A Critical Review on Clinical Application of Separation Techniques for Selective Recognition of Uracil and 5-Fluorouracil

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Abstract The most important objectives that are frequently found in bio-analytical chemistry involve applying tools to relevant medical/biological problems and refining these applications. Developing a reliable sample preparation step, for the medical and biological fields is another primary objective in analytical chemistry, in order to extract and isolate the analytes of interest from complex biological matrices. Since, main inborn errors of metabolism (IEM) diagnosable through uracil analysis and the therapeutic monitoring of toxic 5-fluorouracil (an important anti-cancerous drug) in dihydropyrimidine dehydrogenase deficient patients, require an ultra-sensitive, reproducible, selective, and accurate analytical techniques for their measurements. Therefore, keeping in view, the diagnostic value of uracil and 5-fluorouracil measurements, this article refines several analytical techniques involved in selective recognition and quantification of uracil and 5-fluorouracil from biological and pharmaceutical samples. The prospective study revealed that implementation of molecularly imprinted polymer as a solid-phase material for sample preparation and preconcentration of uracil and 5-fluorouracil had proven to be effective as it could obviate problems related to tedious separation techniques, owing to protein

binding and drastic interferences, from the complex matrices in real samples such as blood plasma, serum samples.

Keywords Bio-analytical applications · Dihydropyrimidine dehydrogenase (DPD) · 5-Fluorouracil · Separation techniques · Therapeutic drug monitoring · Uracil

Introduction

The requirement to analyze biomolecules in biological samples and pharmaceutical products is becoming frequent with the development of selective and sensitive separation techniques. Scientists from clinical and medical disciplines have worked out different extraction methods that meet their needs, based on their background and expertise. Being a molecule of biological importance, uracil (Ura) and its laboratory derivative i.e., 5-fluorouracil (5-FU) have attracted the attention of several research groups who have attempted to selectively recognize these analytes by means of molecular imprinting and hybrid techniques. Ura is a naturally occurring pyrimidine derivative and it is used for drug delivery in pharmaceuticals [1]. Dihydropyrimidine dehydrogenase (DPD) deficiency with a defect of the pyrimidine degradation pathway is critically important in the clinical diagnosis of Ura concentration because this is directly associated with several inborn errors of metabolism (IEM) processes [2]. 5-FU is a chemotherapeutic agent, used in the treatment of common malignancies such as cancers of breast, prostate, ovaries, skin and gastrointestinal tract. Around 80 % of administered dose of 5-FU is catabolized by DPD enzyme. Patients suffering with DPD deficiency (a pharmacogenetics syndrome) or insufficient activity of this enzyme are at great risk of the development of severe 5-FU toxicity [1, 3]. Knowledge of 5-FU concentration levels in

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body fluids, such as blood and urine, allows pharmacotherapy to be optimized and provides the basis for studies on patient compliance, bioavailability, and pharmacokinetics, in addition to the influences of co-medication. Therefore, rapid and accurate determination of Ura and 5-FU is important for clinical diagnosis of IEM disorders and 5-FU drug monitoring in DPD deficient patients.

Owing to the need of extracting analyte of interest (Ura/5-FU) from biological and pharmaceutical samples, current review is primarily addressed to the development of efficient separation techniques for Ura and 5-FU analysis which involves liquid–liquid extraction (LLE), column chromatography, high performance liquid chromatography (HPLC), liquid chromatography (LC), gas chromatography (GC), solid-phase extraction (SPE), solid-phase microextraction (SPME), and affinity sorbent extraction via molecular imprinting technique (MIT). Quantification of Ura/5-FU was made possible by using several analytical detectors (tandem mass spectroscopy (MS/MS), ultraviolet (UV) detector, electrochemical detection (ECD), capillary electrophoresis (CE)) with minimum interferences. Traditional detection of disease biomarkers depends upon pretreatment of biological fluids, e.g., determination of Ura in plasma requires protein removal. The removal of nonspecific protein from clinical samples is often the most time consuming process in clinical analysis, dictating the time required for complete assay procedure. However, this is crucial because the proteins present in the sample often change the morphology of sensing element in modified sensors, which in turn bias the analytical result. Although routine clinical methods indicate levels of biomolecules in urine/blood, they often lack specificity due to severe complex matrix interferences. Recently, molecularly imprinting technique (MIT) is continuing as a developed methodology which has an additional advantage of providing molecular assemblies of desired structures and properties. It is being frequently used in separation processes such as chromatographic material, chemosensors, biosensors and protein recognition [4–7].

While significant improvements in our understanding of molecular imprinting process have been achieved and many new types of polymer formats along with application areas have been investigated. Many opportunities for applications particularly molecularly imprinted polymer (MIP) as chromatographic, LLE, SPE, and SPME materials for Ura and 5-FU analysis are still to be exploited. Depending on the origins of samples and analytical objectives, Ura and 5-FU analysis have been carried out using various analytical instruments in many situations such as clinical control for diagnosis and treatment of diseases, pharmacology, and toxicology [1–4, 8–10]. In the next section, we would like to contemplate on the current chromatographic methods developed for the purposes of separating the Ura and 5-FU

from complex samples containing interferences and detecting them once separation is complete (Fig. 1).

Analytical Separation Techniques Used for Ura and 5-FU Analysis

This survey recapitulated chromatography, LLE, SPE, MIT, and SPME analyses for Ura and 5-FU, which are representative targets, from the viewpoint of therapeutic drug monitoring in DPD deficient patients. The purpose of this review is to give overviews on the significant attempts carried out for a selective and sensitive analysis of Ura/5-FU in recent years. MIPs and their applications as chromatographic, SPE, and SPME materials have found to be advantageous as it selectively recognizes Ura/5-FU, from complex biological mixtures and also reduces the false-positive results. The analytical separation techniques developed for analysis of Ura and 5-FU in human bio-fluids have been summarized in Tables 1 and 2 respectively.

Hybrid Separation Techniques Used for Sensitive Analysis of Ura in Biological Samples

In past decades, an HPLC–MS/MS technique has been developed for measurement of endogenous Ura and dihydrouracil (UH2) in human plasma and urine samples. Separations of analyte from plasma were performed on a Discovery Amide C16 column and pretreatment of urine sample was done by centrifugation. This method was helpful for the evaluation of DPD enzyme activity and for the adjustment of clinical dosages in pyrimidine chemotherapeutics with a limit of quantification (LOQ) of 0.5 ng mL^{-1} for Ura [12]. Ura and UH2 have been further quantified by using LC–UV and LC–MS methods of detections after SPE pre-treatment. The LOQs for Ura and UH2 were 2.5 and $6.25 \text{ } \mu\text{g L}^{-1}$, by LC–UV method and 2.5 and $3.1 \text{ } \mu\text{g L}^{-1}$ by LC–MS method, respectively [13]. An HPLC method for analyses of Ura and UH2 in plasma using SPE and UV detection, have recovered 80.4 % Ura within linear detection range from 2.5 to 80 ng mL^{-1} [2]. In recent years, to evaluate the toxicity levels in patients suffering from gastrointestinal cancer, a concurrent and prompt HPLC–ESI–MS/MS method has been described, for measurement of endogenous Ura and UH2 in human plasma. The calibration curve covers the concentration range of 5 – 200 ng mL^{-1} and concentration of Ura varies from 21.8 to 56.6 ng mL^{-1} in cancer patients with recoveries ranges from 90 to 110 % for both analytes [14]. Another sensitive and specific ultra-high-performance liquid chromatography–MS/MS method (waters UPLC–MS–MS) have been developed and validated for the quantification of Ura and (UH2) levels in human plasma. In this

Fig. 1 A schematic view of analysis of biological samples by analytical techniques

Table 1 Analytical separation techniques developed for Uracil (Ura) analysis in human bio-fluids

Matrix	Analytical techniques	Sorbents used for extraction	Recoveries (%) / values of concentrations (ng mL ⁻¹)	References
Human plasma and urine	HPLC–MS/MS	Discovery Amide C16 column	LOQ: 0.5	[12]
Human plasma	LC–UV, LC–MS	SPE	LOD: 2.5 [#]	[13]
Human plasma	HPLC–SPE–UV	SPE	80.4*, 2.5–80.0	[2]
Human plasma	HPLC–ESI–MS/MS	Reversed-phase XTerra C18 column	90–110*, 5–200	[14]
Human plasma	UPLC–MS/MS	Acquity UPLC BEH C18 column	LOQ: 0.625	[1]
Human plasma	MISPME–MIP sensor	Poly(melamine-co-chloranil) SPME-fiber	LOD: 0.0245	[11]
Human plasma	SPE microcolumns and TLC	Adsorbex SPE	–	[17]

LOQ limit of quantification, LOD limit of detection

* Recoveries of Ura

[#] Concentrations of 5-FU converted to ng mL⁻¹ for comparison

method, analytes were extracted by deproteinization and separated out using Acquity UPLC BEH C18 column for simultaneous quantification of Ura, UH₂, 5-FU and 5,6-dihydrofluorouracil (5-FUH₂) in human plasma [1].

In connection to GC analysis, a single original and sensitive multiresidue method has been developed for the detection and quantification of 61 pesticides of toxicological significance where GC–MS was found to be the best for Ura detection in volatile pesticides in human biological matrixes as reported earlier [15].

After the development of several HPLC systems for Ura analysis, a poly (melamine-co-chloranil) polymer has been successfully used as SPME coating material and detection of Ura was achieved by MISPME fiber coupled with complementary MIP-modified hanging mercury drop electrode (HMDE) sensor. This MISPME hyphenated with MIP-sensor method revealed nanomolar concentrations with a limit of detection (LOD) of 0.0245 ng mL⁻¹ for Ura, without any false positives [11].

Selective Extraction Technique for Ura Analysis

Solid Phase Extraction (SPE)

Solid-phase extraction (SPE) is a method for the isolation and concentration of selected analytes from a fluid sample

by their transfer on a solid-phase. The analytes are recovered by elution or thermal desorption. This method has high recovery, uses less organic solvent than LLE, does not produce foaming and it is easy to automate, but lengthy because it necessitates several steps like sorbent activation, removal of the activation solvent, application of sample, washing, desorption, expensive organic solvents are still required. About two decade ago, an anion-exchange SPE column has been applied for extraction of Ura in rodent diet and quantification of Ura was done by HPLC method using an ODS microbore column with 76–90 % Ura recovery [16]. Adsorbex SPE microcolumns and horizontal and ascending thin layer chromatography (TLC) in various developing systems have been used for the quantitative separation of Ura from plasma samples [17].

Solid Phase Microextraction (SPME)

Solid-phase microextraction (SPME) was developed to address the need for rapid sample preparation both in the laboratory and on-site where the investigated system is located. Its simplicity of operation, solvent-less nature and the availability of commercial fibers have made SPME to become a tool routinely used for certain clinical applications (Fig. 2). The SPME analysis of Ura is highly scarce. So far, two SPME analyses—one for substituted Ura

Table 2 Analytical separation techniques developed for 5-fluorouracil (5-FU) analysis in human bio-fluids

Matrix	Analytical techniques	Sorbents used for extraction	Recoveries (%) / values of concentrations (ng mL ⁻¹)	References
Human plasma	Chromatography with UV detection	C18 reversed-phase column	0.20 ^a	[31]
Human plasma	HPLC–UV detection	Reversed phase Kromasil C18 column	35*, LOD: 10	[32]
Human plasma	HPLC–UV detection	SPE using C18 bondpack column	96.2 ± 0.5*, LOD: 5	[34]
Human plasma	HPLC–UV	Genesis C18 analytical Column	96–101*, 30–1000	[36]
Human lymphocytes and plasma	HPLC	–	100–2000 [#]	[37]
Human plasma	GC–MS	–	82.4 ± 0.44*, 18.5 ± 10.7*	[38]
Human plasma	MS	–	0.4 [#]	[9]
Human plasma	HPLC–UV detection	–	5–500	[40]
Human plasma	LC–MS/MS	Hypercarb column	–	[41]
Human plasma	LC–MS	Off-line SPE	–	[42]
Human plasma	LC–MS/MS	Hydrophilic interaction chromatography (HILIC)	96.0–102.2*, 10–10,000	[43]
Human plasma	LC–MS/MS	–	5–5000	[45]
Human plasma	LC–MS/MS	C18 reverse phase column	–	[48]
Human plasma	LC–MS/MS	–	8.61–1080	[49]
Human plasma	LC–MS/MS	Synergi hydro-RP column	2–500	[50]
Human plasma	LC–MS/MS	–	1.84 ± 0.34 ^b	[52]
Human plasma	LC–MS/MS and LC–UV	LLE	86–91*, 8–200	[54]
Human plasma	LC–ESI–MS/MS	Precipitation	50–5000	[55]
Human plasma	LC–MS/MS	Atlantis dC(18) column	0.1–75 ^a	[56]
Human plasma	MISPME–MIP sensor	Poly(melamine-co-chloranil) SPME-fiber	0.0484	[11]
Human serum	Reversed phase chromatography with UV detection	Copper modified strong cation exchange stationary phase	70–108*	[59]

LOQ limit of quantification, *LOD* limit of detection

* Recoveries of 5-FU

[#] Concentrations of 5-FU converted to ng mL⁻¹ for comparison

^a Values of concentrations in μM

^b Values of concentrations in μg per gram protein

pesticides [18] and other for substituted Ura herbicide [19] have been reported.

Affinity Sorbent Extraction via MIT

The affinity sorbent extraction (ASE) consists of an immobilized antibody or MIP. However, immobilized antibody suffers from poor stability and difficulties associated with their modification and design to suit a specific analyte. On the other hand, MIPs are cross-linked synthetic polymers obtained by copolymerizing a monomer with a cross-linker in the presence of a template molecule. After polymerization, the template is removed from porous network by washing, leaving cavities in the polymeric matrix that are complementary in size, shape, and chemical functionality to the template. Thus, the imprinted molecule is able

to rebind the analyte (template) selectively under certain experimental conditions via non-covalent interactions, e.g. hydrophobic, ionic, and hydrogen bonding [20].

In previous studies, 2, 6-bis(acryloamido) pyrimidine (BAP) was used as a functional monomer for molecular imprinting of Ura mimicking multiple hydrogen bonds between nucleotide bases [21]. This MIP showed 7.9 μmol/g binding capacity for Ura. Again, water-compatible MIPs have been synthesized which were capable of selectively binding with target molecules such as Ura and riboflavin [22]. Earlier, MIPs were synthesized for Ura using BAP as a functional monomer exploring its binding performance through chromatography [4]. Ura recognition was also made possible by using molecularly imprinted copolymer membranes [23]. Latter, MIPs were synthesized by using acrylonitrile (AN) co-polymers, poly (AN-co-MAA) and

Step1: Elution of analyte A for synthesis of MISPME Fiber analyte A with a larger view of biological matrix **Step2: Analysis of specific analyte A by introduction of MISPME fiber in biological matrix**

Fig. 2 A diagrammatic representation of selective recognition and analysis of specific analyte A (Ura/5-FU) by MISPME fiber

poly (AN-*co*-AA), with methacrylic acid (MAA) and acrylic acid (AA) for phase inversion imprinting of Ura [24]. The imprinted poly (AN-*co*-MAA) membrane could bind 7.9 $\mu\text{mol/g}$ of Ura with $8.4 \times 10^{-6} \text{ m}^3/\text{m}^2 \text{ s}$ of volume flux. MIPs appended onto CdSe/ZnS core–shells have been synthesized by utilizing MAA as a monomer and ethylene glycol dimethacrylate (EGDMA) as a cross-linker, to perform photoluminescence assay of Ura [25]. MIP-copolymer membranes functionalized by phase inversion imprinting have been synthesized for Ura recognition via permselective binding [26]. MIPs synthesized for nucleic acid bases using aniline as a monomer have capability to recognize Ura [27]. Ura targeted MIP membranes was also prepared by using polystyrene-*co*-maleic acid matrix obtained in supercritical CO_2 [28]. Imprinting effect was observed to induce crystallization of Ura when a poly (AN-*co*-MAA) membrane was prepared in supercritical CO_2 using phase inversion imprinting technology. The supercritical CO_2 media was found to be an effective solvent for the formation of Ura crystals on the imprinted membrane. However, no attempt was made on the utilization of Ura crystals on an imprinted membrane in this assay. For the first time, dual imprinting protocols have been developed for selective recognition of Ura and 5-FU in human plasma.

Poly (melamine-*co*-chloranil) polymer has been synthesized with melamine and chloranil precursors for selective recognition of Ura/5-FU. Ura has been bound to the cationic polymer via electrostatic interactions and sensed as doubly charged anion (predominantly exists at pH 7.0) on to MIP—modified HMDE technique revealed a LOD value of 0.34 ng mL^{-1} [29]. Recently, multiwall carbon nanotubes—MIP have been synthesized for Ura by using MAA as monomer and EGDMA as cross-linker with AIBN as an initiator. The developed nanostructured Ura imprinted polymer shows highly specific and selective monolayer coverage of Ura on the EGDMA-crosslinked MAA polymer [30].

Although several MIPs for Ura have been reported, only one of them applied as chromatographic material [4] and another as SPME material [11]. This was primarily due to the sensitivity and non-specific complications which are yet to be resolved.

Hybrid Instrumental Techniques for 5-FU Analysis in Biological and Pharmaceutical Samples

For simultaneous determination of 5,6-dihydrofluorouracil (5-FUH2) and 5-FU concentrations, a HPLC–UV method

has been previously reported. Both analytes were extracted and analyzed by isocratic chromatography using C18 reversed-phase column with UV (268 nm for 5-FU, 220 nm for FUH2) detector and LODs for both analytes were 0.005 nmol on column (for aqueous sample) and 0.20 μM (in 1 mL of plasma sample) [31]. 5-FUH2 is the product of 5-FU, catalyzed by DPD enzyme. A relatively simple and sensitive HPLC–UV detection following SPE has been described for 5-FU and its two main metabolites in plasma of patients with metastatic colorectal cancer using reversed-phase Kromasil C18 column. The LODs of three analytes were 10 ng mL⁻¹ with the average recovery of 35 % for 5-FU, 42 % for Ura, and 48 % for 5-fluorodioxuridine from plasma [32]. In last decades, an HPLC–UV method has been validated for the determination of 5-FU in wipe samples by the direct analysis of aqueous solutions and air samples exploiting SPE on styrene-DVB resin. The amount of 5-FU ranged from 0.043 to 0.23 $\mu\text{g}/\text{m}^3$ in air samples and from 0.2 to 470.1 $\mu\text{g}/\text{dm}^2$ in wipe samples [33]. A clinical pharmacokinetic study of 5-FU drug has been performed using HPLC (C18 Bondapak column)–UV detection method in human plasma with a LOD value of 5 ng mL⁻¹ (with 96.2 ± 0.5 % extraction yield) [34]. An HPLC method has been proposed for the determination of 5-FU in hospital effluents where the waste-water samples were enriched by SPE on ENV (+) columns and analyzed by capillary electrophoresis. This technique yielded a recovery of 5-FU up to 80–96 % with a detection limit of 1.7 $\mu\text{g L}^{-1}$ [35]. A sensitive HPLC technique have been further developed for the quantitative determination of 5-FU in human plasma. This method was validated using isocratic elution and UV detection [36]. Recently, 5-FU assessment was made easy by HPLC (within 30 min) for the purpose of study of 5-FU accumulation in lymphocytes as well as its absence in lymphocytes and blood plasma. The method is validated, and the response is found to be linear in the drug concentration range of 0.1–2.0 $\mu\text{g mL}^{-1}$ [37].

A GC–MS technique have been described for quantifying 5-FU in the presence of 2'-deoxy-5-fluorouridine in plasma and urine. 5-FU was converted into tertiary-butyldimethylsilyl derivative. 5-FU was assessed by electron-impact ionization GC–MS technique within the linear range of 0–100 ng mL⁻¹, without any interference of 2'-deoxy-5-fluorouridine. This method was validated in rat plasma samples (LOD: 0.5 ng mL⁻¹) [10]. Plasma 5-FU concentrations have been determined by GC–MS technique in gastric and colorectal cancer patients receiving Ura and tegafur or oral doxifluridine. The UH2 and Ura in urine were assessed by HPLC using column switching [38]. Another GC–MS/MS method have been developed for quantification of 5-FU in hospital waste water. The method was validated over the concentration range from 0.09 to 4.0 $\mu\text{g L}^{-1}$ [39].

Extraction of 5-FU from plasma of hepatic tumor patients and successful synthesis of 5-FU derivative by treatment with di-*N*-ditrifluoromethylbenzyl compound has been carried out in last decade. The 5-FU extract was analyzed by a negative ion chemical ionization MS method. The LOD was found to be as low as 400 pg mL⁻¹, for 5-FU analysis [9]. An assay of 5-FU and Ura–UH2 ratio has been performed in human plasma by HPLC system coupled with UV detector. 5-FU was quantified within the range of 5–500 ng mL⁻¹. This concentration range covers the exposure levels presently found in clinical practice. The method was found to be sensitive, simple, cost effective and capable of identifying DPD metabolic phenotype in cancer patient [40]. 5-FU and 5-FUH2 have been measured by LC/MS–MS method, for pharmacokinetics studies in patients treated with Ftorafur (FT). Separations of analytes were performed on a Hypercarb column [41]. LC–MS analysis of capecitabine and its metabolite (5'-deoxy-5-fluorocytidine, 5'-deoxy-5-fluorouridine, 5-FU) have been performed simultaneously, after an off-line SPE from human plasma. As far as SPE is concerned, Ura and 5-FU were reportedly co-eluted with other endogenous substances of plasma [42]. A hydrophilic interaction liquid chromatography (HILIC) LC–MS/MS technique have been reported for an accurate and precise quantification of 5-FU in human plasma. Recovery from plasma sample was 46.0–72.6 %, and ion suppression method was 9.8–25.7 %. The accuracy was 96.0–102.2 %, and precision was 2.1–7.5 % within the concentration range of 10–10,000 ng mL⁻¹ [43]. An HPLC–MS/MS analysis after SPE has been performed for detection of the cytostatics 5-FU, cytarabine, gemcitabine, human metabolites, uracil- β -d-arabinofuranoside and 2',2'-difluorodeoxyuridine in waste water. The LOQ for 5-FU was 5.0 $\mu\text{g L}^{-1}$ and the maximum concentration detected was 27 $\mu\text{g L}^{-1}$ [44]. A LC–MS/MS method has been described for the determination of 5-FU along with α -fluoro- β alanine and capecitabine in human plasma. The method was validated over the concentration ranges of 10–10,000 ng mL⁻¹, 5–5000 ng mL⁻¹, and 1–1000 ng mL⁻¹ for FBAL, 5-FU, and Cape, respectively. The proposed method was rugged, precise, accurate, and well suited to support [45]. 5-FU have also been quantified in cultured cell model by LC–MS coupled to MS detection technique over a concentration range of 2.5–150 ng mL⁻¹, without any matrix effect [46]. A reliable, simple, isocratic stability indicating HPLC–UV method have been developed for 5-FU analysis using thymine as an internal standard within the concentration range of 0.1–2.0 $\mu\text{g mL}^{-1}$ [47]. A chromatographic analysis for capecitabine and its metabolites (5-FU) was performed on a C18 reverse phase column with detection by atmosphere pressure chemical ionization LC–MS/MS. The developed technique showed high pharmacokinetic inter-patient variability in human plasma [48]. 5-FU concentrations have

been determined in human plasma over a dynamic concentration range from 8.61 to 1080 ng mL⁻¹ by LC–MS/MS technique [49]. Another 5-FU concentration has also been determined using LC–MS/MS in human plasma [50]. In recent years, a simple LC–MS technique have been described for the simultaneous quantification of floxauridine (5-fluoro-2'-deoxyuridine), 5-FU and their metabolites. This method was found to be efficient for achievement of the mass balance of compounds, which could not be attained by conventional UV absorptions based HPLC analysis [51]. 5-FU concentrations have also been determined by LC–MS/MS technique for evaluation of DPD enzyme activity [52]. The severe toxicity owing to the 5-FU treatment has been anticipated for the assessment of DPD status in cancer patients [53]. Low plasma levels of 5-FU and relatively higher tegafur (a 5-FU prodrug) levels have been measured in colorectal cancer patients simultaneously. A coupled LC–MS/MS and LC–UV method have been developed using negative ion atmospheric pressure ionization (API) for the purpose of pharmacokinetics studies. The recoveries of 5-FU and tegafur were 86–91 and 97–110 % respectively. The low concentration of 5-FU extracted by LLE method, ranges from 8 to 200 ng mL⁻¹ and higher tegafur concentration ranges from 800 to 20,000 ng mL⁻¹ in human plasma [54]. LC coupled to electrospray MS/MS detection technique have been developed for quantification of capecitabine and its metabolites in human plasma using reversed phase chromatography which assessed 5-FU and FUH2, using hydrophilic interaction chromatography. This method requires the extraction of analyte by precipitation method before analysis. The linear range for 5-FU detection technique was 50–5000 ng mL⁻¹, and it support pharmacokinetics studies in capecitabine or 5-FU treated patients [55]. A LC–MS/MS method has been described for simultaneous analysis of Ura, 5-FU and UH2 in human plasma. The developed method combined the analysis of 5-FU pharmacokinetics and DPD activity into a single assay [56]. These assays were found to be suitable for monitoring 5-FU plasma levels in routine clinical practice and may contribute to improved efficacy and safety of 5-FU-based chemotherapies for therapeutic drug monitoring and toxicity prediction in cancer patients [57].

A simple, rapid, portable, cost-effective, and direct sensing MISPME–MIP-senor for 5-FU recognition has been developed using poly (melamine-co-chloranil) polymer as MIP motif. It was observed that MIP selectively recognized 5-FU via hydrogen bindings exclusively, when applied, both as a solid phase microextraction coatings as well as a sensitive conducting film on electrode, and achieved nanomolar detection range from 0.14 to 80.0 ng mL⁻¹ (LOD: 0.0484 ng mL⁻¹) for 5-FU analysis, without any matrix interferences [11].

Extraction Techniques Used for 5-FU Separation

Solid-Phase Extraction (SPE)

Several SPE protocols have been developed for 5-FU analysis as reported in past two decades. The second-order derivative spectrophotometric method has been utilized for selective determination of flucytosine in the presence of 5-FU, its synthetic precursor and degradation product. Traces of 5-FU in flucytosine were also determined by the derivatized UV spectroscopy; flucytosine was removed by the selective SPE procedure using a strong cation-exchange sorbent [58]. With advancement of surface modification techniques, a copper-modified strong cation exchange stationary phase was also effective when used for SPE of the antineoplastic drug 5'-deoxy-5 fluorouridine, 5-FU and some of its main metabolites from human serum and it showed average recoveries ranged from 70 to 108 %. In this work, the reversed-phase chromatography with UV detection was used for the separation and quantification of the analytes [59]. By using C18, diol, and ion-exchange sorbents for SPE followed by UV spectrophotometric assay, analysis of basic, acidic and neutral drugs commercially available in creams such as 5-FU were found to be quite useful in obtaining a practical and reliable sample clean-up [60].

Affinity Sorbent Extraction via MIT

As far MIP concern, several MIPs have been developed for 5-FU recognition in last decade. MIPs for 5-FU have been synthesized by using BAP or 2-(trifluoromethacrylic acid) as a functional monomers and EGDMA as a cross-linker [61]. However, these polymers showed higher binding affinity for Ura than 5-FU. For controlled release of 5-FU in biological fluids, a 5-FU imprinted polymer has also been synthesized using MAA as a functional monomer and EGDMA as a cross-linker that showed ability to recapture template selectively, both in organic and aqueous media [62]. Attempts were made to prepare 2-hydroxyethyl methacrylate an acrylic acid based 5-FU imprinted hydrogels using *N,N'*-methylene bisacrylamide ammonium persulfate and *N,N,N',N'*-tetramethyl ethylenediamine as cross-linkers, initially for an accelerator and later for use as drug discovery systems [63]. Molecularly imprinted hydrogel nanospheres have been reported as devices for the controlled release of 5-FU in biological fluids using one-pot precipitation technique of polymerization method with MAA as a functional monomer and EGDMA as a cross-linker. The in vitro release studies in the present instance were carried out in plasma simulating fluids [64]. The re-binding studies was performed in acetonitrile and water.

Again, poly (melamine-*co*-chloranil) MIP-sensor was found to be highly selective for 5-FU analysis, which confirms 5-FU recognition via exclusively hydrophobically driven hydrogen binding. The method represents a LOD value of 0.26 ng mL^{-1} for 5-FU quantification in human plasma [29]. Recently, a 6-aminopurine (adenine) derivative of bis(2,2'-bithienyl) methane, vis-4-[2-(6-amino-9H-purin 9-yl)ethoxy] phenyl-4-[bis(2,2'-bithienyl)methane] have been designed for 5-FU recognition by RNA-type (nucleobase pairing)—driven molecular imprinting. For sensing application, three different transduction platforms (differential pulse voltammetry; LOD 56 nM, capacitive impedimetry; LOD 75 nM, piezoelectric microgravimetry; LOD 0.26 nM) achieved the nanomolar detection limits for 5-FU analysis [65].

A large number of hybrid chromatographic techniques have been reported for 5-FU analysis, in human plasma and pharmaceutical drugs. At the same time, a number of MIT have also been synthesized and used as 5-FU drug delivery systems, but, a miniaturized, simple, sensitive and cost-effective version of advanced separation techniques likes SPE and SPME were rarely reported for 5-FU analysis.

Conclusions

To date, an array of hyphenated techniques, e.g. GC–MS, LC–MS, HPLC–UV and LC–MS/MS-derived methods and extraction techniques like SPE, SPME have been found more advanced, for the separation and quantification of Ura and 5-FU, in human plasma, pesticides, herbicides and pharmaceutical samples. Hyphenated tools greatly reduces the sum of time required compared to using standard sample preparation techniques, and limits the contact of the analytical personnel to infectious biofluids. However, these existing technologies suffer from few demerits such as they often require removal of co-components before analysis, expert training in their use, data analysis and may be too complex to facilitate rapid identification of the disease with obvious potentially negative outcomes. MIPs developed for Ura and 5-FU recognition is ideally suited to SPE and SPME applications for ultratrace level detection, in complex biological samples, yet their application in separation science is still to be limited due to the sensitivity and non-specific complications related to the Ura/5-FU leakage. The application of modern hybrid separation techniques may enhance therapeutic drug monitoring of DPD deficient patients and hence the disease management.

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