JOURNAL OF PHARMACEUTICAL SCIENCE AND BIOSCIENTIFIC RESEARCH (JPSBR)

(An International Peer Reviewed Pharmaceutical Journal that Encourages Innovation and Creativities)

Recent Trends in Effective Extraction Techniques of Bioanalytical Sample Preparation

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Article history:

Received 10 Jan 2019
Accepted 27 Jan 2019
Available online 01 Feb 2019

Citation:
Dhoru M., Prajapati K., Patel P., Detholia K. Recent Trends in Effective Extraction Techniques of Bioanalytical Sample Preparation **J Pharm Sci Bioscientific Res**. 2019. 9(1):111-118

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ABSTRACT:

This review article is giving an idea regarding the newer recent and advanced extraction techniques for sample preparation in bioanalysis.In sample preparation Liquid-Liquid extraction (LLE), Solid phase extraction (SPE) and Protein Precipitation technique (PPT) is well known method. There are more and rapid developments of novel sample preparation techniques in bioanalysis. In SPE techniques there is area of solid-phase microextraction (SPME) and its different versions are available. Techniques such as dispersive solid-phase extraction, disposable pipette extraction, molecularly imprinted polymer SPE and micro-extraction by packed sorbent offer a kind of extraction phases and gives unique advantages to bioanalytical methods. On-line SPE using column-switching techniques is rapidly gaining acceptance in bioanalytical applications. Newer approaches to conventional LLE techniques like salting out LLE and supported liquid techniques are also covered in this review article and also super critical fluid techniques is covered in it.

KEY WORDS: Bioanalysis, Sample preparation, Solid phase micro-extraction, Molecular imprinting solid phase extraction, super critical fluid techniques.

INTRODUCTION:

Sample preparation

Sample preparation techniques in bioanalysis are multistep, time-consuming, and labour-intensive process that can take up 60–80% of the total analysis time. Sample preparation is often the limiting step of fast bioanalysis and the most error coming part of the bioanalytical method. The main objective of the sample preparation process is to provide a suitable sample, usually for chromatographic analysis, which will not contaminate the instrumentation and where the concentration in the prepared sample is reflective of that found in the original. [1] The sample preparation portion of the analysis is often the most critical and difficult part, both in terms of time involved and the difficulty of extracting the desired analyte from the matrix.

The method of sample preparation selected is generally dictated by the analytical technique available and the physical characteristics of the analytes under investigation. [2]

Sample preparation is necessary for at least two reasons [3]
a. To remove as many of the endogenous interferences from the analyte as possible
b. To enrich the sample with respect to the analyte, thus maximizing the sensitivity of the system.
It also serves to ensure that the injection matrix is compatible with the selected column and
mobile phase. In simple terms, sample preparation is a process which aims at selective isolation of the analyte of interest from the matrix, minimization/elimination of matrix components in the processed sample and, if required, concentration of the analyte of interest.

Extraction Techniques Used For Separation of Drug From Biological Fluids

Protein Precipitation (PP)

Protein precipitation involves denaturation of proteins present in biomatrix by external stress such as a strong acid/base/heat or, most commonly, the use of an organic solvent such as acetonitrile/methanol. It is also depends on solubility of analyte in particular solvent which is present in the matrix.[4] Protein precipitation method is used to separate the drug from biological matrixes by means of precipitating protein. The precipitation is carried out by using the suitable organic solvents which has good solubility of the analyte and protein precipitating properties. It is good for bioanalytical sample when at higher range concentration.[5] Precipitation is increased by adding of organic solvents like acetonitrile. There is adding of protein precipitation additives and after it mixing and centrifugation occur, after this the supernatant can be directly injected into the HPLC and LCMS/MS system. Protein precipitation is very popular sample pre-treatment method because it is a very fast and almost generic approach.

Protein Precipitation Plates

One of the recent approaches to overcome the disadvantages of the conventional PP method is development of membrane-based protein precipitation filter plates. PP plates have been developed because of, after precipitation of protein; filtration can be carried out in the same well without centrifugation and supernatant transfer steps. Structurally, these plates contain tubes with membrane/depth filter and are attachable to vacuum filtration. The filtration collection plates are made to be compatible with sampler for easy automation of the whole process. [4] Use of PP plates in high throughput bioanalysis has been compared with manual PP. It has been shows that, in an LCMS/MS method, results from PPT plates showed more reproducible result with accuracy and precision than manual PP. Some of plates show solvent leaking. Among the PP solvents tested, acetonitrile showed higher leaking than methanol. It has been suggested that the precipitating solvent, filter material, pore size, vacuum strength, non-specific binding of analyte to the plate and matrix effects (ion suppression) should be considered when choosing a protein precipitation filter plate for an assay. We get conclusion after reviewing this it shows that, PP plates when compared with manual PP, offers several advantages such as no centrifugation or filtrate transfer steps, reduced processing time, higher solvent recovery, usefulness with very low sample volume and cleaner extracts. This new process showed 90% removal of plasma proteins when compare to the old method 60-65%. [3]

Liquid–Liquid Extraction

One of the most useful techniques for isolating desired components from a mixture is liquid liquid extraction (LLE).Making of aqueous and bioanalytical sample the liquid –liquid extraction is widely used.[1] Principle of this method is based on different solubility and partition equilibrium of analyte between two phases. Polar aqueous phase prefer hydrophilic compound and organic solvents mainly prefer hydrophobic compound. Resulted analyte which is in organic phase will be recovered by evaporating method of the solvent and analyte in aqueous phase can be direct injected on column. It is simple, quick and efficient in the removal of non-volatile substance.[4] LLE technique is simple, rapid is relative cost effective per sample as compared to other techniques and near quantitative recoveries (90%) of most drugs can be obtained by multiple continuous extraction. Most of the LLE procedures require the evaporation of nonpolar organic solvent and its reconstitution in the mobile phase to reach sample preconcentration and to obtain a sample compatible for injection into the LC system. [6] This step substantially prolongs the extraction time and may be critical for sample recovery because of solubility issues. In order to improve the extraction efficiency, salting-out (SALLE), sugaring-out (SULLE), or in-vial derivatization approaches may be applied in LLE.[6]

Conventional LLE uses large amounts and sometimes toxic organic solvents. In order to enhance recovery, enrich the sample, and reduce extraction time and organic solvent consumption, different improvements have been developed with ME.

- **SDME**- Single-drop microextraction (SDME) is liquid-based ME are derived from, in which a single drop of water-immiscible solvent suspended from the tip of a syringe is immersed in the aqueous sample.
- **HFLPME-** hollow fiber liquid-phase microextraction in which a hollow polymeric fiber is used as a support for the acceptor phase.
- **DLLME-** Dispersive liquid-liquid microextraction belongs to the first family and enables high surface contact between the two immiscible phases by creating a microemulsion using a dispersive solvent. A quick equilibrium state is

achieved, followed by a centrifugation step to break the microemulsion. Because the recovered solvent is often nonmiscible with water and therefore incompatible for a RP-LC injection, evaporation is usually performed and the extracted compounds are recovered in a low volume of compatible solvent, allowing for high enrichment.

- **EME-** Electromembrane extraction is another remarkable liquid-based ME, from the second family A typical EME device is composed by two compartments, the donor compartment, which is the biological sample, and the acceptor compartment. They are separated by a polymeric membrane impregnated by an organic solvent immiscible with water, also called supported-liquid membrane (SLM). An electric field is applied between the two compartments, enabling migration of compounds through the SLM. With this approach, passive diffusion is not the only phenomenon but an active migration occurs and high recovery can be obtained in a very short time. Two types of selectivity are afforded by EME: (i) according to the electric field polarity, anions or cations are preferentially extracted and (ii) according to the SLM composition (i.e., nature of the organic solvent and presence of carrier compounds), analytes with different lipophilicity are preferentially extracted. While EME has been widely used for basic and acidic LMW drugs, it still remains a challenge for HMW analytes such as peptides, due to their low mobility and lipophilicity. [1, 4, 6, 14]

Supported liquid extraction (SLE)

Even though LLE is mostly a very effective sample preparation method, it has limitations, in particular low sample throughput. Several extractions are required to improve analyte recovery, sample handling is labor-intensive and time consuming, and emulsions can form at the intrface between liquid layers. [5] These limitations can be overcome by using supported liquid extraction (SLE), where aqueous samples are adsorbed on a porous solid support material, e.g. diatomaceous earth. Some studies have shown analyte recovery from SLE that was comparable or higher than LLE. The SLE technique has been implemented in various LC-MS/ MS methods recently. It was particularly powerful for normal phase separation systems, since the high percentage of organic solvent in the eluate did not need to be evaporated prior to injection into the LC-MS/MS system.

Salting-out Assisted LLE (SALLE)

When an inorganic salt is added to mixture of water and a water miscible organic solvent, it leads to the separation of the solvent from the mixture and forms a biphasic system, a phenomenon commonly termed as 'salt-induced phase separation. Salting out technique is not only useful in separating water-miscible organic solvent, but is also used to enhance extractions into non-polar, immiscible organic solvents.[4] The effectiveness of the salting-out depends on the physicochemical properties of the analyte and the type of environmental analyses of various trace elements salt used. The principle of salting is commonly used in environ. Overall, SALLE offers several advantages over conventional LLE such as applicability to a broad range of drugs from low to high lipophilicity, improved recoveries of drugs and the availability of a range of salts (chemical nature, molecular weight and volatility), making it possible to comply efficiently with LC-MS-MS methods. Importantly, SALLE methods are cost-effective and can be automated.[6]

Cartridge used in Liquid-Liquid extraction

MAX Cartridge

MAX is a mixed-mode polymeric sorbent that has been optimized to achieve higher selectivity and sensitivity for extracting acidic compounds with anion-exchange groups.[2]

WCX Cartridge

WCX is a polymeric reversed-phase, weak ion exchange

mixed-mode sorbent that has been optimized for fast, simple, and highly selective sample preparation of strong basic compounds and quaternary amines. [2]

WAX Cartridge

WAX is a polymeric reversed-phase, weak anion exchange mixed-mode sorbent that has been optimized for fast, simple, and highly selective sample preparation of strong acidic compounds. [2]

Solid-Phase Extraction (SPE)

Solid phase extraction is the important technique use in sample pre-treatment for HPLC.The principle of SPE is work on the partition of the analytes between two phases where sample which have a high affinity to solid phase than compound. Selective adsorption mechanism is mainly found in SPE.If the targeted analyte get adsorb on the solid phase, they can selectively remove by using an appropriate elution solvent. In SPE different cartridges are used. It is a very powerful technique. It includes following four steps [4]

- **Conditioning:** Conditioning is mainly for the activation of the column. Solvents that work as a wetting agent on packing materials are used for conditioning of the column

and solvents. Water and buffer is adding for proper adsorption, which leads to activation of the column.

- **Loading**: In this step there is adjustment of pH and after that the compound analyte is loading on the column by gravity, pump. Sample loading on cartridge at slow rate without break and must care is there no drop remain on the wall of the cartridge, for interaction of analyte with adsorbent it is necessary flow rate will be slow.
- **Washing**: In washing interference from the matrix are removed and analyte will retain.
- **Drying**: With the help of vacuum pump drying can be done .It is necessary to remove excess solvent and buffers to which interpreting like precipitation and also for avoiding any block formation in cartridges.
- **Elution**: There is use of appropriate solvents or buffer for elution, which elutes the analyte from the SPE bed for analysis. In this step solvents are going through cartridge. Rate of flow is also slow so, soaking time on packing get increased which gives maximum extraction.

Now days SPE also develop as, flat disks and µSPE. Disks are conditioned and used in a similar way as packed columns. Higher flow rates can be easily applied by disks as compared to packed columns is one of the main advantages of disks.

Depending upon the type of solvent, SPE methods can be divided into three categories:
Normal phase
Reversed phase
Ion exchange SPE

- **Reversed phase-**It **involves** a polar or moderately polar sample matrix (mobile phase) and a non-polar stationary phase. The analyte of interest is typically mid- to non-polar. Retention occurs via non-polar interaction between carbon-hydrogen bonds of the analyte and carbon-hydrogen bonds of the sorbent function groups due to Van der Waals or dispersion forces. The materials that are used as reversed phases are carbon-based media, polymer-based media, polymer-coated and bonded silica media.[4,6,8]
- **Normal phase-**It involve a polar analyte, a mid- to non-polar matrix (e.g. acetone, chlorinated solvents and hexane) and a polar stationary phase. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. Ex. Hydrogen bonding, dipole-dipole, induced dipole-dipole and pi-piths phases can offer a highly selective extraction procedure capable of separating molecules with very similar structures. The main drawback is that the analyte must be loaded onto the sorbent in a relatively non-polar organic solvent such as hexane.[4,6,8]
- **Ion exchange SPE-**It can be used for compounds that are in a solution. Anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface. Biofluids can usually be applied directly to ion-exchange sorbents following dilution of the sample with water or a buffer, and possibly adjustment of pH. However, elution from strong ion-exchange sorbents can be a problem as high ionic strength or extremes of pH, may be required which may affect analyte stability or further processing of sample. Anionic (negatively charged) compounds can be isolated on LC-SAX or LC-NH2 bonded silica cartridges. Cationic (positively charged) compounds are isolated by using LC-SCX or LC-WCX bonded silica cartridges.[1,3,4]

Table 1 Features of new techniques of on-line biological sample processing. [7]

Pretreatment technique	Mechanism	Application	Characteristic
SPE	Non-polar effect, polar effects, ionic and covalent effect	Firstly extraction of urine samples of drug abuse; analyzing prostaglandin level in human serum	Good accuracy, precision and recovery; no emulsification and damage compared with conventional LLE
SPME	Evolved from SPE technique; a fused silica fiber surface coated with adsorbed material	Extremely complex mixture such as blood plasma, whole blood, urine and tissue	Small volume of solvent compared with LLE and SPM; promoting the development for the analysis of endogenous and exogenous compounds
Column switching	Change through a valve to the mobile phase	Complex biological matrices such as serum, blood, plasma,	Good resolution and higher selectivity compared with

technique	system, then eluent go from the pre-column into the analytical column	urine; environmental protection, pesticide residue monitoring, food inspection	traditional method
RAM	Based on the size exclusion principle	Removal of endogenous macromolecules in biological matrice	Longer lifetime, higher efficiency, higher analyte recovery, lower organic waste, lower total costs compared to traditional SPE sorbents
TFC	Column filled with adsorption material of large particle size	Removal of macromolecular proteins	A high flow rate to extract and remove the interference in the matrix
MIP	Synthesizing materials with specific molecular recognition properties	Identification and determination of low concentration compounds in complex matrices	High mechanical, thermal and pressure stability, but influenced by sample solvent
MD	Perfuse MD probe under the non-equilibrium conditions	Monitoring physiological levels of the active substances in the animal and human	A novel miniaturized sample pretreatment technique; consuming no solvent and achieving good sensitivity Compared with SPE and LLE.

Types of cartridge used in SPE

HLB cartridge

HLB is Hydrophilic-Lipophilic balanced, water- wetteble, reversed phase sorbent. It is made up from specific ratio of two monomers, the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. Universal for acidic, basic and neutral compound. **[2]**

MCX Cartridge

MCX is a novel, mixed-mode polymeric sorbent that has been optimized to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups. Since the MCX is water wettable, it maintains its capability for higher retention and excellent recoveries even if the sorbent runs dry, which means there is no need to take extraordinary steps to keep the sorbent beds from drying out during the critical steps prior to sample loading. **[2]**

Solid Phase Microextraction (SPME)

It is a new sample preparation technique using a fused-silica fibre coated on the outside with an appropriate stationary phase and is termed Solid phase micro-extraction (SPME). Physically, it is a modified syringe that contains stainless steel microtubing within its syringe needle. This micro tubing has about a 1 cm fused-silica fibre tip which is coated with an organic polymer. This

coated silica fibre can be moved backwards and forwards with the plunger of the syringe.[3] In contrast to conventional SPE with packed-bed cartridges, SPME syringe assembly design allows

combination of all the steps of sample preparation into one step, thus reducing sample preparation time, use of organic solvents and disposal cost. Solid phase microextraction (SPME) consists of a stationary phase grafted on a probe. As for SPE, a large variety of stationary phases and probe dimensions are available. Alternatives to the probe are also used, with syringe needles for instance, as found in microextraction by packed sorbent (MEPS). The principal assets of these techniques are (i) application to volatile compounds by head space extraction, (ii) ready hyphenation with GC, and (iii) probe recycling by thermal desorption.[5] Recently, reports have been made using SPME for *in vivo* sampling: SPME probe was surgically inserted in tissues and organs (e.g. liver, brain) enabling a

rapid monitoring of a treated area without tissue lesions.[6] The foremost advantage of the technique is improved detection limits.There are two types of extraction modes for SPME: first, direct immersion (DI) of SPME fibre into liquid sample matrix, simply termed as DI-SPME) and second, head-space (HS) extraction in which the liquid sample matrix is heated in a vial to volatilize the analytes and the fibre is placed just above the sample matrix. This process is usually referred to as HS-SPME.[3]

Success of SPME is determined by physicochemical properties and the thickness of the fibre

coating. Various commercially available fibre coatings are polydimethylsiloxane for extraction

of non-polar analytes, polyacrylate for extraction of polar analytes (especially phenols), polydimethylsiloxane–divinylbenzene for extraction of polar analytes.Other interesting SPE formats have been developed. The first one is the zip-tip format, in which the stationary phase is contained in the end of a 10 µL pipette tip. It has been particularly used in RP mode in the proteomics field for peptide desalting and enrichment. With disposable pipette extraction (DPX), pipette tips incorporate loosely contained sorbent material, which is mixed with the sample solution. Turbulent air bubble mixing creates a suspension of sorbent in the sample ensuring optimal contact and efficient extraction.[5,3.8,16]

Micro-extraction by Packed Sorbent

The purpose of MEPS is to reduce the sorbent bed volume, making it suitable for large sample volume range (from as low as 10–1000 mL), reducing the number of steps typically involved in conventional SPE and providing easy automation. Typical MEPS is designed in the syringe format, wherein approximately 1mg of the sorbent is packed inside a syringe (100–250 mL) as a plug or between the barrel and the needle as a cartridge. Similar to other sample preparation techniques such as MISPE/dSPE/DPX, novel sorbent materials such as silica-based material carbon, polystyrene–divinylbenzene copolymer (PS-DVB) or MIPs can be used in the MEPS cartridge. It is also possible to use coated sorbents to improve selectivity for a given analyte.[17] it can be seen that there is significant reduction in the amount of sorbent bed, solvents and sample preparation times. One of the most significant advantages of the MEPS is that the same syringe (sorbent bed) can be reused many times just by washing with water (three or four cycles) and for four or five cycles with appropriate solvent.[5,4,8]

Molecularly Imprinted Polymer SPE

A molecular approach towards developing more specific and selective stationary phases for SPE is molecularly imprinted polymer SPE (MISPE). Molecularly imprinted polymers (MIPs) are highly cross-linked polymers which have artificially generated recognition sites that are intentionally engineered and specific to the chemistry of a target analyte or class of analytes to provide high selectivity.[4] MISPE is powerful sample preparation tool. The ability to use MISPE columns directly with the detection system leads to very simple analytical methods. One of the important advantages of MISPE is that, being highly selective in nature, detection and quantitation can be carried out with simple analytical techniques such as HPLC-UV rather than costlier LC-MS systems. [5,4,8]

Dispersive Solid-phase Extraction

Dispersive solid-phase extraction (DSPE), popularly known as, QuEChERS (quick, easy, cheap, effective, rugged and safe), is a novel sample preparation technique which offers unique advantages such as applicability to wide variety of analyte chemistries, low solvent use, little use of glassware/plasticware, high cost effectiveness and automation. The dSPE sample preparation approach involves the solvent extraction of the sample with organic solvents such as acetonitrile–ethyl acetate–acetone and partitioning with magnesium sulfate alone or in combination with other salts (such as sodium chloride) followed by clean-up using dSPE. Essentially, a typical procedure will involve a homogeneous biomatrix sample being transferred to plastic/glass tube, followed by addition of organic solvent followed by mixing. To this mixture, salt is added. The purpose of salt addition is to induce phase separation, which also results in the salting out effect (as discussed earlier in SALLE) and to influence the analyte partitioning behavior. In this step, if required, pH adjustment based on analyte p*K*a is also recommended so as to improve the liquid extraction efficiency. The sample, acetonitrile and salt mixture is further vortex mixed followed by addition of internal standard. The whole mixture is then centrifuged and supernatant is transferred to a clean glass/plastic tube. To this supernatant, the sorbent phase in powder form is added and vortex mixed/shaken. This is stage where contaminants are adsorbed and analyte of interest is present in

supernatant/solvent. The supernatant is separated by centrifugation, leaving solid residue. The supernatant can be evaporated and reconstituted or can directly injected into HPLC.[5,14]

Disposable Pipette Extraction

Disposable pipette extraction (DPX) is another variant of traditional SPE and dSPE. From the bioanalyst's perspective; sample preparation technology should be simple, require minimum

solvent, have a speedy extraction process, and be precise and cost effective. DPX claims to be an environmental friendly yet simple and effective sample preparation technique for separation and extraction of analytes from the various matrices.[4] In its simplest original form, it is modified standard pipette tip which is loaded with free flowing sorbent powder, which is free to disperse. Being a standard tip, is it easy to take the solvent in or out through this dispersive sorbent.

In its modified form, the standard tip contains a dispersible sorbent loosely placed between two frits In case of conventional SPE cartridges which contain packed bed sorbent, sample is loaded from the top; every sorbent particle is used once , thus much sorbent material is required to retain analytes. Also, the success of conventional SPE depends on flow control of the sample loading, washing and elution to achieve good repeatabilities. in DPX sample is mixed with material, and every sorbent particle faces analytes several times. This leads to fast and efficient extraction and not much material needed to retain analytes, although contact time (in tip mixing/aspiration) control is important to achieve good repeatabilities.[5,4]

Supercritical fluid extraction

Supercritical fluid extraction is becoming a very popular technique for the removal ofcnon-polar to moderately polar analytes from solid matrices. Supercritical fluids (SCFs)are increasingly replacing the organic solvents that are used in industrial purificationcand recrystallization operations because of regulatory and environmental pressures on hydrocarbon and ozone-depleting emissions. SCF processes eliminate the use of organic solvents, so it has attracted much attention in the industrial sectors like pharmaceuticals, medical products and nutraceuticals. Pharmaceutical chemists have found SCFs useful for extraction of drug materials from tablet formulation and tissue samples. Supercritical fluids exhibit a liquid-like density, while their viscosity and diffusivity remain between gas and liquid values. The recovery of a supercritical solvent after extraction can be carried out relatively simply by reducing the pressure and evaporating the solvent. Above the critical temperature the liquid phase will not appear even the pressure is increased.[3] The compressibility of a supercritical fluid just above the critical temperature is large compared to the compressibility of ordinary liquids. A small change in the pressure or temperature of a supercritical fluid generally causes a large change in its density. The unique property of a supercritical fluid is that its solvating power can be tuned by changing either its temperature or pressure. The density of a supercritical fluid increases with pressure and becomes liquid-like, the viscosity and diffusivity remain between liquid-like and gas-like values. Additionally, supercritical fluids exhibit almost zero surface tension, which allows facile penetration into microporous materials leads to more efficient extraction of the analyte than the organic solvents. Carbon dioxide is a relatively good supercritical solvent will dissolve many relatively volatile polar compounds. In the presence of small amounts of polar co-solvents like water and short-chain alcohols to the bulk, the carbon dioxide gas can enhance the solubility of polar, non-volatile solutes in supercritical carbon dioxide. Supercritical fluids can be used to extract analytes from samples.[3,5,10]

Column switching

Column switching techniques afford an interesting and creative form of sample preparation. This approach depends on the selectivity of appropriately chosen HPLC stationary phase to retain and separate the analyte of interest while allowing unretained components to be eliminated from the column.[7] The benefits of this technique include total automation and quantitative transfer of targeted compounds within the column switching system. In the heart cut mode a narrow retention time region containing a desired components is cut from the chromatogram and transferred onto another HPLC column for further separation. In this instance, quantitative transfer of the components without adsorptive or degradative losses can be assured.[3,7]

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