Device for Quantitative Analysis of a Metabolite Profile

Technical Field

The present invention relates to a device, in particular to a sample preparation device for the quantitative analysis of a drug and/or metabolite profile in a biological sample. Moreover, the present invention relates to an insert for such a device being impregnated with at least one internal standard, to the internal standard itself, and to a kit comprising the device. Furthermore, the present invention also relates to an apparatus containing the device, and to a method for the quantitative analysis of a drug and/or metabolite profile in a biological sample employing the device.

Background Art

Metabolomics is generally defined as the analysis of a substance or group of substances necessary for or taking part in a particular metabolic process in a human or animal body. It's also known as the metabolome analysis. Metabolomics is an evolving discipline that studies unique chemical fingerprints reflecting metabolic changes related to disease onset and progression. Metabolite profiling, an area within metabolomics, measures small molecules or metabolites, contained in a human cell, tissue or organ, which are involved in primary and intermediary metabolism. The biochemical information resulting from metabolite analysis reveals functional end-points associated with physiological and pathophysiological processes, influenced by both genetic predisposition and environmental factors, such as nutrition, exercise or medication (Harrigan, G.G. & Goodacre, R. (2003) Metabolic profiling: Its role in biomarker discovery and gene function analysis. Kluwer Academic Publishers, Boston/Dordrecht/London; Schmidt, C. (2004), *Journal of the National Cancer Institute*, 96, 732-734 ; Raudys, S. (2001) *Statistical and neural classifiers*, Springer-Verlag, London; Daviss, B. (2005) *The Scientist*, 19, 25-28).

Metabolite profiling in combination with data mining approaches have the potential to revolutionize clinical diagnosis and drug development. In particular, big pharma companies are under continuous pressure to discover new targets and novel, more efficacious and safer compounds, and expedite

biomarker and drug discovery, and generally lower costs of pharmaceutical development. Therefore they rely increasingly on biotech companies to fill this innovative gap and future pipelines. In this context, innovative bioanalytical and data mining techniques will play a fundamental role in saving costs by reducing time to market and drug attrition rates.

Recently, due to significant advances in high-throughput technologies, a wider set of the human metabolome - a thus far largely unexplored source of bioinformation - is now accessible (Beecher, C. (2003). In Harrigan, G.G., Goodacre, R. (Ed). *Metabolic profiling: Its role in biomarker discovery and gene function analysis* (pp. 311-319). Kluwer Academic Publishers, Boston/ Dordrecht/Londong ; Dunn,, W.B., Bailey, N.J. & Johnson, H.E. (2005) *Analyst*, 130, 606-625). Statistical comparison of metabolite profiles can expose multivariate patterns that have the potential to revolutionize the health care system by specifically capturing latent warning signs of up-coming diseases before any disease symptoms show up. Early disease screening and prevention, opposed to late disease detection and expensive therapeutic interventions, is probably the primary solution to afford health care coverage in the future. By definition, these so called biomarkers are "objectively measured indicators of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention, and intend to substitute for a clinical endpoint (predict benefit or harm) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence" (Biomarkers Definitions Working Group. (2001) *Clinical Pharmacology and Therapeutics*, 69, 89-95). Interest in the discovery of novel biomarkers originates from their broad range of potential applications and fundamental impact on pharmaceutical industry dynamics and current health care sector principles. Successful implementation of biomarkers in drug discovery can reduce the time and cost of drug development while the application to molecular diagnostics will improve patient compliance in clinical settings and reduce unnecessary costs resulting from false diagnosis in addition to late disease detection (Stoughton, R.B. & Friend, S.H. (2005) *Nature Reviews. Drug Discovery*, 4, 345-350; Morris, M., & Watkins, S.M. (2005). *Current Opinion in Chemical Biology.*, 9, 407-412 ; McCandless, S.E. (2004). *Primary Care*, 31, 583-604).

Qualitative and quantitative metabolite profiling technologies comprise a range of advanced analytical and data processing tools, with the objective of utilizing potential markers as a result of comparison of small molecule components of biological systems. Tandem mass spectrometry (MS), for example, detects hundreds of metabolites simultaneously from micro liter quantities of biological samples, such as whole blood, serum, plasma, urine or other body fluids from minute amounts, with high precision and sensitivity (Roschinger, W., Olgemoller, B., Fingerhut, R., Liebl, B. & Roscher, A.A. (2003). *European Journal of Pediatrics*, 162 (Suppl 1), S67-76; Strauss, A.W. (2004). J Clin Invest 2004; 113:354-356; Kaltashov, I.A. & Eyles, S.J. (2005) *Mass spectrometry in biophysics: Conformation and dynamics of biomolecules*. Wiley). Quantification is achieved by reference to a wide range of appropriate internal standards.

For example, WO 03/005628 describes a method for generating, viewing, interpreting and analyzing a quantitative database of metabolites. Further, US 2002/0009740 describes methods for drug discovery, disease treatment and diagnosis using metabolomics. US 6,455,321 describes a method for interpreting tandem mass spectrometry data for clinical diagnosis. US 6,258,605 describes an analytical method to screen the newborn populations' acylcarnitine and amino acids from blood samples. US 6,627,444 describes a sampling device to aid in calibrating a field instrument.

Furthermore, US 2006/0057554 describes a sample collection device comprising a support bearing an inert absorbing matrix for a fluid sample, wherein the matrix preferably comprises pre-calibrated selected inorganic analytes as internal standards. Moreover, US 2003/0199102 discloses a testing tray comprising a plurality of cells, wherein the cells contain a tried internal standard. The testing tray is for conducting a plurality of tests on a biological fluid.

In order to handle the biological samples to be evaluated further sample devices are known in the prior art. For example, Tanaka et al., Clinical Chemistry 47:10, 1829-1835 (2001) describes a microvolume blood-sampling device with low hemolysis and high consistent yield of serum components.

However, the known sample devices show several disadvantages. In particular, the device described in US 6,627,444 is designed for releasing calibration compounds by way of heating only. It is also designed solely for calibrating an instrument.

Other devices like the device disclosed in US 2006/0057554 fail to use identical organic compounds labelled with stable isotopes preernbedded in a specially designed device for extracting and derivatising.

In view of the problems of the prior art as cited above it is an object of the present invention to provide for an improved quantitative analysis of a drug profile or a metabolite profile in a biological sample, i.e. of concentrations of primarily endogenous, but not excluding exogenous compounds like drugs and metabolites thereof and metabolites from various biological samples, being highly efficient and reliable. Moreover, it is an object to provide for an improved analysis as mentioned above being relatively salt free which is a requirement for mass spectrometry analysis. In particular, it is an object of the present invention to provide for a sample preparation device which may be used for the quantitative analysis of a drug and/or metabolite profile in a biological sample. Moreover, it is also an object of the present invention to provide for an insert for such a device, a kit comprising the device and an apparatus containing the device.

Summary of the Invention

The problems underlying the present invention have surprisingly been solved in accordance with the present invention comprising the following aspects.

In a first aspect, there is provided an insert for a device suitable for the quantitative mass spectrometric analysis of a drug profile and/or a metabolite profile in a biological sample comprising (a) a support impregnated with (b) at least one internal standard wherein the internal standard is a reference material of known absolute amount which belongs to the same family of compound to be analyzed in the biological sample and is either labelled with isotopes or constitutes a non-naturally occuring compound.

In a second aspect, there is provided a device for the quantitative analysis of a drug and/or metabolite profile in a biological sample comprising (a) one or more wells, and (b) one or more inserts according to the first aspect located in the wells, and (c) optionally, a retainer for the insert in the well.

In one embodiment of the invention, there is provided an internal standard for the quantitative analysis of a drug and/or metabolite profile in a biological sample being encapsulated and suitable for being employed according to the first aspect.

In a third aspect, there is provided a kit for the quantitative analysis of a drug and/or metabolite profile in a biological sample comprising the device according to the second aspect.

In a fourth aspect, there is provided an apparatus for the quantitative analysis of a drug and/or metabolite profile in a biological sample comprising (a) a treatment unit for preparing the drug and/or metabolite to be screened comprising (al) an automated liquid handling system, and (a2) at least one device according to the second aspect disclosed above for derivatisation of the drugs and/or metabolites present in the sample and for subsequent extraction of the derivatives; (b) a mass spectrometer for the quantitative targeted mass spectrometry-based analysis, and (c) database for storing results of the analysis.

In a fifth aspect, there is provided a method for the quantitative analysis of a drug and/or metabolite profile in a biological sample employing the insert and/or the device and/or the kit and/or the apparatusas disclosed above.

Reference to the Attached Figures

Figure 1 describes a cross-sectional view of a singular device according to the invention containing wells or vials and its assemblage from individual components. Reference number (1) shows a well/vial. Reference number (2) shows an insert according to the present invention comprising an immobilising stationary phase of glass, celluloses or other suitable material (i.e. a porous support) containing internal standards with optional (micro)encapsulation; reference number (3) shows a retainer to hold the porous support in the well or vial, which is chemically inert to derivatives and solvent; reference number (4) shows a filter; reference number (5) shows an outlet, which opens under pressure of centrifugal or gravitational force or vacuum.

Figure 2 describes a neutral loss scan of 135 in negative mode using ion tandem mass spectrometry of phenylthiorurea amino acid derivatives (PTU), showing amino acids from a red blood cell sample and their corresponding stable isotope internal standards prepared with the multi-device described in Example 2.

Figure 3A describes a precursor scan 184 in positive ion mode (A), showing the multi-devices ability to extract phospholipids from a red blood cell sample of Example 2. For example sphingomyelins and phosphatidylcholines are observable in the *m/z* range 700 - 840, and lyso phosphatidylcholines in the *m/z* range 400 - 650.

Figure 3B describes an MRM scan (multiple reaction monitoring) in positive ion mode of Example 2.

Figure 4 describes an example of how immunotherapy drugs Sirolimus, Everolimus, Cyclosporin A, Tacrolimus, and internal standards Ascomycin and Cyclosporin D from a quality control blood sample are analysed with LCMS to generate quantitative data. The area under the integrated peaks of the internal standard Cyclosporin D and Ascomycin, of known concentrations, are used for comparison against the area under the peak of the immunosuppressants in the five quality control samples containing known concentration amounts. This provides a measure of accuracy for all four drugs.

Figure 5 describes a calibrator curve from calibrators for Sirolimus obtained from multi-device with cellulose insert (Example 3).

Figure 6 describes a calibrator curve from calibrators for Everolimus obtained from multi-device with cellulose insert (Example 3).

Figure 7 describes a calibrator curve from calibrators for Cyclosporin A obtained from multi-device with cellulose insert (Example 3).

Figure 8 describes a calibrator curve from calibrators for Tacrolimus obtained from multi-device with cellulose insert (Example 3).

Detailed Description of the Invention

The present invention will be described in more detail hereinbelow by making reference to its particularly preferred embodiments.

The present invention basically relates to a simple device containing pre-measured internal standards (reference compounds in general) in a vial or well, to which biological samples to be measured can be added for further treatment and eventual analysis by e.g. mass spectrometry. The device comprises an insert that can be porous and contains one or more internal standards of known molar amounts. Additionally, these internal standards may also be encapsulated/embedded into a protective matrix to prolong shelf-life. The protective matrix comprises, but not limited to, either a non-naturally occuring phosphatidylcholine, a polyethylene glycol polymer or a viscous glycerol or sorbitol solution. The device can be used for analyzing a metabolite profile and/or for analyzing a drug profile (i.e. therapeutic drug monitoring, TDM) in a biological sample. Thus, it should be understood that even if the present invention will be described in the following for the analysis of a metabolite profile, it is not limited thereto. Quite in contrast, the same considerations also apply for the analysis of a drug profile.

In the following the constituting components of the device according to the invention as well as the use of the device will be explained.

The device according to the invention contains (A) one or more wells/vials, and (B) at least one insert. Said insert comprises (a) a support which is (b) impregnated with at least one internal standard. The insert is indicated as reference number (2) in Figure 1.

Insert

The term 'insert' as used in the invention should be understood to be the porous support containing the internal standards with an optional chemical protectant. The insert may have any geometrical form as long as the insert fits into the well or vial of the device. In a preferred embodiment the insert is arranged within the well or vial of the device by using a retainer. Said retainer is indicated as reference number (3) in Figure 1. In a particular

preferable embodiment the retainer (3) allows the insert to be arranged within the well without any direct contact between the insert and the well. Thus, the insert is located above the bottom of the well preferably within a distance of 2 to 10 mm, more preferably of 3 to 5 mm by using the retainer. In other words, in a preferred embodiment there is a so-called *"gap"* or *"distance"* between the bottom of the well and the insert and/or between the walls of the well and the insert. As the retainer in the preferred embodiment any retainer is suitable as long as it allows the formation of the gap between the bottom of the well and the insert. Such an arrangment allows for maximum surface area of the support to precipitate the samples onto. The design further ensures the insert is fully accessed by flow of air or other drying gas around the insert to enable rapid drying of sample after application. This design principal also ensures the insert is fully accessed by flow of solvent from all sides enabling metabolite or drug extraction from sample with minimized protein or salt contaminants. Thus, the pores of the support allow a reaction (derivatisation) to proceed within the support itself, minimizing solvent usage and also subsequent removal as evaporation of excess derivative and solvents is provided by maximum surface area to circulating drying gases (air or nitrogen) around the sample. The increased surface area and solvent mobility around the entire support also ensures high extraction efficiencies using appropriate solvents. In other words, the above-mentioned gap allows an almost free arrangement of the insert within the well and an improved circulation of fluids flowing through the well.

Moreover, according to a preferred embodiment of the invention the device may comprise more than one insert arranged in stacks, wherein the respective inserts are more preferably arranged with the above-mentioned gap between each other in order to allow the circulation of fluids.

Support

The support as used in the invention may be any support preferably with at least medium degree, preferably a high degree of porosity. Such a support in principle is known in the prior art and also commercially available.

The porosity "Φ" of a medium (i.e. the support) is defined to be the proportion of the non-solid volume to the total volume of material, and is

defined by the ratio:

$$\Phi = V_p / V_m$$

where V_p is the non-solid volume (pores and liquid) and V_m is the total volume of material, including the solid and non-solid parts.

Thus, porosity is a value between 0 and 1, typically ranging from less than 0.01 for solid granite to more than 0.5 for peat and clay, although it may also be represented in percent terms by multiplying the fraction by 100 %. The porous support of the invention has a porosity of at least 30 %, more prefablypreferably at least 50 %, even more preferably at least 70 %, and most preferably at least 90 %.

The porous support as used in the insert may be of any suitable material, but it is preferably a solid support. More preferably the porous support is comprised of a sorbent material for liquids (also named liquid sorbent material). Still more preferably the support is consisting of the liquid sorbent material. The sorbent material may be an adsorbent or an absorbent material.

A liquid sorbent material as used in the invention should be understood to be any material that allows solutions of internal standards and subsequent samples for analysis to be adsorbed or absorbed uniformally throughout the pores additionally allowing carrier solvent removal by evaporation.

The liquid allowed to being ad- or absorbed by the support material can be any kind of liquid, but it is preferably a volatile liquid at atmospheric pressure, for example a liquid having a boiling point less than about 250 degrees Centigrade (C) at atmopspheric pressure.

More preferably the liquid sorbent material according to the invention comprises at least one of a carbohydrate material, such as cellulose material, glass fibres, glass beads, polyacrylamide gel, porous plastic inert polymer and porous graphite. The said porous sorbent material may more preferably be comprised of a carbohydrate material or derivative thereof, such as agarose, agar, cellulose, dextran, chitosan, or konjac, carrageenan, gellan, or

alginate. The liquid sorbent material is, however, most preferably made of cellulose or glass fibres. The shape of the support or liquid sorbent material is not particularly limited but preferably is of a circular, square or scroll or nautilus dimension. According to the invention the shape of the support or sorbent material is adapted to the shape of the well or vial of the device. As mentioned, the porous support or sorbent material may be fixed or secured in its position in the well or vial by a fixing structure such as a retainer (indicated as (3) in Figure 1).

The porous support comprising the liquid sorbent material mainly has two functions. The first is to embed the internal standards (reference material) as descibed below at predefined concentration ready for addition of the biological sample. The second is the immobilizing of the contents of each sample. This immobilizing step induces cell lysis, protein immobilisation/ precipitation and salt and many other drug or metabolite retention from each of the samples. The porosity of the support is then essential for maximal exposure to both the derivatising agents and also the extraction solvent to be added for the analysis.

Internal standard

An internal standard as used in the invention should be understood to be any reference materials of known absolute amounts that are used for comparisons to similar or identical compounds in order to quantify unknown amounts of compounds present in a given sample. Preferably the internal standard is an organic internal standard. Internal standards as used in the present invention may belong to the same group or family of compounds to be analyzed in the biological sample. However, they are preferably labelled with isotopes in order to properly allow a distinction between the metabolites of the sample and the internal standard. Any other way of distinguishing the metabolites of the sample from the internal standards, however, may also be used. For example, non-naturally occuring compounds may also be used as internal standards.

Specific examples for the internal standard as used in the present invention are listed in Table 1 below.

Table 1

Lipids

abbreviation	Full name	Comments
		Fatty acid chain length
SM(d18:1/6:0)	N-Hexanoyl-sphing-4-enine-1-phosphocholine	6
GPCho(9:0/0:0)	1-Nonanoyl-sn-glycero-3-phosphocholine	9
GPCho(14:0/14:0)	1,2-Ditetradecanoyl-sn-glycero-3-phosphocholine	28
GPIns(16:0/16:0)	1,2-Dihexadecanoyl-sn-glycero-3-phospho-(1'-myo-inositol)	32
GPCho(20:0/20:0)	1,2-Di-(3,7,11,15 tetramethyl hexadecanoyl)-sn-glycero-3-phosphocholine	40
GPSer(20:0/20:0)	1,2-Di-(3,7,11,15 tetramethyl hexadecanoyl)-sn-glycero-3-phosphoserine	40
GPSer(6:0/6:0)	1,2-Dihexanoyl-sn-glycero-3-phosphoserine	12

Amino acids

Abbreviation	Full name	Comments
13C2-15N-Gly	13C2-15N-Glycine	
D4-DL-Ala	D4-DL-Alanine	
15N2-L-Argl	15N2-L-Arginine HCl	
D3-DL-Asp	D3-DL-Aspartic Acid	
15N2-L-Asn	15N2-L-Asparagine H2O	
D3-L-Glu	D3-L-Glutamic Acid	
D5-L-Gln	D5-L-Glutamine	
13C6-L-His	13C6-L-Histidine H2O	
13C6-L-Ile	13C6-L-Isoleucine	
13C-L-Lys	13C-L-Lysine 2HCl	
D3-L-Met	D3-L-Methionine	
D6-L-Orn	D6-L-Ornithine HCl	
D5-L-Phe	D5-L-Phenylalanine (ring 5-phe)	
D7-L-Pro	D7-L-Proline	
D3-DL-Se	D3-DL-Serine	
13C4-L-Thr	13C4-L-Threonine	
15N2-L-Trp	15N2-L-Tryptophan	
D4-L-Tyr	D4-L-Tyrosine (ring 4-tyr)	
D8-DL-Val	D8-DL-Valine	

Acylcarnitines

Abbreviation	Full name	side chain length
D3-C0	[d3-methyl]-Carnitine.HCl	C=0
D9-C0	[d9-trimethyl]-Carnitine.HCl	C=0
D3-C2	[d3]-Acetyl-L-carnitine.HCl	C=2
D3-C3	[3,3,3-d3]-Propionyl-L-carnitine.HCl	C=3
D3-C4	[4,4,4-d3]-Butyryl-L-carnitine.HCl	C=3
D7-C4	[d7]-Isobutyryl-L-carnitine.HCL	C=4
D3-C5	[5,5,5,-d3]-Valeryl-L-carnitine.HCl	C=4
D9-C5	[d9]-Isovaleryl-L-carnitine.HCl	C=5
D3-C6	[6,6,6-d3]-Hexanoyl-L-carnitine.HCl	C=6
D3-C8	[8,8,8-d3]-Octanoyl-L-carnitine.HCl	C=8
D3-C10	[10,10,10-d3]-Decanoyl-L-carnitine.HCl	C=10
D3-C12	[12,12,12-d3]-Dodecanoyl-L-carnitine.HCl	C=12
D3-C14	[14,14,14-d3]-Tetradecanoyl-L-carnitine.HCl	C=14
D3-C16	[16,16,16-d3]-Hexadecanoyl-L-carnitine.HCl	C=16
D3-C18	[18,18,18-d3]-Octadecanoyl-L-carnitine.HCl	C=18

Reducing monosaccharides

Abbreviation	Full name	Comments
13C6-Glc	13C6-Glucose	

Pyruvate/Lactate

Abbreviation	Full name	Comments
13C3-Pyr	13C3-Pyruvate	

Creatinine

Abbreviation	Full name	Comments
	[d3-methyl]-Creatinine	

Immunosuppressants

Abbreviation	Full name(s)	Comments
	Ascomycin	
	Cyclosporin D	
	32-Desmethoxyrapamycin	

Biological Sample

A biological sample as used in the invention should be understood to be any sample of, relating to, caused by, or affecting life or living organisms, biological processes, such as growth and digestion.

Examples of a biological sample may include, but are not limited to blood, cell culture supernatant, saliva, tears, urine, blood, serum, plasma, sweat, vaginal fluids, semen, feces, mucous, breast milk, ascites, lymph, pleural effusion, synovial fluid, bone marrow, cerebro-spinal fluid, and washings from bodily cavities (e.g., bronchial lavage), hair, tissue, bones, or teeth.

Preferably, the biological sample is a liquid sample. More preferable, the biological sample is blood, and most preferable human blood. Liquid means a state of matter with definite volume but no definite shape at 25°C, like water.

Metabolite profile

A metabolite profile as used in the invention should be understood to be any defined set of values of quantitative results for metabolites that can be used for comparison to reference values or profiles derived from another sample or a group of samples. For instance, a metabolite profile of a sample from a diseased patient might be significantly different from a metabolite profile of a sample from a similarly matched healthy patient.

Metabolites, such as, but not limited to amino acids, peptides, acylcarnitines, monosaccharides, lipids and phospholipids, prostaglandins, hydroxyeicosatetraenoic acids, hydroxyoctadecadienoic acids, steroids, bile acids and glyco- and phospholipids can be detected and/or quantified.

Examples of metabolites that are amenable to mass spectrometic analyses according to the invention are listed in Table 2. In particular, lipid species from C4:X to C46:X (where X, the degree of saturation, ranges from 0 to 8) in any given fatty acid residue are shown. The lipids include also sphingolipids and glycosphingolipids.

Amino acids, which can be detected and quantified, are proteogenic or non-proteogenic amino acids. The proteogenic amino acids and the non-proteogenic amino acids, as indicated in Table 2, are preferred.

Acylcarnitines from C4:X to C18:X (wherein X is the degree of saturation and ranges from 0 to 8 in any given acid residue) can be detected and/or analyzed. Examples for acylcarnitines which are preferred are also listed in Table 2.

Monosaccharides are preferably reducing or non-reducing carbohydrates. Examples of monosaccharides are also listed in Table 2.

Table 2

Lipids:

abbreviation	**Full name of lipid subtype**	**Comments**
	Glycerophospholipids, sphingolipids and glycosphingolipids	**Fatty acid chain length**
Sph	sphingosine	None
Cer	ceramide	C6:X – C36:X
SM	sphingomyelin	C6:X – C36:X
Sph pchol	sphingosylphosphorylcholin	None
Sph dh	dihydrosphingosine	None
PC	phoshatidylcholine	C4:X – C46:X
PI	phosphatidylinositol	C4:X – C46:X
PS	phosphatidylserine	C4:X – C46:X
PC (a)	lysophoshatidylcholine	C4:X – C32:X
PI (a)	lysophosphatidylinositol	C4:X – C32:X
PS (a)	lysophosphatidylserine	C4:X – C32:X
PC (e)	plasmenylphoshatidylcholine	C4:1 – C32:X
PC (e)	plasmanylphoshatidylcholine	C4:0 – C32:0

Amino acids

Proteinogenic amino acids

	abbreviation	Full name	Comments
A	Ala	Alanine	
D	Asp	Aspartic acid	
E	Glu	Glutamic acid	
F	Phe	Phenylalanine	
G	Gly	Glycine	
H	His	Histidine	
	Xle	Leucine/Isoleucine	
K	Lys	Lysine	
M	Met	Methionine	
P	Pro	Proline	
R	Arg	Arginine	
S	Ser	Serine	
T	Thr	Threonine	
V	Val	Valine	
W	Trp	Tryptophan	
Y	Tyr	Tyrosine	
	ADMA	Asymmetrical dimethyl arginine	LC MS method
	SDMA	Symmetrical dimethyl arginine	LC MS method
Q	Gln	Glutamine	
N	Asn	Asparagine	
		Nitrotyrosine	LC MS method
		Hydroxyproline	LC MS method
		Kynurenine	LC MS method
		3-Hydroxy kynurenine	LC MS method

Non-proteinogenic amino acids

	abbreviation	Full name	Comments
O	Orn	Ornithine	
	Cit	Citrulline	

Acylcarnitines

abbreviation	Full name	Comments
C0	Carnitine (free carnitine)	C0
C2:X to C18:X	Acylcarnitine	C0:X to C26:X
C3:X-OH to C 18:2-OH	Hydroxylacylcarnitine	C3-OH to C18:2-OH
C3:0-DC to C18:2-DC	Dicarboxylacylcarnitines	C3:0-DC to C12:0-DC

Reducing monosaccharides

abbreviation	Full name(s)	Comments
H	Hexose	
P	Pentose	
dH	Deoxyhexose	

Others

abbreviation	Full name	Comments
Cr	Creatinine	
	Spermidine	LC MS method
	Spermine	LC MS method
	Putrescine	LC MS method
	Dopamine	LC MS method
	Serotonin	LC MS method

	Prostaglandins	LC MS method
	Hydoxyeicosatetraeneoic (HETEs)	LC MS method
	Hydroxyoctadecadienoic (HODEs)	LC MS method
	Leukatrienes	LC MS method
	Thromboxanes	LC MS method
	Bile acids	LC MS method
	Sterols	LC MS method
	Cholesterols	LC MS method
	Vitamins and cofactors	
	Drugs and drug metabolites	LC MS method

Drug profile

A drug profile as used in the invention should be understood to be any defined set of values of quantitative results for one or more drugs or drug metabolites in a specified sample. Moreover, immunosuppressants as specific examples can also be detected and quantified. For example, a drug profile of a transplant patient would give the physician the immediate circulating amounts of one or more drug therapies in use, and future dosages could therefore be increased or decreased according to the quantities measured to achieve best therapeutic range. Such an analysis is designated as therapeutic drug monitoring (TDM). Immunosuppressants in accordance with the present invention are to be understood as drugs that may be used in immunosuppressive therapy to inhibit or prevent activity of the immune system. Clinically they are used to prevent the rejection of transplanted organs and tissues and in treatment of autoimmune diseases such as rheumatoid arthritis, myasthenia gravis, systemic lupus erythematosus, Crohn's desease, and ulcerative colitis. Immunosupperessants as defined herein basically can be classified into four groups: glucocorticoids, cytostatics, antibodies, and drugs acting on immunophilins. Preferred examples of immunosupperessants as used in the present invention are Cyclosporin A, Sirolimus, Everolimus and Tacrolimus.

Encapsulation of the Standards

The internal standard according to the invention is preferably encapsulated with a covering material or protecting material protecting the internal standard from degradation and chemical reactivity prior to use. The protection of the internal standard from degradation and chemical reactivity can prevent many forms of breakdown or chemical modification of the internal standard, such as prevention of the action of sunlight, temperature, and microorganisms, in particular prevention from any process that transforms the internal standard into breakdown or degradation products, thereby influencing the outcome of a quantitative analysis.

A protecting/covering material as used in the invention should be understood to be any material for shielding or designed to shield the internal standard(s) against degradation.

The protecting/covering material according to the invention can be any material suitable for protecting the internal standard from an environmental influence as mentioned above. The covering material according to the invention preferably comprises at least one of a polymer, a micelle-forming compound, a liposome-forming compound and a polyhydroxy compound, or any mixtures thereof.

If the covering material is a polymer, said polymer as used in the invention is not particularly limited and understood to be a high molecular weight organic compound, such as having a weight average molecular weight of at least 500 g/mol or at least 1,000 g/mol or at least 5,000 g/mol or at least 10,000 g/mol, which is either natural or synthetic, whose structure can be represented by a repeated small unit of a monomer. A synthetic polymer is formed in a manner known in the art such as by addition or condensation polymerization reaction of monomers. The polymer of the invention may also be a co-polymer, when two or more different monomers are involved. A homopolymer is a polymer which is formed from only one type of monomer.

The polymer according to the invention is preferably a polyalkylene glycol homopolymer or copolymer or a mixture thereof. The weight average molecular weight is preferably about 1000 daltons (Da). More preferably the

polymer according to the invention is a polyethylene glycol (PEG) or polypropylene glycol (PPG), preferably PEG 1000 having a weight average molecular weight of about 1000 Da, as it is soluble or miscible with highly polar and less polar to unpolar solvents.

If the covering material is a micelle-forming compound said compound as used in the invention should be understood to be any compound which can induce submicroscopic aggregation of molecules, as droplets in a colloidal system. The micelle-forming compound according to the invention is preferably a surfactant.

A surfactant as used in the invention is understood to be any chemical compound that reduces the surface tension between two liquids; or any surface-active agent which increases the emulsifying, foaming, dispersing, spreading and wetting properties of a product, in particular any organic compound whose molecules contain a hydrophilic group at one end and a lipophilic group at the other end. Suitable surfactants comprise cationic, anionic, nonionic, and amphoteric surfactants. Preferably the surfactant is phosphatidyl $(C17:0)_2$.

If the covering material is a liposome-forming compound said compound as used in the invention should be understood to be any compound which can build artificial microscopic vesicles consisting of an aqueous core enclosed in one or more phospholipid layers, used to convey vaccines, drugs, enzymes, or other substances to target cells or organs.

A phospholipid as used in the invention is understood in the general way in the art and should comprise phosphorous containing lipid, such as lecithin and cephalin, made of glycerol and fatty acids, with a phosphate group attached. More preferably the liposome forming compound is a phospholipid, such as a phosphatidyl choline or a phosphatidyl ethanolamine or derivatives thereof.

If the covering material is a polyhydroxy compound said compound as used in the invention should be understood to comprise at least two hydroxy groups. Most preferably the polyhydroxy compound is sorbitol and/or glycerol.

Preferably the encapsulation according to the invention is a microencapsulation. A microencapsulation as used in the invention should be understood to be any encapsulation of microcapsules, which are small, preferably microscopic capsules designed to release their contents when broken by pressure, dissolved or melted. In particular, the capsules of the invention preferably have a diameter of less than 100 micrometer, more preferably less than 10 micrometer and most preferable less than 1 micrometer.

Microencapsulated internal standards are robust in terms of storage and shipping and are stable regarding oxidation and degradation processes, and they have a relatively long shelf-life. The microencapsulation is preferably standardized to prepare synthetic quality control material based on microencapsulated components. This is typically achieved by drying internal standards and other protected samples down with the covering material in a solvent that is a suitable solvent for these compounds like a chloroform/ methanol mixture for phospholipids. Typically addition of water to these samples induces micelle and/or liposome formations to occur, and embedding of these internal or external standard lipophilic protected compounds is then made possible in water.

For example, the device is prepared as follows: The internal standard, dissolved in a suitable solvent, is pipetted in a known amount onto a porous support and dried. This procedure is repeated for every internal standard or class of internal standards to be employed in the device. If an encapsulation is provided, as the final step, the encapsulating/covering material, preferably in a suitable solvent, is put onto the support including the internal standards (i.e. the insert) and dried. The insert is then inserted into the well, preferably by using a securing means or fixing structure such as a retainer. As an alternative, the support may be inserted into the well before pipetting the internal standards and the optional covering material onto the support.

Multiple Device

A multi-device as used in the invention should be understood to be any multiple devices joined together to form a multi-device such as a microtitre plate standard format.

A microtiter plate as used in the invention should be understood to be any plastic sample holder used in biology or chemistry research facilities. The microtitre plate standard was formalized by the Society for Biomolecular Screening (SBS) in 1996. It typically has 6, 24, 96, 384 or 1536 sample wells arranged in a 2:3 rectangular matrix. The standard governs well dimensions (e.g. diameter, spacing and depth) as well as plate properties (e.g. dimensions and rigidity).

For the multi-device the same description of constituting components as mentioned above applies. Therefore, also the multi-device includes a porous support such as cellulose or glass fibre as examples, preferably retained in at least one well by a chemically inert retaining structure. The porous support has embedded into it internal standards in a dry state; optionally microencapsulated (coated) with a protective or covering material or mixture of chemicals, for example polyethylene glycol 1000, Phosphatidylcholine, glycerol or sorbitol.

The device in multiple format herein named a multi-device may also have a different format. Pre-embedding several vials, as an example 6 wells, give a 6 point calibration with multiple calibrating compounds. Quality control samples containing known metabolites and/or multiple drug concentrations are also pre-embedded.

Well

A well as used in the invention should be understood to be any vial or tube consisting of a material, which is preferably solvent and derivative resistant, wherein an extraction or chemical reaction can take place.

The one or more wells (indicated as reference number (1) in Figure 1) of the device preferably comprise at least one filter (indicated as (4) in Figure 1) for separating micron size solids, more preferably exactly one filter (4) for separating micron size solids. The one or more wells of the device preferably comprise at least one outlet (5) in Figure 1 for discharging the filtrate.

A filter contained in the well as used in the invention should be understood to be any porous material a liquid or gas is passed through in order to

separate the fluid from suspended particulate matter. The filter (4) has preferably a pore size of 50 to 0.01 micrometer, more preferably 5 to 0.1 micrometer, and even more preferable 1 to 0.3 micrometer. Most preferable, the filter (4) has a pore size of 0.45 micrometer.

Preferably according to the invention, the filter (4) is located between the insert (2) and the outlet (5).

Moreover, the outlet (5) according to the invention preferably opens under applied centrifugal force or reduced pressure, preferably below 500 mbar. The reduced pressure is preferably applied on the side of the outlet (5) of the well (1). Alternatively, an increased pressure on the side of the insert (2) can be applied in order to ensure a flow from the insert (2) to the outlet (5).

Kit

The device according to the present invention may be further used in a kit for the quantitative analysis of a drug and/or metabolite profile in a biological sample. A kit as used in the invention should be understood to be any system of reagents, solvents, software inclusive of the device enabling preparation of metabolites for quantitative targeted analysis of a range of metabolites usually in conjuction with an analytical instrument.

Apparatus

Furthermore, the device according to the present invention may also be used in an apparatus for the quantitative analysis of a drug and/or metabolite profile in a biological sample. Said apparatus comprises (a) a treatment unit for preparing the drug and/or metabolite to be screened comprising (a1) an automated liquid handling system, and (a2) at least one device as defined above for derivatisation of the drugs and/or metabolites present in the sample and for subsequent extraction of the derivatives; (b) a mass spectrometer for the quantitative targeted mass spectrometry-based analysis, and (c) database for storing results of the analysis.

The apparatus (or platform) as used in the invention should be understood to be any apparatus that enables the complete preparation of a biological

sample ready for analysis by mass spectrometry. This encompasses processes of extraction, derivatization, desalting and concentrating. This also includes all possible combinations of some or all of these processes in a fully automated method, preferably incorporating a liquid handling system in combination with a sample centrifugal device, a sample heating and cooling device, a sample shaking device, a sample drying device, a sample pipetting device and a sample homogenization device.

A liquid handling system as used in the invention should be understood to be any mechanical device that enables accurate aspiration and dispensing of many types of solvents in and out of vials and microtitre plates. A liquid handling system may be be operated via a computer and software in such a liquid handling system.

A database or data bank as used in the invention should be understood to be any collection of data arranged for ease and speed of search and retrieval.

A targeted mass spectrometry analysis as used in the invention should be understood to be mass spectrometry analysis, wherein one or more preset ion pairs are used, specifically defining and representing a known metabolite by a known fragmentation pattern that is characteristic for the corresponding analyte, for identification of the targeted metabolite. The obtained ion intensities are used together with the appropriate internal standard to calculate the concentration of the targeted metabolite. The internal standard is identified by using a characteristic ion pair (or several), their obtained ion intensities are related to the known concentration of the internal standard allowing the quantification of a corresponding targeted metabolite. The set of targeted metabolites is known in advance and can be pre-annotated. Therefore, detected and quantified metabolites are already annotated allowing a fast and direct interpretation. A tandem mass spectrometer is particularly preferred as a mass spectrometer capable of MSMS analyses to distinguish more specifically ion species. Preferably, the apparatus allows for automated standardized sample preparation and high-resolution tandem mass analytics procedures. In particular, the automated sample preparation procedure increases day to day reproducibility of reliable results and lower coefficients of variance (CVs). When, for example, analyzing a derivatized sugar with a precursor ion scan, the derivative itself can be detected by the

mass spectrometer. In positive ion mode this is preferably the formation of the phenylmethylpyrazolone (PMP) (MH)+ ion at m/z 175. The composition of the carbohydrate itself or discret isomers are detectable.

When carrying out a metabolome analysis using the device according to the invention a quantity of hundreds of metabolites can be analysed simultaneously from microtiter quantities of biological material with high speed, precision and sensitivity using pre-analytical steps. Quality assured (QA) data is generated from individual samples in the matter of minutes and interpreted employing cutting edge statistical software tools. This method also overcomes hitherto to existing analytical bottlenecks through pre-analytical standardization and automation, and user-friendly statistical and biochemical data interpretation. This integration of all components in the method of the invention into a new technology platform will make "biochemical fingerprinting" accessible for widespread application and will expedite the spread of metabolomics.

Examples

The present invention will be further illustrated by the following non-limiting examples.

Preparation and conditions of the multi-device

One multi-device was prepared using 7 mm cellulose spots (cut from generic card - 10 539 859, Schleicher Schuell Biosciences GmbH, Dassel, Germany) as the porous support in each of the 96 wells of a Solvinert microtiter plate (MSRP N04, Millipore Corp. MA, USA). These were fixed into place with manufactured retainers made from polypropylene (Biocrates, Tirol, Austria).

To analyse a selected subset of metabolites, in this case, amino acids, acylcarnitines and phospholipids from a sample, a selection of suitable internal standards of amino acids, acylcarnitines and lipids labelled with stable isotopes to represent all the twenty proteogenic phosphatidylcholines, sphingomyelins, and lyso species of each were used. These were pre-imbedded into the porous support of the multi-device by pipetting known amounts of each internal standard class, allowing each to dry within the

porous support before adding the next mixture of internal standards, allowing to dry and so forth. In this example there were added acylcarnitines followed by amino acids and last, a mixture of phospholipid internal standards in a water solution containing 0.1 % w/w polyethyleneglycol 1000 (PEG 1000), a compound which served dual purposes. As a surfactant, PEG 1000 resides in the pores of the porous support coating all internal standards offering a protective barrier to otherwise degradative actions of exposure to oxygen and water.

When completely dry the multi-device technical validation samples were then added to the first five wells of the multi-device.

Well 1: a blank,

Wells 2 and 3: control mixtures of unlabelled metabolites,

Well 4: a quality control with low concentration metabolites (normal levels or 1 times), and

Well 5: a quality control with high concentration metabolites (levels 10 times normal).

The multi-device containing pre-embedded internal standards with additional control samples in wells 1 to 5 is then stored ready to use at 4 °C.

Method of Using the Multi-Device

[Example 1]

For example purposes only, the following is a description of how the device as specified above is used to process samples for analysis of a selection of metabolites.

To analyse a subset of metabolites, amino acids, acylcarnitines and phospholipids from a sample, a selection of suitable internal standards of amino acids, acylcarnitines and lipids, stable isotope labelled to represent all the twenty proteogenic amino acids, the most abundant acylcarnitines and

phospholipids including phosphatidylcholines, sphingomyelins, and lyso species of each were used. Upon addition of a predefined amount of sample, typically 10 µl of plasma, the internal standards and amino acids of the sample are mixed within the confines of the pores of the insert. Any subsequent treatment that causes loss or degradation of metabolites will therefore be correlated for by the internal standard. Derivatisation of the amino acids can then take place within the confines of the pores of the insert. The derivatising reagent in this example consists of 15 µl of a 5% phenylisothiocyante in a 1:1:1 solution of pyridine, water, ethanol. This derivatisation process occurs at room temperature in less than 20 minutes. As the derivatising solution is completely volatile it can be simply removed under a gentle stream of nitrogen or vacuum at room temperature. The addition of a methanol solution containing 10mM ammonium acetate extracts the derivatised amino acids, acylcarnitines and the phospholipids simultaeously from the porous device into the methanol solvent. The microtitre plate of choice for this purpose has additional properties. It has a 0.45 micron filter and a liquid outlet, that only opens under centrifugal force or vacuum, built into the bottom of each well. The methanol extract from the sample is then simply collected via centrifugation into a capture-microtitre plate, placed under the microtitre plate containing device. Mass spectrometry analysis of the solution from each well can then take place, typically using an autosampling instrument to deliver the sample to the mass spectrometer.

[Example 2]

The following will demonstrate that the device can be used to process samples for analysis of a selection of metabolites.

The multi-device upon accurate addition of 10 µl of blood samples from one patient to each well is mixed with the internal standards within the confines of the pores of the porous support (insert). Any subsequent treatment that causes loss or degradation of metabolites will therefore be correlated to the internal standard. Derivatization was carried out as in Example 1 and the resulting solutions from each well are then analyzed by mass spectrometry methods, typically using an autosampling instrument to deliver the sample to the spectrometer.

Results from the mass spectrometric measurements of the metabolites derivatized and extracted with the multi-device are graphically depicted in Figure 2 and Figure 3 showing the amino acids, the phospholipids and the acylcarnitines, respectively.

The quantities of the amino acids and acylcarnitine metabolites are shown in Table 3 below, also showing the accuracy and the variance of the values obtained using the multi-device

Table 3 - The quantities accuracy and reproducibility of the amino acids, lipids, lactate, creatinine and glucose from a single sample measured 10 times are shown in Table 3 and were obtained using the multi-device.

Amino Acids

Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [µmol/l]	std	CV [%]
Arginine-PTC	64.8	71.0	67.5	68.7	62.3	63.1	67.9	65.6	67.4	66.5	63.7	66.2	2.6	4.0
Phenylalanine-PTC	74.0	71.6	72.1	81.5	70.1	69.2	70.4	73.6	72.5	72.9	76.2	73.1	3.4	4.7
Proline-PTC	114.4	117.1	124.5	120.4	120.2	120.6	137.2	118.2	127.5	126.3	120.6	122.5	6.2	5.1
Lysine-PTC	102.2	94.4	108.4	110.5	93.0	102.1	102.1	98.1	98.9	101.8	102.1	101.2	5.2	5.1
Histidine-PTC	90.0	97.0	95.4	89.4	93.0	89.2	97.7	82.5	88.5	95.3	100.6	92.6	5.2	5.6
Tryptophane-PTC	35.8	39.0	42.7	38.2	36.6	36.6	37.4	38.9	34.8	34.8	35.7	37.3	2.3	6.2
Tyrosine-PTC	93.4	99.9	95.7	92.7	94.8	103.7	95.2	97.2	86.1	87.4	83.6	93.6	6.0	6.4
x-Leucine-PTC	174.5	181.2	158.1	191.9	164.1	153.8	167.5	157.2	150.2	158.6	157.1	164.9	12.8	7.8
Valine-PTC	117.0	96.9	108.7	121.7	116.3	114.6	115.7	134.2	111.7	116.9	121.0	115.9	9.1	7.9
Ornithine-PTC	78.7	81.7	58.8	67.9	69.3	68.9	71.6	68.9	74.8	74.9	74.4	71.8	6.2	8.6
Methionine-PTC	44.4	38.2	34.9	41.5	44.7	38.8	37.8	43.2	36.2	36.0	36.3	39.3	3.6	9.1
Citrulline-PTC	28.3	24.8	28.0	23.5	20.6	25.2	26.1	25.0	28.0	29.4	25.8	25.9	2.5	9.7
Glutamine-PTC	455.8	455.6	445.7	364.4	352.0	372.8	369.0	374.2	322.0	355.7	369.1	385.1	45.6	11.8
Serine-PTC	182.1	153.7	176.7	177.1	191.9	188.5	173.4	137.8	132.6	134.0	154.9	163.9	22.1	13.5
Threonine-PTC	21.3	26.0	31.0	34.4	23.5	29.5	25.7	29.4	25.0	24.2	29.0	27.2	3.8	14.0
Alanine-PTC	219.6	316.7	227.8	282.3	298.7	267.5	205.8	177.8	203.0	245.4	210.9	241.4	44.4	18.4
Asparagine-PTC	236.4	250.9	204.5	179.4	168.4	136.5	170.1	192.4	230.5	206.7	157.0	193.9	35.7	18.4
Glycine-PTC	320.9	282.0	272.0	297.9	412.6	422.9	252.8	268.5	225.1	226.8	278.0	296.3	66.2	22.4
Glutamic Acid-PTC	2115.4	1508.6	1694.6	1220.8	2118.8	1560.0	2137.9	2770.8	2137.9	1563.6	1984.8	1892.1	431.0	22.8

Acylcarnitines

Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [µmol/l]	std	CV [%]
C2	8.487	9.674	9.529	9.007	8.845	9.105	9.847	9.477	9.928	8.552	9.137	9.24	0.49	5.4
C18:1	0.161	0.233	0.218	0.216	0.193	0.193	0.207	0.225	0.205	0.191	0.180	0.20	0.02	10.4
C8:1	0.161	0.233	0.218	0.216	0.193	0.193	0.207	0.225	0.205	0.191	0.180	0.20	0.02	10.4
C0	38.351	48.949	51.179	53.867	53.869	52.218	61.745	60.195	56.018	52.762	56.524	53.24	6.21	11.7
C12-DC	0.021	0.015	0.017	0.013	0.015	0.017	0.015	0.017	0.016	0.017	0.017	0.02	0.00	12.4
C14:2	0.041	0.050	0.044	0.035	0.047	0.052	0.041	0.050	0.035	0.047	0.045	0.04	0.01	13.1
C8	0.232	0.306	0.352	0.274	0.200	0.212	0.264	0.250	0.258	0.276	0.299	0.27	0.04	16.4
C12	0.043	0.051	0.071	0.047	0.043	0.043	0.044	0.048	0.046	0.045	0.055	0.05	0.01	16.7
C12:1	0.028	0.025	0.030	0.031	0.027	0.035	0.035	0.030	0.040	0.034	0.019	0.03	0.01	18.6
C16:1	0.038	0.016	0.036	0.037	0.030	0.035	0.030	0.033	0.024	0.030	0.035	0.03	0.01	20.2
C3	0.293	0.401	0.524	0.376	0.274	0.324	0.309	0.435	0.423	0.473	0.423	0.39	0.08	20.5
C14:1	0.110	0.106	0.103	0.113	0.113	0.141	0.174	0.118	0.172	0.108	0.117	0.13	0.03	20.6
C4:1	0.110	0.106	0.103	0.113	0.113	0.141	0.174	0.118	0.172	0.108	0.117	0.13	0.03	20.6
C7-DC	0.050	0.036	0.044	0.052	0.040	0.039	0.066	0.055	0.036	0.064	0.041	0.05	0.01	22.5
C5-M-DC	0.192	0.204	0.184	0.202	0.191	0.209	0.210	0.316	0.177	0.136	0.160	0.20	0.05	22.7
C4-OH	0.106	0.080	0.100	0.167	0.138	0.129	0.170	0.151	0.175	0.115	0.121	0.13	0.03	23.7
C11	0.006	0.008	0.010	0.010	0.013	0.008	0.010	0.007	0.012	0.008	0.006	0.01	0.00	23.9
C16	0.136	0.204	0.188	0.169	0.127	0.096	0.132	0.137	0.100	0.124	0.178	0.14	0.04	24.6
C4:1-DC	0.148	0.182	0.241	0.167	0.111	0.097	0.165	0.146	0.226	0.127	0.218	0.17	0.05	28.3

Lipids

Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [µmol/l]	std	CV [%]
GPCho 36:3a	190.12	219.29	195.68	224.00	250.66	236.77	231.16	199.46	207.93	210.60	198.76	214.9	19.2	8.9
SM d18:1/16:0	311.11	387.86	330.25	339.33	346.71	381.94	419.57	307.61	337.20	348.34	380.74	353.7	34.8	9.8
GPCho 36:2e	22.84	25.00	20.99	22.67	21.71	21.29	23.19	23.91	27.44	19.20	21.12	22.7	2.2	9.9
GPCho 32:1a	55.79	50.54	58.33	55.17	58.33	67.42	64.89	62.50	67.03	56.25	48.00	58.6	6.4	10.9
GPCho 32:0a	7.69	7.37	7.73	5.20	7.87	6.71	7.82	6.63	7.74	9.23	7.07	7.4	1.0	13.6
LGPCho 18:2a	6.17	6.43	6.17	6.00	8.55	6.45	6.52	4.35	7.93	7.28	6.21	6.6	1.1	16.7
GPCho 34:1p	76.84	76.34	87.50	93.10	73.96	82.02	80.85	127.50	82.42	83.33	82.00	86.0	14.7	17.2
GPCho 36:1p	243.16	268.82	354.16	327.59	300.00	277.53	288.30	425.00	290.11	269.79	259.00	300.3	51.8	17.3
LGPCho 18:1p	160.00	174.19	215.28	168.97	165.62	180.90	156.38	257.50	180.22	138.54	164.00	178.3	32.4	18.2
LGPCho 18:0e	40.24	47.37	51.38	43.35	47.19	65.10	53.63	59.64	50.97	67.69	37.88	51.3	9.7	18.8
GPCho 38:1a	18.95	21.51	29.17	17.24	25.00	22.47	28.72	25.00	26.37	25.00	34.00	24.9	4.8	19.3
LGPCho 18:0p	17.28	28.57	21.61	16.67	24.34	21.94	27.54	19.02	17.68	26.49	18.01	21.7	4.4	20.2
GPCho 30:0a	11.73	21.43	12.35	12.67	14.47	14.19	15.22	9.78	13.41	15.89	16.15	14.3	3.0	21.2
GPCho 38:2a	41.86	56.25	63.42	80.00	65.85	87.50	78.12	82.35	70.27	79.31	50.00	68.6	14.6	21.3
GPCho 34:0e	6.79	8.57	6.79	9.33	11.84	9.03	11.59	13.04	12.20	10.60	9.32	9.9	2.1	21.3
GPCho 32:1p	25.26	15.05	30.56	19.54	19.79	22.47	22.34	23.75	16.48	17.71	16.00	20.8	4.6	22.3
GPIns 38:4	13.52	20.27	15.11	14.77	10.75	17.97	16.27	15.24	15.62	13.51	11.61	15.0	2.7	18.0
GPIns 36:2	7.32	5.48	4.00	8.00	3.58	5.22	6.78	6.35	6.88	4.32	3.23	5.6	1.6	29.2

Lactate, Glucose and Creatinine

Sample Name	QAlow C2	QAlow C3	QAlow C4	QAlow C5	QAlow C6	QAlow C7	QAlow C8	QAlow C9	QAlow C10	QAlow C11	QAlow C12	mean [µmol/l]	std	CV [%]
Lactate	21513	24282	25798	25443	22912	22519	23778	21673	22400	24968	23625	23637	1473	6.3
Glucose	3826	4250	4383	4104	4235	4288	4486	4156	3949	3983	3981	4149	202	4.9
Creatinine	213.62	270.39	247.02	243.48	242.64	233.51	280.19	263.16	262.74	238.50	244.91	249.1	18.7	7.5

[Example 3]

Therapeutic drug monitoring

Immunosuppressants are required to inhibit organ rejection after transplantation. The immunosuppressants used are Everolimus, Cyclosporin A, Tacrolimus, Sirolimus, and Mycophenolic acid. Therapeutic drug monitoring results prepared from a suitably prepared multi-device similarly as described above is shown here to further illustrate the use of the multi-device and support the claims of the invention.

Preparation and conditions of multi-device

This multi-device was prepared with exactly the same method as described above, but instead using a single 8 mm cellulose spot (cut from generic card - 10 539 859, Schlicher Schuell, Biosciences GmbH, Dassel, Germany) as the porous support.

Into the two multi-devices wells were placed a methanol solution (20 µl) containing Everolimus (200 ng/mL) (Sigma, Vienna, Austria), an internal standard for Sirolimus and Tacrolimus, and Cyclosporin D (400 ng/mL) (Sigma, Vienna, Austria), an internal standard for Cyclosporin A, was pipetted (Gilson 20 µl pipette) onto the porous supports of the multi-device and allowed to dry at room temperature for 30 minutes. Calibrator mixture and quality control levels I-V (whole blood calibrator set (level 0-6) for immunosuppressants, ClinChek R whole blood control for immunosuppressants, Recipe Chemicals and Instruments GmbH, Munich, Germany) were reconstituted according to manufactures instruction and both stored at -20 °C. Prior to use, six calibrator solutions with increasing concentrations of Cyclosporin D and Everolimus and five quality control solution with various concentrations of Cyclosporin A, Tacrolimus, Sirolimus and Everolimus were thawed and allowed to reach room temperature around 23 °C. Into six wells 20 µl of each six calibrators were pipetted (20 µl Gilson pipette) onto porous supports of multi-device. The five quality controls were pipetted into five separate wells porous supports of multi-device. To the multi-device was added acetonitrile (HPLC grade) immediately(Gilson 200 ml pipette) onto the multi-devices porous supports and instantly shaken with an

orbital shaker at less than 600 rpm for 30 minutes. The eluant was collected by placing a 300 µl capacity microtiter capture plate under the device and then centriguation of the two at 500 g for 6 minutes. The eluant was then analyzed by mass spectrometric technique based on a published method (T. Koal, M. Deters, B. Casetta, V. Kaever, Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry, J. Chromator. B, Analyt. Technol. Biomed. Life Sci. 2004 Jun 15, 805(2); 215-222). A representative example of how the results are obtained and calculated are presented in Figure 4 for Cyclosporin A analysis with LCMS to generate quantitative data. The areas under the integrated peaks of the internal standard Cyclosporin D were used for comparison against the area under the peak of the immunosuppressant Cyclosporin A in the five quality control samples containing known amounts.

Figures 5 to 8 show linear standard curves for all four immunosuppressants Cyclosporin A, Tacrolimus, Everolimus and Sirolimus using cellulose supports as inserts within the multi-device. Table 4 shows the calculated concentrations and the actual for accuracy comparisons of the five quality assurance materials analysed.

Table 4

		Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)				Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
Cyc A	Calibrator 0	0.2	0.201	101	**Sirolimus**	Calibrator 0		0	No Peak	N/A
	Calibrator 1	46.7	43.8	93.7		Calibrator 1		2.4	2.81	117
	Calibrator 2	115	116	101		Calibrator 2		6.6	6.38	96.7
	Calibrator 3	304	315	103		Calibrator 3		12.7	12.8	101
	Calibrator 4	483	472	97.7		Calibrator 4		19.6	19.6	99.8
	Calibrator 5	777	820	106		Calibrator 5		29	31.5	109
	Calibrator 6	1940	1900	97.9		Calibrator 6		49.4	46.7	94.4
	QA1	61	45.3	74.2		QA1		3.04	2.46	80.8
	QA2	116	95.5	82.3		QA2		8.65	10.1	117
	QA3	254	220	86.7		QA3		15.3	12.6	82.4
	QA4	474	391	82.6		QA4		0	No Peak	N/A
	QA5	1340	1310	98.1		QA5		0	No Peak	N/A
Tacrolimus	Calibrator 0	0.1	No Peak	N/A	**Everolimus**	Calibrator 0		0	< 0	N/A
	Calibrator 1	2.1	2.17	103		Calibrator 1		2.1	2.46	117
	Calibrator 2	5.6	5.38	96		Calibrator 2		6	5.71	95.1
	Calibrator 3	10.9	10.5	95.9		Calibrator 3		12.3	12.5	101
	Calibrator 4	15.8	16.1	102		Calibrator 4		18.2	18.3	100
	Calibrator 5	21.9	22	101		Calibrator 5		25.3	27.1	107
	Calibrator 6	38.8	38.9	100		Calibrator 6		46.5	44.4	95.5
	QA1	3.23	3.69	114		QA1		3.48	3.18	91.3
	QA2	6.6	7.35	111		QA2		11.1	10.8	97.3
	QA3	13.2	14.8	112		QA3		18.2	18.5	102
	QA4	0	0.246	N/A		QA4		0	No Peak	N/A
	QA5	0	0.355	N/A		QA5		0	No Peak	N/A

Industrial Applicability

The invention makes possible a versatile and standardized analysis of various biofluids and tissues. For example, current in-house capacities can demonstrate simultaneous and fully automated sample preparation and analysis, generating more than 1000 quantitative and annotated data points from 10 µl of dried blood within 6 minutes of MS machine time covering various classes of metabolites within more than 100 annotated pathways. Thereby the invention for the first time overcomes most of the bottlenecks in (pre)analytics, automatization and data processing and interpretation that have prohibited so far wide-spread quantitative metabolomics mining.

Compared to prior art analytical methods and devices, the quantitative analysis of the invention is extremely rugged and the results are highly reproducible. In particular, the metabolite data is much superior to comparable proteome or transcriptome data. Only 10 µl blood or serum or 20 µl urine or less than 100,000 cultured cells are needed.

The performance features of the analytical method and the device can meet both research (discovery) application and subsequently clinical diagnostic standards. This ensures or makes possible quality assured data, standardized data, which is comparable from laboratory to laboratory, rapid turn-around time, "ready to go" implementation (hits), easily received data interpretation and visualization and a very high degree in automatization and standardization (SOPs). The overall costs/data point makes the metabolome information orders of magnitudes less expensive than proteome information.

The quantitative information obtained by the method or the device of the invention covers pathways and metabolites in a systemic (system biology) context and scalable fashion. Thereby, a representative functional picture or screen shot or metabolic fingerprint of intermediary metabolism can be finally derived from arrays of marker metabolites.

Moreover, functional end-point information that is annotated and can conveniently be linked to information sources of the proteome, transcriptome and genome, recruiting metabolome information for system biology needs.

The device and the method can be used in an integrated tool (software and analytical) suitable to establish a new "standard" for simultaneous generation of large scale quantitative identified and annotated metabolite profiles and the study of complex and dynamic multiple biomaker patterns. Moreover, commercially available hardware components, consisting of a liquid handling system for automated and standardized sample preparation and a mass spectrometer for MS-MS analytics, can be integrated by proprietary and protected designed consumable-based products and application software, comprising (pre-) analytical procedures and innovative modules for quality controlled data processing, technical validation and documentation, statistical analysis and biochemical interpretation.

The sample preparation time in the present invention (hit-based in batch of 90 samples/microtiter tray) is only roughly 2 h, and will be further reduced by means of parallalization through the scheduling software. A wide range of specific internal standards for quantification is pre-formulated in proprietary chemistry as integral part of usually one or two step reaction preparation and application hits, as is contained all necessary material for QC and QA in combination with software and SOPs.

Industrial applications include biomarker discovery and commercialization with the objective to utilize validated biomarkers for disease diagnosis, treatment efficacy or toxicity. The main applications in pharmaceutical development include the areas drug metabolism and pharmacokinetics, toxicology and safety, drug efficacy and pharmacodynamics. Other fields comprise clinical diagnostics and theranostics, where, for example, early, sensitive and specific diagnosis and accurate staging facilitates disease prevention instead of costly interventions and allows personalized treatment, and where therapeutic effects can be specifically monitored supporting personalized treatment. Further application areas include, but are not limited to, nutrition industry, wellness, homeland security, and basic biology.

In a preferred embodiment of the invention the device comprises a support comprising a sorbent material; and a plurality of mass spectrometry, organic, metabolite standards impregnated in the support and dried. More preferably, the mass spectrometry, organic, metabolite standards comprise

amino acids labelled with stable isotopes, polypeptides labelled with stable isotopes, lipids labelled with stable isotopes, or acylcarnitines labelled with stable isotopes.

In another preferred embodiment of the invention the device comprises a support comprising a sorbent material; and a plurality of mass spectrometry, immunosuppressant drug standards impregnated in the support and dried. More preferably, the immunosuppressant drug standards comprise one or more of Everolimus and Cyclosporin D.

Claims

1. An insert for a device suitable for the quantitative mass spectrometric analysis of a drug and/or a metabolite profile in a biological sample comprising

(a) a support impregnated with

(b) at least one internal standard,

wherein the internal standard is a reference material of known absolute amount which belongs to the same family of compound to be analyzed in the biological sample and is either labelled with isotopes or constitutes a non-naturally occuring compound.

2. The insert according to claim 1, wherein the support comprises a sorbent material for liquids.

3. The insert according to claim 2, wherein the sorbent material comprises at least one of a cellulose material, glass fibres, glass beads, polyacrylamide gel, porous plastic inert polymer and porous graphite.

4. The insert according to any one of claims 1 to 3, wherein the internal standard is encapsulated with a covering material protecting the internal standard from degradation and chemical reactivity prior to use.

5. The insert according to claim 4, wherein the covering material comprises at least one of a polymer, a micelle-forming compound, a liposome- forming compound and a polyhydroxy compound.

6. The insert according to claim 5, wherein the encapsulation is a microencapsulation.

7. The insert according to claim 5 and/or claim 6, wherein the polymer is a polyalkylene glycol homopolymer or copolymer or a mixture thereof, and/or the polyhydroxy compound is sorbitol and/or glycerol.

8. The insert according to any one of claims 5 to 7, wherein the polymer is a polyethylene glycol (PEG) or polypropylene glycol (PPG), preferably PEG 1000.

9. The insert according to claim 5 and/or claim 6, wherein the micelle-forming compound is a surfactant and/or the liposome forming compound is a phospholipid, preferably a phosphatidyl choline or a phosphatidyl ethanolamine or derivatives thereof.

10. A device for the quantitative analysis of a drug and/or a metabolite profile in a biological sample comprising

(a) one or more wells, and

(b) one or more inserts as specified in any one of claims 1 to 9 located in the wells; and

(c) optionally, a retainer for the insert in the well.

11. The device according to claim 10, wherein the one or more wells comprise a filter for separating micron size solids, preferably above 5 micron, and an outlet for discharging the filtrate.

12. The device according to claim 11, wherein the filter is located between the insert and the outlet.

13. The device according to claim 11 and/or claim 12, wherein the outlet opens under applied centrifugal force or reduced pressure, preferably below 500 mbar.

14. A kit for the quantitative analysis of a drug and/or a metabolite profile in a biological sample comprising the device as defined in any one of claims 10 to 13.

15. An apparatus for the quantitative analysis of a drug and/or a metabolite profile in a biological sample comprising

(a) a treatment unit for preparing the drugs and/or metabolites to be screened comprising

a1) an automated liquid handling system, and

a2) at least one device as defined in any one of claims 10 to 13 for derivatisation of the drugs and/or metabolites present in the sample and for subsequent extraction of the derivatives;

(b) a mass spectrometer for the quantitative targeted mass spectrometry-based analysis, and

(c) database for storing results of the analysis.

16. Method for the quantitative analysis of a drug and/or a metabolite profile in a biological sample employing the insert as defined in any one of claims 1 to 9 or the device as defined in any one of claims 10 to 13 or the kit as defined in claim 14 or the apparatus as defined in claim 15.