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Review Article

SAMPLE PREPARATION AND BIOANALYSIS VALIDATION FOR NATURAL PRODUCT SAMPLE

IDA AYUMIATI, FEBRINA AMELIA SAPUTRI, MUCHTARIDI MUCHTARIDI

Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, University of Padjadjaran, Sumedang, Indonesia 45363

Email: muchtaridi@unpad.ac.id

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ABSTRACT

Bioanalysis study and its validation are important for the application in natural drug discovery and preparation for natural medicine products with safe drug development approaches. Bioanalysis data for natural products can provide quantitative information on active compounds from natural products for pharmacokinetics, pharmacodynamics, bioavailability, bioequivalence, and toxicokinetics. Bioanalysis validation provides useful guidance for obtaining accurate data on drug discovery, preclinical and clinical testing of natural drug product development, and to ensure the methods used are suitable for application in testing the samples. In general, sample preparation and validation of the bioanalysis method are part of the pharmacokinetic characterization of a chemical compound from the discovery to the development stage of natural material products. Therefore, this paper aims to discuss how to prepare samples and validate bioanalysis for natural products.

Keywords: Bioanalysis, Drugs, Natural product, Validation

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INTRODUCTION

Natural product or natural material product is a preparation or product derived from plant material or plant products, including leaves, stems, flowers, roots, and seeds. Plants like this are known as crude extracts. In general, crude extracts are sold as an extract. Usually, the crude extracts are processed first by the extraction process. The extraction process that is commonly used for extraction is maceration. This maceration method can use water, ethanol, or other solvents that can be used to extract some of the chemicals substances in the plant. The resulting product will contain chemical compound metabolites such as fatty acids, sterols, alkaloids, flavonoids, glycosides, saponins, and others. At present, many pharmaceutical dosages are formulated with ingredients from natural products or commonly called herbal medicines. Herbal medicines must also be standardized and tested for safety to obtain efficacy and safety [1].

Most of the medicines used are derived from natural ingredients. Treatment is carried out by the community back to nature due to several things and considerations such as the price of modern drugs or expensive synthetic chemical drugs and consideration of the side effects caused. Many studies have been reported the pharmacological effects of various classes of natural substances. These natural ingredients include flavonoids, terpenoids, steroids, phenylpropanoids, polyketides, and alkaloids. The bioactivity of a candidate compound for the discovery of new drugs. In the pharmaceutical field, plants are one of the raw materials used for the discovery and development of new medicines, both traditional and modern medicine. Associated with the use of natural ingredients as medicinal materials and use for treatment in the community, it is necessary to be assessed and analyzed regarding the efficacy the safety of natural medicinal products that can be scientifically justified [1].

Many natural medicine products currently available in Indonesia are produced based on pharmaceutical standardization references [1]. The development of natural products refer to those stipulated by the health department, must be scientifically justified. The development of natural products must go through six stages, namely selection, screening of biological substances, pharmacodynamic testing, toxicity testing, and development of preparations of drug formulations as well as preclinical and clinical testing in humans. Determination of the composition of natural ingredients as ingredients for medicinal products that conform to standardization by setting tolerance limits for variations in compounds needs to be done to ensure the quality and efficacy of natural medicine products [2]. Active compounds of natural products can be in the form of crude extract or extracts derived from one or several plant species. Both crude extracts and extracts contain many active compounds with various chemical characteristics such as molecular weight, polarity, solubility, volatility, and concentration, ranging from pico to micromolar concentrations [3]. So, to guarantee the quality and efficacy of natural medicinal products, it is necessary to conduct an evaluation analysis of all the components of the compounds in the product [4-7].

Many researchers are currently focusing their research on developing, improving and optimizing analytical methods for testing natural products in the biological matrix to ensure quality, efficacy. Bioanalysis is a sub-discipline of analytical chemistry used to determine drug concentrations or quantitative levels of xenobiotic substances (drugs and their metabolites) and biotic substances such as macromolecules, proteins, DNA, large drug molecules [8]. The biological matrix that is often used in bioanalysis to determine drug levels are blood, serum, plasma, urine, saliva, and cerebrospinal fluid [9]. The bioanalysis method was developed to improve sensitivity, specificity, accuracy, efficiency, throughput testing, data quality, handling and processing of analytical data, analytical costs, and impacts on the environment. Bioanalysis methods are widely used to quantitatively determine drugs and their metabolites in biological matrix and bioanalysis can be applied to clinical pharmacological studies, pharmacokinetics, and toxicity profiles of drug substances (table 1) [7].

The bioanalysis method at the stage of drug analysis in the biological matrix has several stages, namely the selection and collection of biological samples; sample preparation and extraction of analyte from the biological matrix; and analyte detection by several quantification methods. In most bioanalysis studies, biological samples cannot be tested directly but require pretreatment to eliminate endogenous proteins, carbohydrates, salts, and lipids. To get the drug analyte contained in the biological matrix accurately, it must be carried out in the pretreatment stage, namely the extraction stage. The extraction stage becomes the critical success stage in bioanalysis to get good and accurate results. Extraction is the process of separating the material from the mixture using an appropriate solvent [1].

The bioanalysis method used must be validated. Validation of the bioanalysis method usually refers to the rules of the Food and Drug Administration (FDA) or the European Medicine Agency (EMA). To ensure the safety and effectiveness of natural products or herbal

medicines, all processes and methods in making medicines must be properly controlled or validated, especially the analytical methods used to determine the levels of active substances in the preparations. Where the validation of the method is must be able to determine the levels of the active substance. Quantitative analysis methods must be able to meet the requirements of several parameters, namely accuracy, precision, range and linearity, the limit of detection (LOD), the limit of quantitation (LOQ), and specificity. Besides that, there is also a need for a drug analysis method that guarantees the accuracy of research results that can be trusted in the biological matrix. Selective and sensitive bioanalysis methods for quantitative assessment of a drug and its metabolites are essential for successful pre-clinical and clinical pharmacological testing. The measurement of analyte from the biological matrix must be validated. Validation of the bioanalysis method includes all procedures which show that the specific method used for quantitative measurement of analyte originating in the biological matrix. The basic parameters of validation aim to determine quality which includes criteria such as selectivity, sensitivity, linearity, accuracy, precision, stability, quantification limit (LOQ), recovery (recovery), limit of detection (LOD), reproductivity and roughness [10-21]. Therefore, this paper aims to discuss various types of sample preparation techniques and bioanalysis validation for natural products, which include information on various biological sample collection and preparation techniques, discussion of biological sample preparation techniques, and discussion of the application of bioanalysis to various products natural compound.

Sample preparation method in bioanalysis

Sample collection

The first step in bioanalysis is to determine which biological matrix will be used. The biological matrix that is most often used in bioanalysis to determine drug levels are blood, serum, plasma, urine, saliva, and cerebrospinal fluid. Blood sampling is usually done intravenously, through arms or capillaries, sampling from the fingertips, or using a biological matrix of urine [22].

The selection of a biological matrix for sampling is determined by the nature of the analyte in the study. To determine drug levels in pharmacokinetic studies, the selection of the biological matrix blood and urine, is used to determine the elimination profile of compounds from the kidney [18]. Data on drug profiles in the blood are also needed in bioavailability testing. The use of plasma blood samples is important to describe the profile of drugs in the blood in studies of bioavailability or pharmacokinetics [12**,** 23, 24]**.**

Venous blood sampling is usually was taken into a tube with anticoagulants, such as EDTA or heparin. Meanwhile, to get blood serum, it is drawn into a tube without anticoagulants [25]. Blood plasma is obtained when all collected blood is centrifuged at 4000-14,000 rpm for 5-15 min and as much as 30% to 50% of blood volume can be collected. Centrifugation for 15 min at 2,000 x g depletes platelets in the plasma sample. Plasma is the biggest component of blood. Blood plasma is a liquid part of blood [26]**.** Blood plasma fills around 55-60% of the volume of blood in the body. Blood plasma is composed of±92% water, and the remaining 8% is carbon dioxide, glucose, amino acids (protein), vitamins, fats, and mineral salts. After absorption of the drug, the drug is distributed, then undergoes metabolism. The bond of the drug with plasma protein or tissue occurs during the drug distribution process. This bond is usually reversible, meaning that the drug can again be free from bonds with plasma proteins. Its reversible ties indicate that its chemical bonds are weak, for example, hydrogen bonds, van der walls force.

The main components of plasma proteins that bind to drugs are albumin, globulin, and lipoprotein. Drugs that are bound to plasma proteins cannot penetrate biological membranes and are not pharmacologically active or produce effects. Of the various doses of the drug given, some will be bound to plasma proteins, some in free form, depending on how much the drug's affinity for plasma or tissue protein.

Blood was sampled and collected from human subjects or test animals using 5 to 7 ml hypodermic syringe punctures (depending on the sensitivity of the test and the total number of samples taken for the study conducted) [27]. Blood from veins is drawn into tubes that contain anticoagulants such as EDTA, heparin, and others. If plasma and serum blood samples are used, the blood that has been sampled should be immediately centrifuged at 5000-14,000 rpm for 5-10 min [10], then transferred to another tube and clean using a pipette.

Sample preparation

Sampling and sample preparation are very important stages for the analysis of drugs and their metabolites from the biological matrix. Sample preparation is carried out to take analyte and clean samples before analyzing to improve detection without interference from the endogenous content of the biological matrix and does not interfere the detection of an analyte by the introduction of biological matrix or compounds from natural drugs themselves [9] [table 1]. The biological matrix can usually interfere the detection process of analyte or drugs. Materials in the biological matrix that can interfere the analysis process and block chromatographic columns or detectors including proteins, salts, endogenous macromolecules, small molecules, and metabolic byproducts. Therefore, the method of extracting with suitable solvents and the selection of methods for separating drug components from effective biological endogens is very important.

In plasma or serum, the drug is bound to the surface of the protein and must be released. The purpose of the analyte extraction process from a biological matrix is to first eliminate interference in the matrix such as proteins, nucleotides, salts, and others. Secondly to concentrate analyte so that they can be detected properly and more sensitive, thirdly to improve specificity or selectivity, fourthly to improve the analytical performance of the instrument. The choice of the method of isolating an analyte compound from the biological matrix must be precise because it greatly influences the recovery value of the medicinal compounds to be analyzed.

To support the success of bioanalysis, the sample quality factor is very influential on the results of the analysis. Success factors for bioanalysis include parameters for collecting biological samples, handling and sending samples to the laboratory, sample preparation, and storage conditions for the samples during and until the analysis process. The separation parameters of the drug from plasma are part of the sample preparation stage. Some extraction methods of drugs from plasma, (1) Precipitation of proteins (Protein Precipitation, PPT) (2) Liquid-liquid extraction (LLE) (3) Solid-phase extraction (SPE) [table 1].

Precipitation of proteins (PP)

Protein precipitation is the simplest separation method for sample pretreatment. Protein precipitation is often used for routine analysis to remove proteins in a sample or biological matrix. Precipitation occurs due to the addition of organic substances, salt accompanied by changes in pH that can affect the protein. The principle of precipitation of this protein depends on the solubility of the analyte in the specific solvent present in the biological matrix [58]. Samples that have been mixed with the appropriate solvent are then vortexed and centrifuged. The obtained supernatant can be directly injected into the HPLC system or evaporated until dry, usually carried out at 40-50 °C under nitrogen flow and then dissolved in the appropriate solvent until the sample concentration is reached [28].

The protein precipitation method has advantages compared to SPE as a method for removing interference from the biological matrix. The protein precipitation method is more efficient because in time, it only requires a short time so the process is faster, the number of solvents used is even less. Besides, even then, there are disadvantages of this protein precipitation method, which is that samples usually still often contain residues from protein and this is a non-selective sample cleaning method. There may be a risk of endogenous compounds or from medicinal compounds that can interfere in the instrument system as well as in HPLC. The protein precipitation technique is often combined with the SPE technique to produce a cleaner supernatant [25].

In this method, there are organic solvents that are commonly used as protein precipitators depending on the size of the molecule, the size of the protein molecule, and the concentration of the organic

solvent used to precipitate the protein. Organic solvents are often used to precipitate proteins in plasma. Organic solvents are solvents that can be mixed with water such as methanol, ethanol, acetonitrile, and acetone [25]. These solvents can reduce the dielectric constant of the solution, which causes a decrease in solubility resulting in protein deposition. Some studies, methanol, and acetonitrile are organic solvents that are often used for the extraction of samples in biological fluids. Methanol is used as a protein depositor in plasma with the same volume or twice the sample volume. Methanol is also the organic solvent most often used among other organic solvents because it can produce a clear supernatant which is suitable for direct injection into the HPLC system [29]. Ethanol is also used as a protein depositor in plasma with the same volume or twice the plasma volume. Compared to methanol, ethanol is more effective because the longer the chain of alcohol, the easier it is to denaturation protein compared to short-chain alcohol. Acetonitrile is often used as a protein precipitate. The use of acetonitrile is used in the same volume as the plasma volume [1]. Acetonitrile is the best solvent that provides the highest depositional presentation with a low volume to plasma ratio [30].

Liquid-liquid extraction (LLE)

Liquid-liquid extraction is also included as a simple extraction method because this method requires a fairly efficient cost and the use of small solvents. This method also has advantages because it can extract standard analyte or internal efficiently enough [31]. This separation by liquid-liquid extraction is a technique in which a solution (usually in water) is made in contact with a second (usually organic) solvent, which is essentially incompatible with the first solution so that one or more solutes of the first solution move into the second solvent [32]. This is based on the selective extraction of analytes in liquid samples through mixed organic solvents [9]. This separation method is very simple, efficient, fast, and easy to do. Separation with this method can be done by shaking the two solutions in a separating funnel for several minutes. This technique is very suitable for lipophilic compounds. Solvents that are often used in liquid-liquid extraction methods include methyl butyl ether (MTBE), dichloromethane (DCM), ethyl acetate (EC), diethyl ether (DEE), hexane, and others [33-35].

Solid-phase extraction (SPE)

Solid-phase extraction (SPE) is a solid-phase extraction method that can be used for analysis, separation, purification of samples in industrial, pharmaceutical, and toxicological analyzes such as in biological liquids (blood, serum, plasma, urine) and food. This separation method is where the compound dissolved in the liquid mixture is separated from other compounds in the mixture according to its physicochemical properties. This solid-phase extraction is used to concentrate and purify the sample for analysis. SPE is a fairly difficult method for sample preparation, where the analyte is bound to the solid phase, the disturbance will disappear and the analyte is selectively eluted in the final stage [25].

The application of solid-phase extraction is very broad in scope, including for sample preparation aimed at the analysis of new drug compounds, analysis of metabolites in various biological fluids such as blood, serum, and urine, and to separate heavy metal ions from other types of metal ions. SPE is a sample separation method that uses solid particle material, material for chromatography which is usually made in cartridge type packaging which is chemically used to separate the various components of a mixed sample. SPE is a sample preparation technique used to prepare liquid samples and analytic extracts of semi-volatile or non-volatile, but solid samples can also be used. The results of quantitative extraction by the SPE method are easy, fast, and automatic. The use of solvents and time is more effective and efficient [36].

The principle of Solid Phase Extraction (SPE) is extraction carried out by flowing the solution through the solid phase. The analyte dissolved in a solvent that has a low elution power is inserted into the cartridge and then retained on the SPE medium. The analyte is then rinsed with other solvents which have low elution power and is finally eluted using solvents with strong volumes of small volumes [12, 37].

This SPE method is a selective method for sample preparation in which the analyte is bound to the solid phase, the dirt is then washed and the analyte is selectively eluted. Because of the many different sorbent choices, solid-phase extraction is a very powerful technique. Solid-phase extraction consists of four main steps, namely the conditioning, sample loading or retention, washing, and elution. The choice of absorbent in solid-phase extraction is based on its ability to bind to the analyte. Where the bond between the analyte and the absorber must be stronger than the bond of the analyte with the sample matrix so that later the analyte will be held in the absorbent. Then the solvent is selected, which can release the bonds between the analyte and the absorber at the elution stage [38].

Table 1: Application of extraction methods in biological samples

No	Title	Extraction technique	Solvent	Biological samples	Quantification technique	Author
1	Simultaneous estimation of mangiferin and four secoiridoid glycosides in rat plasma using liquid chromatography-tandem mass spectrometry and its application to the pharmacokinetic study of herbal preparation	Protein precipitation	Acetonitrile	Plasma	LC-MS	[1]
2	Rapid and sensitive liquid chromatography-tandem mass spectrometric method for the quantitation of metformin in human plasma	Protein precipitation	Acetonitrile	Plasma	LC-MS	[18]
3	Determination of metformin in human plasma by high-performance liquid chromatography with spectrophotometric detection	Protein precipitation	Acetonitrile: dichloromethane	Plasma	HPLC	[33]
4	Ion-pair vortex assisted liquid-liquid microextraction with back extraction coupled with high-performance liquid chromatography-UV for the determination of metformin in plasma	Ion-pair VALLME followed by back extraction	The obtained extraction recovery was higher than the reported results using bromothymol blue (BTB) which has been previously used as an ion-pair reagent for the LLE of metformin from plasma samples	Plasma	HPLC-UV	[39]
5	Determination of metformin in human plasma by high-performance liquid chromatography	Liquid-liquid extraction	1 butanol: n-hexane (1:1)	Plasma	HPLC	[33]

6	Simple, sensitive, and rapid LC-MS method for the quantitation of indapamide in human plasma—application to pharmacokinetic studies	Liquid-liquid extraction	Diethyl ether Methanol	Plasma	LC–ESI–MS	[31]
7	Simultaneous quantification of antidiabetic agents in human plasma by a UPLC±QToF-MS method	Protein precipitation	Acetonitrile: formic acid 1%	Plasma	UPLC-QToF-MS	[40]
8	Determination of mangiferin in rat plasma by liquid-liquid extraction with UPLC–MS/MS	Liquid-liquid extraction	Acetic Acid: n butanol: isopropanol (24: 5: 1)	Plasma	UPLC-MS/MS	[13]
9	Pharmacokinetic study of mangiferin in human plasma after oral administration	Liquid-liquid extraction	Acetoacetate: n butanol: isopropanol (24: 5: 1)	Plasma	HPLC-MS	[14]
10	Development and validation for the high selective quantitative determination of metformin in human plasma by cation exchanging with normal-phase LC/MS/MS	SPE (cation exchange)	Methanol, water	Plasma	LC-MS/MS	[38]
11	Salting-out homogenous extraction followed by ionic liquid/ionic liquid-liquid micro-extraction for determination of sulfonamides in blood by high-performance liquid chromatography	ionic liquid-liquid micro-extraction	Acetonitrile	Serum	HPLC	[41]
12	Development and validation of a multiplex UHPLC-MS/MS method for the determination of the investigational antibiotic against multiresistant macozinone tuberculosis (PBTZ169) and five active metabolites in human plasma	Protein precipitation	Methanol	Plasma and serum	UHPLC-MS/MS	[29]
13	High-performance liquid chromatographic method for the determination of mangiferin in rat plasma and urine	Liquid-liquid extraction	Acetonitrile: acetic acid (9: 1)	Plasma and urine	HPLC	[42]
14	Simultaneous determination of 17 Toxic alkaloids in human fluids by Liquid chromatography coupled with electrospray ionization tandem mass spectrometry	Liquid-liquid extraction	Chloroform: ether	Plasma	LC-ESI-MS/MS	[19]
15	Quantification of fexofenadine in human plasma by liquid chromatography coupled to electrospray tandem mass spectrometry using mosapride as the internal standard	solid-phase extraction (SPE) OASIS™ HLB cartridge	Methanol, water	human plasma	LC-MS	[43]
16	Strawberry anthocyanins are recovered in urine as glucuroand sulfoconjugates in humans	Solid-phase extraction (SPE) cartridge (Sep-Pak C18 Plus; Waters, Milford, MA)	Methanol, water, chloride acid	Urine	HPLC-ESI-MS-MS and HPLC with UV-visible	[12]
17	Simultaneous quantitation of 3-n-butylphthalide (NBP) and its four major metabolites in human plasma by LC-MS/MS using deuterated internal standards	Protein precipitation	Methanol, chloride acid	plasma	LC-MS	[44]
18	Comparative pharmacokinetics of baicalin, wogonoside, baicalein and wogonin in plasma after oral administration of pure baicalin, *Radix Scutellariae* and *Scutellariae-Paeoniae* Couple Extracts in Normal and Ulcerative Colitis Rats.	Protein precipitation	Methanol-Acetonitrile-5 mmol Ammonium acetate	Plasma	LC-MS	[45]
19	Identification of Puerarin and its metabolites in rats by liquid chromatography-tandem mass spectrometry	Precipitate proteins	Methanol	blood and urine	LC-MS	[46]
20	Sensitive analysis of aconitine, hypaconitine, mesaconitine and jesaconitine in human body fluids and aconitum tubers by LC/ESI-TOF-MS	Solid-phase extraction (SPE)	Methanol, water, and buffer phosphate (pH 5.5)	Plasma	LC/electrospray (ESI)-time-of-flight (TOF)-MS	[37]
21	Development and validation of a liquid Chromatography coupled with atmospheric-pressure chemical ionization ion trap mass spectrometric method for the simultaneous determination of Triptolide, Tripdiolide, and Tripterine in human serum.	Liquid-liquid extraction	Ethyl acetate	Serum	LC-MS/MS	[47]
22	UPLC-MS/MS method for the simultaneous quantification of eight compounds in rat plasma and its application to a pharmacokinetic study after oral administration of veratrum (*Veratrum nigrum* L.) extract	Protein precipitation	Methanol	Plasma	UPLC–MS/MS	[48]

23	Application of a hybrid ion trap/time-of-flight mass spectrometer in metabolite characterization studies: structural identification of the metabolism profile of antofloxacin in rats rapidly using MSn information and accurate mass measurements	Solid-phase extraction with a C18 cartridge (Waters C18 Sep-Pak, 500 mg)	Methanol, water	The bile and urine samples	LC-IT-TOF/MS	[36]
24	Separation and identification of Aconitum alkaloids and their metabolites in human urine	SPE C18 cartridge pre-equilibrated with distilled water	Methanol, water	Urine	LC-ESI-MS	[49]
25	Determination of artemether and dihydroartemisinin in human plasma with a new hydrogen peroxide stabilization method	Micro-elution HLB plate	Methanol, water	Plasma	LC-MS/MS	[15]
26	Determination of capsaicin, nonivamide, and dihydrocapsaicin in blood and tissue by liquid chromatography-tandem mass spectrometry	Liquid-liquid extraction	n-butyl chloride	Blood and tissue	LC-MS/MS	[50]
27	Identification and quantification of flavonoids in human urine samples by column-switching liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry	Liquid-liquid extraction	10% Methanol, DMSO	Urine	HPLC	[51]
28	Determination of apomorphine in canine plasma by liquid chromatography-electrospray ionization mass spectrometry and its application to a pharmacokinetic study	Liquid-liquid extraction	Diethyl ether	Urine	LC-MS	[34]
29	Simultaneous determination of b-Artemether and its metabolite dihydroartemisinin in human plasma and urine by a high-performance liquid chromatography-mass spectrometry an assay using electrospray ionization	Liquid-liquid extraction	2,2,4 trimethylpentane: ethyl acetate (7:3 v/v)	Plasma and urine	HPLC-MS	[52]
30	Quantitative analysis of artemether and its metabolite dihydroartemisinin in human plasma by LC with tandem mass spectrometry	Liquid-liquid extraction	Methyl t-butyl ether	Plasma	LC-MS	[53]
31	Rapid Determination of Nicotine in Saliva by Liquid Phase Microextraction-High Performance Liquid Chromatography	Microextraction (LPME)	Acetone	Saliva	HPLC	[54]
32	Simultaneous determination of nicotine and cotinine in serum using high-performance liquid chromatography with fluorometric detection and post-column UV-photoirradiation system	Liquid-liquid extraction	Chloroform	Serum	HPLC	[55]
33	Nicotine and metabolites determination in human plasma by ultra-performance liquid chromatography-tandem mass spectrometry: a simple approach for solving contamination problem and clinical application	Liquid-liquid extraction	Buffer 10 mmol sodium carbonate-bicarbonate (pH 9.0) dan DCM	Plasma	LC-MS/MS	[56]
34	Simultaneous determination and pharmacokinetic study of gambogic acid and gambogenic acid in rat plasma after oral administration of Garcinia hanburyi extracts by LC-MS/MS	Liquid-liquid extraction	Ethyl acetate	Plasma	LC-MS/MS	[57]
35	Identification and quantitative determination of a major circulating metabolite of gambogic acid in human	Liquid-liquid extraction	Ethyl acetate	Plasma	LC–DAD–MS/MS	[58]
36	HPLC–APCI–MS for the determination of teprenone in human plasma: method and clinical application	Protein precipitation	Ethanol	Plasma	HPLC–APCI–MS	[59]
37	Determination of gambogic acid in dog plasma by high-performance liquid chromatography for a pharmacokinetic study	Liquid-liquid extraction	Ethyl acetate	Plasma	HPLC	[60]
38	Determination of total and unbound sufentanil in human plasma by ultrafiltration and LC-MS/MS: application to a clinical pharmacokinetic study	Solid-phase extraction (SPE)	Methanol, water	Plasma	LC-MS/MS	[61]
39	Simultaneous determination of five alkaloids by HPLC-MS/MS combined with micro-SPE in rat plasma and its application to pharmacokinetics after oral administration of *Lotus Leaf* Extract	Micro SPE	50% methanol in water 0.1% formic acid in water	Plasma	HPLC-MS/MS	[62]

40	Determination of armepavine in mouse blood by UPLC MS/MS and its application to the pharmacokinetic study	Protein precipitation	Acetonitrile	Blood	UPLC MS/MS	[63]
41	High throughput-SPE based elution coupled with UPLC-MS/MS for determination of eluxadoline in plasma sample: Application in pharmacokinetic characterization of PLGA nanoparticle formulations in rats	Micro SPE (Waters 96-well Extraction Plate Vacuum Manifold)	Methanol, water, 2% formic acid	Plasma	UPLC-MS/MS	[23]
42	A rapid method to determine colonic microbial metabolites derived from grape flavanols in rat plasma by liquid chromatography–tandem mass spectrometry	Micro solid-phase extraction (µ-SPE) Oasis HLB µ-Elution Plates 30 µm (Waters, Barcelona, Spain)	Methanol, 0.2% acetic acid	Plasma	HPLC-ESI-MS/MS	[64]
43	Application of a rapid µ-SPE clean-up for multiclass quantitative analysis of sixteen new psychoactive substances in whole blood by LC-MS/MS	Micro solid-phase extraction (µ-SPE)	Water, methanol, acetonitrile	Whole blood	LC-MS/MS	[65]
44	Pharmacokinetics of Nuciferine and N-Nornuciferine, two major alkaloids from *Nelumbo nucifera Leaves*, in rat plasma and the brain	Liquid-liquid extraction	Ethyl acetate	Plasma	UPLC coupled with photodiode array detection	[66]
45	Plasma and brain pharmacokinetics of ganoderic acid A in rats determined by a developed UFLC–MS/MS method	Liquid-liquid extraction	Ethyl acetate	Plasma	UFLC–MS/MS method	[67]
46	A sensitive liquid chromatography-tandem mass spectrometry method for pharmacokinetics and tissue distribution of nuciferine in rats	Liquid-liquid extraction	Ethyl acetate	Plasma	LC-MS/MS	[68]
47	Pharmacokinetic profiles of the five isoflavonoids from Pueraria lobata roots in the CSF and plasma of rats	Liquid-liquid extraction	Ethyl acetate-methanol (3:1)	Plasma	UFLC-MS/MS	[20]
48	HPLC method for the determination and pharmacokinetic studies of four triterpenoids in rat plasma after oral administration of *Ganoderma lucidum* extract	Liquid-liquid extraction	Dichloromethane–ethyl acetate (90:10)	Plasma	HPLC	[69]
49	Pharmacokinetics of ganoderic acid D and its main metabolite by liquid chromatography-tandem mass spectrometry	Protein precipitation	2% hydrochloric acid, methanol, acetonitrile	Plasma	LC-MS/MS	[70]
50	A new quantitation method of protodioscin by HPLC-ESI-MS/MS in rat plasma and its application to the pharmacokinetic study	Protein precipitation	Acetonitrile	Plasma	HPLC-ESI-MS/MS	[71]
51	Metabolism and pharmacokinetics in rats of ganoderiol F, a highly cytotoxic and antitumor triterpene from *Ganoderma lucidum*	Solid-phase extraction (SPE)	Methanol, water	Plasma	LC/MS/MS	[72]
52	Analysis of anthocyanins in plasma for determination of their bioavailability	Solid-phase extraction (SPE)	0.01M o phosphoric acid at a pH of 5.0 set with triethyl ammonia as eluent. And water/ Formic acid/acetonitrile (350/50/50)	Plasma	HPLC	[24]
53	Simultaneous determination of different flavonoids in human plasma by a simple HPLC assay	Liquid-liquid extraction	Aceton, acetic acid	Plasma	HPLC	[73]
54	The identification of flavonoids as glycosides in human plasma	Protein precipitation	Methanol	Plasma	HPLC	[74]
55	Determination of steroid hormones in human plasma by GC–triple quadrupole MS	Liquid-liquid extraction	Chloroform	Plasma	GC–triple quadrupole MS (GC-MS/MS)	[75]
56	A UPLC-MS/MS method for simultaneous determination of free and total forms of phenolic acid and two flavonoids in rat plasma and its application to comparative Pharmacokinetic Studies of *Polygonum capitatum* Extract in rats	Protein precipitation	Methanol	Plasma	UPLC-ESI-MS/MS	[16]
57	LC-MS/MS determination and pharmacokinetic study of seven flavonoids in rat plasma after oral administration of *Cirsium japonicum* DC. Extract	Protein precipitation	Methanol	Plasma	LC-MS/MS	[76]
58	Simultaneous determination of four	Protein	Methanol	Plasma	LC-MS/MS	[17]

	flavonoids and one phenolic acid in rat plasma by LC-MS/MS and its application to a pharmacokinetic study after oral administration of the *Herba Desmodii Styracifolii* extract	precipitation				
59	Pharmacokinetic study of four flavones of glycyrrhiza in rat plasma using HPLC–MS	Protein precipitation	Methanol	Plasma	HPLC–MS	[77]
60	Determination of chamaechromone in rat plasma by liquid chromatography-tandem mass spectrometry: Application to a pharmacokinetic study	Liquid-liquid extraction	Ethyl acetate	Plasma	LC-MS/MS	[78]
61	Simultaneous quantification of paeoniflorin, nobiletin, tangeretin, liquiritigenin, isoliquiritigenin, liquiritin, and formononetin from Si-Ni-San extract in rat plasma and tissues by liquid chromatography-tandem mass spectrometry	Solid-phase Extraction (SPE) Oasis HLB SPE cartridges (30 mg; Milford, MA, USA)	Methanol, water	Plasma	LC-MS/MS	[79]
62	Structural pharmacokinetics of polymethoxylated flavones in rat plasma using HPLC-MS/MS	Liquid–liquid extraction	Ethyl acetate	Plasma	UHPLC-MS/MS	[80]
63	Simultaneous determination of seven active components in rat plasma by UHPLC-MS/MS and application to a quantitative study after oral administration of Huang-Lian Jie-Du Decoction in high fat-induced atherosclerosis rats	Protein precipitation	Methanol and acetonitrile	Plasma	UHPLC–MS/MS	[81]
64	Determination of acacetin in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study	Protein precipitation	Acetonitrile	Plasma	UPLC-MS/MS	[35]
65	Simultaneous determination of vitexin-4”-O-glucoside, vitexin-2”-O rhamnoside, rutin and vitexin from hawthorn leaves flavonoids in rat plasma by UPLC–ESI-MS/MS	Protein precipitation	Methanol	Plasma	UPLC–ESI-MS/MS	[82]
66	UPLC-QTOF-MS Identification of the chemical constituents in rat plasma and urine after oral administration of *Rubia cordifolia L*. Extract	Protein precipitation	Methanol	Plasma and urine	UPLC-QTOF-MS	[83]
67	Determination and pharmacokinetic study of pirfenidone in rat plasma by UPLC-MS/MS	Protein precipitation	Acetonitrile	Plasma	UPLC-MS/MS	[84]
68	UPLC–MS/MS method for determination of avicularin in rat plasma and its application to a pharmacokinetic study	Protein precipitation	Acetonitrile: Methanol (9:1, v/v)	Plasma	UPLC-MS/MS	[21]
69	Determination of cephalomannine in rat plasma by gradient elution UPLC-MS/MS method	Protein precipitation	Perchloric acid: methanol (1:9, v/v)	Plasma	UPLC-MS/MS	[85]
70	Determination of songorine in rat plasma by UPLC–MS/MS: Assay development and application to the pharmacokinetic study	Protein precipitation	Acetonitrile	Plasma	UPLC-MS/MS	[86]
71	Determination of parthenolide in rat plasma by UPLC-MS/MS and its application to a pharmacokinetic study	Protein precipitation	Acetonitrile	Plasma	UPLC-MS/MS	[87]
72	Determination of kurarinone in rat plasma by UPLC–MS/MS	Liquid-liquid extraction	Ethyl acetate	Plasma	UPLC–MS–MS	[88]
73	Simultaneous determination of seven components from Hawthorn Leaves flavonoids in rat plasma by LC-MS/MS	Protein precipitation	Methanol	Plasma	LC-MS/MS	[89]
74	Rapid determination of ruscogenin in rat plasma with application to pharmacokinetic study	Liquid-liquid extraction	Ethyl acetate	Plasma	UPLC–MS–MS	[90]
75	Quantification of liensinine in rat plasma using ultra-performance liquid chromatography-tandem mass spectrometry and its application to a pharmacokinetic study	Protein precipitation	Acetonitrile	Plasma	UPLC-MS/MS	[91]
76	A Validated HPLC-DAD method for simultaneous determination of etodolac and pantoprazole in rat plasma	Protein precipitation	Acetonitrile	Plasma	HPLC-DAD	[92]
77	Liquid chromatography/negative ion electrospray tandem mass spectrometry method for the quantification of rosuvastatin in human plasma: Application	Liquid-liquid extraction	Ethyl ether	Plasma	UPLC-MS/MS	[93]

	to a pharmacokinetic study					
78	High-Performance Liquid Chromatographic Method for the determination and pharmacokinetic study of mangiferin in plasma of rats having taken the traditional Chinese medicinal preparation Zi-Shen Pil	Protein precipitation	Acetonitrile	Plasma	HPLC	[94]
79	Development and validation of an HPLC method for simultaneous determination of trimethoprim and sulfamethoxazole in human plasma	Protein precipitation	Acetonitrile	Plasma	HPLC	[95]
80	Determination of mangiferin in rat eyes and pharmacokinetic study in plasma after oral administration of mangiferin-hydroxypropyl-beta-cyclodextrin inclusion	Protein precipitation	acetonitrile-glacial acetic acid (9:1, v: v)	Plasma	HPLC	[96]
81	Method for analysis of tannic acid and its metabolites in biological samples: Application to tannic acid metabolism in the rat	Liquid-liquid extraction	Ethyl Acetate	Serum and urine	HPLC	[97]
82	Evaluation of polyphenol bioavailability in rat small intestine	Protein precipitation	trichloroacetic acid	Tissue	Spectrophotometer UV-Vis	[98]
83	Preparation of Penta-O-galloyl-β-d-glucose from tannic acid and plasma pharmacokinetic analyses by liquid-liquid extraction and reverse-phase HPLC	Liquid-liquid extraction	Ethyl Acetate	Plasma	HPLC	[99]
84	Human pharmacokinetics of ginkgo terpene lactones and impact of carboxylation in blood on their platelet-activating factor antagonistic activity	Liquid-liquid extraction	Ethyl Acetate	Plasma	HPLC	[100]
85	Simultaneous determination of flavonols and terpene lactones in beagle dog plasma by ultra-performance liquid chromatography-tandem-mass spectrometry: Application to pharmacokinetic studies on *Ginkgo Leaf* extract	Liquid-liquid extraction	Ethyl Acetate	Plasma	UPLC-MS	[101]

Sample preparation for natural product

Flavonoids are important secondary metabolites in plants. Flavonoids are phenolic compounds, providing pharmacological effects such as antioxidants, cytotoxics, antimicrobials, and antivirals [51], therapy for diseases such as cardiovascular and neurodegenerative [73]. Examples of polyphenol groups include catechin, routine, baicalein, quercetin, apigenin-7-glucoside and luteolin-7-glucoside are all flavonoids [73]. In 2011 more than 9000 types of flavonoids were reported used for food supplements. Nowadays many supplements or herbal medicines are made by utilizing the properties of flavonoid compounds. From one study of *Swertia chirata* (family Gentianaceae) plants. Generally, this plant is known by the name "*Chirata*". This plant is often used for medicines because it has pharmacological effects such as antimalarial, a bitter stomachic, anthelmintic, and as a remedy for urine scanty, epilepsy, ulcer, bronchial asthma, and certain types of mental disorder. Herbal medicine based on *Polygonum capitatum* extract also containing phenolic acid and flavonoids is thought to contribute greatly to the clinical therapeutic efficacy of *P. Capitatum* [16].

Flavonoid compounds in biological fluids are usually extracted using ethyl acetate after acidification, whereas alkaloids are usually extracted with chloroform or ether after the alkalinization process [10, 50]. Saponin and sesquiterpenoids are extracted with n-butanol, methylene chloride, ether, or ethyl acetate. The selection of sample pretreatment techniques for each analyte in biological fluids depends on the expected concentration of the analyte and the required detection limits. In the biological type of urine, to obtain metabolites with high concentrations, simple sample dilution is performed before analysis. With the method of protein precipitation using acetonitrile will give good results and removal of proteins for quantitative analysis of an analyte in biological fluids. In testing analyte in biological liquids, matrix suppression usually occurs; the SPE method or liquid-liquid extraction is needed before the bioanalysis process is carried out using LC-MS. In other cases, the supernatant observed after extraction of protein deposition can be combined with continuing SPE procedures or with liquid-liquid extraction techniques.

At the beginning of the study, it was found that the content in chirata plants contained compounds of flavonoids, xanthones, terpenoids, iridoid, and secoiridoid glycosides. One product from this plant extract (Chirata) has been developed as an antidiabetic agent by the Central Drug Research Institute (CDRI), Lucknow, from aerial part of *S. chirata*. To find out and realize the pharmacological effects of these chirata-based extracts, pharmacokinetic testing of bioactive compounds is needed. Mangiferin and amarogentin are two bioactive markers of the chirata plant used for standardization. Determination of these bioactive compounds from flavonoids by the development of LC-MS-based testing. LC-MS/MS detect and measure chirata (xanthone glycosides and sekoiridoid) in plasma in a sensitive manner, for use in herbal pharmacokinetic testing with compounds from chirata in mouse plasma. Analysis with relatively low concentrations is usually found in analyzes based on the biological sample matrix. The UPLC-ESI-MS/MS system is used to measure levels of a compound in plasma or biological matrix. HPLC is widely used to separate and analyze flavonoids [73]. The analytical method for determining gallic acid, quercitrin, and quercetin compounds which are phenolic acids and flavonoids in biological samples, is high-performance liquid chromatography (HPLC) with MS [16]. The addition of acid to the mobile phase in the LC system causes satisfactory peak symmetry and good resolution and can significantly increase sensitivity and reduce analysis time with columns along with the 50 mm. It was also reported that the LC-MS/MS method was quite sensitive and selectively used to evaluate the pharmacokinetics of various flavonoid compounds in plasma [89].

The preparation used for pharmacokinetic testing of flavonoid bioactive compounds is by precipitation protein method. The precipitation protein method process can be used organic solvents such as acetonitrile, acetone, methanol, and ethanol. The extracting solvent used for flavonoid compounds is acetonitrile. These organic

solvents are widely used as extracting solutions because they correspond to the solvents used in the analysis of high-performance liquid chromatography or LC-MS/MS. The selection of suitable internal standards is also a key factor in the analysis of biological samples [16, 17]. Internal standards are used to reduce unknown errors that can occur during quantitative testing [17]**.**

One of the main groups of flavonoids that have antidiabetic effects is mangiferin. Mangiferin, 1,3,6,7-tetrahydroxyxanthone-C-2-d-glucoside is a natural glucosyl xanthone from the Chinese medicinal herb Mangifera indica [13]. Many researchers report that the pharmacological effects of mangiferin are antibacterial, antidiabetic, and anticancer. Mangiferin is also reported as an oral treatment for antidiabetic. To evaluate this mangiferin-based herbal drug, pharmacokinetic and bioavailability testing is performed to determine absorption in the body and to determine its effectiveness. So before testing, proper biological sample preparation is required. Measurement of mangiferin in biological samples is very important for drug development and a fast and reliable bioanalytic method is needed. Blood samples taken from test animals or volunteers are immediately centrifuged for separation to avoid damage or hydrolysis samples. Blood samples are centrifuged, then plasma or serum are transferred to another clean plastic container. Plasma and serum that have been separated are stored at-20 °C until used for the next analysis stage. After that in extracting mangiferin in biological samples, the protein precipitation method is used and the solvent used is acetonitrile [94, 96]**.**

Unlike the case with the method of extracting mangiferin samples from biological samples is done by the liquid-liquid extraction method. This extraction solution uses 2 ml acetate: n-butanol: isopropanol (24: 5: 1, v/v/v). Another procedure also used the way the sample was prepared according to the method described by Han *et al.* [13], with slight modifications. 100 µl of plasma was extracted using 2 ml of acetoacetate: n-butanol: isopropanol (24: 5: 1, v/v/v) after adding 50 µl of hydrochloric acid solution (1 mol/l^{-1}). Polyhydroxylated flavonoids which have health effects as anticancer, anti-inflammatory, neuroprotective properties. Three polymethoxylated flavonoids, kumatakenin, pachypodol, and retucine, were isolated from the methanol extract of *Melicope semecarpifolia*. To extract the analytes, a liquid-liquid extraction method with ethyl acetate solvent was used and 0.1% formic acid was added. Pharmacokinetic measurements of gambogic acid and gambogenic acid are also used the liquid-liquid extraction method using ethyl acetate with the addition of acidification conditions with hydrochloric acid [57, 58]. Precipitation of protein with acetic acid is the ratio of acetonitrile-acetate (9: 1, v/v) for pretreatment of plasma samples before being analyzed using HPLC. This extraction method shows adequate sensitivity, selectivity, and accuracy. Determination of catechin, routine, baicalein, quercetin, apigenin-7-glucoside, and luteolin-7-glucoside in plasma used the method of extracting precipitation protein with methanol [73]. Development and validation for the quantification of seven flavonoids namely pectolinarin, linarin, pectolinarigenin, hispidulin, diosmetin, acacetin, and apigenin in rat plasma after oral administration of *Cirsium japonicum* DC. Extracts used a plasma sample preparation method by precipitation protein using methanol solvent [76]. The solvent that previously added acetic acid [73]**.** Gallic acid, quercitrin, and quercetin, which is a group of phenolic acids and flavonoids, were analyzed with the performance of ultra-liquid chromatography-ionization electrospray-tandem mass spectrometry (UPLC-ESI-MS/MS) extracted in biological samples by precipitation protein methods using plasma solvents using solvents methanol and 1% formic acid added as much as 50 ul [16]**.** The precipitation protein method was initially used in this flavonoid study, there was ion suppression and a lower recovery (<50%) was obtained when the plasma concentrations exceeded 1000 ng/ml. From the discussion of several studies of flavonoids also reported that the use of acetonitrile solvents provides a good peak shape for all analytes of flavonoids compounds analyzed from plasma samples. Separation is shorter [89]**.** Mangiferin is also a weak organic acid so that a hydrochloric acid solution is added at the beginning to maximize the conversion of mangiferin into its union form. Besides this mangiferin has a tricyclic aromatic ring. Such compounds have poor solubility in most non-polar solvents. For testing five types of flavonoids used different cellular phase conditions to obtain the best analysis results. The acetone solution gives the highest recovery results compared to using methanol, acetonitrile, ethanol, diethyl ether, and ethyl acetate [73]. The addition of acetic acid at the beginning of the preparation by extraction of a precipitation protein method is also one of the things that causes the best recovery results compared to using hydrochloric acid. The addition of acid to the precipitation protein method aims to improve extraction efficiency. Types of acids that can be used are 1% acetic acid, 1% formic acid, 0.1% hydrochloric acid; 0.2; 0.4 mol/l. Addition of formic acid 1% 50 µl to 100 µl into the plasma obtained high analytic recovery results [16]. Simultaneous determination of seven flavonoid compounds, i.e. routine, vitexin-4 "-O-glucoside, vitexin-2"-O-rhamnoside, hyperoside, vitexin, shanyenoside A, and quercetin in rat plasma after administration of hawthorn flavonoid (HLF) leaves using IV lisionotin as an internal standard (IS). This target compound was extracted by precipitation protein method using a methanol solvent [89]**.**

Research conducted by Dongxiao sun *et al.*, for the simultaneous determination of four flavonoids and one phenolic acid in rat plasma by the LC-MS/MS method and its application for pharmacokinetic studies after oral administration of *Desmodii Styracifolii* herbal extract [17]**.** *Desmodii Styracifolii* has been tested by phytochemicals where the results are known to contain many elements, such as flavonoids, phenolic acids, polysaccharides, and alkaloids. The herb *Desmodii Styracifolii* (Guangjinqiancao), has been used as traditional Chinese medicine (TCM), which comes from the *Desmodium styracifolium* (Osb.) Merr. (Leguminosae) dried. The four types of flavonoids are schaftoside, isovitexin, luteolin, and apigenin as well as one phenolic acid which is ferulic acid in the biological matrix of rat plasma and sulfamethoxazole is used as an internal standard (IS) [17]**.** Another natural product, Glycyrrhiza, which is a popular drug in China that has been used for years as an anti-cancer, anti-ulcer, anti-inflammatory, antiviral, and for improving liver function [19]**.**

The development of quantitative methods to measure the constituents of herbal samples in biological samples needs to be done to obtain information and a better understanding of the pharmacological and clinical effects of a product of natural ingredients (herbs). This herbal remedy is used as a treatment for kidney stones. The extraction sample preparation used was the protein deposition method [17, 19]. A methanol solvent was used to extract four flavonoids and phenolic acids from the extract of Herba *Desmodii Styracifolii* in plasma. For liquid-liquid extraction and precipitation, protein methods are performed to compare which method produces good and high recovery results. In this research for flavonoid measurement, the liquid-liquid extraction method produces a low recovery value. Ethyl acetate, chloroform, diethyl ether, or n-butanol solvents were optimized as extract solvents in the liquid-liquid extraction method. For the use of the precipitation protein method it produces a better recovery value, can eliminate plasma protein, the resulting supernatant is cleaner, and results in a good separation between peaks without any disturbance. This effective result was found when using methanol in the precipitation protein method. As explained in the previous discussion that the addition of acid into biological samples before the precipitation protein process can produce a much higher analyte response value. This is because the results of the research show that acidification can suppress protein adsorption and promote the release of analyte constituents from proteins [17]**.**

In addition to precipitation protein methods and liquid-liquid extraction, many studies to measure natural compounds in biological matrix use the Solid Phase Extraction method. One of them is in the pharmacokinetic determination of five types of alkaloids (nunciferine, O-nornuciferin, liriodenine, armepavine, and pronuciferine) in *Lotus Leaf* Extract [62] samples of 100 µl and standard internal solutions at vortex were then extracted using SPE columns (Cleanert PEP-2, 40–60 µm, 60Å 10 mg /1 ml; Phenomenex, Torrance, CA, USA). In the first stage, the SPE column is poured with 50% methanol solution in water and 0.1% formic acid. The second stage is used methanol to wash the column. The third step was carried out elution with acetonitrile solution after adding a plasma sample. The last step of the eluent is centrifuged

and the solution obtained is injected or continued to the analysis stage [62] n the SPE method, the selection of sorbents is one of the key factors of extraction ability because physicochemical properties affect the absorption capacity for different types of sorbents. The SPE method is used to extract anthocyanin in plasma to measure bioavailability. Anthocyanin shows great potential as a natural food antioxidant. Four anthocyanins are known in elderberry two main components (cyanidin-3-Oglucoside and cyanidin 3-O-sambubioside). For protein removal, in this study (LiChrospher ADS) with a hydrophilic surface (diol silica) and the hydrophobic inner surface of the pores (C18) was used [24].

Research methods for the analysis of Tannic Acid and its metabolites in biological samples: Application for Tannic Acid Metabolism in mouse also uses the ECC method to prepare samples. The procedure is done for 0.5 ml serum or 2 ml urine added 2 ml buffer acetate 0.1 M (pH 5.0), 0.1 ml 4% ascorbic acid solution (in air), 0.1 ml 4% solution Na_2EDTA solution (in water), and 0.2 ml of a solution of $CaCl_2$ 0.6 M (in water) [97]. In this procedure hydrochloric acid was used as a solution of that acid with 0.5 ml HCl 1 N. The metabolite was extracted three times with a 5 ml ethyl acetate extraction solvent after obtaining PA (39.33 mmol) as an internal standard (IS). After evaporation to dryness, the residue is dissolved into 2 ml of methanol to use the HPLC method [97]. The clinical effects of tannin recently have antimutagenic, cardiovascular, anticancer [17, 97, 102], antioxidant, anti-bacterial [102], cholesterol and serum triglyceride levels, and lipogenesis by insulin. Chemically reactive tannins and forming inter-and intra-molecular hydrogen bonds that facilitate can precipitate macromolecules, such as proteins and carbohydrates. For the preparation of *penta-O-galloyl--d-glucose* samples from tannic acid in the plasma matrix for pharmacokinetic testing the liquid-liquid extraction technique and the incorporation of quantification with reverse-phase HPLC [100]. Gallotannin *penta-O-galloyl-beta-d-glu*cose (PGG) has many biological activities as an anti-cancer. The procedure used is plasma and internal standards mixed with 20% ascorbic acid, which functions as an antioxidant. Then 2% acetic acid solution is added to acidify the sample. Samples with acetyl as extraction solution [100].

Other herbal compounds are terpene lactone. Terpene lactone is a class of bioactive constituents of standard preparations of *Ginkgo biloba leaf* extract, which is widely used as adjunctive therapy in patients with ischemic cardiovascular and cerebrovascular diseases. In a study conducted by this investigation evaluated human pharmacokinetics of lactone terpene *ginkgo* and the effects of carboxylation in the blood [100]. *Ginkgo biloba* leaf extract is often used as an herbal medicinal product as a food supplement and cardiovascular treatment [101]. Previous studies of potential terpene pharmacological therapeutic agents showed as antioxidant, anti-inflammatory, anticancer, analgesic, immune modulation, and wound healing [103]. Analysis of lactone terpenes in biological matrices was analyzed using a mass spectrometer. Preparation of biological samples is used liquid-liquid extraction method with the addition of hydrochloric acid before extraction using ethyl acetate solvent. The extraction product is then dried by nitrogen gas flow and the residual results obtained are dissolved in acetonitrile for analysis by mass spectra [100]. Another study reported on the study of pharmacokinetic evaluations of the main compounds in the dosage formulation of *Ginkgo Biloba leaves.* Effective compounds evaluated include kaempferol, quercetin, isorhamnetin, ginkgolides A, ginkgolides B, ginkgolides C and bilobalide. This compound was detected by using ultra-performance tandem liquid-chromatography mass-spectrometry (UPLC-TMS), which was validated [101]. All prepared plasma samples were extracted using liquid-liquid extraction techniques. In this extraction process, hydrochloric acid was added to the plasma sample to convert the flavonoid glycosides into the form of aglycone. In other studies also reported preparation techniques for terpene bioavailability of protein precipitation techniques with acetonitrile [103].

Gallic acid (GA) is an endogenous product found in plants, in free or bound form; gallic acid is found in large quantities in tea leaves, which are extracted in hot water. Gallic acid is a powerful antioxidant that has antimutagenic effects and anti-cancer activity. Bioavailability studies were carried out on gallic acid in the plasma of test animals such as mice and rabbits. The sample extraction method used is the liquid-liquid extraction method. The separated plasma sample was centrifuged at 1800 g for 10 min after which extraction was carried out with ethyl acetate solvent and the separation of the organic fraction [104].

The application of biological sample extraction techniques is also often used in pharmacokinetic tests for xanthone group compounds. One of them is evaluating mangosteen compounds in biological samples, as a study Pharmacokinetic characterization of mangosteen (*Garcinia mangostana*) fruit extract standardized to α-mangosteen in C57BL/6 mice. For this study, we evaluated the PK profile of α-mangosteen using a standardized mangosteen extract in C57BL/6 mice. Mangosteen has antioxidant activity both *in vitro*. The sample preparation used is the protein precipitation technique with the addition of acetonitrile [105]. Consumption of mangosteen products is currently increasing as a food supplement because it has high antioxidant properties [106]. So research to evaluate pharmacokinetic studies of mangosteen has been carried out. In other studies, methanol is used as a solution for extraction with protein precipitation techniques. LC-MS/MS is used to measure the quantification. For some antioxidants, it is reported that the extraction technique that can be done is the solid phase extraction technique in plasma samples [107]. Active compounds that have efficacy as other antioxidants include methyl gallate (MG) and Penta alloys glucopyranose (PGG). It is a phenolic bioactive compound that has the potential as an antioxidant, anti-inflammatory, anti-cancer, anti-bacterial, and anti-viral [108]. This study developed an ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method to simultaneously measure MG and PGG in rat blood samples. The biological sample preparation technique used is liquid-liquid extraction using ethyl acetate. Before adding the ethyl acetate plasma sample mixed with 1% formic acid solution (10 µl) to acidify the sample, which is then vortexed.

Bioanalysis sample quantification techniques

Bioanalytic methods are commonly used for qualitative and quantitative analysis of drug substances, drug products, and biological samples such as plasma, serum, urine, saliva. Bioanalytic methods play an important role in pharmacokinetic studies, evaluation, and interpretation of bioavailability and bioequivalence data. Furthermore, the method needs to be validated to ensure that the method used is accurate, specific, and can be done repeatedly. Current technology in the field of drug development, liquid chromatography-mass spectrometry (LC-MS) techniques are used as bioanalytic techniques that utilize liquid chromatography with mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS/MS) is a technique that uses liquid chromatography (or HPLC) with mass spectrometry. LC-MS/MS is commonly used in laboratories for qualitative and quantitative analysis of drug substances, drug products, and biological samples. This technique is usually used to identify, qualitative and quantitative analysis of various dosage forms of pharmaceutical products or compounds in biological samples including natural pharmaceutical products, due to their high sensitivity to the identification of compounds in biological complex samples [31]. Chromatographic techniques are usually chosen for pharmacokinetic studies of a drug or to look at the bioavailability and bioequivalence of a drug compound.

LC-MS can be used to analyze pharmacokinetic parameters testing for several compounds (table 1). The development and validation of bioanalytic methods are very important to determine the pharmacokinetic parameters and the toxicokinetic profile of drug substances. This technique has been widely used for the study of bioavailability and bioequivalence. This method uses a set of instruments that are more sophisticated and complex when compared to other methods that are often used for the analysis of drugs in biological fluids. *In vivo* testing in plasma using test animals. In the research process, it will be observed whether in the analysis process a drug compound is not disturbed either by the metabolites of the compound itself or other endogenous components contained in plasma (table 1).

Validation of bioanalytic methods involves specific laboratory documentation and validation process tested and verified for

quantitative measurements of a drug substance in the biological matrix. Basic validation parameters consist of determinations for data quality such as selectivity, sensitivity, model calibration, accuracy, precision, stability, quantification limit (LOQ), recovery, linearity, the limit of detection, reproducibility, and roughness.

In analyzing drug concentrations in plasma, high-performance liquid chromatography (HPLC) methods are generally used because they can separate substances that can interfere with analysis and can detect and determine drug levels in plasma that are very small. Currently, reverse-phase partition chromatography with column C18 is a method commonly used for the analysis of drugs in plasma, because it is simpler, selective, and shorter analysis time. Several liquid chromatography methods with mass spectrometry [1, 18, 19], UV-Vis [22], and fluorescence, DLC HPLC [37], Sensitive analysis of aconitine, hypaconitine, mesaconitine and jesaconitine in human body fluids and Aconitum tubers by LC/ESI-TOF-MS also be used for the analysis of several drug compounds in plasma. The use of liquid chromatography with mass spectrometry requires sophisticated equipment, high accuracy, and expensive materials, while liquid chromatography with UV-Vis spectrophotometer is less sensitive and selective for analyzing drugs in small concentrations. Therefore, high-performance liquid chromatography with a more sensitive and selective fluorescence spectrophotometer is used in several studies depending on the physicochemical properties of each drug. The principle of pharmacokinetics is the study of changes in drug concentration with time. The application of pharmacokinetics in optimizing drug therapy and evaluating bioavailability is made possible by the skills of analytical chemists who pioneered the development of HPLC (Waters).

Validation method for bioanalysis

Validation of the bioanalysis method is important so that the bioanalysis method used produces accurate results. Validation is the process used to establish that quantitative analysis methods are suitable for bioanalysis applications. Bioanalysis techniques will continue to experience changes by developments and will continue to increase with the development of current technology. The bioanalysis technique also has its characteristics for each analyte, and specific validation needs to be developed for each analyte [104, 109].

The importance of validating the bioanalysis method is that it plays an important role in showing validation results that are reliable, accurate, and trustworthy to support the registration of new or reformulated drugs from existing ones. The validation of the bioanalysis method refers to agreed-upon Food and Drug Administration [110] or The European Medicines Agency (EMEA) guidelines. In pharmacokinetic test studies, the method of analyzing drug levels in biological samples is the main key to the validity of data. Validation of bioanalysis methods is very important to ensure the creation, processing of quality data during drug discovery and development.

The US Food and Drug Administration (FDA) guidelines for this industry provide general recommendations for validation of bioanalytic methods because the validation parameters of the bioanalytic methods in this guide are quite complete and suitable for determining the validation of analytical methods. This method contains a calibration curve, accurate, precision, the lower limit of quantification (LLOQ), matrix effects, recovery, and bound.

Calibration curve

The calibration curve is the ability of the method to obtain test results that are proportional to the concentration in the range of expected concentration, while the range is a result of the highest and lowest analyte content which shows acceptable accuracy, precision, and good linearity [80]. The instrument response to the analyte concentration must be known and must be evaluated by determining the concentration range. According to the ICH definition, the linearity of analytic procedures is its ability (within a given range) to obtain test results that are directly proportional to the concentration (number) of analyte in the sample. If the total range cannot be explained by a single calibration curve, two calibration ranges can be validated. The correlation coefficient is most used to test linearity. Although the correlation coefficient is useful for showing a high level of relationship between concentration and response data, it is of little value in making linearity [111].

In its application, the making of a calibration curve is done by diluting the parent standard to various concentrations. This method can also be done by spiking with internal standards. Usually, 5-6 concentration points are used. The results can be calculated from the standard peak and internal area ratio values. A good calibration curve value is the correlation coefficient must be more than 0.995.

Accuracy and precision

Accuracy test is an analytical method that illustrates the closeness of the average test results obtained by the method with the actual value of the analyte The two most common methods used to determine the accuracy or bias of analytic methods are (i) analyzing control samples spiked with the analyte and (ii) by comparing analytic methods with reference methods. Accuracy is determined by the analysis of the sample containing the number of the analyte that is known and replicated. The most commonly used method for determining accuracy is recovery studies.

While the precision test is the accuracy of analytic methods that describe the closeness of the value of individual analytes that are repeated. Precision is expressed as a relative standard deviation (coefficient of variation,% KV). Precision testing is done by using four levels of concentration (LLOQ, QCL, QCM, and QCH). This precision is the proximity of the individual steps of the analyte when the procedure is repeatedly applied to several aliquots of a homogeneous volume of biological matrix.

There are various parts for precision, such as repetition, intermediate precision, and reproducibility (roughness). Repetition or repeatability describes the precision in the same working conditions that is carried out by analysts in the same laboratory and equipment. Intermediate precision is precision in different laboratory conditions, done by different analysts, done on different days, and using different equipment at the same time. Whereas reproducibility is the accuracy between various laboratories and determined by the transfer method.

Accurate and precise measurements are obtained by replication for each concentration six times per day (intra-day) and more than six consecutive (inter-day), at least three times within the expected concentration range [112].

Matrix effects and recovery

The extraction of this parameter can be calculated by comparing the response of the analyte after sample preparation with the response of the solution containing the analyte at the maximum theoretical concentration. Therefore, the absolute acquisition value cannot usually be determined if the sample inspection includes the derivatization step, because the derivative is usually not available as reference material.

The matrix and recovery effects were analyzed at three concentrations (low, medium, and high) on the standard and sample and internal standards. Liquid biological blanks are processed by liquid-liquid extraction or other extraction methods depending on the type and nature of the analyte to be analyzed. After that, it is mixed with a standard solution. After the supernatant is produced the remaining solvent is evaporated and dissolved with the appropriate solvent.

Specificity/selectivity

Selectivity is the ability of analytical methods that only measure certain substances carefully and thoroughly to distinguish analyte and internal standards with other components such as endogenous compounds such as proteins, amino acids, fatty acids, and others present in biological fluid samples such as plasma and serum. The measurement of selectivity is carried out by measuring six blanks with different sources by looking at interference in LLOQ. An analytical method is said to be specific if it produces a single response to one analyte. In the selectivity analysis, it is also necessary to analyze the extent of the disturbance caused by drug

metabolites, the disruption of the degradation product formed during sample preparation, and the disruption of the possibility of the drug being given simultaneously.

Detection limits and quantization limits

The limit of detection (LOD) is the smallest analyte concentration test parameter in a sample that can be detected on an instrument without having to meet accuracy and precision criteria. The detection limit is the smallest concentration value of the analyte in the sample which still shows the absorption at the instrument. And also for LLOQ is the smallest concentration of analytes in the sample but is determined quantitatively that meets the criteria of accuracy and precision.

Range

The range is the interval between the highest and lowest analyte content in a given sample and shows acceptable values of accuracy, precision, and linearity.

Recovery

The extraction of this parameter can be calculated by comparing the response of the analyte after sample preparation with the response of the solution containing the analyte at the maximum theoretical concentration. Therefore, absolute recovery cannot usually be determined if the sample inspection includes the derivatization step, because the derivative is usually not available as reference material.

Stability

Stability evaluations are carried out to ensure that each step was taken during sample preparation and sample analysis, as well as the storage conditions of the samples used, do not affect the concentration of the analyte. Stability is carried out to ensure each step in the analytical method, ensuring the conditions applied to the stability test, such as the sample matrix, anticoagulants, materials and containers, storage, and analytical conditions are the same as those used for the actual research sample. The purpose of the stability test is to detect any degradation of the analyte analyzed during the process of sample collection, processing, storage, preparation, and analysis. All stability can be done during the method validation process except for long-term stability.

There are 3 types of stability tests, namely standard solution stability, freeze-thaw stability, short-term stability, and long-term stability.

a. Stability of Freeze

Thaw Freeze thaw stability is carried out by the way the sample is stored and frozen in the freezer at the desired temperature, then thawed at room temperature or the temperature at which the sample is frozen again by applying the same conditions. In each cycle, the sample must be frozen for±12 h before thawing. The number of cycles in freeze-thaw stability must equal or exceed the study sample cycle. This stability test compares the response of the analyte 34 after being stored to the response of the analyte after it has been thawed and frozen for 3 cycles to the response of the analyte in a fresh sample solution.

b. Long-term stability

Long-term stability is tested by the way the sample is stored in the freezer under the same storage conditions and has the same duration as the study sample. For small molecules that are considered to be able to apply the bracketing approach ie case stability has been proven for example at-700 and-200 C. For large molecules such as peptide and protein stability must be studied at each temperature where the research sample will be stored. This stability test compares the response of the analyte after being stored at the time of analysis to the response of the analyte after storage of the response of the analyte in a sample solution that is still new.

c. Short-term stability

Samples are stored at room temperature or the temperature at which they will be processed. The length of the test is adjusted to the length of the sample at room temperature. A minimum of 3 samples was used at low and high concentrations in the biological matrix stored at room temperature for 24 h. This stability test compares 35 responses of the analyte after being stored to the response of the analyte after storage of the response in a sample solution that is still new.

d. Stability of standard solutions

The stability of analytes and internal standards is assessed using a minimum of 6 h at room temperature and 30 d in cold temperatures. This stability test compares the response of the analyte after being stored in the response of the analyte in a fresh stock solution [39].

CONCLUSION

Bioanalysis part of important role in therapeutic evaluation studies, interpretation of bioavailability and bioequivalence studies, pharmacokinetic studies, pharmacodynamics. The development of natural medicinal products according to those stipulated by the health department must be scientifically justified. Selective and sensitive bioanalysis methods for quantitative assessment of a drug and its metabolites are essential for successful pre-clinical and clinical pharmacological testing. The selection of extraction methods with suitable solvents and the selection of methods for separating drug components from effective biological endogens is very important. Some separation of drugs from plasma are (1) Protein Precipitation (PPT) (2) Liquid-liquid extraction (LLE) (3) Solid-phase extraction (SPE). The measurement of analytes from the biological matrix must be validated. Validation of the bioanalysis method includes all procedures which show that the specific method used for quantitative measurement of analytes originating in the biological matrix, such as blood, plasma, serum, or urine, can be trusted and can be repeated repeatedly for the intended use. The basic parameters of validation aim to determine quality which includes criteria such as selectivity, sensitivity, linearity, accuracy, precision, stability, quantification limit (LOQ), recovery, the limit of detection (LOD).

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

There is no conflict of interest from all the authors.

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