Detection and development of a quantitation method for undeclared compounds in antidiabetic biologically active additives and its validation by high performance liquid chromatography

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Received 22 October 2021 ♦ Accepted 7 December 2021 ♦ Published 5 January 2022

Citation: Kirakosyan VG, Tsaturyan AH, Poghosyan LE, Minasyan EV, Petrosyan HR, Sahakyan LY, Sargsyan TH (2022) Detection and development of a quantitation method for undeclared compounds in antidiabetic biologically active additives and its validation by high performance liquid chromatography. Pharmacia 69(1): 45–50. https://doi.org/10.3897/pharmacia.69.e76247

Abstract

An isocratic, high-performance liquid chromatography (HPLC) quantitation method was developed for the quantitative determination of metformin, glibenclamide, gliclazide, glimepiride in some antidiabetic biologically active additives. A Nucleosil C18, 5 μm, 4.6 mm × 150 mm, column with mobile phase containing buffer (10 mm Na2HPO4, 10 mm sodium dodecyl sulfate): acetonitrile = 68 : 32 (V/V), pH = 7.5 was used. The flow rate was 1.0 mL/min, and effluents were monitored at 226 nm. The retention times of gliclazide glibenclamide, glimepiride and metformin, were 2.203, 4.587, 5.667 and 10.182 min, respectively. Linearity was studied by preparing standard solutions of gliclazide, glibenclamide, glimepiride and metformin at the concentration range of 50% to 150% of working concentration from a stock solution. The method was successfully applied to the estimation of gliclazide, glibenclamide, glimepiride and metformin in some antidiabetic biologically active additives. This method was validated to confirm its system suitability, selectivity, linearity, precision and accuracy according to international conference on harmonization (ICH) guidelines.

Keywords

HPLC, biologically active additives, metformin, glibenclamide, gliclazide, glimepiride

Introduction

Biologically active additives (BAA) contain bioactive elements - vitamins, minerals, proteins, are often supplemented with organ tissues, plants, etc., which in contrast to drugs are in less quantities than therapeutic dosages and affect the body within physiological norms, are intended to strengthen the health, the body's resistance to pathogenic factors and to improve the quality of life (Gabrielyan et al. 2002; Zemtsova et al. 2020; Makhmudov et al. 2021).

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There is no reasonable guarantee that BAA are absolutely safe, as there are many cases when undeclared substances such as hormones, analgesics, antidiabetic and other drugs are added to BAA to stimulate their effect, the long-term uncontrolled use of which is extremely hazardous (Peng et al. 2013; Mihaylova et al. 2020).

The correct selection of the method of analysis is of great importance for the detection of undeclared chemicals in BAA. The selected method should have high sensitivity, the ability to work with small quantities of samples, high selectivity, be distinguished by the rate of expertise, the simplicity of sample preparation, ease of maintenance of analytical equipment, reliability and reproducibility of the method, universality, automation of the analytical process. In practice, the most widely used method (95% of studies) is the high performance liquid chromatography (HPLC) with different detection techniques (Watson 2012; Kloos et al. 2014).

High performance liquid chromatography is a highly precision physical method that meets the modern requirements of drug quality control and is used for the separation, quantitative and qualitative identification of compounds. In this study an available reversed-phase HPLC method for identification of metformin, glibenclamide, gliclazide, glimepiride with UV detection and isocratic elution mode was developed, evaluation of the method applicability and determination of validation indicators were carried out (Attimarad et al. 2011; Monzón et al. 2016; Mahmoud et al. 2019). It has been shown that the developed method meets the current international requirements. For the detection of metformin, glibenclamide, gliclazide, glimepiride, the developed method has been used in some BAA (“Dialevel”, “Sugar Balance”, “Blood Sugar”, “Karela Capsules”).

Taking into account that quite large quantities of BAA are consumed by the population in the Republic of Armenia and there is no permanent control over it, the following tasks have been set forward:

- Development of a detection method for undeclared chemicals in antidiabetic BAA and its validation,
- Confirmation of the applicability of the selected method for daily use in the laboratory,
- Implementation of research on some of the most common antidiabetic BAA in the Republic of Armenia (“Dialevel”, “Sugar Balance”, “Blood Sugar”, “Karela Capsules”) by the newly developed method.

For the mobile phase methanol (HPLC Grade, AppliChem), disodium hydrogen phosphate dihydrate (Na2HPO4·2H2O HPLC Grade, Sigma Aldrich), sodium dodecyl sulfate (HPLC Grade, ≥ 99%, Carl Roth), orthophosphoric acid (HPLC Grade, 85%, Carl Roth) were used. Dialevel (Walmart, Czech Republic), Sugar Balance (Velt Farma, Germany), Blood Sugar (Nature’s Way, Australia), and Karela Capsules (Himalaya, India) were chosen as research samples.

As a standards metformin hydrochloride (BN-M0605000; 99.81%; E.Ph. RS), glibenclamide (BN-G0325000; 99.9% E.Ph. RS), gliclazide (BN-M0605000, 99.81%, E.Ph. RS), glimepiride (BN-Y0000515, 99.46%, E.Ph. RS) were used and 10 mm Na2HPO4, 10 mm SDS (sodium dodecyl sulfate) have been used as buffer.

The chromatographic conditions are presented in Table 1. Standard preparation: Methanol was used as a solvent, and for the preparation of the standard solutions (Table 2) the corresponding weights of the standards were dissolved in 25 ml of solvent, after which they were placed in an ultrasonic bath for 10 minutes. The resulting solutions were then stirred with a VORTEX core stirrer for 5 minutes.

Table 1. Chromatographic conditions for the expertise of BAA method used in diabetes.

| Chromatographic column | Nucleosil C18 5 μm, 4.6 mm × 150 mm, (Macherey-Nagel, Germany) | Nucleosil C18 5 μm, 4.6 mm × 150 mm, (Macherey-Nagel, Germany) |
|------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Detector wavelength    | 226 nm                                                        | 226 nm                                                        |
| Flow rate              | 1 ml/min                                                      | 1 ml/min                                                      |
| Injection volume       | 20 μl                                                        | 20 μl                                                        |
| Column temperature     | 35 °C                                                        | 35 °C                                                        |
| Pump operating mode    | Isocratic                                                    | Isocratic                                                    |
| Mobile phase           | Buffer: acetonitrile = 68 : 32 (V/V), pH = 7.5               | Buffer: acetonitrile = 68 : 32 (V/V), pH = 7.5               |

Mobile phase preparation: After mixing the organic and inorganic components of the mobile phase, the pH of the solution has been adjusted to 7.5 with orthophosphoric acid, filtered with a 0.45 μm membrane, again filtered and degasified.

Three injection were made from each solution.

Preparation of standard mother solution (STD-5). 1 ml of each of the above solutions was transferred to a 10 ml volumetric flask and adjusted to the mark in a mobile phase. The theoretical concentration of standards in this solution is presented in Table 3.

Preparation of solutions for the construction of the calibration curve. A mixture of 5 standards of different

Table 2. Preparation of standard solutions.

| Sample                          | STD-1 Metformin (99.81%) | STD-2 Glibenclamide (99.90%) | STD-3 Gliclazide (99.80%) | STD-4 Glimepiride (99.46%) |
|---------------------------------|--------------------------|-------------------------------|--------------------------|---------------------------|
| Standard sample quantity (mg)   | 5.1                      | 5.2                           | 5.0                      | 5.1                       |
|                                 | 5.0                      | 5.1                           | 5.0                      | 5.1                       |
|                                 | 5.0                      | 5.2                           | 5.1                      | 5.0                       |

Table 3. Concentration of standards in standard mother solution.

| C (μg/ml) | Metformin | Glibenclamide | Gliclazide | Glimepiride |
|-----------|-----------|---------------|------------|-------------|
| 0.020362  | 0.020779  | 0.019892      | 0.020359   |

Materials and methods

All measurements were made with a “Shimadzu LC-20MS” (Japan) equipped with an automatic injection system (SIL-20A), a detector (SPD-M20A), a chromatographic column (Nucleosil C18, 5 μm, 250 × 4.6 mm), and a column thermostat (Shimadzu). Analytical balance (Shimadzu), deionized water system (Purelab, ELGA), “Vortex” core stirrer (Stuart, BioCote, UK), 0.45 m membrane filters (E-chrom Tech, Taiwan), glass volumetric flasks, measuring cylinders, cups, and pipettes of various capacities (Normax, Portugal) were used for the samples preparation.
concentrations was selected. For each preparation, the volumes shown in Table 4 were taken from STD-1, STD-2, STD-3, STD-4 solutions and diluted in a mobile phase up to 20 ml.

**Preparation of solutions for interlaboratory accuracy and precision.** QCL (calib.1 - 0.01 mg/ml), QCM (calib.3 - 0.02 mg/ml) and QCH (calib.5 - 0.03 mg/ml) solutions were prepared. Three injections were made from each solution.

### Table 4. Preparation of calibration solutions.

| Primary solutions Standard solutions | Calib. 1 | Calib. 2 | Calib. 3 | Calib. 4 | Calib. 5 |
|--------------------------------------|---------|---------|---------|---------|---------|
| STD-1 Metformin                      | 0.010181| 0.015227| 0.020362| 0.025453| 0.030543|
| STD-2 Glibenclamide                  | 0.0103895| 0.015584| 0.020779| 0.025974| 0.0316485|
| STD-3 Glimepiride                    | 0.009946| 0.014919| 0.019892| 0.024865| 0.029830 |
| STD-4 Gliclazide                     | 0.0101795| 0.0152693| 0.020359| 0.025449| 0.0305385|

### Results and discussion

Validation of the method for quantitation of gliclazide, glibenclamide, glimepiride and metformin in antidiabetic biologically active additives was performed by evaluating the following indicators:

- Selectivity
- Accuracy
- Precision
- Linearity range

The most common representatives of chemical origin of this group of drugs are metformin from biguanides and glibenclamide, gliclazide, glimepiride derivatives of sulfonylurea.

The pharmacopoeia does not specify test methods for the associations of these substances. So, such a method should be selected that is universal for detecting substances from these two different chemical groups.

**System suitability test of the chromatographic system with the expertise BAA method used in diabetes.**

It is to be implemented to perceive whether the selected method for the simultaneous determination of metformin and glibenclamide can be used for the simultaneous detection of other representatives of this series.

The retention times of the substances under the selected chromatographic conditions are shown in Figure 1.

### Acceptance criteria:

1. the tailing factor of the peak due to gliclazide, glibenclamide, glimepiride and metformin is not more than 2.0,
2. the number of theoretical plates of the peak due to gliclazide, glibenclamide, glimepiride and metformin is more than 2500,
3. resolution between peaks to gliclazide, glibenclamide, glimepiride and metformin is not less than 2.0. The results are presented in Table 5.

### Table 5. System suitability test of the chromatographic system.

| SST | Resolution (Distribution between peaks) | Tailing Factor | Theoretical Plate |
|-----|----------------------------------------|---------------|-------------------|
| Gliclazide | 1.32 |                                   |               | 2500              |
| Glibenclamide | 10.4 | 1.00 | 4300              |
| Glimepiride | 3.6  | 0.97 | 4900              |
| Metformin | 8.9  | 1.67 | 3400              |

Selectivity is the ability to access unequivocally the analyte in presence of components, which may be expected to be present. To prove selectivity, the following experiment is carried out. Selectivity was tested on blank, active ingredient and finished product.

**Acceptance Criteria:** no interference of the blank at the retention time of gliclazide, glibenclamide, glimepiride and metformin. The results are presented in the Figure 2 and Table 6.

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity was studied by preparing standard solutions of gliclazide, glibenclamide, glimepiride and metformin at the concentration range of 50% to 150% of working concentration from a stock solution and each concentration was injected in triplicate and chromatographed as per procedure. The results are presented in Tables 7 and 8 and Figure 3.

**Conclusion:** a method is linear in the range from 50 percent to 150 percent of gliclazide, glibenclamide, glimepiride and metformin concentration in standard solution chromatogram.

**Acceptance Criteria:** correlation coefficient is not less than 0.995.
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Table 6. Evaluation results of the indicator of "Method selectivity".

| Sample code | Gliclazide | Glibenclamide | Glimepiride | Metformin |
|-------------|------------|---------------|-------------|-----------|
|             | Retention time (min) | Surface of stress point | Retention time (min) | Surface of stress point | Retention time (min) | Surface of stress point | Retention time (min) | Surface of stress point |
| Sel.-1      | 2.203      | 466511        | 4.587       | 592230    | 5.667       | 481690        | 10.182     | 541124    |
| Sel.-2      | 2.198      | 468454        | 4.575       | 591999    | 5.651       | 481894        | 10.190     | 542061    |
| Sel.-3      | 2.206      | 468542        | 4.577       | 592152    | 5.648       | 482539        | 10.205     | 545546    |
| Sel.-4      | 2.200      | 469074        | 4.563       | 592244    | 5.631       | 482160        | 10.206     | 543073    |
| Sel.-5      | 2.194      | 468743        | 4.550       | 592877    | 5.614       | 482381        | 10.209     | 542536    |
| Sel.-6      | 2.189      | 467345        | 4.538       | 592617    | 5.599       | 482414        | 10.213     | 544059    |
| Average     | 2.19833    | 468094.8      | 4.565       | 592319.8  | 5.635       | 482150.8      | 10.2083    | 543066.8  |
| SD          | 0.00562    | 874.906       | 0.01676     | 245.2206  | 0.023101    | 285.8306      | 0.011037   | 1425.881  |
| RSD, %      | 0.25553    | 0.186908      | 0.36721     | 0.041400  | 0.40996     | 0.059282      | 0.108193   | 0.262561  |

Table 7. Linearity range: Evaluation results of the indicator of "Linearity range" for gliclazide and glibenclamide.

| Calibration curve code | Gliclazide | Glibenclamide |
|------------------------|------------|---------------|
|                        | Concentration (mg/ml) | Surface | Concentration (mg/ml) | Surface |
| Calib.1                | 0.0101795  | 240666       | 0.0103895 | 295663 |
| Calib.2                | 0.0152893  | 359533       | 0.015584 | 443067 |
| Calib.3                | 0.020359   | 464247       | 0.020779 | 592182 |
| Calib.4                | 0.025449   | 573331       | 0.025974 | 741294 |
| Calib.5                | 0.0305385  | 686518       | 0.031185 | 889448 |
| Correlation coefficient | R (NLD 0.995) | 0.9998101   | 0.999979 |

Table 8. "Linearity range" results for glimepiride and metformin.

| Calibration curve code | Glimepiride | Metformin |
|------------------------|-------------|-----------|
|                        | Concentration (mg/ml) | Surface | Concentration (mg/ml) | Surface |
| Calib.1                | 0.009946    | 240467    | 0.010181 | 265471 |
| Calib.2                | 0.014919    | 362123    | 0.015272 | 406362 |
| Calib.3                | 0.019892    | 482007    | 0.020362 | 543058 |
| Calib.4                | 0.024865    | 603238    | 0.025453 | 687018 |
| Calib.5                | 0.029380    | 723869    | 0.030543 | 823807 |
| Correlation coefficient | R (NLD 0.995) | 0.9999982 | 0.9999730 |

Table 9. Accuracy: Evaluation results of the indicator of "Accuracy".

| Sample code | Gliclazide | Glibenclamide | Metformin |
|-------------|------------|---------------|-----------|
| Sel.-1      | 0.020359   | 0.020779      | 0.020362  |
| Sel.-2      | 0.020500   | 0.020780      | 0.020161  |
| Sel.-3      | 0.020504   | 0.020773      | 0.020215  |
| Sel.-4      | 0.020524   | 0.020783      | 0.020125  |
| Sel.-5      | 0.020513   | 0.020798      | 0.020106  |
| Sel.-6      | 0.020449   | 0.020796      | 0.020161  |
| Average     | 0.0204835  | 0.0207855     | 0.020125167 |
| SD          | 4.0103E-05 | 8.63616E-06   | 5.15636E-05 |
| RSD, %      | 0.19781934 | 0.041548985   | 0.256214558 |
| Average recovery, % | 100.61 | 100.03 | 98.84 |

Acceptance Criteria: the mean recovery should be in the range of 98.0 percent to 102.0 percent. RSD should be less than 2 percent. The results are presented in Table 9.

Acceptance Criteria: difference between the RSD results of Day 1 & Day 2 is not more than 2.0%. The results are presented in Tables 10 and 11.

Similar results were obtained with glibenclamide and glimepiride.

In large pharmacy chains surveys were conducted for the detection of the most common BAA in these groups, which resulted in the selection of "Dialevel", "Sugar Balance", "Karela Capsules", "Blood Sugar" additives used in diabetes.
As shown in Figure 4 and 5, no undeclared substance in additives “Dialevel” and “Sugar Balance” were detected by automatic registration system as a result of expertise. Similar results were obtained during the expertise of BAA in “Karela Capsules” and “Blood Sugar”.

### Conclusion

A method for detecting undeclared chemicals in antidiabetic BAA has been developed and introduced by us. The
developed new method was used to study some of the most common antidiabetic BAA in the Republic of Armenia (Dialevel, Sugar Balance, Blood Sugar, Karela Capsules). The applicability of the selected method for daily use in the laboratory has been confirmed.

Conflict of interest

The authors declare no conflict of interest.

Author’s contribution

H. Petrosyan participated in the preparation, writing and editing of the manuscript. V. Kirakosyan, E Minasyan performed quantitative determinations using Shimadzu LC-20-MS, as well as processing and interpretation of the manuscript. L. Poghosyan took part in obtaining test samples, conducting experiments, as well as processing and interpreting data. L. Yu. Sahakyan, participated in the design and implementation of experiments, as well as in the processing and interpretation of data. A. Tsaturyan, T. Sargsyan participated in the design and implementation of experiments, as well as in the processing and interpretation of data.

Acknowledgements

The authors would like to thank the Scientific Centre of Drug and Medical Technology Expertise after Academician E.Gabrielyan, for their technical assistance, which is highly appreciated.

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