Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 79 (10) 1279–1293 (2014)
JSCS–4664
UDC 615.2clopidogrel+546.226+
541.8–039.7+543.544.3+519.248
Original scientific paper

Application of gas chromatography analysis to quality control of residual organic solvents in clopidogrel bisulfate

ALEKSANDAR D. PAVLOVIĆ[1], LJUBIŠA M. IGNJATOVIĆ[1*#], SAŠA Z. POPOV[1], ALEKSANDAR R. MLADENOVIĆ[2] and IGOR N. STANKOVIĆ[3]

[1]*University of Belgrade, Faculty of Physical Chemistry, Studentski trg 12–16, Belgrade, Serbia,* [2]*University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, Serbia and* [3]*University of Novi Sad, Faculty of Sciences, Trg Dositeja Obradovića 3, Novi Sad, Serbia*

(Received 20 November 2013, revised 4 February, accepted 23 February 2014)

Abstract: A direct-injection, split-mode capillary gas chromatographic procedure with flame ionization detection was developed for the analysis of eight solvents used in the synthesis and purification of the anti-thrombotic drug clopidogrel bisulfate. The solvents analyzed were methanol, acetone, dichloromethane (DCM), 2-butanol, cyclohexane, toluene, acetic acid and *N*,*N*-dimethylformamide (DMF). In addition, because of dehydration of 2-butanol during the drying process, significant amounts of 2-butanol dehydration products (1-butene, *cis*- and *trans*-isomers of 2-butene, 2,2′-oxybis[butane] and 1-(1--methylpropoxy)butane) may be detected in clopidogrel bisulfate samples. The content of each of these volatile products can be evaluated using the same gas-chromatographic method, with quantification based on the response factor established for the chromatographic peak of 2-butanol. Based on a large number of result sets, retrospectively, from many different batches analyzed, conclusions were made about process variations and reliability and a lack of consistency was identified in the quality of the active substance from a particular producer source. Multivariate analysis was used as the statistical technique to classify the samples. From the analyzed set of 11 solvents, 6 of them were preselected based upon their occurrence in the samples and both Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed.

Keywords: volatile impurities; validation; chemometrics; multivariate analysis; GC.

* Corresponding author. E-mail: ljignjatovic@ffh.bg.ac.rs
# Serbian Chemical Society member.
doi: 10.2298/JSC131120013P

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INTRODUCTION

Clopidogrel bisulfate (structural formula is shown in Fig. 1) is a potent antithrombotic drug used for the prevention of vascular thrombosis in patients with coronary artery disease, peripheral vascular disease, and cerebrovascular disease.[1–4]

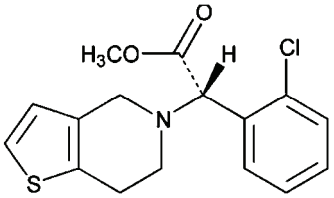

Fig.1. The structural formula of clopidogrel bisulfate, methyl (2*S*)-2-(2-chlorophenyl)-2-(6,7-dihydro-4*H*-thieno[3,2-*c*]pyridin-5-yl)-acetate hydrogen sulfate.

Clopidogrel is a dihydro thieno pyridine derivative pro-drug which is inactive *in vitro* and is only active after intravenous or oral administration.[5] *In vivo* studies have demonstrated that for its activation, clopidogrel has to undergo CYP2C19 metabolization to obtain an intermediate metabolite.[6] This intermediate metabolite is hydrolyzed and produces the active form.[7]

Regardless of the efficiency drying techniques, it is impossible to remove completely organic solvents routinely used in the synthesis and purification of active pharmaceutical ingredients. The solvents remaining in pharmaceutical products are designated as “residual solvents” or “volatile organic impurities”.[8] The residual organic solvents have no therapeutic function, can be toxic and may also accelerate the degradation of the active substance and thereby threaten the stability of the drug. Moreover, they are not desirable in the final product because of their odor or taste, which could be unpleasant for patients. Testing of drug substances, excipients, and drug products for residual solvents should be performed when production or purification processes are known to result in the presence of such residual solvents. Compendial methods of testing for the content of residual solvent are described in USP-NF general chapter.[9] However, as it is only necessary to test for residual solvents that are used or produced in the manufacture or purification of drug substances, the use of other alternative methods is encouraged.[10,11] Gas chromatography (GC) was the natural method of choice for residual solvent analysis. It is a relatively old analytical technique, well documented in the literature, but still irreplaceable in this issue.[12–18] Modern capillary-column GC can separate a large number of volatile components, permitting identification through retention characteristics and detection at ppm levels using a broad range of detectors.[19] However, flame ionization detection (FID) is by far the most preferred because of its universality, low detection limits, robustness, ease of operation, and general accessibility and reliability.[20,21] Residual solvent determination using direct-injection sample preparation is the oldest technique, and it was preferred because of its simplicity, reliability and ease of operation.[22–

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[25] The drug substance is dissolved in or extracted with a high-boiling-point solvent, such as water, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide (DMA) and benzyl alcohol, and then directly injected. Using high-boiling-point solvent has the advantage that the diluent solvent peak elutes later, thus not interfering with the earlier eluting analyte peaks. The aim of this study was to set up a method for the determination of residual solvents in clopidogrel bisulfate that uses the simplest GC instrumentation that is available to almost every laboratory.

Chemometric methods were used for the classification and comparison of several different samples of clopidogrel bisulfate according to the profiles of the residual solvents obtained by using this GC method of analysis. The application of chemometry to monitoring data enables these data to be compared with data for older samples in order to obtain a complete overview of the quality and reliance of a particular clopidogrel bisulfate source. The applications of chemometric pattern recognition techniques (principal component analysis – PCA and hierarchical cluster analysis – HCA) were used to reduce the complexity of the large data sets and to achieve a better interpretation and understanding of the quality of the samples.[26–28]

Taking the above-mentioned consideration into account, the aim of this study was to develop and validate a simple analytical method that allows the determination of residual solvents in clopidogrel bisulfate and to obtain a complete overview of the quality and reliance of a particular source of clopidogrel bisulfate.

EXPERIMENTAL

Chemicals and reagents

Analytical grade solvents were obtained from the following suppliers: 2-butanol, cyclohexane, DMA and acetic acid were purchased from Merck (Darmstadt, Germany); methanol, acetone, DMF were purchased from Sigma–Aldrich (Steinheim, Germany), and DCM from J. T. Baker (Deventer, The Netherlands). The samples of clopidogrel bisulfate, under investigation were kindly provided by Hemofarm (Vršac, Serbia). All the solvents and reagents were commercial products, suitable for GC analysis and more than 99 % pure, were used without further purification. Nitrogen, hydrogen and hydrocarbon-free synthetic air were of 6.0 purity purchased from Messer Tehnogas (Belgrade, Serbia).

Equipment

All experiments were performed on an Agilent Technologies 6850 series gas chromatograph (Santa Clara, CA, USA), which was equipped with a standard oven for temperature ramping, split/splitless injection ports, 6850 series automatic liquid sampler and flame ionization detector (FID). An analytical balance CPA 225D from Sartorius, (Göttingen, Germany) was used for weight measurements. Variable micropipettes (20–200 μL and 10–100 μL from Carl Roth (Karlsruhe, Germany) and 0.5–10 μL from Biohit (Helsinki, Finland) were used.

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Chromatography conditions

Chromatographic separation was performed using a low to medium polarity, megabore capillary column DB-624, Agilent Technologies, with a stationary phase composition: 6 % cyanopropylphenyl/94 % dimethylpolysiloxane, with an internal diameter of 0.53 mm, film thickness of 3.0 μm and length of 30.0 m. The initial oven temperature of 40 °C was maintained for 10 min, then raised at a rate of 6 °C min^{-1} to 130 °C and maintained for 5 min, increased at a rate of 35 °C min^{-1} to reach a final temperature of 260 °C and maintained for 16 min.

The temperature of the injection port was maintained at 220 °C. The samples were injected by the direct injection method in the split mode at a split ratio of 1:5, a split flow rate of 20.2 cm^3 min^{-1}, and a total flow rate of 26.7 cm^3 min^{-1}. The injection volume was 1 μL, injected in GC injection port automatically by the Agilent 6890 series auto sampler. Nitrogen was used as the carrier gas at a constant flow rate of 4.0 cm^3 min^{-1} with the pressure maintained at 19.3 kPa. The average velocity of the gas through the column was 30 cm s^{-1} at 45 °C. The FID temperature was 250 °C, and the FID flow rate was 30 cm^3 min^{-1} for hydrogen, 400 cm^3 min^{-1} for air. Nitrogen was used as the makeup gas at a constant flow rate of 25 cm^3 min^{-1}. Chromatographic data were collected and processed by the ChemStation chromatography data management system (rev. B.02.01. Agilent Technologies). The data were stored in data organizing and storage module ChemStore C/S (rev. B03.03, Agilent Technologies).

Preparation of the standard and test solutions

A common standard stock solution in DMA containing all the known residual solvents of clopidogrel bisulfate (*i.e.*, methanol, acetone, DCM, 2-butanol, cyclohexane, toluene, acetic acid and DMF) was prepared in such a way that after dilution it had a final concentration of 500 μg g^{-1} for methanol, 500 μg g^{-1} for acetone, 600 μg g^{-1} for DCM, 5000 μg g^{-1} for 2-butanol 2000 μg g^{-1} for cyclohexane, 890 μg g^{-1} for toluene, 2600 μg g^{-1} for acetic acid and 880 μg g^{-1} for DMF each with respect to 20 mg cm^{-3} of the respective test concentration. About 13 μL of methanol, 13 μL of acetone, 9 μL of DCM, 124 μL of 2-butanol, 51.3 μL of cyclohexane, 21 μL of toluene, 50 μL of acetic acid and 19 μL of DMF were transferred by suitable autopipettes into a 10-mL volumetric flask partially filled with DMA and diluted to volume with the same solvent. The calibration standard solution was prepared by diluting 100 μL of the standard stock solution to 10.0 mL with DMA. The test solution was prepared as follows: accurately weighed 200 mg sample of clopidogrel bisulfate was dissolved with DMA in a 10-mL volumetric flask.

Quantification

The concentration c_i of *i*-th residual solvents in μg per g of the drug substance sample (μg g^{-1}) was calculated by using the external standards method. The employed equation was:

$$c_i = \frac{10^4 r_{t,i} v_i \rho_i}{r_{s,i} m_t} \quad (1)$$

where $r_{t,i}$ is the area response of solvent *i* in an injected sample solution, $r_{s,i}$ is the average area response of solvent *i* in six injected standard solutions, ρ_i and v_i are the density and volume, respectively, of solvent *i* in the standard solution, and m_t is measured mass in mg of the clopidogrel bisulfate sample. The densities of methanol, acetone, DCM, 2-butanol, cyclohexane, toluene, acetic acid and DMF used in the calculation were 0.79, 0.78, 1.32, 0.81, 0.78, 0.87, 1.05 and 0.95 g cm^{-3}, respectively. All degradation products of 2-butanol (1-butene, *cis*-

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-2-butene, *trans*-2-butene, 2,2′-oxydibutane and 1-(1-methylpropoxy)butane) were quantified using the same response as that of the 2-butanol peak.

Data analysis

PCA and HCA were realized using Statgaphics Plus 5.1 software. All data were mean centered and scaled to the unit standard deviation prior to any multivariate analysis.

RESULTS AND DISCUSSION

Method development

The boiling points for 1-butene, –6.5 °C, *cis*-2-butene, 0.9 °C, and *trans*-2--butene, 3.7 °C, are lower than room temperature and for this reason, short retention times were obtained for these substances. As the oven had no cryogenic cooling option to cool the column to under room temperature, it was not possible to achieve the higher resolution that would be obtained at lower temperatures. Therefore, an isocratic part of the temperature ramp at 40 °C was chosen to ensure robustness of the method, regardless of the ambient temperature, and satisfactory separation of the low boiling solvents was attained. After this isothermal part, two temperature ramps of 6 and 35 °C min^{-1} were used to speed up the chromatographic analysis for the late-eluting peaks and for fast elution of DMA. DMA was selected as the sample diluent as it has a high boiling point of 165 °C that does not interfere with the more volatile analytes. Clopidogrel bisulfate is freely soluble in DMA allowing 1 g of substance to be dissolved in less than 10 cm^{-3} of this solvent.

There was no noticeable degradation of the matrix components in the injection port or on the column, which would generate products that could interfere with the components of interest. Accordingly, direct-injection sample preparation was selected as an entry-level in terms of the necessary instrumentation.

Method validation

Using a well-designed experiment and statistically relevant analysis, method validation was performed and accomplished in accordance with relevant guidelines.[29,30] The method validation was realized by evaluating the specificity, limit of detection (*LOD*) and limit of quantification (*LOQ*), linearity, accuracy, precision and robustness. The range of the method was determined by in-house specification limits given in Table S-I of the Supplementary material to this paper.

System suitability test. System suitability test was developed for the routine application of the method based on the results obtained in several representative performances of the method. Prior to each analysis, the chromatographic system must satisfy requirements (resolution and repeatability) of the suitability test. System suitability was determined from six replicate injections of the standard solution. The peak-to-peak resolution between each peak measured on a reference solution must be above 1.0 and the relative standard deviation (*RSD*) must be less than 15.0 % for the peak area for each solvent. All the system suitability

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criteria during validation of the study and batch analysis study were within the acceptance limits.

Specificity. Clopidogrel bisulfate samples were spiked with all the solvents individually and each sample was chromatographed to examine interference, if any, of the residual solvents peak on each other. The selectivity was confirmed by injecting a blank solution of DMA, the standard solution (Fig. S-1 of the Supplementary material), the test solution, and the test solution spiked with residual solvents at the level of the specifications (Fig. S-2 of the Supplementary material). The relative retention times for methanol, acetone, DCM, 2-butanol, cyclohexane, toluene, acetic acid and DMF were found to be 0.14, 0.22, 0.26, 0.47, 0.52, 0.86, 0.93 and 1.00, respectively. The resolution between each two adjacent chromatographic peaks in test solution was found to be less than 1.0, as given in Table I.

TABLE I. Some of the achieved chromatographic parameters

Peak origin	Retention time, min	*RRT*	USP tailing	Resolution
1-Butene	2.526	0.055	1.030	–
trans-2-Butene	2.661	0.062	1.085	2.2
cis-2-Butene	2.794	0.069	1.035	1.9
Methanol	2.931	0.071	1.055	1.7
Acetone	4.448	0.158	1.095	10
DCM	5.282	0.202	1.003	6.2
2-Butanol	9.463	0.426	1.086	18
Cyclohexane	10.503	0.482	1.010	3.2
Toluene	17.263	0.844	1.014	26
2,2′-Oxydibutane	17.855	0.876	1.098	3.1
1-(1-Methylpropoxy)butane	18.080	0.888	1.069	1.1
Acetic acid	18.658	0.919	1.313	2.5
DMF	20.180	1.000	1.459	6.8

Limits of detection and quantification. For predicting the limit of detection (*LOD*) and limit of quantification (*LOQ*) values of each residual solvent, a standard solution was prepared with all residual solvents at the level of about 30 μg g^{-1} with respect to 20 mg cm^{-3} of the test concentration. This standard solution was injected into the chromatographic system and the *LOD* and *LOQ* values were predicted from the signal to noise (*S*/*N*) ratio data. The *LOD* and *LOQ* correspond to the concentration with signal of 3 and 10 times the noise level, respectively. Solutions containing all the residual solvents at the predicted *LOQ* concentration levels were prepared and analyzed six times to evaluate the precision of the method at this concentration level, detailed in Table II.

Linearity. The linearity of the method was confirmed by injecting solutions at twelve standard concentration levels, corresponding approximately to 0.5–150 % of the specification level for each of the residual solvents. The concentrations

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studied were within the ranges 2.5–750 μg g^{-1} for methanol and acetone, 3–900 μg g^{-1} for DCM, 25–7500 μg g^{-1} for 2-butanol, 10–3000 μg g^{-1} for cyclohexane, 4.5–1300 μg g^{-1} for toluene, 13–3900 μg g^{-1} for acetic acid and 4.4–1300 μg g^{-1} for DMF. The linearity was evaluated by linear regression analysis, which was calculated by least-square regression analysis. The area and concentration were treated by least square linear regression analysis plot (area count in terms of intensity, pA s, on the *y*-axis *vs.* concentration, μg g^{-1}, on the *x*-axis). The statistical parameters, slope, intercept, residual standard deviation and correlation coefficient values, were calculated and are given in Table III. The standard mixture showed good linearity for all residual solvents in the tested ranges. The area response obeyed the equation $y = ax + b$, where the intercept b was zero within 95 % confidence limits and the square correlation coefficient (R^2) was always greater than 0.9997 (Table III).

TABLE II. *LOQ* and *LOD* values and the precision at the *LOQ* values

Compound	*LOD* / μg g^{-1}	*LOQ* / μg g^{-1}	Precision at *LOQ*, *RSD* / % (*n*=6)
Methanol	5	22	3.0
Acetone	7	25	5.6
DCM	21	65	6.2
2-Butanol	23	75	4.8
Cyclohexane	14	45	2.3
Toluene	6	22	2.2
Acetic acid	10	40	7.2
DMF	12	35	5.7

TABLE III. Linearity data for standard mixtures: $y = ax + b$, where x is the concentration of residual solvent (μg g^{-1}), y is peak area count (pA s), *RRSD* is the residual relative standard deviation ($S\Delta y/y$, n–2)

Compound	R^2	Regression equation	Slope pA s μg $^{-1}$ g	Intercept pA s	*RRSD* / %
Methanol	0.999956	$y = 1548x + 438$	1548±7	438±1991	0.99
Acetone	0.999984	$y = 3885x + 355$	3885± 11	355±2998	0.59
DCM	0.999747	$y = 145x + 353$	145±2	353±535	2.37
2-Butanol	0.999970	$y = 300x + 1420$	300±1	1420±3192	0.82
Cyclohexane	0.999705	$y = 999x + 4824$	999±12	4824±13289	2.56
Toluene	0.999988	$y = 901x + 918$	901± 2	918±1079	0.52
Acetic acid	0.999982	$y = 263x + 1721$	263±1	1721±2115	0.63
DMF	0.999980	$y = 932x – 308$	932±3	–308±1433	0.67

Precision. The system precision was evaluated with replicate injections of the standard and spiked sample solutions. The percent relative standard deviation (*RSD*) was found to be 2.04 % for methanol, 2.27 % for acetone, 2.63 % for DCM, 1.72 % for 2-butanol, 3.31 % for cyclohexane, 2.10 % for toluene, 1.34 % for acetic acid and 8.73 % for DMF. The repeatabilities were the intra-day vari-

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ation (method precision) and the inter-day variation (ruggedness). The repeatability of the method was studied by analyzing six sample solutions, separately, by addition of solvents at known concentration levels (100 % of specification limits). The *RSD* was found to be 0.98 % for 1-butene, 0.90 % for 2-butene, 2.28 % for methanol, 1.33 % for acetone, 0.89 % for 2-butanol, 0.93 % for cyclohexane, 0.90 % for toluene, 4.89 % for acetic acid, 6.82 % for DMF and 1.99 % for the butyl ethers. The degree of reproducibility, known as ruggedness, was obtained by the analysis of the same sample concentration (which is used for method precision determination) under a variety of conditions using different column series, with a different analyst on different days using new standards and calibration. The overall *RSD* from such a measurement series of twelve runs was found to be 3.85 % for 1-butene, 2.49 % for 2-butene, 2.04 % for methanol, 7.49 % for acetone, 1.87 % for DCM, 1.05 % for 2-butanol, 1.70 % for cyclohexane, 1.12 % for toluene, 8.08 % for acetic acid, 6.99 % for DMF and 1.66 % for the butyl ethers.

Accuracy. The accuracy of the method was evaluated by recovery experiment using the standard addition technique. The recoveries were determined by spiking the respective residual solvents at five different levels ranging from the *LOQ* values to 150 % of the specification level into clopidogrel bisulfate drug substance. The samples were prepared as per the methodology, and analyzed in triplicate and percentage recoveries were calculated. The average recovery values are summarized in Table IV.

Robustness. For the determination of the robustness of the method, a number of method parameters, such as flow rate, initial column temperature, FID temperature and split ratio, were varied within a realistic range, and the quantitative influences of the variables were determined. The method was challenged by varying the following parameters within the limits: flow rate of carrier gas ±10 %, initial temperature of the column oven 40±5 °C, split ratio (5±1):1 and FID temperature 240±10 °C. For each set of variations, six replicate injections of the standard solution were performed. The system suitability results met the acceptance criteria at each of the deliberately varied conditions. The *RSD* of the solvent obtained at conditions deliberately varied from those of the developed methodology did not vary much. In all the varied conditions, the chromatographic resolution between any of the two components was not less than 1.0. Hence, the test method is robust for all the varied chromatography conditions.

Result of the analysis for multiple batches of clopidogrel bisulfate

The validated method was used for the analysis of 18 clopidogrel bisulfate samples originating from a single production source (Table S-II of the Supplementary material). From the eight solvents used in the process of synthesis and purification of clopidogrel bisulphate, it was found that the following organic sol-

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TABLE IV. Accuracy of the method expressed as the percent recovery (*R*) at different levels, for: I – methanol, II – acetone, III – DCM, IV – 2-butanol, V – cyclohexane, VI – toluene, VII – acetic acid and VIII – DMF

Parameter	I	II	III	IV	V	VI	VII	VIII
Level *LOQ*	106.2	102.9	105.3	103.5	103.6	105.6	105.3	98.3
	105.3	107.7	110.2	105.2	98.9	102.2	99.8	103.8
	104.2	101.6	101.4	101.3	98.9	103.7	108.4	107.0
Average	**105.2**	**104.1**	**105.6**	**103.3**	**100.5**	**103.8**	**104.5**	**103.0**
RSD / %	0.95	3.10	4.17	1.89	2.71	1.63	8.88	4.27
	99.5	100.3	103.7	100.4	97.8	99.7	97.5	97.5
Level 25 %	101.8	101.1	105.3	102.3	98.3	100.9	93.9	105.7
	100.6	100.4	104.1	98.8	97.9	98.3	100.3	99.6
Average	**100.6**	**100.6**	**104.4**	**100.5**	**98.0**	**99.6**	**97.2**	**100.9**
RSD / %	1.16	0.42	0.82	1.77	0.26	1.31	3.30	4.22
	104.6	103.0	106.8	100.3	99.7	100.1	97.5	102.7
Level 50 %	101.2	97.9	98.7	97.3	99.0	97.0	97.0	99.0
	103.6	101.9	102.4	99.0	98.7	99.3	98.1	102.5
Average	**103.1**	**100.9**	**102.6**	**98.9**	**99.1**	**98.8**	**97.5**	**101.4**
RSD / %	1.68	2.62	3.99	1.53	0.52	1.65	0.55	2.03
	102.1	100.7	100.8	101.6	100.2	101.2	99.4	104.9
Level 100 %	99.8	100.1	99.9	100.1	100.2	100.2	96.7	104.3
	97.1	96.7	95.9	97.1	96.3	96.8	98.4	95.6
Average	**99.7**	**99.2**	**98.9**	**99.6**	**98.9**	**99.4**	**98.2**	**101.6**
RSD / %	2.53	2.22	2.65	2.32	2.32	2.28	1.39	5.09
	100.8	101.7	101.7	101.0	101.5	101.2	101.2	101.5
Level 150 %	99.7	100.2	100.4	100.7	101.1	100.9	101.2	98.5
	98.6	98.6	98.1	99.1	99.8	99.1	100.4	98.3
Average	**99.7**	**100.2**	**100.0**	**100.3**	**100.8**	**100.4**	**100.9**	**99.4**
RSD / %	1.14	1.54	1.83	1.02	0.86	1.16	0.44	1.77

vents were frequently present in the samples: 2-butanol, cyclohexane and acetic acid. Due to intramolecular and intermolecular dehydration of 2-butanol, significant amounts of products derived from 2-butanol, 1-butene, *cis*-2-butene, *trans*-2-butene, 2,2′-oxydibutane and 1-(1-methylpropoxy)butane, could be found in the samples. Intramolecular dehydration of 2-butanol results in a mixture containing: 1-butene, *cis*-2-butene and *trans*-2-butene, predominantly the last two in a 50:50 ratio. Intermolecular dehydration of two 2-butanol molecules results in a mixture containing 50:50 ratio of 2,2′-oxybis[butane] and 1-(1-methylpropoxy)-butane. Although *cis*-2-butene and *trans*-2-butene were chromatographically separated from each other, for regulation reasons, they shall be reported as 2-butene by the sum of their contents. A similar approach was used in the case of 2-butan-2-yloxybutane and 1-(1-methylpropoxy) butane, where the total amount was designated as dibutyl ethers. Validation tests of the accuracy showed that 2-butanol products were not formed during GC analysis but were already present

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in the clopidogrel samples. The presence of these is likely to be caused by the course of the drying process in clopidogrel bisulfate production.

Substantial contents of acetic acid and 2-butanol (further products derived from 2-butanol, likewise) were found. However, in all the samples they were within the regulatory tolerance limits and without much impact on the quality and stability of final drug product. It should also be taken into account that the contents of residual solvents in the final product were lower than those in the active substance, because only 75 mg of clopidogrel bisulfate is present in 350 mg of total tablet mass.

Multivariate analysis and pattern recognition

Generally, there are two main causes of variation in the quality of products or processes: random causes and patterns that could be recognized. As no deeper insights into the production and purification process of active pharmaceutical ingredients are available, continuous quality control of purchased materials is the only option remaining. Using this approach, based on the result sets of multiple batches of clopidogrel bisulfate (Table S-II), some conclusions may be drawn on the reliability of the sources for providing high-quality material. Multivariate analysis is a commonly used statistical technique to classify samples. From the analyzed set of 11 solvents, 6 of them were preselected based upon their occurrence in the samples and both PCA and HCA were performed. PCA is a method that projects multi-dimension space to a lower dimensional space, reducing the number of variables and enabling graphical interpretation of the results. It could be assumed that a correlation exists between the amount of 2-butanol and its products, or between the amount of 2-butanol and the presence and quantity of the other used solvents. Thus, the implementation of PCA is justified, and in the present case, two principal components were extracted (PC1 and PC2), since these two components had eigenvalues greater than or equal to 1.0. The first principal component had the equation:

$$\begin{aligned}\text{PC1} = {} & 0.5481\times[\text{2-Butanol}]-0.5234\times[\text{1-Butene}]-0.5621\times[\text{2-Butene}]+ \\ & +0.1726\times[\text{Cyclohexane}]+0.2467\times[\text{Acetic acid}]+0.1376\times[\text{Butyl ethers}] \end{aligned} \quad (2)$$

The second principal component had the equation:

$$\begin{aligned}\text{PC2} = {} & -0.6281\times[\text{Cyclohexane}]-0.4282\times[\text{Acetic Acid}]+ \\ & 0.1430\times[\text{2-Butanol}]-0.1329\times[\text{1-Butene}]-0.2558\times[\text{2-Butene}]- \\ & -0.5644\times[\text{Butyl ethers}] \end{aligned} \quad (3)$$

In Eqs. (2) and (3), the values of the solvent concentrations were standardized by subtracting their means and dividing by their standard deviations. Thus, the PCs are dimensionless quantities. The PCA results showed that the first component accounted for about 41.5 %, and second component for 27.1 % of total variance in the data set. These two components together explained 68.6 %

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of total variance accounting for most of the variability in the original data. Since the eigenvalues were greater than 1.0 (2.48 for PC1 and 1.62 for PC2), the discussion is focused only on these two components. PC1 had a relatively high positive weight for 2-butanol, and almost the same high negative weights for 1-butene and 2-butene (the weights are the coefficients from Eq. (2)). PC2 had relatively high weights for cyclohexane, acetic acid and butyl ethers (the weights are the coefficients from Eq. (3)). A weight close to 0 indicates little contribution of the variable to the component. Vectors pointing in the same direction are positively correlated and those pointing in the opposite direction are negatively correlated (Fig. 2). Based on these findings, it was concluded that PC1 could be denoted as being the part dependent on 2-butanol and its main products. On the other hand, PC2, which has high weights for cyclohexane, acetic acid and butyl ethers, can be designated as being the component dependent on the other relevant solvents.

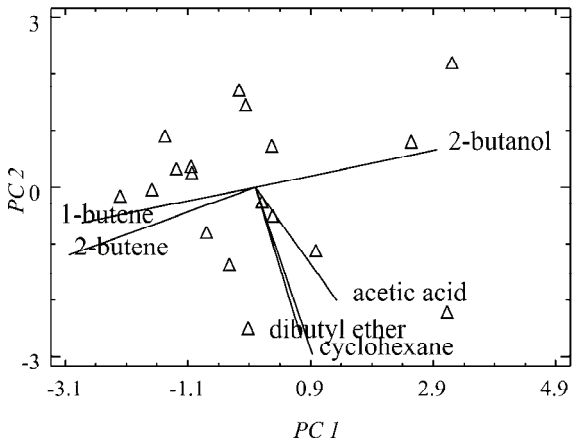

Fig. 2. A bi-plot which involves superimposition of the scores and the loadings plot, with solvents (variables) and samples represented on the same diagram with two selected principal components PC1 and PC2 that are standardized (dimensionless quantities).

The dendrogram analysis (Fig. 3) showed the result of the clustering the 18 batches using the furthest neighbor method and the squared Euclidean distance indicated 6 different classes exit within the data. Strictly speaking, there is only one true class of samples within 12 batches of the population. In this class, the samples were very closely clustered, and were well separated from the other samples. The remaining 6 samples are not similar to any other sample in the all data set. The highest degree of similarity in these 6 samples was observed between two samples that form the same cluster, the similarity was lower than that expressed in the separate 12-member cluster. Generally, it could be assumed that six of the samples are unusual, but that does not help in locating the cause. The

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observed dissimilarities were linked with the appearance of cyclohexane, acetic acid or 2-butanol at unusually high levels, in various mutual combinations. In the case of acetic acid, there is a strong variation of its contents from batch to batch (over all, and within the most populated cluster), which is likely to be a problem in controlling the process by which it is removed. Therefore, the manufacturer used the option to set the specifications of 5000 μg g^{-1}, the highest permissible limit for this USP class of residual solvent. Contrary to this, the total content of 2-butanol and its dehydration products are relatively consistent (average of 2800 μg g^{-1} with *RSD* of 14.1 %), indicating a good control of the process (Fig. S-III of the Supplementary material). The content of residual solvents could be changed with time due to a further dehydration of 2-butanol and evaporation of all the solvent present, which is dependent on the aging time, packaging and storing conditions, as well as by some of the significant factors.

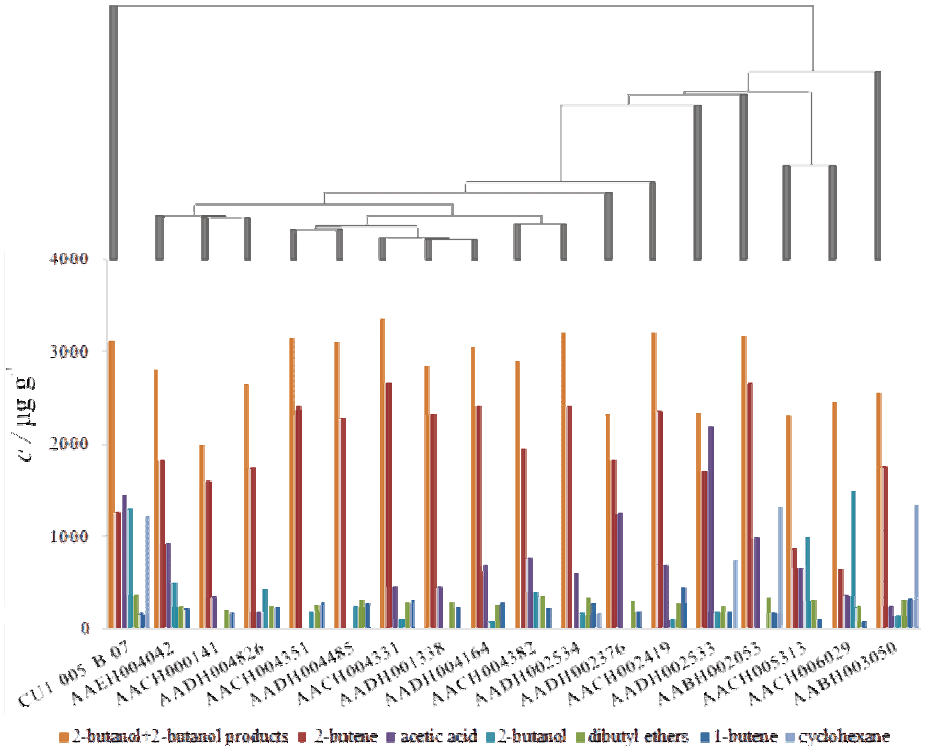

Fig. 3. Graph of variation of content from batch to batch of the most frequent and most abundant organic solvents: 2-butene, acetic acid, 2-butanol, dibutyl ethers, 1-butene and cyclohexane. In the first column, the total amount of 2-butanol and its dehydration products is presented. In the upper part of the image, a dendrogram obtained by the means of HCA is suitably placed. A horizontal line connecting two groups shows that the groups were combined at the distance shown on the vertical axis.

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The general conclusion could be that the observed variations in the content of the detected residual solvents between the different batches are probably due to an on-going process of optimization attempts (in 6 of the 18 batches). However, the remaining 12 batches constitute one cluster with similar characteristics and a negligible variation within the group. Other five solvents used in the process, methanol, acetone, DCM, toluene and DMF are present at low levels or completely absent, which are well below the required regulatory limits. In this case, it is possible to narrow the limits of their presence in the specification of the clopidogrel bisulfate samples. Generally speaking, the fact that the manufacturer remained within acceptable limits for all residual solvents in all 18 batches is encouraging, but the observed variations indicate the need for permanent control of material from this source.

CONCLUSIONS

In this work, a GC method for the evaluation of residual solvents in clopidogrel bisulfate samples is presented and validated. Methanol and acetone have a linear response from 2.5 to 750 μg g^{-1} (with respect to a drug concentration of 20 mg cm^{-3}), DCM from 3 to 900 μg g^{-1}, 2-butanol from 25 to 7500 μg g^{-1}, cyclohexane from 10 to 3000 μg g^{-1}, toluene from 4.5 to 1300 μg g^{-1}, acetic acid from 13 to 3900 μg g^{-1} and DMA from 4.4 to 1300 μg g^{-1}. Calibration line intercepts were zero within the 95 % confidence limit and the square correlation coefficients (R^2) were at least 0.9997. Average recovery values ranged from 97.2 to 105.6 %. Relative standard deviations for precision were not more than 8.08 %. The quantification limits (in μg g^{-1}) were as follows: methanol, 22; acetone, 25; DCM, 65; 2-butanol, 75; cyclohexane, 45; toluene, 22; acetic acid, 40; DMA, 35.

The proposed analytical method coupled with the chemometrics data analysis technique was used as a powerful tool for quality control purposes to differentiate the content of residual solvents among samples of clopidogrel bisulfate. The application of chemometry to monitoring data enables a complete overview of the quality and reliability of a particular clopidogrel bisulfate source to be obtained.

SUPPLEMENTARY MATERIAL

Tables S-I and S-II, and Figs. S-1–S-3 are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ПРИМЕНА ГАСНО-ХРОМАТОГРАФСКЕ АНАЛИЗЕ ЗА ОДРЕЂИВАЊЕ САДРЖАЈА ЗАОСТАЛИХ ОРГАНСКИХ РАСТВАРАЧА У КОНТРОЛИ КВАЛИТЕТА КЛОПИДОГРЕЛ-БИСУЛФАТА

АЛЕКСАНДАР Д. ПАВЛОВИЋ[1], ЉУБИША М. ИГЊАТОВИЋ[1], САША З. ПОПОВ[1], АЛЕКСАНДАР Р. МЛАДЕНОВИЋ[2] и ИГОР Н. СТАНКОВИЋ[3]

[1]*Универзитет у Београду, Факултет за физичку хемију, Студентски трг 12–16, Београд,* [2]*Универзитет у Београду, Технолошко–металуршки факултет, Карнегијева 4, Београд и* [3]*Универзитет у Новом Саду, Природно-математички факултет, Трг Доситеја Обрадовића 3, Нови Сад*

Развијена је капиларна гасно-хроматографска метода, у *split* моду, уз директно инјектовање и пламено-јонизациону детекцију, за анализу растварача који су коришћени у синтези и пречишћавању антитромботске активне фармацеутске супстанце, клопидогрел-бисулфата. У процесу производње коришћено је осам растварача: метанол, ацетон, дихлорметан, 2-бутанол, циклохексан, толуен, сирћетна киселина и диметилформамид. Додатно, као резултат дехидратације 2-бутанола током процеса сушења, у испитиваним узорцима клопидогрел-бисулфата, у значајним количинама се могу наћи дехидратациони производи: 1-бутен, *cis* и *trans* изомери 2-бутена, 2-ди-*sec*-бутил-етар и *sec*-бутил-*n*-бутил-етар. Садржај сваког од ових испарљивих производа може се проценити коришћењем исте гаснохроматографске методе, уз квантификацију засновану на фактору одговора успостављеном за хроматографски пик 2-бутанола. За сваки од ових растварача метода је валидирана на селективност, линеарност, тачност, прецизност, робусност, лимит квантификације и лимит детекције. На основу комплексне групе резултата анализа, ретроспективно, за већи број различитих производних серија, закључено је о степену одступања у процесу производње и његовој поузданости, и препознат је недостатак доследности у квалитету активне супстанце која води порекло од једног од комерцијалних произвођача. Мултиваријантна анализа је коришћена као статистичка техника у међусобном разврставању узорака. Од анализираног скупа од 11 растварача, 6 растварача је било унапред одабрано на основу њихове редовне појаве у узорцима. Изведене су мултиварјантне статистичке технике: анализа главних компоненти (PCA) и хијерархијска кластер анализа (HCA).

(Примљено 20. новембра 2013, ревидирано 4. фебруара, прихваћено 23. фебруара 2014)

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