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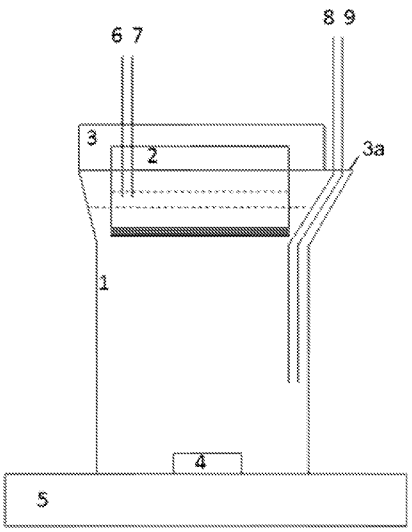
(54) **Title:** MODULE FOR ON-LINE MONITORING OF PERMEATION TESTS

FIG. 2



(57) **Abstract:** The invention provides a module for an on-line permeation test monitoring system, comprising an assembly of two plastic containers, namely a larger main container for culture medium, into which a smaller container forming a cell cultivation insert, filled on at least one side of its inner surface by a continuous cell monolayer, is inserted. The smaller container forms a donor compartment and the interspace between the inserted smaller container and the larger container defines an acceptor compartment. A marker and/or tested substance are added into the donor compartment or the acceptor compartment. The main container further comprises a lid for closing the main container, the lid is provided with at least one inlet and outlet channel for the donor compartment and at least one inlet and outlet channel for the acceptor compartment. Preferably, the plastic module is the 3D-printed module.

WO 2021/185394 A1

Module for on-line monitoring of permeation tests

FIELD OF TECHNOLOGY

The present invention relates to the area of analytical chemistry with application in the field of pharmacology and toxicology. In particular, it relates to the real-time monitoring of the course of the permeation tests. The invention will allow detailed monitoring of the interaction of tested exogenous substances with membrane transporters, which protect cells from the entry of these substances. The present invention uses a plastic module, preferably a 3D-printed module, with an insert for cultivating the epithelial cells in a monolayer together with an automated flow analysis technique based on a closed system, in which automatic sampling from the donor and acceptor compartments of the plastic module, preferably 3D-printed module, takes place, and at the same time on-line determination of the fluorescent marker that enters cells and crosses the cell monolayer. The content of the marker in both compartments then corresponds to the activation or inhibition of a given membrane transporter.

BACKGROUND OF THE INVENTION

Currently, permeation tests are performed in commercially available cultivation plates with inserts, on which tested cell lines, usually genetically modified to contain a membrane transporter under study, are cultivated. Both compartments can be monitored, but the analysis of the substances is carried out repeatably in a few rather long-time intervals, e.g., 1 hour period in a test lasting 2-4 hours. Hence, it is not possible to monitor the interaction of the substances with membrane transporters in detail. However, only a difference between the final concentration of the fluorescence marker in a given compartment is evaluated.

Rhodamine 123 (Rho123) is most often used as a marker of the P-glycoprotein transporter (P-gp), the most important transporter of the so-called drug transporters, which is also used in the testing of the 3D-printed module. The transport of said marker is monitored across a cell monolayer cultivated on the polycarbonate insert, which is also used for testing in the case of the present invention - a plastic module, preferably a 3D-printed module. The active passage

of the marker enabled by the membrane transporter into the cell or across the cell monolayer then corresponds to the inhibition of the P-gp transporter, which protects the cells from entering of the exogenous substances.

Permeation tests in this arrangement serve to study the interactions of the exogenous substances with P-gp transporter, which, when active, does not allow Rho123 transfer into the cell and, on the contrary, keeps it out of the cell. In the case of an interaction with a substance that is an inhibitor of the P-gp transporter, Rho123 penetrates into the cell and possibly across the entire cell monolayer. This process can be monitored due to the difference in concentration of Rho123 in the donor compartment - where in the case of the inhibition of the P-gp transporter its content decreases. When the test substance is P-gp substrate, then Rho123 (also a substrate) competes for interaction with P-gp transporter and usually does not pass into the cell then its content in the donor compartment remains unchanged even after the permeation test termination.

Monitoring of the progress of the permeation test in connection with an automated flow technique, in particular sequential injection analysis, was performed by the inventors of the present invention in the previous step. For monitoring, automatic dosing from the acceptor compartment of the so-called Franz cell was employed. Franz cell is used to test a release of the active substances from semi-solid pharmaceutical formulations of the ointment and gel types. The on-line determination of Rho123 was also performed in a flow system. Thus, a principle of permeation test monitoring was preliminarily tested out, which identified a number of drawbacks, such as a small volume of the donor compartment, a large volume of the acceptor compartment, inability to dose from the donor compartment, and consequently incomplete information on Rho123 transport into the cells. [Zelená L., Marques S., Segundo M., Miro M., Pávek P., Sklenářová H., Solich P., Anal. Bioanal. Chem. 408 (2016) 971-981]. A similar arrangement was disclosed in the U.S. Patent Application No. 005198109A, which describes the use of a diffusion cell very similar to Franz cell, with special stirring based on a spiral coil. This diffusion cell was used to monitor the kinetics of the active substance permeation across the skin. In this case, the invention was based on special stirring and also on modified sampling from the acceptor compartment.

Another similar arrangement was used in the case of a microfluidic platform for monitoring a passage of the substances across the skin in a modified Franz cell, in which it was possible to continuously monitor a content of the active substance in the acceptor compartment with the flowing acceptor solution. [Alberti M., Dancik Y., Sriram G., Wu B., Teo Y.L., Feng Z., Bigliardi-Qi M., Wu R.G., Wang Z.P., Bigliardi P.L., Lab Chip 17 (2017) 1625-1634].

Definitions of the terms

A compartment is generally a part of a whole, partially or wholly separated, eventually a part of a whole with a set of certain specific features, particularly with a clear external boundary that selectively influences the exchange of the substances between external and internal environment.

For the purposes of the present invention, said boundary is given by the presence of the cell monolayer selectively affecting the exchange of the substances.

An insert is a commercially available component that is used for cultivating cells in the form of the monolayer - it is a plastic component with a membrane of a given material and with specific porosity.

Epithelial cells are a cell type that can be cultivated in the form of a monolayer. Epithelial tissue (epithelium, covering tissue) is a tissue formed by the cells which are adjoined tightly, and thus a passage across the paracellular spaces being very restricted.

SUMMARY OF THE INVENTION

The key subject-matter of the invention is an arrangement according to the invention for on-line monitoring of permeation tests, which is based on the plastic module, preferably on the 3D-printed module, intended for connection to a flow system.

Another subject-matter of the invention is a flow system for on-line monitoring of permeation tests where the plastic module is incorporated.

According to the invention, it provides a module for a system for on-line monitoring of permeation tests comprising an assembly of two plastic containers, a larger main container for a culture medium, into which a smaller container forming a cell cultivation insert is incorporated, wherein the smaller container is covered on at least one side of its inner surface with a continuous cell monolayer, and wherein smaller container forms a donor compartment and the space between the inserted smaller container, and larger container defines an acceptor compartment. A marker and/or tested substance are added into the donor compartment or the acceptor compartment. The main container further comprises a lid for enclosing the main container. The lid is provided with at least one channel for an inlet and an outlet for the donor compartment and at least one channel for an inlet and an outlet for the acceptor compartment, wherein the channels being simultaneously adapted to ensure a connection of these compartments to external flow system.

The advantage according to the invention is the module which further comprises a collar enclosing circumferentially the main container, the collar comprising at least one channel for an inlet and an outlet for the acceptor compartment instead of at least one channel in the lid for its connection to the external flow system.

A preferred embodiment of the plastic module is the 3D-printed module.

According to the invention, any kind of plastic that is compatible with living cells can be used as a material for the module, which means the material compatible with living cells in the meaning that it does not adversely affect living cells or does not adsorb lipophilic substances.

Preferably, the material is polyacrylate- or polycarbonate-based resin, most preferably in the form of the filament.

The 3D-printed module, according to the invention, is adapted for monitoring both donor and acceptor compartments and allows an insertion of the commercially available insert, on which the cells are routinely cultivated in the form of the monolayer for permeation tests in commercial plates.

Construction of the lid, namely a shape of the lid of the 3D-printed module according to the invention, prevents evaporation and at the same time prevents condensation of the evaporated culture medium in contrast to state of the art represented by the so-called Franz cell, including its structural modifications.

Commonly performed tests do not allow the high frequency of sampling. Consequently, the tests are based on only a few concentration points, which are obtained by manual sampling and off-line determination. The 3D-printed module allows the sampling with a minimum dead volume at a pre-programmed period, on-line and fully automatic due to the aspiration of the samples directly into the flow system.

The term dead volume, according to the invention, means the volume at the site of connection of the inlets and outlets in the lid and/or the collar of the module, defined by both the donor and the acceptor compartments. At the connection site, a larger space is created, which cannot be sufficiently rinsed/washed by the dispensed liquid. The minimization of the volume in this connection according to the present invention thus results in a reduction in the dead volume, which means that rinsing is fast, efficient, and very short.

The 3D-printed module also allows the temperature control that is necessary for the test. Further, it allows a mixing of the internal contents of the module - by means of a magnetic stirrer in the acceptor compartment and in both compartments by means of the flow of donor/acceptor medium by a continuous flow driven by a peristaltic pump in a closed loop. It is then possible to sample into the flow system and on-line determine the monitored substance in both loops. Consequently, it is possible to obtain a very detailed kinetic profile of the passage of tested substance across the cell membrane, most often for testing various membrane transporters and their interaction with exogenous substances.

The 3D-printed module, according to the invention, differs from the commercial solution (i.e., cultivation inserts on cultivation plates) by the possibility of on-line monitoring. It is similar to the so-called Franz cell, which was used in the past for on-line monitoring of liberation tests to monitor a release of the active substance from semi-solid pharmaceutical formulations. It was also used for a pilot study of the automation of the permeation test. The drawbacks of this arrangement have been eliminated, and the new 3D-printed module,

according to the invention, differs not only in internal volume and the size of commercial inserts used but mainly in sampling options from both compartments, which is essential for monitoring of the profile of given substance in either compartment or the difference of which allows determining a concentration of a given substance which is located inside the cells. These are the fundamental advantages of the invention over the prior art.

Another preferred embodiment of the 3D-printed module according to the invention is the module that comprises a separate inlet and outlet from both the donor and acceptor compartments by means of channels in the lid and/or in the collar for connection to the external flow system. An advantage is therefore detailed monitoring of the interaction of exogenous substances with the membrane transporter in real time and on the base of the kinetic profile of fluorescent marker content in either compartment or thus also inside the cells of the tested cell line. The system in which the 3D-printed module is integrated allows the automatic implementation of the entire test with sampling at pre-programmed intervals and on-line determination of Rho123. In the course of the entire test, the system is closed, thus eliminating the risk of cell contamination, the risk of human error in sampling during their off-line determination, and the risk of an operator exposition to the effect of exogenous substances. There is also no risk of breaking the cell monolayer during sampling and contaminating the solutions in both compartments.

Another advantage according to the invention is the possibility of using a commercially available sequential injection analysis system (FIG. 4), to which the 3D-printed module can be connected, and monitoring of permeation test under common laboratory conditions can be performed using an incubator for setting test temperature (37 °C), a stirrer for mixing the content of the acceptor compartment, a peristaltic pump for mixing of the content of closed donor and acceptor loops and a fluorescence detector for determination of Rho123. Only the 3D-printed module is a made-for-purpose device.

A drawback may be integrating only a small number of permeation units (max. 3) to one flow system and thus the parallel carrying out of only 3 tests. A limitation is time-shifted sampling into one system, which is limited by the time of one analysis, usually 3-5 min. Typically, 6-12

parallel tests can be carried out within the permeation test in the plates with inserts, however, at the expense of the limited number of the analyses of a fluorescent substrate.

The plastic module is preferably attached to a holder from below in a predetermined position and orientation.

An economically advantageous and important aspect of the invention is the possibility to easily redesign the spatial arrangement (construction) of the 3D-printed module, as shown for example in FIG. 3A, 3B. Therefore, easily repeatable production of the module using 3D printing is possible.

Another subject-matter of the invention is a flow system for on-line monitoring of permeation tests, as demonstrated in FIG. 3, which includes:

thermostated incubator/water bath (10);
a magnetic stirrer (5);
a pump (11), preferably a peristaltic pump, for mixing of the content using closed donor and acceptor loop;
a sequential injection analyzer (12) for monitoring of permeation test under common laboratory conditions;
multiport selection valve (13);
a piston pump (14) for pumping distilled water;
a fluorescence detector (15) for determining a fluorescence marker;
a computer (16) with a control program;
a distilled water reservoir (17) for washing the entire system;
a culture medium reservoir (18) for replenishing of the plastic module compartments with the medium;
a waste (19) for draining the culture medium after washing the plastic module;
wherein the flow system further comprises a plastic module (2), preferably 3D-printed module (2), with a connection to the peristaltic pump (11), more preferably in the thermostated incubator/water bath (10) with the magnetic stirrer (5).

One of the preferred embodiments is that in the flow system, at least two plastic modules, more preferably two 3D-printed modules, are integrated.

Procedure for monitoring of the permeation test

1. Stabilization of conditions – a temperature of the culture medium in the 3D-printed module is maintained by placing it in an incubator with a set temperature (37 °C). Alternatively, a water bath with the same temperature can be used

2. Placing the insert with cell monolayer into the 3D-printed module

3. Application of Rho123 solution with or without additional tested substance into the donor compartment

4. Initiation of the test - the first sampling from the donor compartment takes place after washing a sampling tube with a small amount of culture medium circulating in it and releasing it to the waste. This is followed by aspiration of the sample into flow system and fluorescent detection of Rho123 (twice) with replenishment of the volume in given dosing loop with pure culture medium.

5. Delay between sampling - the setting of the time intervals to 5, eventually 10 min (according to test requirements)

6. Further sampling from the acceptor compartment - same procedure as for the donor compartment

7. Completion of the test after 2 hours - sufficiently long time to monitor the interaction of a tested substance with P-gp transporter. Longer test duration is also possible depending on the type of cell line tested.

8. Washing of the 3D-printed module and both sampling loops with pure culture medium

Processing of the obtained data

To determine Rho123 content correctly, it is necessary to calculate a dilution of the content of the sampling loop with pure culture medium to maintain a constant volume of both loops/compartments - it proceeds according to formula 1.1. which is used to monitor a penetration of active substance across the skin in liberation tests

Formula 1.1. - Calculation of Rho123 concentration

$C_{n,\,corrected} = C_{n,\,measured} +$ sample volume / loop volume $\times C_{n-1,\,measured}$

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Drawing of the 3D-printed module with a lid;

FIG. 2: Drawing of the 3D-printed module with lid and collar;

FIG. 3A, 3B: One particular embodiment of the 3D-printed module housed in a holder;

FIG. 4: Schematic view of the entire flow system with the 3D-printed module.

FIG. 5: Plot No. 1 - passive diffusion of Rho123 sample with a concentration of 50 µmol/L across the insert with polycarbonate membrane in 3D-printed module according to the invention

Plot No. 2 - passive diffusion of Rho123 sample with a concentration of 100 µmol/L across the insert with polycarbonate membrane in 3D-printed module according to the invention

FIG 6: Plot No. 3 - permeation test of Rho123 sample with a concentration of 50 µmol/L in 3D-printed module according to the invention

FIG. 7: Plot No. 4 - permeation test of Rho123 sample with a concentration of 20 µmol/L in 3D-printed module according to the invention

Plot No. 5 - permeation test of Rho123 sample with a concentration of 10 µmol/L in 3D-printed module according to the invention

FIG. 8: Plot No. 6 - interaction of Rho123 sample with a concentration of 10 µmol/L and verapamil with a concentration of 50 µmol/L with P-gp transporter in 3D-printed module according to the invention

Plot No. 7 - interaction of Rho123 sample with a concentration of 10 µmol/L and verapamil with a concentration of 100 µmol/L with P-gp transporter in 3D-printed module according to the invention

FIG. 9: Plot No. 8 - interaction of Rho123 sample with a concentration of 20 µmol/L and verapamil with a concentration of 50 µmol/L with P-gp transporter in 3D-printed module according to the invention

Plot No. 9 - interaction of Rho123 sample with a concentration of 50 µmol/L and verapamil with a concentration of 50 µmol/L with P-gp transporter in 3D-printed module according to the invention

EXAMPLES OF THE INVENTION

EXAMPLE 1

Monitoring of passive diffusion of Rho123 across the insert with polycarbonate membrane performed in the 3D-printed module according to the invention in the embodiment with the lid (see FIG. 1)

The lid of the 3D-printed module loosely closed the larger main container from above and the channels for separate inlet and outlet from donor and acceptor compartments, respectively, passed through it and, from the outside, the channels were connected to the flow system containing culture medium.

Testing of the 3D-printed module was carried out first without the cell monolayer, only with an empty polycarbonate insert. Thus, the system was optimized for sampling from both compartments. At the same time, the rate of passive diffusion was confirmed, which could

also occur if the permeation test conditions (temperature 37 °C, the composition of the culture medium) were not kept constant, which, consequently, would lead to disruption of the monolayer (tight connections between individual cells) or even cell necrosis. Therefore, it is necessary to monitor any deviations from the beginning of the test, which would clearly show a failure of the monolayer due to passive diffusion. During diffusion, Rho123 is rapidly transported across the polycarbonate membrane of the insert.

The test was carried out in the same manner as when the insert with cell monolayer was used. The volumes of culture medium in both compartments (4 and 9 ml) were also the same. Rho123 concentrations for this test were 100 and 50 µmol/L, and in both cases, the sampling was performed alternately from the donor and the acceptor compartment in 5 min intervals. Using 50 µmol/L Rho123, the concentrations in both compartments equilibrated after 45 min. In the case of a higher concentration, equilibration took 60 minutes, but this concentration was chosen to be extremely high and would pose a toxicity risk to living cells.
Due to the spatial arrangement of the 3D-printed module according to the invention, the dead volume inside the module was reduced, thus allowing the operation with a lower concentration of Rho123, which further led to reduced toxicity risk for tested cell lines.

Plots No. 1 and 2 (FIG. 5) demonstrate sampling points (as crosses) from the donor compartment and from the acceptor compartment (as circles).

EXAMPLE 2

Monitoring of the interaction of Rho123 with P-gp transporter (Rho123 as substrate) in 3D-printed module according to the embodiment with lid and collar (see FIG. 2)

The collar encircled the larger main container at a level below the lid, securing the lid to the main container with a hinge. However, it will be appreciated that an attachment of the lid may also be achieved by other ways commonly known in the art.
The lid closed the larger main container from above and the separate channels both for the inlet and outlet passed through it, however, only from the donor compartment.

The collar was provided with separate channels both for the inlet and outlet from the acceptor compartment.

From the outside, the channels in both the lid and the collar were connected to a flow system containing the culture medium.

The 3D-printed module was better handled by means of the collar, also due to separate inlets to the donor compartment (through the lid) or acceptor compartment (through the collar), in comparison to the embodiment without the collar, i.e., by connecting the flow system to 3D-printed module only through the lid itself.

The 3D-printed module was additionally attached to the holder from below (see FIG. 3A, 3B).

To monitor the permeation test with cell monolayer, the concentration of Rho123 was reduced to 50, 20, and 10 µmol/L. Thus, the interaction with P-gp transporter was ensured without a risk of toxicity manifestations of this substance. If the concentration were too high, the obtained profiles would show a passive diffusion, which would lead to a rapid equalization of the concentrations in both compartments. This phenomenon was not observed, so it can be confirmed that these concentrations are not toxic to the cells.

The implementation of the test was exactly the same as in Example 1, donor and acceptor compartment volumes of 4 and 9 ml were maintained, temperature 37 °C, and Opti-MEM culture medium was used, which ensured optimum conditions throughout the test. The sampling was carried out in 5 min intervals alternately from the donor and acceptor compartment. Each sampling was repeated twice, 30 µL at a flow rate of 50 µL/s. The data were evaluated as the average of these two consecutive determinations at the same time point and compared with a linear calibration equation in the range of 2.5 - 50 µmol/L. It was also verified that the culture medium does not contain any substances that would affect obtained fluorescence signal.

For all three concentrations of Rho123, no penetration of Rho123 into the cells or across the cell monolayer (into the acceptor compartment) was observed. The concentrations in this

compartment were below or just around the quantitation limit of Rho123 determination (2.5 µmol/L).

In plots No. 3, 4, and 5 (FIG. 6 and 7), the concentrations of Rho123 in the donor compartment are represented by crosses, and the concentrations in the acceptor compartment are represented by circles. A change in the total concentration of Rho123 results from diluting the tested solution in an amount of 3 mL into the insert with cell monolayer at a total volume of 4 mL in a dosing loop of the donor compartment.

EXAMPLE 3

Monitoring of the interaction of Rho123 and verapamil with P-gp transporter (verapamil as inhibitor) in the 3D-printed module according to the embodiment with lid and collar, as in Example 2

First, various concentrations of verapamil were tested, and by adjusting the excitation and emission wavelengths for selective detection of Rho123, it was shown that verapamil did not affect its signal.

To test the effect of the P-gp transporter inhibitor, verapamil was selected as a well-described inhibitor of this transporter, which has been tested many times in the conventional permeation tests. The ratio of Rho123 to verapamil had to be maintained so that verapamil was in excess for sufficient interaction with P-gp transporter. A ratio of 1:5 was chosen in combination with 10 µmol/L Rho123 and 50 µmol/L verapamil (see plot No. 6). A ratio of 1:10 was also tested for the combination of 10 µmol/L Rho123 and 100 µmol/L verapamil (see plot No. 7), where a risk of the toxicity of concentrated verapamil solution is already possible. This toxicity was not demonstrated as it would be manifested by passive diffusion, which was not found.

In contrast, at a lower ratio of 2:5 with the combination of 20 µmol/L Rho123 and 50 µmol/L verapamil (see Plot No. 8), no inhibitor effect was visible, as in the case of 1:1 ratio for the combination of 50 µmol/L Rho123 and 50 µmol/L verapamil (see plot No. 9). The conditions of the permeation test were exactly the same as in the previous case.

A time lag of interaction of verapamil in combination with Rho123 with P-gp transporter was observed, which caused the penetration of Rho123 into the cells after a time delay of 90 min. At the same time, no increase in the concentration of Rho123 in the acceptor compartment was observed, which means that Rho123 remains inside the cells throughout the test. In the plots No. 6, 7, 8, and 9 (FIG. 8 and 9), crosses represent sampling points from the donor compartment, and circles represent sampling points from the acceptor compartment.

It is clear from Examples 1 to 3 that the 3D-printed module used according to the invention met all requirements for on-line monitoring of permeation studies.

However, it is to be noticed that the exemplary embodiments are intended to be illustrative only and are not intended to limit the scope of the invention, which is defined by the appended claims.

INDUSTRIAL APPLICABILITY

The plastic module, preferably the 3D-printed module according to the invention for on-line monitoring of permeation tests, is useful in pharmaceutical and analytical laboratories and in preclinical drug development laboratories.

Interaction and inhibition with drug transporters is also the cause of so-called drug interactions. Therefore usability can also be sought in pharmacological and toxicological laboratories.

List of the reference numbers:

1 – larger main container - dashed line represents a level of culture medium
2 – smaller container with an insert with cell monolayer - dashed line represents a level of culture medium with a marker and/or tested substance
3 – lid of the 3D module with inlets and outlets
3a – collar of the 3D module with inlets and outlets
4 – magnetic stirring bar
5 – magnetic stirrer
6 – inlet into donor compartment
7 – outlet from donor compartment
8 – inlet into acceptor compartment
9 – outlet from acceptor compartment
10 – thermostated incubator/water bath
11 – peristaltic pump
12 – sequential injection analyzer
13 – multi(6-)port selection valve
14 – piston pump
15 – fluorescence detector
16 – computer with control programme
17 – distilled water reservoir
18 – culture medium reservoir
19 – waste
20 – holder

PATENT CLAIMS

1. A module for on-line monitoring of the permeation tests, **characterized in that** it comprises an assembly of two plastic containers (1, 2), namely a larger main container (1) for a culture medium, into which a smaller container (2) forming a cell cultivation insert, covered at least on one side of its inner surface with a continuous cell monolayer, is inserted, wherein the smaller container (2) forms a donor compartment and a space between the inserted smaller container (2) and the larger container defines an acceptor compartment, whereas a marker and/or tested substance are added into the donor compartment or the acceptor compartment, and

wherein the main container (1) further comprises

a lid (3) for closing the main container (1),

wherein the lid (3) is provided with at least one channel for inlet (6) and outlet (7) for the donor compartment and at least one channel for inlet (8) and outlet (9) for the acceptor compartment, the channels being configured to ensure a connection of these compartments with an external flow system.

2. The module, according to claim 1, **characterized in that** the module further comprises a collar (3a) enclosing circumferentially the main container, wherein the collar (3a) comprises at least one channel for inlet (8) and outlet (9) for the acceptor compartment instead of the channel in the lid for connecting it to the external flow system.

3. The module, according to claim 1 or 2, **characterized in that** the plastic module is a 3D-printed module.

4. The module, according to any one of the preceding claims 1 to 3, **characterized in that** the plastic of the module is compatible with living cells, wherein preferably the plastic is a polyacrylate-based resin.

5. The module, according to any one of claims 1 to 4, **characterized in that** the module comprises a separate inlet (6, 8) and outlet (7, 9) from both the donor and acceptor

compartment by means of the channels in its lid and/or collar for connecting to the external flow system.

6. The module, according to any one of the preceding claims 1 to 5, **characterized in that** the main container (1) of the module contains a magnetic stirring bar (4) in the acceptor compartment for stirring by a magnetic stirrer (5).

7. The module, according to any one of claims 1 to 6, **characterized in that** both the donor and acceptor compartment of the plastic module is configured by an interconnection through channels in the lid and/or the collar for continuous supply and mixing by the external flow system by means of a pump in a closed-loop.

8. A flow system for on-line monitoring of the permeation tests, comprising:
a thermostated incubator/water bath (10);
a magnetic stirrer (5);
a pump (11), preferably a peristaltic pump, for mixing of the contents using a closed donor and acceptor loops;
a sequential injection analyzer (12) for monitoring of the permeation test under common laboratory conditions;
a multiport selection valve (13);
a piston pump (14) for pumping distilled water;
a fluorescence detector (15) for determining a fluorescence marker;
a computer (16) with a control program;
a distilled water reservoir (17) for washing the entire system;
a culture medium reservoir (18) for replenishing of the plastic module compartments with culture medium;
a waste (19) for draining the culture medium after washing the plastic module;
characterized in that
it further comprises the plastic module, preferably the 3D-printed module, according to any one of claims 1 to 7, which is connected to the pump (11).

9. The flow system, according to claim 8, **characterized in that** at least two plastic modules according to claim 1 to 7 are integrated into the flow system.

AMENDED CLAIMS
received by the International Bureau on 17 August 2021 (17.08.2021)

1. A module for on-line monitoring of the permeation tests, **characterized in that** it comprises an assembly of two plastic containers (1, 2), namely a larger main container (1) for a culture medium, into which a smaller container (2) forming a cell cultivation insert, covered at least on one side of its inner surface with a continuous cell monolayer, is inserted, wherein the smaller container (2) forms a donor compartment and a space between the inserted smaller container (2) and the larger container defines an acceptor compartment, whereas a marker and/or tested substance are added into the donor compartment, and
wherein the main container (1) further comprises
a lid (3) for closing the main container (1) and preventing evaporation and at the same time preventing condensation of the evaporated culture medium, and a collar (3a) enclosing circumferentially the main container,
wherein the lid (3) is provided with separate channels for inlet (6) and outlet (7) for the donor compartment and the collar (3a) comprises separate channels for inlet (8) and outlet (9) for the acceptor compartment, the channels being configured to ensure a connection of these compartments with an external flow system for continuous mixing in a closed-loop, supply of the medium and sampling of samples of the medium for measurement of the marker.

2. The module, according to claim 1, **characterized in that** the plastic module is a 3D-printed module.

3. The module, according to claim 1 or 2, **characterized in that** the plastic of the module is compatible with living cells, wherein preferably the plastic is a polyacrylate-based resin.

4. The module, according to any one of the preceding claims 1 to 3, **characterized in that** the main container (1) of the module contains a magnetic stirring bar (4) in the acceptor compartment for stirring by a magnetic stirrer (5).

5. A flow system for on-line monitoring of the permeation tests with a fluorescence marker, comprising:

a thermostated incubator/water bath (10);

a magnetic stirrer (5);

a pump (11), preferably a peristaltic pump,;

a sequential injection analyzer (12) for monitoring of the permeation test under common laboratory conditions;

a multiport selection valve (13);

a piston pump (14) for pumping distilled water;

a fluorescence detector (15) for determining a fluorescence marker;

a computer (16) with a control program;

a distilled water reservoir (17) for washing the entire system;

a culture medium reservoir (18) for replenishing of the plastic module compartments with culture medium;

a waste (19) for draining the culture medium after washing the plastic module;

characterized in that

it further comprises the plastic module, preferably the 3D-printed module, according to any one of claims 1 to 4, which is connected to the pump (11), wherein the plastic module is connected to the pump (11) by means of the multiport selection valve (13) to form closed donor and acceptor loops used for mixing a content of the acceptor compartment (1) and the donor compartment (2) and for sampling of the sample into flow system for fluorescent detection of the marker in the fluorescence detector (15).

6. The flow system, according to claim 5, **characterized in that** at least two plastic modules according to claim 1 to 4 are integrated into the flow system.

FIG. 1

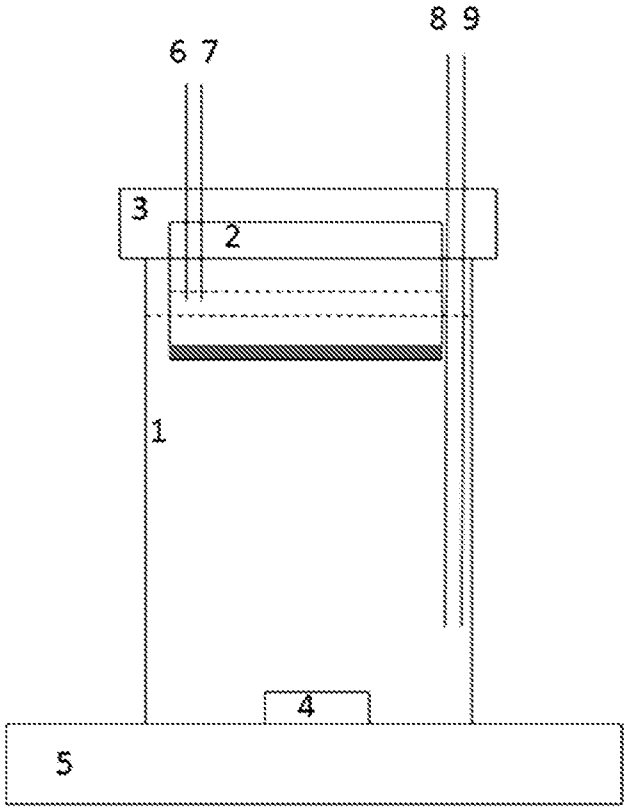


FIG. 2

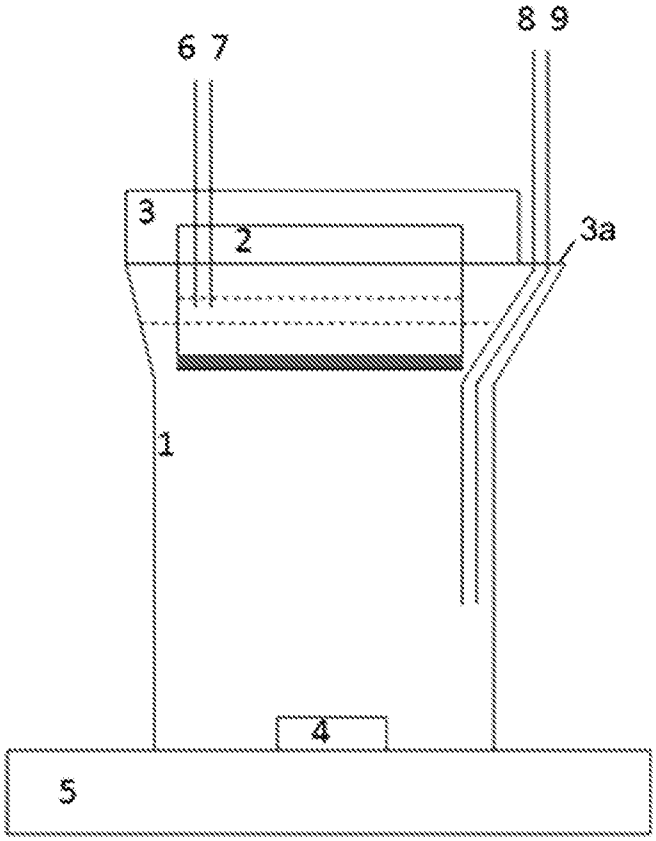


FIG. 3A

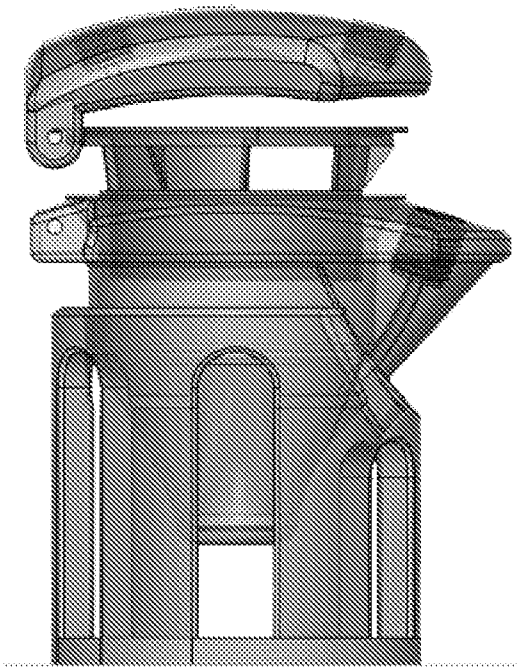


FIG. 3B

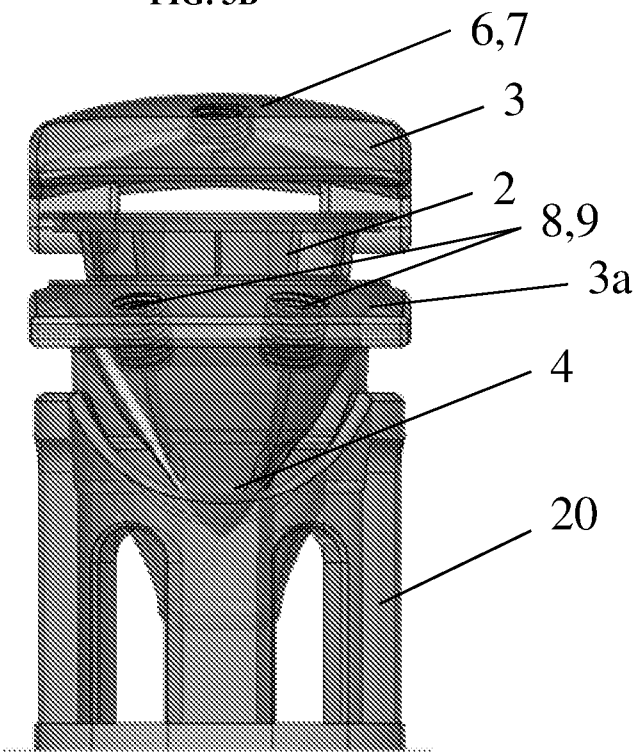


FIG. 4

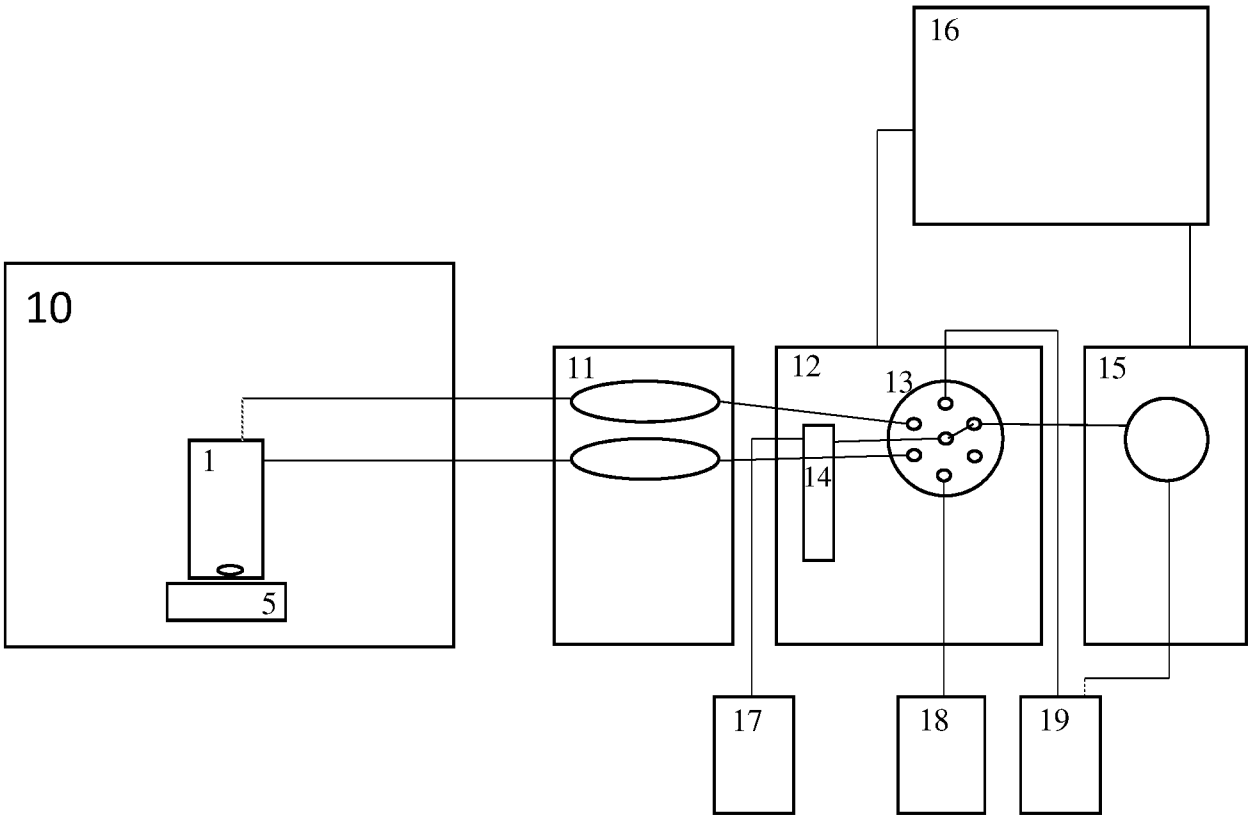
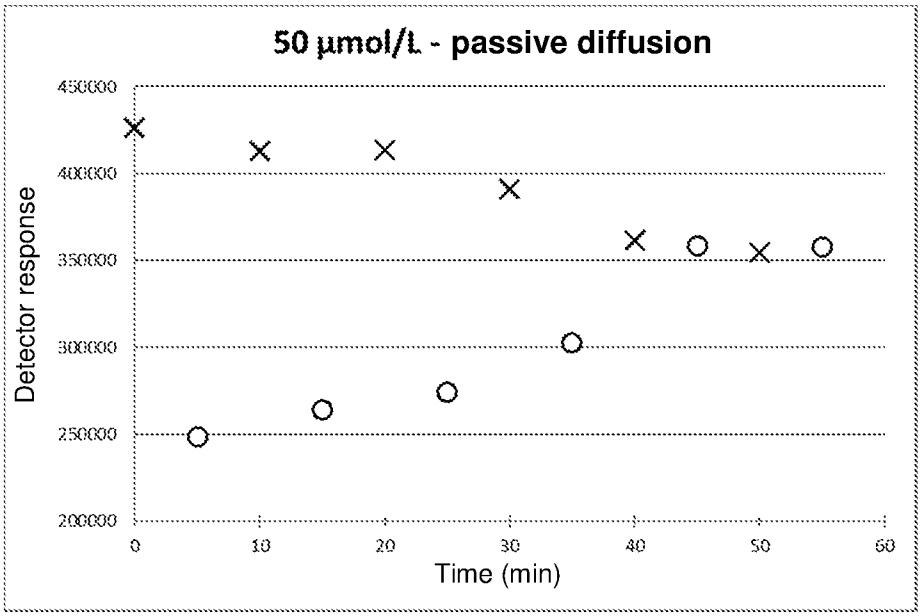


FIG. 5

Plot No. 1



Plot No. 2

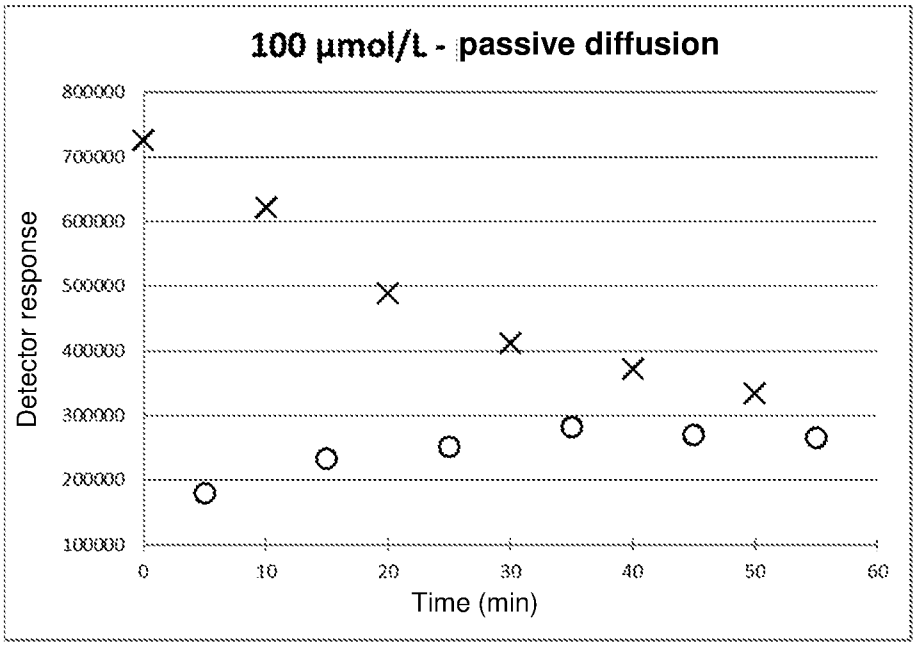
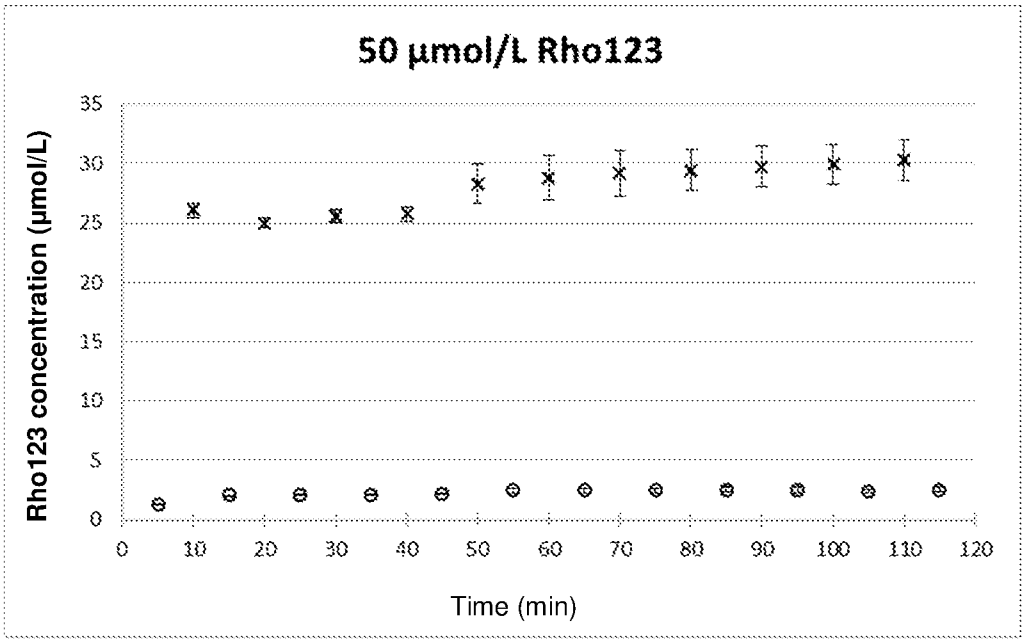


FIG. 6

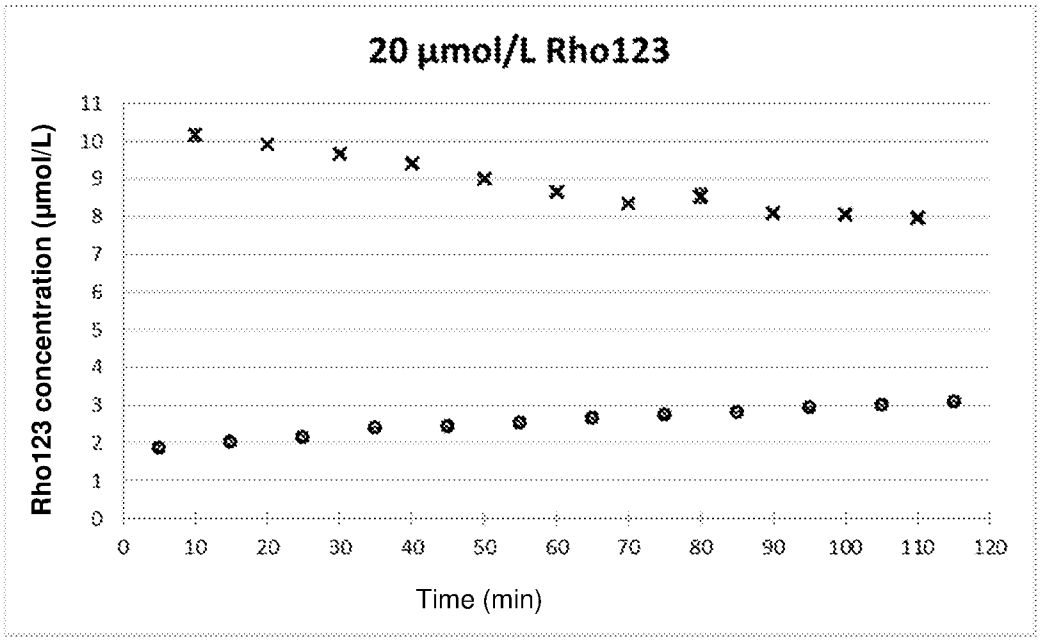
Plot No. 3



5

FIG. 7

Plot No. 4



Plot No. 5

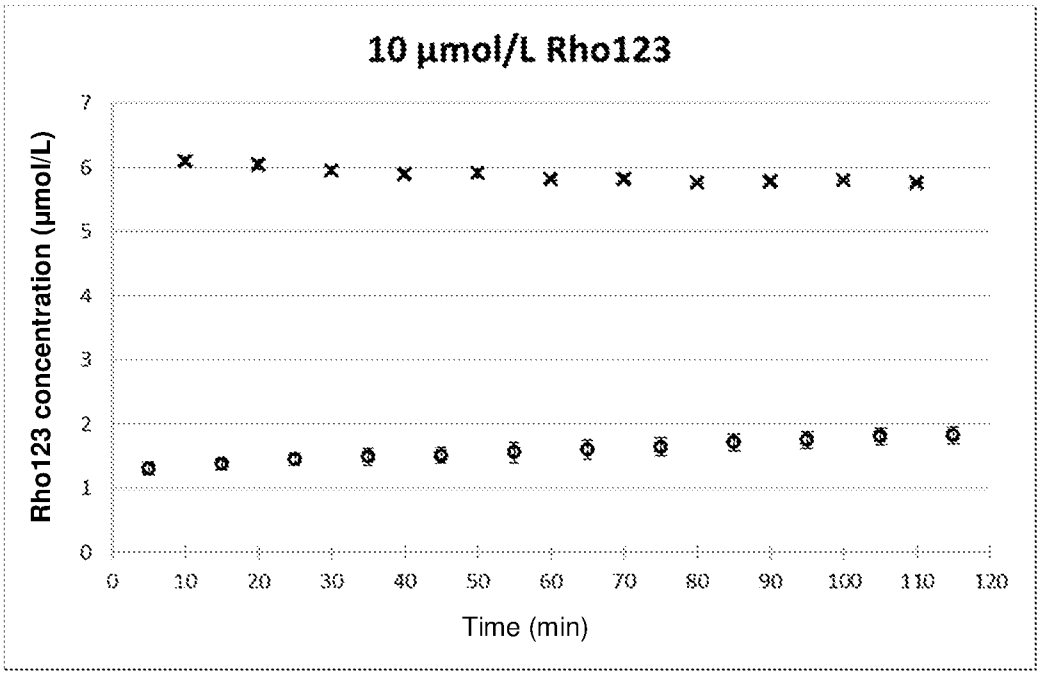
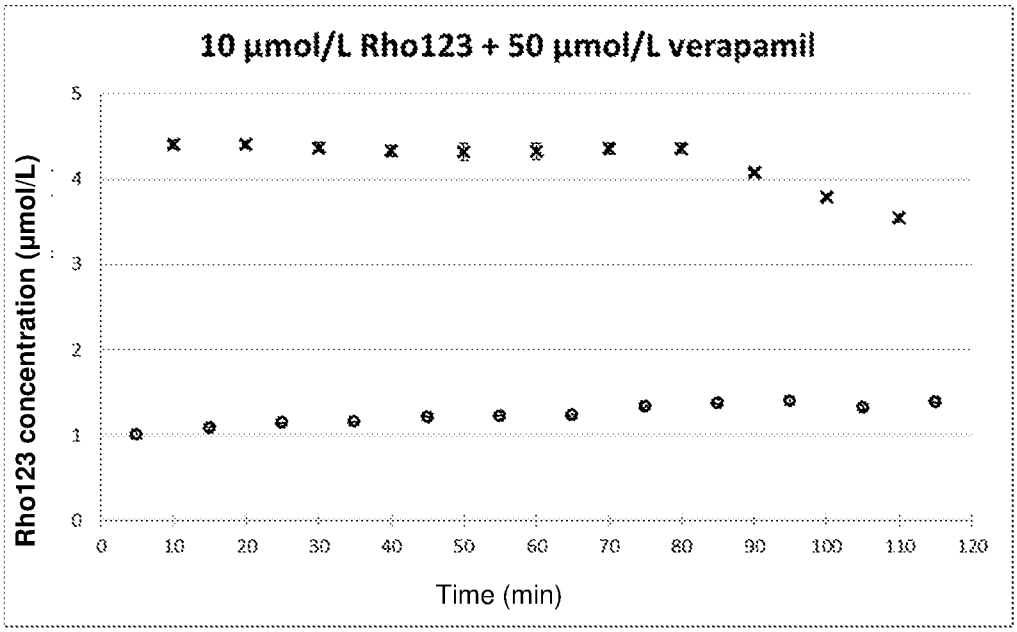


FIG. 8

Plot No. 6



Plot No. 7

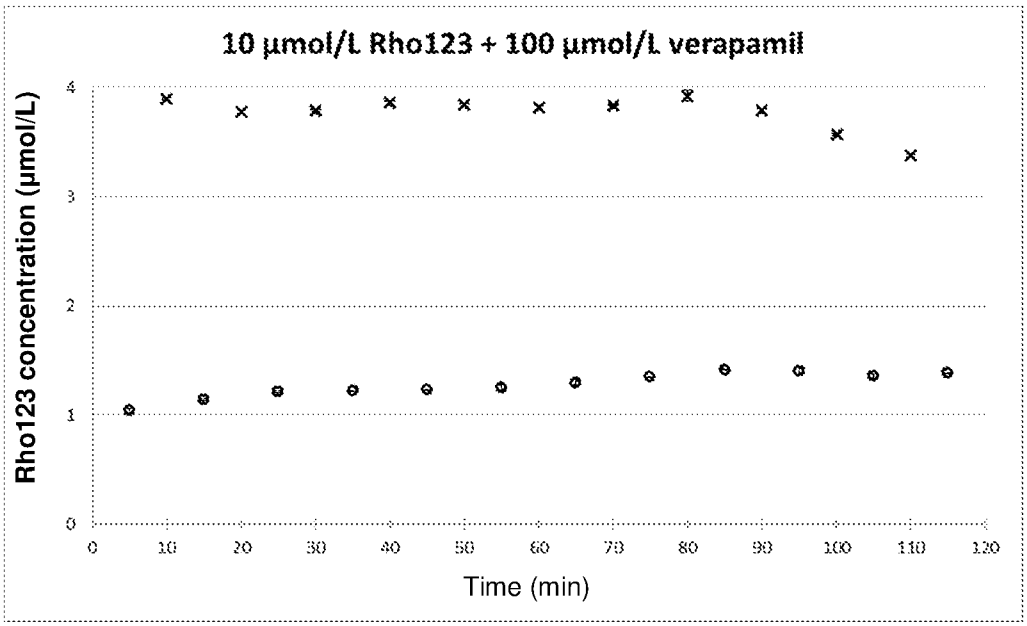
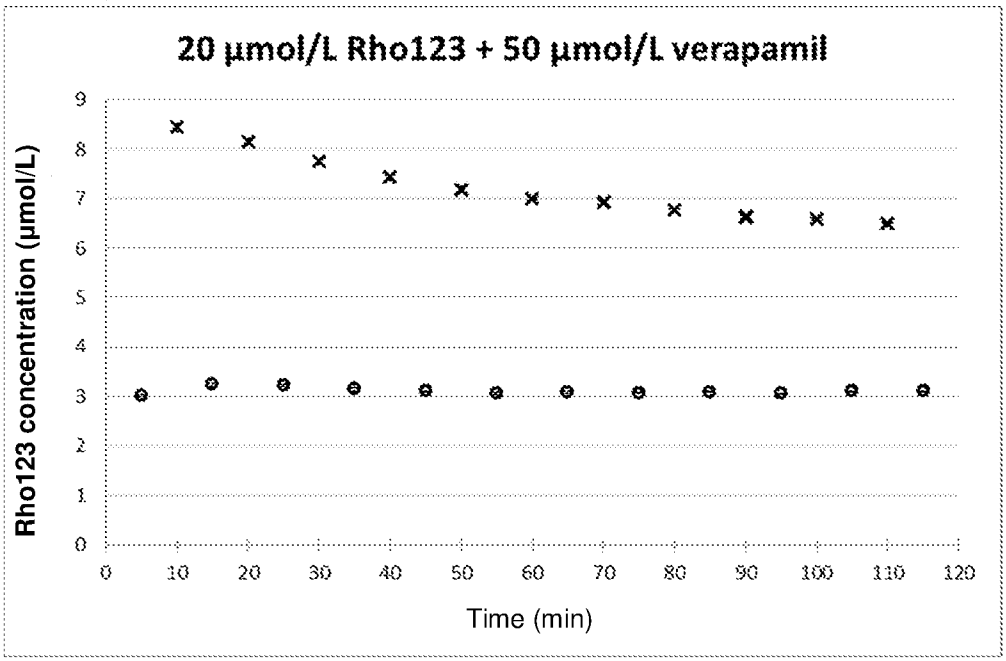
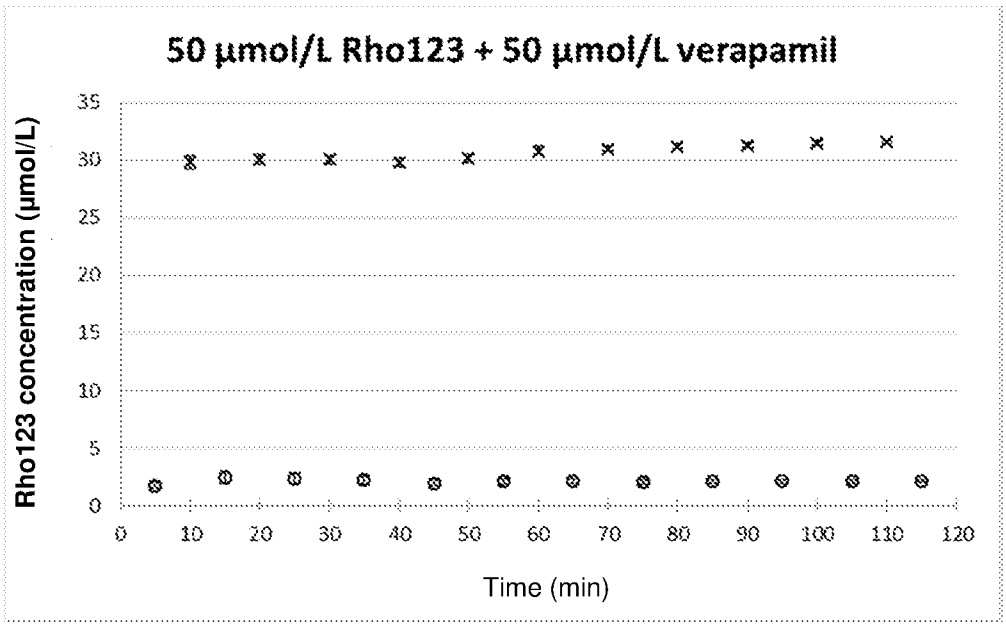


FIG. 9

Plot No. 8



Plot No. 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2021/050031

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/15 G01N13/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/305922 A1 (DOHODA D J; JENNINGS S P ET AL.) 20 October 2016 (2016-10-20) paragraphs [0004], [0053] - paragraph [0071]; claim 19; figure 2	1,3-6
X	US 4 686 190 A (CRAMER EVA B [US] ET AL) 11 August 1987 (1987-08-11) column 2, line 24 - line 57; claims 16, 17, 19, 23 column 3, line 1 - column 4, line 65; figures 1-8	1-4,7

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 June 2021	23/06/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pagels, Marcel

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2021/050031

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZELENÁ LUCIE ET AL: "Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer", ANALYTICAL AND BIOANALYTICAL CHEMISTRY, SPRINGER BERLIN HEIDELBERG, DE, vol. 408, no. 3, 28 November 2015 (2015-11-28), pages 971-981, XP035867890, ISSN: 1618-2642, DOI: 10.1007/S00216-015-9194-0 [retrieved on 2015-11-28] cited in the application the whole document -----	1-9
A	M. ALBERTI ET AL: "Multi-chamber microfluidic platform for high-precision skin permeation testing", LAB ON A CHIP, vol. 17, no. 9, 3 April 2017 (2017-04-03), pages 1625-1634, XP055669400, UK ISSN: 1473-0197, DOI: 10.1039/C6LC01574C cited in the application sections "Introduction" and "Material and methods" -----	1-9

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/CZ2021/050031

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016305922 A1	20-10-2016	US 2016305922 A1	20-10-2016
		WO 2016168445 A1	20-10-2016
US 4686190 A	11-08-1987	NONE	