromatograms of the diluent, test solution and specificity solution

Table 2. Validation results of the acetic acid, DMSO and ethylene glycol determination.

Parameter		Acetic acid	DMSO	Ethylene glycol	
System precision	6 × 10% of spec. limit, RSD%	peak area	1.13	0.57	1.50
		t _R	0.01	0.07	0.08
	6 × 100% of spec. limit, RSD%	peak area	0.49	0.50	0.72
		t _R	0.006	0.009	0.007
Method precision – repeatability	3 × 10%, 50%, 100%, 120% of spec. limit, RSD%	6.39	6.38	6.54	
	6 × 100% of spec. limit, RSD%	6.29	8.08	4.87	
Method precision – intermediate precision	6 × 100% of spec. limit, RSD%	2.42	3.44	4.79	
	Snedecor's F-test ($F_{5,5,exp.}$)	3.17	2.01	1.63	
Accuracy	3 × 10%, 50%, 100%, 120% of spec. limit	$\bar{x} \pm \Delta x$	91.6 ± 3.7%	97.0 ± 3.9%	99.4 ± 4.1%
		RSD%	6.39	6.37	6.54

Table 3. The characteristic of regression lines ($y = ax + b$).

Parameter	Acetic acid	DMSO	Ethylene glycol
R ²	0.9994	0.9994	0.9991
SD _{xy}	1061.23	1716.30	190.91
y-intercept, b	-1612.13	-841.45	-183.62
SD _b	717.25	1159.98	129.03
t _{b,exp.}	2.25	0.73	1.42
Slope, a	216.97	351.50	252.55
SD _a	1.86	2.99	2.63
t _{a,exp.}	116.89	117.60	96.08

Figure 2 presents the comparison of the chromatograms obtained from the blank, test solution and specificity solution, respectively.

Precision

The precision of the method was established as the repeatability, system and intermediate precision. The repeatability was established by measuring triplicate independent preparations of the solutions I–IV with the analytes at 10, 50, 100 and 120% of the specification limit, 6 independent preparations of the solution III with the analytes at about 100% of the specification limit – prepared according to the solution preparation presented in *Additional validation solutions*. The system precision was established by measuring the response of six replicate injections

of the standard solution – the solution with the AA, DMSO and EG at 100% of the specification limit – and six replicate injections of the standard solution – the solution with AA, DMSO and EG at 10% of the specification limit. The intermediate precision was established by performing a repeatability test on a different day and by a different analyst. The intermediate precision was determined by comparing the results obtained by both analysts using Snedecor's F-test.

The acceptance criteria were set up as the RSD value below 15, 10 and 15%, respectively. An additional criterium based on Snedecor's F-test was set up as: if $F_{\text{experimental}} \leq F_{\text{critical}}$ (for $\alpha = 0.05$, $f_1 = n_1 - 1$, $f_2 = n_2 - 1$), then, the difference between the results obtained by both analysts is insignificant. The criti-

cal parameter F ($\alpha = 0.05$; $f_1, f_2 = 5$) is 5.05. The results of the method precision are presented in Table 2. It has been pointed out that all criteria were fulfilled and the method is precise.

Accuracy

The accuracy was assessed on the samples spiked with known amounts of the analytes (sample spiked with the analytes at 10, 50, 100 and, 120% of the specification limit). The accuracy of the method was established by assaying 12 sample solutions (triplicate independent preparations of solutions I–IV), blank (ACN) and test solution against the standard solution (three replicate injections). Then, the recovery results were calculated. The acceptance criteria were set up as the RSD value below 15% and the recovery value $100\% \pm 20\%$. The recovery results are presented in Table 2. All set up criteria were fulfilled. The method is accurate.

Limits of detection and quantitation

The sensitivity of the method was demonstrated by low LOD values obtained for the analytes. The limit of detection (LOD), calculated as the concentration which generated a peak about 3 times as high as the noise's height, and the limit of quantitation (LOQ) calculated as the concentration which generated a peak about 10 times as high as the noise's height, were found as 14 and 46 $\mu\text{g/mL}$ for AA, 6.3 and 20 $\mu\text{g/mL}$ for DMSO and 9.2 and 29.2 $\mu\text{g/mL}$ for the EG, respectively.

Linearity

The linearity of the method was evaluated by analyzing 3 replicates of the standard solutions at ten concentration levels in the range between 10 to 120% of the specification limit. The AA concentrations ranged from 50.22 to 602.64 $\mu\text{g/mL}$, which corresponds with approximately 50.2–6026.4 $\mu\text{g/mL}$ in the tested substance. The DMSO concentrations ranged from 50.44 to 605.28 $\mu\text{g/mL}$, which corresponds with approximately 504.4–6052.8 $\mu\text{g/mL}$ in the tested substance and for EG they ranged between 6.38–76.56 $\mu\text{g/mL}$, which corresponds to approximately 63.8–765.6 $\mu\text{g/mL}$ in the tested substance. The results of the statistical evaluation of the linearity experiments (correlation coefficient, y -intercept (b), slope of regression line (a), residual standard deviation (SD_{xy}), standard deviation of b (SD_b), standard deviation of a (SD_a)) are all summarized in Table 3. The obtained correlation coefficients (R^2) of the linear regression for the determined solvents were above 0.999. This indicates a linear relationship between the analyte con-

centrations and the detector response. The critical parameter t (95%, 8) is 2.306. Our results show that the parameters a and b are statistically important, the method is characterized by a very good precision and is free from systematic errors.

Robustness

In order to evaluate the robustness of the method, the influence of the variations of such method parameters as the pressure, temperature and rate were investigated to ensure the separation of the solvents with the use of different chromatographic conditions. System suitability (SST) requirements were checked for the variations of ± 10 kPa on the carrier gas flow, $\pm 5^\circ\text{C}$ on the initial oven temperature, $\pm 1^\circ\text{C/min}$ on the rate. Under all the deliberately changed chromatographic conditions, all system suitability criteria were within the limits (the resolutions (R_s) between two analyte peak > 1.5 , and RSD $< 10.0\%$). The obtained results indicate that the studied variations of the GC conditions do not cause any significant changes in the resolutions and the method is robust.

CONCLUSIONS

A direct injection GC is the preferred method to determine AA, DMSO and EG. A preliminary direct injection GC method was developed using a DB-WAX column (stationary phase: poly(ethylene glycol); 60 m \times 0.32 mm i.d., 0.5 μm film thickness) at 240°C of the inlet heater. During the method, evaluation matrix effects were observed, resulting in an unknown impurity – a decomposition peak with the retention time of EG. Reports on drug matrix effects in the determination of residual solvents are rare. Kersten (9) reported a study on matrix effects in the GC determination of residual solvents in a drug substance (acidic, basic and neutral). It was claimed that no matrix effects were observed. In this paper, attempts were made to overcome the matrix effects and it was found that they can be eliminated by changing the inlet heater temperature. Bosentan is a polar compound that contains hydroxy group. The melting point of bosentan is 110°C and it is therefore possible that a higher injector temperature results in the decomposition of bosentan, which represents itself as a peak with the retention time of EG. The method described above represents an interesting tool for the analysis of high boiling VOCs, namely A, DMSO and EG in matrices which are unstable at higher temperatures. Its considerable advantage is that the specificity enormously increased for the investigated solvents after reducing

the injector temperature. This is the first method ever reported in the literature, which concerns the separation and quantitation of AA, DMSO and EG.

In this study, the validation of a new GC method for the simultaneous control of residual AA, DMSO and EG in bosentan was performed. During the validation procedure, which was carried out according to the ICH guidelines Q2R1, the specificity, precision, accuracy, limits of detection and quantitation and robustness were evaluated. All set up criteria were fulfilled. The method is specific, accurate, linear and shows a satisfactory level of precision. The determined solvents can be detected and quantified at $\mu\text{g/mL}$ level. As a consequence, these solvents can be quantified in substance bosentan far below their respective official limit concentrations. The validation procedure shows that the method is suitable for its intended purpose.

Acknowledgments

The study was supported by the European Union under the European Regional Development Fund No. UDA-POiG.01.03.01-14-062/09-00 "Innovative technologies of cardio-vascular medicines of special therapeutic and social importance". We wish to thank Ms. Maria Puchalska, Ph.D., Ms. Joanna Zagrodzka, Ph.D. and Ms. Katarzyna Sidoryk, Ph.D. from PRI for their support in this study.

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Project co-financed by the European Regional Development Fund under the framework of the Innovative Economy Operational Programme.

UDA-POiG contract no 01.03.01-14-062/09 „Innovative technologies of cardio-vascular medicines of special therapeutic and social importance”: www.ifarm.eu/poig/cardio