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A novel 3D-printed sample preparation method for benzodiazepine quantification in human serum

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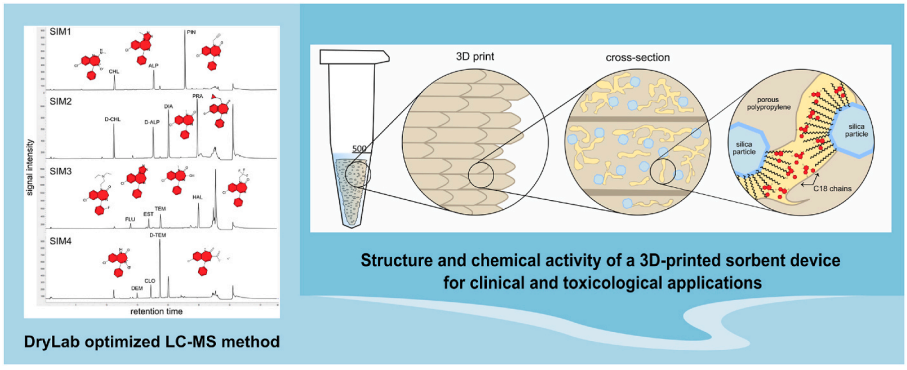
HIGHLIGHTS

- Extraction utilizing 3D-printed sorbent devices.
- Devices made with a thermoplastic material with incorporated C18 silica.
- Sample preparation fully validated in human serum according to ICH guideline.
- A DryLab optimized LC-MS method for separation of 11 benzodiazepines.
- A protocol suitable for toxicological and medical analyses of real samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Benzodiazepine abuse remains a significant public health concern. Current sample preparation methods for benzodiazepine analysis from human serum often involve complex procedures that require large sample volumes and extensive organic solvent use. To address these limitations, this study presents a novel and efficient sample preparation method utilizing 3D-printed sorbent devices.

Results: The 3D-printed devices, fabricated from a thermoplastic composite incorporating C18-modified silica, demonstrated exceptional performance in extracting benzodiazepines from human serum. The method was optimized and validated according to ICH guidelines, ensuring its reliability for quantitative benzodiazepine analysis. Notably, the method required minimal sample and solvent volumes, eliminating the need for protein precipitation, evaporation, and reconstitution.

Significance: This novel sample preparation approach offers significant advantages over traditional methods, providing a more efficient and environmentally friendly solution for benzodiazepine analysis. The versatility of 3D printing allows for the customization of sorbent devices for various analytes and matrices, expanding the potential applications of this method. Coupled with a rapid and robust LC-MS method optimized with DryLab,

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this approach presents a valuable and sensitive tool for benzodiazepine monitoring in clinical and toxicological settings.

1. Introduction

Benzodiazepines are well-established pharmaceuticals used in treatment of wide range of illnesses such as anxiety disorders, seizures, insomnia, and muscle spasms [1]. Although their intake decreases, they are still some of the most commonly prescribed medicines in high-income countries [2]. Through binding with GABA receptor, benzodiazepines enhance the calming effect of a γ-aminobutyric acid (GABA) on the central nervous system, resulting in sedative, somnifacient, anxiolytic, anticonvulsant, diastolic, and muscle relaxant effect on the organism. Aside from their therapeutic effect, benzodiazepines are also referred to as some of the so called "date rape drugs" - substances used to facilitate sexual assaults [3]. The therapeutic and illegal use of benzodiazepines, as well as their release into the environment, necessitates the continuous development of analytical methods leading to their identification and quantification in the human body.

To date, benzodiazepines are most commonly determined analytically with a use of liquid chromatography and gas chromatography with various detection methods, especially mass spectrometry. However, with each year novel analytical techniques, such as immunoassays, play more important role [4,5]. Novel sample preparation methods are being developed even more dynamically, including using non-conventional biological samples such as exhaled breath or vitreous humour [6]. Among more usual sample matrices, there is whole blood, serum, plasma, urine, and saliva [7]. The most widely used sample preparation methods used to determine benzodiazepines in biological matrices are liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [8] with its modifications, for example renowned solid-phase microextraction (SPME) [9] or less widely used pipette-tip micro-solid phase extraction (PT-μSPE) [10]. As sample preparation is a critical step preceding instrumental analysis, it is essential to develop protocols that are both instrument-compatible and environmentally friendly. 3D-printed sorbent devices for sample preparation, containing immobilized sorbent and produced in exact required amount, adjusted in size and shape to the needs of a certain analysis, are some of the novel sample preparation tools worth further observation [11,12].

3D printing is a well-known manufacturing technology, which is also continuously evolving, especially in the fields of improvement of printing parameters and creating novel materials. It is a common name for a number of different additive manufacturing technologies, characterized with completely distinct materials and properties of manufactured objects. Their wide application in analytical chemistry includes, among others, creating sensors [13], membranes [14], sorbent devices [15], systems facilitating certain sample preparation techniques [16], and microfluidic devices [17]. Some of the applications of 3D printing in combination with determination of benzodiazepines include electrochemical devices assembled on a reusable 3D-printed holder [18] and wearable electronic finger [19], both applied for beverage screening.

One particularly interesting field related to 3D printing in analytical chemistry is the development of extraction devices. Novel materials, some including biomass, can be utilized to create these devices [20]. They can be used to clean up samples, limit contamination, and recover target analytes, even at low concentrations [21,22]. 3D-printed sorbent devices have broad applications in the determination of drugs and biomarkers in various sample matrices [23–25].

Authors' field of interest is focused primarily on porous materials suitable for pharmaceutical analysis and analytical chemistry applications [26–28]. Recently a 3D-printable thermoplastic material consisting of polypropylene (PP), acrylonitrile-butadiene-styrene (ABS) and C18-functionalized silica was developed and characterized [29]. This study is a direct continuation of the previous work, focusing on the evaluation of a novel material for the extraction of benzodiazepines from human serum. The sorbent devices are manufactured with a use of a fused deposition modeling (FDM) 3D printer. The size and geometry of the sorbent device, as well as parameters are carefully optimized and discussed. The validation study for quantification in human serum is performed.

2. Materials and methods

2.1. Chemicals

Polypropylene (PP, product No.: PP306320) was purchased from Goodfellow Cambridge Ltd. (Huntingdon, UK) in the form of 3 mm granules, whereas the acrylonitrile−butadiene−styrene (ABS) pellet was provided by Felfil (Turin, Italy). The applied polymers have a density of 0.90 and 1.04 g/cm3, respectively. Octyldecyl-functionalized silica gel 9−13 % carbon loading (product No.: 553522; LOT: MKCQ2192) was from Merck (Darmstadt, Germany). The analytes (chlordiazepoxide, alprazolam, diazepam, lorazepam, prazepam, flurazepam, estazolam, temazepam, halazepam, demoxepam, potassium clorazepate) as well as internal standards (D-chlordiazepoxide, D-alprazolam, D-temazepam) in methanol were obtained from LGC Standards Ltd (Teddington, United Kingdom). Human serum (product number H3667), acetone, methanol, acetonitrile, isopropanol, and dichloromethane were provided by Merck, and water was purified by Merck−Millipore to obtain 18 MΩ deionized water.

2.2. Optimization of LC-MS method with DryLab

Quantitative analysis was performed on Agilent Technologies (Santa Clara, CA, USA) liquid chromatograph (model 1260) coupled with a single quadrupole mass spectrometer (model 6120). The process was monitored with Agilent ChemStation software. The Poroshell EC-C18 column (3 mm × 100 mm; 2.7 μm) was obtained from Agilent Technologies (Santa Clara, CA, USA). Conditions of the ion source were as follows: a nebulizer pressure of 50 psig, a drying gas feed of 10 L/min (N_2) at a temperature of 350 °C, a capillary voltage of 3.0 kV, and a fragmentor voltage of 150 V. The *m/z* values of the analytes are listed in Table 1. Mobile phases A: deionized water with 0.1 % of formic acid and B: acetonitrile with 0.1 % of formic acid were used.

LC-MS method allowing separation of 11 analytes with similar structure was optimized with assistance of Dry Lab software (Molnár-Institute, version 3.9.0). Four separate analytical runs with 5–100 % phase B gradient (15 min and 40 °C; 30 min and 40 °C; 15 min and 50 °C; 30 min and 50 °C) provided input data to model optimal gradient length and temperature.

Table 1
List of analytes with corresponding internal standards, *m/z* value and retention time.

Analyte	Internal standard	*m/z*	Retention time [min]
chlordiazepoxide	D-temazepam	300	11.24
alprazolam	D-alprazolam	309	17.63
pinazepam	D-temazepam	309	22.77
diazepam	D-temazepam	285	20.06
prazepam	–	325	24.82
flurazepam	D-chlordiazepoxide	388	13.96
estazolam	D-chlordiazepoxide	295	16.81
temazepam	D-temazepam	301	18.76
halazepam	–	353	25.01
demoxepam	D-chlordiazepoxide	287	14.93
potassium clorazepate	D-temazepam	271	17.17

In the final method phase B linearly increased from 5 % to 55.4 % over the first 24.5 min. Subsequently, phase B was rapidly increased to 100 % and held for 3 min before equilibrating back to 5 % phase B. The total analysis time was 34 min at 47 °C and a flow rate of 0.5 mL/min, using an injection volume of 5 μL.

2.3. Fabrication of extraction devices

2.3.1. Composite material

The composite material used to 3D print the extraction devices has been comprehensively described in the previous work [29]. Briefly, a 3D-printable thermoplastic material consisting of polypropylene (PP), acrylonitrile-butadiene-styrene (ABS) and C18-functionalized silica was designed, compounded, and assessed. Activation (rinsing with acetone to eliminate ABS) creates porosity and enables access to incorporated silica particles and enhances active surface.

2.3.2. CAD model of extraction devices and 3D printing

External shape of the model was prepared using Autodesk Tinkercad online software (San Francisco, CA, USA) and its internal structure was obtained through choice of gyroid fill pattern in PrusaSlicer (version 2.7.1) during slicing. Other significant slicing parameters included 50 % infill density, layer height of 0.2 mm, extrusion width of 0.4 mm, no perimeter or solid bottom/top layers, printing temperature of 230 °C, print bed temperature of 50 °C, fan speed of 100 %, and 2 mm of retraction. Sorbent devices were printed on a ZMorph 3D printer (ZMorph 2.0S, Wroclaw, Poland). The print bed was covered with polypropylene tape to ensure sufficient adhesion.

3D-printed sorbent devices were then activated through rinsing in acetone, dried, and stored in a moisture-proof container until they were taken out directly for analysis.

2.4. Optimization of extraction

All tests were performed in triplicates unless stated otherwise. For all the tests until examination of kinetics, 45 min of sorption and 30 min of desorption were applied and methanol was used as desorption solvent.

2.4.1. Matrix modification

Firstly, the relationship between dilution of the sample and extraction efficiency was assessed. Samples consisting of 500 μL of serum spiked with analytes were compared to those consisting of 250 μL of serum spiked with analytes and 250 μL of water. Total concentration of analytes in both options was 100 ng/mL. In all further experiments, concentration of 50 ng/mL and addition of 0.1 % of formic acid to the matrix were applied. Secondly, addition of methanol in amounts of 0, 5, 10, and 15 % was examined.

2.4.2. Extraction parameters

The most suitable solvent for desorption was selected from methanol, isopropanol, acetonitrile, and dichloromethane, as the most commonly used solvents for solid-phase extraction of benzodiazepines.

To evaluate kinetics of sorption, an initial desorption time of 30 min was retained and sorption times varying from 10 to 60 min with 10 min intervals were applied. Highest peak intensity and lowest relative standard deviation within 15 % were the criteria of choice.

For assessment of kinetics of desorption, the optimal sorption time was applied. Desorption was evaluated in the time range varying from 5 min to 45 min with 10 min intervals. Again, highest peak intensity and lowest relative standard deviation within 15 % were the criteria of choice.

2.4.3. Final extraction protocol

Extraction was preceded with activated and dried sorbent devices being shaken in methanol for 10 min in order to expand the silica's carbon chains.

During optimization and validation, the samples consisted of: 250 μL of human serum spiked with mixture of 11 analytes in concentration of 100 ng/mL each and 250 μL water with 20 % of methanol and 0.2 % of formic acid. Ultimately, analyzed samples were 500 μL at concentration of 50 ng/mL with 10 % addition of methanol and 0.1 % of formic acid.

Sorption and desorption steps were performed on a standard laboratory shaker at 360 RPM. Sorption time was 50 min, after which the devices were briefly rinsed with water, and dried from excess moisture with paper towel. Desorption time was 20 min and 500 μL of methanol was used as desorption solvent. Afterwards, the samples were directly injected to LC-MS.

2.5. Validation

Final extraction protocol with all the optimized parameters was applied. Validation was performed according to ICH guideline M10 on bioanalytical method validation and study sample analysis [30]. Peak areas of the analytes were subsequently divided by the values of the proper internal standards added to each sample in concentration of 500 ng/mL. All samples were spiked serum processed with full sample preparation protocol.

Analytical run included extracts obtained from an un-spiked sample, a sample without analytes but containing internal standards (zero sample), calibration samples in concentrations listed below, and QCs necessary to assess precision and accuracy of the method.

Method's linearity was assessed in the range from 5 ng/mL to 1000 ng/mL. Evaluated extracts were obtained according to the final extraction protocol and analyzed with an optimized chromatographic method. Following concentrations were assessed: 5, 10, 50, 75, 100, 250, 400, 600, 800, and 1000 ng/mL.

In accordance with ICH guideline M10, precision and accuracy were assessed with a use of four concentration levels: 5 ng/mL (LLOQ), 15 ng/mL (low QC), 350 ng/mL (medium QC), and 850 ng/mL (high QC). Parameters were assessed within-run (n = 5) and between-run (n = 15), in total of three runs over at least two days, each QC with five replicates at each run. Precision was assessed with the values of relative standard deviation. Accuracy was determined by calculating the concentration using an appropriate regression equation for the analyte and comparing the result to the nominal concentration.

3. Results and discussion

3.1. Extraction devices

The devices were printed from a new batch of a composite containing 15 % w/w of C18 functionalized silica and 85 % of polymers mixed in a 1:2.5 ratio (ABS/PP w/w). Activation procedure of rinsing ABS with acetone created porosity and access to the C18 silica particles incorporated in the polymer matrix. C18 silica is the most popular sorbent used in pharmaceutical analysis and has been proven suitable for extraction of benzodiazepines in numerous articles [31–33] as well as indicated by United Nations Office on Drugs and Crime in 1997 [34].

Shape of the devices was designed in accordance to internal dimensions of a 1.5 mL Eppendorf tube to perfectly fit its pointy bottom (Fig. 1). The length of the model was adjusted so that 300–500 μL of the sample could fully cover the device. Lack of the solid layers and solid perimeters resulted in a characteristic corrugated structure increasing the active surface and allowing penetration of the liquid into the device from all directions. The gyroid infill structure allowed the liquid to flow through the inside of the device during shaking. Overall, the structure and shape were designed to increase the contact surface between the liquid sample and the sorbent device.

3.2. Optimization of LC-MS method with DryLab

Entry data is presented in Table S1, while DryLab Resolution Map is

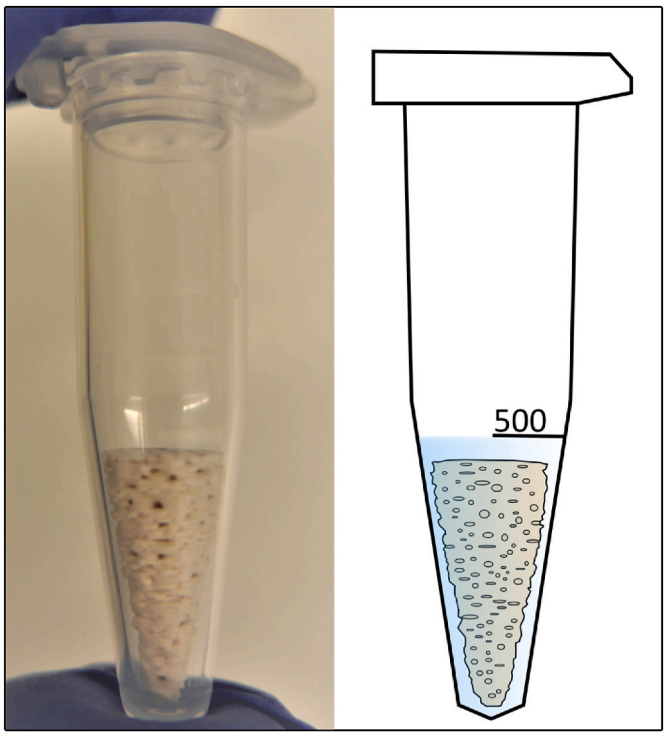

Fig. 1. Sorbent device placed in a 1.5 mL test tube.

available in Fig. S2. Comparison of the DryLab chromatogram with the actual chromatogram from the analytical run performed according to the predicted conditions can be found in Fig. 2. Correlation coefficient R^2 between predicted and experimental retention times was 0.9989. Exemplary chromatograms can be found in Fig. S1.

The modeled method employed gradient elution at 47 °C with a flow rate of 0.5 mL/min, initiating with 5 % phase B and linearly increasing to 100 % in 42 min. As all analytes were eluted within 24 min of this method, the gradient duration was reduced to 25 min while keeping the original slope of the curve.

3.3. Optimization of extraction

In assessment of the effect of matrix modification on extraction efficiency the results indicated the diluted serum was a better choice (Fig. 3-A). Higher viscosity of the sample weakened liquid penetration into the sorbent. Outcome of this experiment reinforced the intention to use as little biological material as possible making it a total of 250 μL of human serum per one sample. 100 ng/mL was chosen due to the concentrations of analyzed compounds occurring in human serum, which for most of them vary from 10 to 300 ng/mL.

Besides of the human serum spiked with analytes and water used for dilution, formic acid and methanol were added as matrix modifiers. 0.1 % of formic acid was added to enhance ionic strength and fix pH of the solution and hence promote sorption of the analytes to the device. Addition of methanol to the sample was supposed to prevent collapsing of the carbon chains decorating silica particles. It turned out that 10 % addition of methanol results in the highest analytical signals compared to the rest of the examined samples (Fig. 3-B). It is worth mentioning that developed procedure did not involve protein precipitation and addition of 10 % of methanol didn't result in any visible changes in the structure of the sample.

When it comes to the most suitable desorption solvent for the developed extraction protocol, although dichloromethane was found to be associated with the highest analytical signal, the relative standard deviation and environmental toxicity aspects determined the selection of methanol as the second best, presenting a lower RSD, and considered a green solvent (Fig. 3-C).

Kinetics of sorption and desorption aimed to determine the most stable and repeatable time for both processes. For sorption, 50 min and 60 min resulted in similar intensity of peaks, however, lower RSD was associated with 50 min, hence it was chosen as an optimal option. The results are summed up in Fig. 4-A. A graph presenting kinetics of desorption is visible in Fig. 4-B. Time-efficiency determined the optimal desorption time, as 15 min and 45 min resulted in similar peak intensity and comparable RSD. Since the desorption kinetics graph peaked at 15 min, 20 min was chosen for the final extraction protocol. This time point resides in a flattened region of the graph, yet close to the peak.

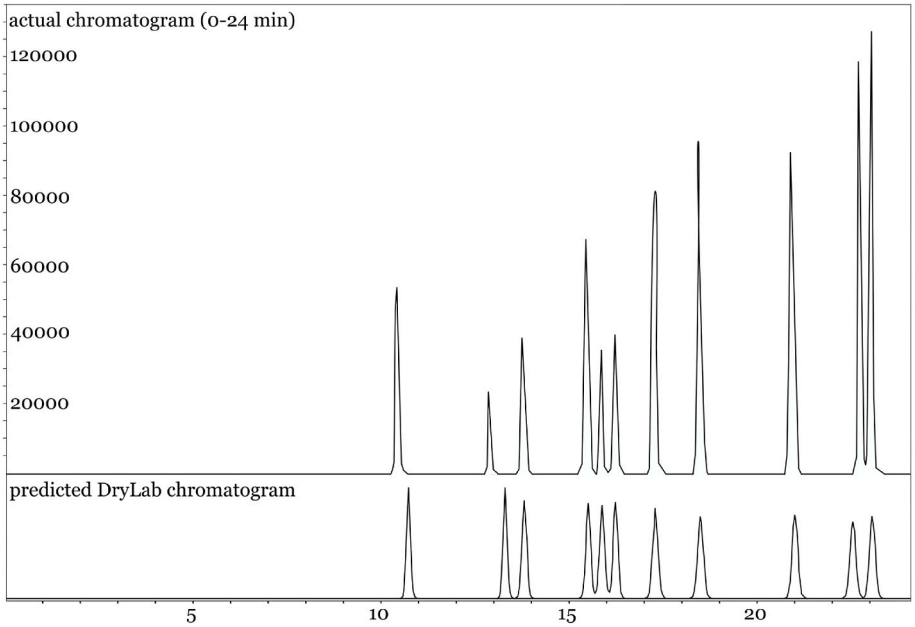

Fig. 2. Comparison of the DryLab chromatogram to the actual chromatogram from the analytical run performed according to the conditions indicated by the software.

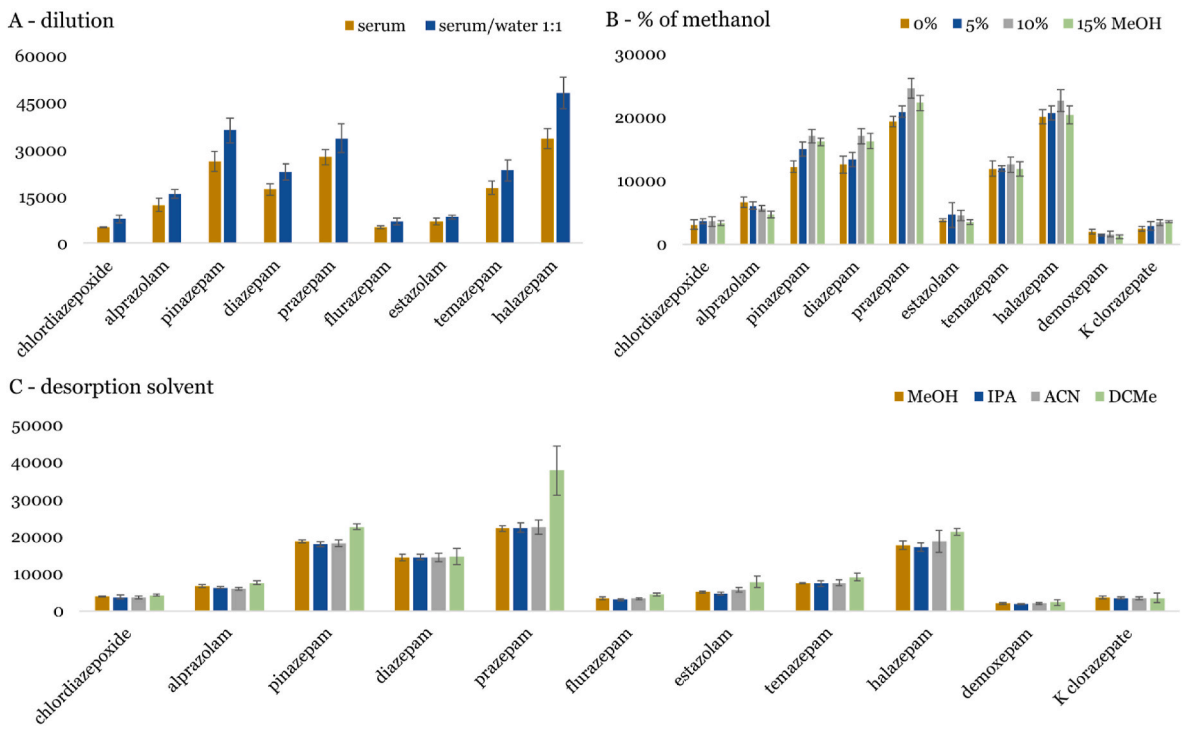

Fig. 3. A – assessment of the effect of matrix dilution; B – evaluation of the optimal amount of methanol as a matrix modifier; C – choice of the most suitable desorption solvent based on signal intensity and standard deviation.

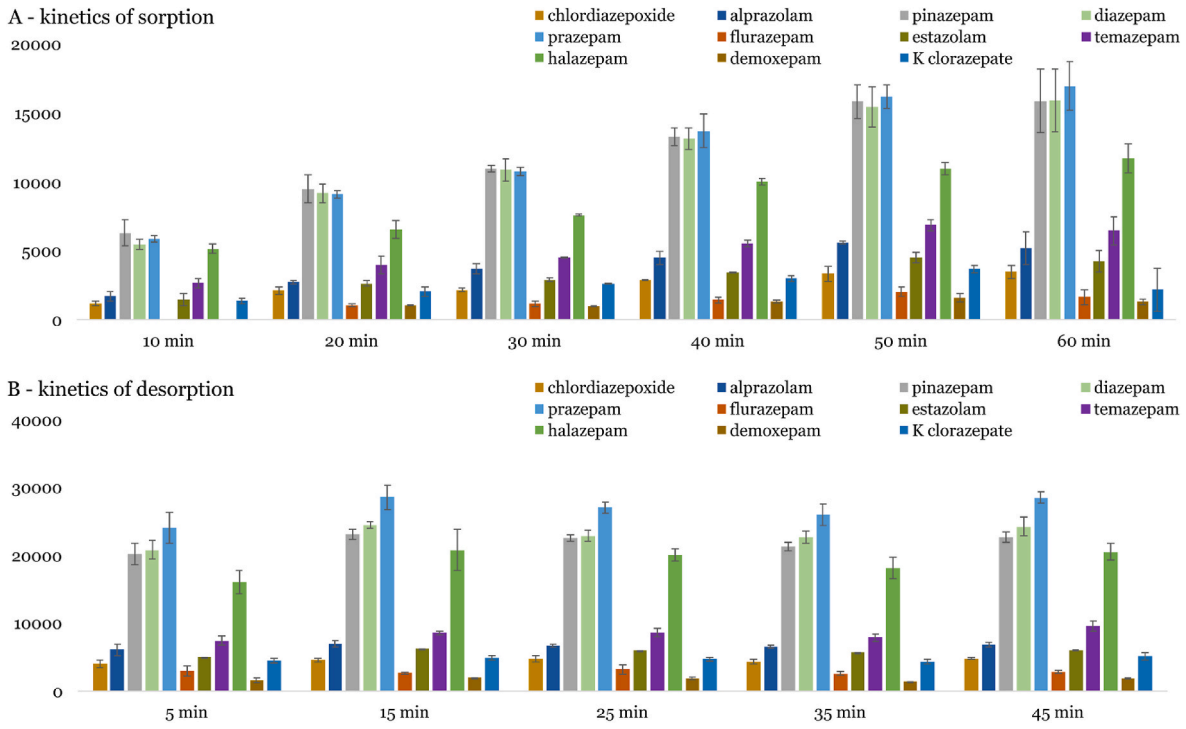

Fig. 4. Kinetics of sorption and desorption presented with peak intensities and respective standard deviation.

3.4. Validation

A blank sample (without analytes and internal standards) showed no signals in analyzed retention times and a zero sample (containing internal standards but not analytes) only presented expected signals.

Based on the results from the extraction of 5, 10, 50, 75, 100, 250, 400, 600, 800, and 1000 ng/mL divided by the area of a respective internal standard, a regression equation for each analyte was obtained. The most suitable regression, due to the wide range of the concentrations, turned out to be a linear regression using $1/x^2$ weighting factor. Regression equations together with correlation coefficients are summed up in Table S2.

Precision and accuracy were considered acceptable when they did not exceed 20 % for LLOQ and 15 % for the remaining QCs. Examined

parameters were acceptable for all the analyzed analytes and concentrations. Detailed data is available in Table 2.

3.5. Method comparison

Similar analytical protocol including determination of six common examined analytes in serum, however utilizing SPE on HLB cartridge as sample preparation [35], also reported 25 min gradient. Despite requiring additional sample preparation steps, including evaporation and reconstitution, this method achieved lower LOQs for five out of six common analytes analyzed also in the current study.

Another relevant study employed 96-blade SPME for extraction of benzodiazepines from human plasma [36]. This method required a total extraction time of 140 min, compared to the 70 min reported in our study. While the 96-blade format allows for simultaneous extraction of 96 samples, this is not possible with the current geometry of 3D-printed sorbent. From the practical point of view it is possible to process 32 samples for a single analytical run without compromising the quality. For diazepam, the current study demonstrated significantly lower inter-day RSD (3.95 %) than the reported 8.3 % at comparable concentrations.

A study by Tomomi Ishida et al. analyzed 43 compounds, including six shared analytes, using LC-MS and a small particle amide-functionalized column [37]. Although requiring four times the plasma volume compared to the current serum-based method, precision was comparable, with a slight advantage for the presented study.

Compared to the abovementioned methods, presented extraction protocol is distinguished by good precision, time-efficiency, small volume of serum used, suitable LLOQ, and no need for evaporation of solvent and reconstitution, which makes it green chemistry compliant.

4. Conclusions

The presented study introduced a novel and efficient sample preparation method for extracting benzodiazepines from human serum. Together with a new application for a thermoplastic composite material with incorporated C18 silica particles, a novel sorbent device, functional in shape and structure, was presented. Proposed sorbent device enabled consistent and reproducible solid-phase microextraction (SPME) of a wide range of analytes compatible with C18-functionalized silica. Low

Table 2
Precision and accuracy calculated for each analyte.

Analyte	Concentration [ng/mL]	Intra-day precision [%] n = 5	Intra-day accuracy [%] n = 5	Inter-day precision [%] n = 15	Inter-day accuracy [%] n = 15
alprazolam	5	10.26	3.48	16.24	17.49
	15	1.70	1.64	12.17	3.01
	350	4.37	4.92	14.68	1.21
	850	4.45	13.04	13.57	10.86
chlordiazepoxide	5	8.62	2.20	16.35	12.00
	15	2.00	3.63	12.25	2.75
	350	4.74	6.57	12.13	11.51
	850	1.83	12.21	10.52	12.60
demoxepam	5	3.32	6.26	19.18	10.93
	15	1.25	8.03	11.05	14.58
	350	1.40	4.19	14.78	8.79
	850	2.18	0.16	11.85	4.35
diazepam	5	7.06	0.53	13.93	6.86
	15	1.44	4.71	10.73	5.66
	350	3.95	4.21	12.88	6.92
	850	4.20	14.61	13.40	10.21
estazolam	5	4.64	10.75	13.31	12.99
	15	0.65	10.66	14.21	8.45
	350	4.06	1.12	14.05	3.81
	850	3.80	14.34	9.87	10.56
flurazepam	5	6.36	3.37	17.35	2.24
	15	2.31	6.08	12.54	8.62
	350	8.97	0.10	13.73	1.77
	850	2.39	9.36	14.16	8.45
halazepam	5	1.84	13.11	17.19	13.08
	15	1.43	14.03	13.95	2.68
	350	3.47	12.14	13.37	13.86
	850	5.99	14.92	14.55	1.52
K clorazepate	5	4.91	19.06	17.96	14.81
	15	3.73	4.53	14.24	5.40
	350	3.50	1.08	14.57	2.60
	850	3.46	13.48	9.20	11.96
pinazepam	5	5.04	1.55	12.49	8.56
	15	1.34	3.36	14.76	3.27
	350	3.41	3.97	13.50	1.53
	850	5.31	12.74	11.60	14.81
prazepam	5	6.05	16.51	13.95	15.82
	15	2.89	14.61	14.05	10.45
	350	2.47	14.20	13.05	5.74
	850	5.64	7.38	13.35	3.59
temazepam	5	5.17	2.72	15.91	19.02
	15	1.90	1.85	9.26	3.55
	350	8.28	0.40	14.62	3.96
	850	1.79	10.79	8.34	8.19

relative standard deviation of the examined samples demonstrated satisfactory repeatability of both the extraction protocol and the 3D printing process. Application of additive manufacturing in the presented work allowed us to achieve gyroid internal structure of the devices, which would not be possible with the use of another technology, such as cast molding or micromachining. Versatility of the 3D printing technology for creating custom-designed sorbent devices tailored to different analytes and matrices made this demonstration valuable example of novelty in pharmaceutical analysis and analytical chemistry.

The sample preparation protocol presented multiple advantages, including no protein precipitation, utilization of low amounts of biological sample, low consumption of organic solvents, no need for special laboratory equipment, and self-efficiency with 3D printing on demand. Accuracy and precision met the criteria outlined in the ICH guideline M10 for all analytes.

To complement the extraction method, an optimized chromatographic method was developed using a DryLab in silico model. This method effectively separated fourteen benzodiazepine compounds, including eleven analytes and three internal standards, within a 25-min runtime. The developed chromatographic method may be used in clinical and toxicological applications involving monitoring of benzodiazepines.

CRediT authorship contribution statement

Dagmara Kroll: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Szymon Ulenberg:** Writing – review & editing. **Paweł Georgiev:** Writing – review & editing, Investigation. **Bartosz Marciniak:** Writing – review & editing, Investigation. **Gert Desmet:** Writing – review & editing. **Tomasz Bączek:** Writing – review & editing, Resources. **Mariusz Belka:** Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.343552.

Data availability

Data will be made available on request.

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