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Review Article

Analytical Techniques in Simultaneous Estimation: An Overview

Pragya Nand Badyal[1], Chetan Sharma[1], Navdeep Kaur[1], Ravi Shankar[2], Abhay Pandey[1] and Ravindra K. Rawal[1]*

[1]Department of Pharmaceutical Analysis, Indo-Soviet Friendship College of Pharmacy (ISFCP), Moga-142001, India

[2]Medicinal Chemistry Division, Indian Institute of Integrative Medicine (CSIR), Jammu-180001, India

***Corresponding author:** Ravindra K. Rawal, Department of Pharmaceutical Analysis, Indo-Soviet Friendship College of Pharmacy (ISFCP), Moga-142002, India

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Abstract

Simultaneous estimation plays a very important role in pharmaceutical world as it is very feasible and time saving. For the multi component analysis various techniques like spectrophotometric techniques (UV-VIS, IR, NMR and MASS spectrometry) and chromatographic techniques (Thin Layer Chromatography, High Performance Liquid Chromatography, Ultra-High Performance Liquid Chromatography, High Pressure Thin Layer Chromatography and Gas Chromatography) is used. These techniques provide high degree of specificity and selectivity and further provide the high degree of assurance that these techniques fit for the simultaneous estimation of the pharmaceutical dosage form. Chromatographic and spectrophotometric techniques together develop new hyphenated techniques which are useful for the simultaneous estimation and impurity profiling. The simultaneous analytical analysis provides specificity and assurance for the identification of the chemical entities in the pharmaceutical formulation. The main objective behind the analytical estimation is to provide the assurance that the particular formulation contains the equal amount of active pharmaceutical ingredient as mentioned in the label.

Keywords: Analytical techniques; Spectrophotometric; HPTLC; Hyphenated techniques; Gas Chromatography

Abbreviations

API's: Active Pharmaceutical Ingredients; UV-VIS: Ultra Violet –Visible; I.R: Infra Red; I.P: Indian Pharmacopoeia; B.P: British Pharmacopoeia; USP: United States Pharmacopoeia; NMR: Nuclear Magnetic Resonance; MALDI: Matrix- Asisted Laser desorption Ionization; GAA: Glacial Acetic Acid; HPLC: High Performance Liquid Chromatography; PAD: Pulsed Amperometric Detection; PDA: Photo Diode Array; NS,CD: Non-suppressed Conductometric Detection; ACN: Acetonitrile; KOH: Potassiun Hydroxide; TLC: Thin layer Chromtography; HPTLC: High Performance Thin Layer Chromatography; UPLC: Ultra Performance Liquid Chromatography; TFA: Triflouro Acetic Acid; GC-MS: Gas Chromatography – Mass Spectrometry; LC-IR: Liquid Chromatography– Infra Red; LC-MS: Liquid Chromatography– Mass Spectroscopy; GC-IR: Gas Chromatography–Infra Red; GC-MS-MS: Gas Chromatography–Mass spectroscopy–Mass spectroscopy; LC-MS-MS: Liquid Chromatography–Mass spectroscopy–Mass spectroscopy; GC-GC-MS: Gas Chromatography–Gas Chromatography–Mass Spectroscopy; GC-NMR: Gas Chromatography–Nuclear Magnetic Resonance; GC-AES: Gas Chromatography–Atomic Emission Spectroscopy.

Introduction

The development of pharmaceuticals and their analysis has brought the world to the revolutionary extent in health sciences. The process of drug molecule discovery and pharmaceutical investigation or analysis of the formulation provides the safety and therapeutic effect to a high extent. To make API and formulation safe a large number of analytical techniques have been developed. During various stages of preparation of pharmaceutical formulation, the addition of impurities or development of impurities makes the preparation harmful for the administration and may cause other disorders and disease like cancer(mainly from solvents like benzene) [1,2]. In formulation the analytical techniques play the great role in the identification of physical and chemical properties of the formulation. The analytical techniques provide the important description for the evaluation of the toxicity and other impurities [1]. Presently various combinations in dosage forms are present in enormous amount and are increasing rapidly. These multi component formulations provide the increased therapeutic index, multiple actions, less side effects and quicker relief. The analytical process deals with two parts of chemical characterization either it is qualitative or quantitative. The qualitative analytical analysis provides quality and identity of the sample and quantitative analytical analysis provides the amount of chemical identities present in the formulation. The main objective behind the analytical estimation is to provide the assurance that the particular formulation contains the equal amount of active pharmaceutical ingredient as mentioned in the label [3].

For the estimation of multi component in formulation the various instrumental techniques like spectrophotometric and chromatographic techniques are used due to their advantages viz. less time consuming, cheap, specific and accurate which provides result up to high extent [3] (Figure 1).

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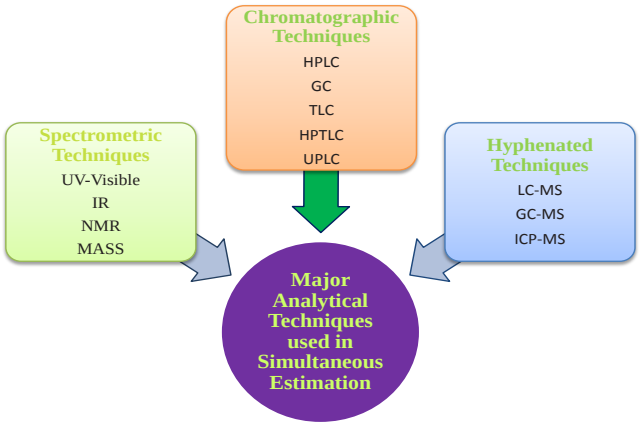

Figure 1: Different types of analytical techniques used in pharmaceutical world.

Spectrophotometric Techniques

Spectrophotometric techniques are the important instrumental techniques which are available to pharmaceutical analyst. The basics of spectrophotometric techniques are that they measure the interaction of electromagnetic radiations with sample in quantized form [4]. There are various spectrophotometric techniques which are used in the pharmaceutical world for the analysis of the API's and pharmaceutical ingredients.

UV/VIS Spectroscopy

It is the cheapest and easiest working analytical tool available that is used in the pharmaceutical laboratories and research. The analytical applications of the UV spectroscopy are qualitative and quantitative. As most of the pharmaceutical contains chromophore they can be determined directly. However UV/VIS spectroscopy is not much suitable for the simultaneous estimations with spectral overlapping. The derivative spectroscopy provides the enhancement of specificity and sensitivity in pharmaceutical formulations [5].

The ultraviolet–visible spectrophotometry techniques is one of the most frequently used techniques in the analysis which involves the measurement of amount of ultraviolet and visible radiations absorbed by the pharmaceuticals in a solution. Various techniques has been used in simultaneous estimation by UV/VIS spectroscopy such as: (i) Simultaneous Equation method; (ii) Absorbance ratio method; (iii) Geometric correction method; (iv) Orthogonal polynomial method; (v) Difference spectroscopy; (vi) Derivative spectroscopy [4]. The advantages of the UV/VIS spectroscopy techniques are that they have low time and labor consumption. The precision and accuracy of analyst by using the UV/VIS spectroscopy is up to a high limit [1].

Derivative Spectrophotometry

It is one of the most highly developed spectrophotometric techniques. The origin of derivative spectrophotometry is linked with appearance of spectrophotometers enabling recording of derivative spectra [6]. The derivative spectrophotometry involves the change of normal spectrum to its first, second or higher derivative spectrum [4]. The derivative spectra can be obtained by optical, electronic and mathematical methods. In optical method there is wave length modulation where the wavelength of incident light is quickly modulated over a narrow wavelength range by electromechanical device [6].

If the derivative spectrum is expressed as absorbance (A) as function of wavelength (λ), the derivative spectra are:

Zero order: $A = f(\lambda)$

First order: $dA/d\lambda = f'(\lambda)$

Second order: $\frac{d^2A}{d\lambda^2} = f''(\lambda)$

The derivative spectra are employed to get the better differences among spectra to resolve the overlapping bands in qualitative analysis and to reduce the outcome of scattering matrix [4].

The strong positive and negative bands with maximum and minimum at same wavelength of an absorption band as inflection point in absorbance band governs the odd (first and second) derivative spectrum whereas the strong positive and negative bands with minimum or maximum at same wavelength as λ_{max} of absorbance band governs the even (second and fourth) derivative spectrum.

Number of bands = derivative order +1

The merits of derivative spectroscopy are to increase the resolution permitting identification of analyte with close $\lambda_{max,}$ to decline the baseline shift arising from instrument or sample handling and diminish the scattering effect thus helpful for analyte present in turbid solution [7] (Table 1,2,3).

IR Spectroscopy

Pharmaceutical quality control and quality assurance depends on monitoring the composition and uniformity of the drug substance during processing and in the pharmaceutical product. Various tests have been used to determine identity, quality and strength. Vibrational spectroscopic techniques, including Mid-infrared, Near-infrared and Raman, have been proposed as other alternative approaches [25]. Infra-red is an important technique which gives sufficient information about the structure and its functional groups of a compound. This technique provides a spectrum containing large number of absorption bands i.e. functional groups can be derived

Table 1: Simultaneous estimation of drugs in pharmaceutical combination by first-order derivative UV spectrophotometric method.

Drug combination	Wavelength (nm)	References
Adrenaline and Noradrenaline	394 and 342	[8]
Amiloride and Furosemide	241.4 and 343.6	[9]
Amitryptyline and Chlorpromazine hydrochloride	254 and 260	[10]
Amoxicillin and Bromohexine hydrochloride	278.8 and 326.2	[11]
Analgin and Adamon	600 and 310.5	[12]
Analgin and Hyoscine N-butyl bromide	291.8 and 219.8	[13]
Atenolol and Nifedipine	276 and 340	[14]
Cephalothin and Cefoxitin	235 and 236.7	[15]
Cilazapril and Hydrochlorothiazide	242.8 and 282.8	[16]

Table 2: Simultaneous estimation of drugs in pharmaceutical formulation by second-order derivative UV spectrophotometric method.

Drug combination	Wavelength (nm)	References
Acrivastine and Pseudoephedrine hydrochloride	288 and 270.2	[17]
Cefatoxime sodium and Cefadroxil monohydrate	257 and 279	[18]
Telmisartan and Metoprolol	224 and 299.5	[19]
Prasugrel and Asprin	252.4 and 267.6	[20]
Ibuprofen and Famotidine	272.8 and 290	[21]
Citicoline and Piracetam	274.6 and 206.8	[22]
Diclofenac sodium and Thiocolchicoside	249 and 246	[23]

Table 3: Simultaneous estimation of drugs in pharmaceutical estimation by third-order derivative UV spectrophotometric method.

Drug combination	Wavelength (nm)	References
Atenolol and Amlodipine	264 and 308	[24]
Amitryptyline and Chlorpromazine hydrochloride	254 and 260	[10]

Table 4: Determination of pharmaceutical drugs by IR spectroscopy.

Drug name	Near IR	Mid IR	Far IR	Property Description	Ref.
Indomethacin–saccharin	✓	✓		Characterization of cocrystals	[27]
Enalapril		✓		Characterization of six salt forms	[27]
Celecoxib		✓		Characterization of polymorphs	[27]
Omperazole sodium		✓		Characterization of API in salt form	[27]
Acyclovir and lactose		✓		Drug excepient compatibility	[27]
Bicifadine HCl		✓		Characterization of polymorphs	[27]
Piroxicam monohydrate	✓			Quantification during isothermal dehydration	[27]
Troglitazone	✓			Drug (crystalline versus amorphous) distribution in solid dispersion	[27]
Theophylline and caffeine	✓	✓		Characterization of hydrate formation during wet Granulation	[27]
Sulfathiazole	✓			Polymorph screening and processing-induced transformation (PIT) screening	[27]
Prediction of DNA			✓	Oligonucleotide of DNA	[28]

from which the structures of organic compound can be studied or demonstrated. The absorption of infra-red radiations (quantized) causes the various bands in a molecule to stretch and bend with respect to one another [26]. The most important region for the organic chemist is 2.5μ to 15μ in which molecular vibrations can be detected. The ordinary infra-red region extends from 2.5μ to 15μ. The region from 0.8μ to 2.5μ is called Near IR region and that from 15μ to 200μ is called Far IR region. When the electromagnetic radiations are passed through sample which are absorbed by the bonds of the molecules in the sample causing them to stretch or bend. The wavelength of the radiation absorbed is characteristic of the bond absorbing it (Table 4).

Shortcomings and limitations of infrared spectroscopy

Infra-red spectroscopy has proved to be one of the most important and needy methods for characterization of both quantitatively and qualitatively of large number of inorganic compounds encountered in research as well as in industry yet it suffers some shortcomings which are mentioned below:

- It is impossible to determine the unknown substance from the individual IR spectrum. Example a mixture of paraffin and alcohol will give the same IR spectrum as by the higher molecular weight alcohols.

- In case of Near IR region technique extensive method development is required before the technique can be used for analysis of pharmaceuticals. Development of method requires a highly trained professional analyst with computing knowledge [29].

Application of IR spectroscopy

Various pharmacopoeias like IP, BP and USP are used to identify API's and to check the purity at short interval of the time which indirectly increases the productivity eg: Amylobarbitone, Betamethasone, Dexamethasone, Cyclophosphamide, Sulphalene. Assay of pharmaceutical dosage form: A few remarkable examples are codeine phosphate in tablet, aspirin-phenacetein–caffeine tablet, meprobamate in tablet have been reported [30].

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance is a division of analytical chemistry and spectroscopy which deals with the radio frequency waves which produces transitions among magnetic energy levels of nuclei of a molecule. The magnetic energy levels are created by keeping the nuclei in magnetic field. The first observation of NMR was observed and studied by Purcell and Bloch at 1945. Ethyl Alcohol was the first compound which was studied and demonstrated by this technique in 1951 [31].

When the energy in the form of radiofrequency is applied, the applied energy is equal to the precessional frequency, the adsorption of energy takes place and NMR spectrum is obtained. On increasing the strength of magnetic field it will cause raise in precessional frequency but it does not cause the transition from ground state to excited state. Without the magnetic field and radiofrequency there will be no reason to cause the formation of NMR spectrum. NMR is used for the quantitative and qualitative analysis to determine the impurities of the drug, to elucidation of natural drug products and to determination metabolites of drugs in body fluids. The enhancement or power of NMR can be increased by following ways:

- Hyper-polarization
- Two dimensional(2D)
- Three dimensional(3D) and higher dimension multi-frequency techniques
- Distortionless enhancement by polarization transfer (DEPT)
- Correlation spectroscopy (COSY)
- Nuclear overhouser enhancement spectroscopy (NOESY)

Incredible natural abundance double quantum transfer experiment (INADEQUATE), and with the addition of chromatographic techniques ie hyphenated techniques [32].

Application of NMR

1. Food Chemistry: It is used for the authentication of the wine aging and identification of the fatty oil's constituents in food and other beverages.

2. Clinical application: It is used for the identification and studies of metabolites in biological fluids *in vivo* or *in vitro* and used for diagnosis and helpful in treatment of diseases [33].

3. Study of the Hydrogen Bonding: Hydrogen bonds in the metal chelates as well as in organic compounds can be determined by this technique. Hydrogen bonding results in the decrease in the electron shield protons and signal is shifted towards low field.

4. Impurity profiling of the pharmaceuticals: NMR play has an important role in the impurity profiling of pharmaceuticals (Table 5).

Mass Spectrometry

In mass spectroscopy the compound or a complex molecule is ionized, the ions are separated on the basis of their mass/charge ratio, and the number of ions generated during the ionization represents mass/charge unit which is recorded as the spectrum. Commonly electron impact mode is applied for bombarding the molecules in vapour phase with high energy electron beam and records as a spectrum of positive ions, which have been separated on the basis of mass/charge [38]. Some important features of mass spectroscopy are such as (i) small quantity of sample is used in this process which is ionized by the ion source which generally produces cations; (ii) the mass analyzers used in the mass spectrometer separate ions according to their mass to charge ratio; (iii) the ions which are formed are detected by the detector and displayed, which is called mass spectrum.

Advantages of mass spectrometry

- It is highly sensitive and accurate technique.
- Small amount of sample is required from nanogram to microgram can be analyzed.
- Resolution time is up to high extent.
- Highly specific due to fragmentation and helps in study of structure.

Disadvantages of mass spectrometry

- Sample recovery cannot be achieved due to destructive nature of the process.
- Very costly and required high maintenance.

Introduction of sample is difficult due to small sample size [29].

Application of mass spectrometry

- Determination of molecular mass of the compounds: peak having highest m/e ratio shows the molecular mass of the compound

Table 5: Simultaneous estimation of drugs in presence of impurity by NMR spectroscopy.

Drugs	Impurity	References
Heparin	Galactosamine	[34]
Fluticasone propionate	Monomeric or dimeric impurity	[35]
Ropivacaine hydrochloride	Drug associated impurity	[36]
Anastrozole	Degradation associated impurity	[37]

- Characterization of polymers: Determination of polymers and elucidation of polymer structure can be done by this technique. The structure of the polymer can be distinguished on the basis of arrangement of atoms.
- Impurity profiling in pharmaceuticals by Mass spectrometers: Mass spectrometry is the finest and latest technique used for the detection of impurities in the complex compounds. If the molecular weights of the impurities are larger than major components their detection is easier due to their higher mass peaks which are free from major constituents. For example: i) Determination of impurity (toxic elements) in Hydroxyapatite [39]; ii) Determination of impurity (Anhydro-simvastatin, Simvastatin dimer) in Simvastatin [40].
- Mass spectrometry is also used for the analysis of simple or complex proteomes using quantitative mass spectrometry [41].
- Analytical biological quantitative estimation: Quantitative analysis of antiretroviral drugs (Ritonavir. Saquinavir, Amprenavir, Indinavir, Nelfinavir, Tipranavir, Carbamazepine. As internal standard, carbamazepine for nevirapine, indinavir for amprenavir, lopinavir for tipranavir, indinavir for nelfinavir, saquinavir for ritonavir, nelfinavir for indinavir, and tipranavir for lopinavir were used) in lysates of peripheral blood mononuclear cells using MALDI-triple quadrupole mass spectrometry have been reported [42].

Chromatography

Chromatography is moderately a new technique which was developed by M. Tswett, a botanist in 1906 in Warsaw. In that year he was successful in elucidating chlorophyll, xanthophylls and several other colored substances from vegetable extracts through a column of calcium carbonate. The calcium carbonate act as an adsorbent and other various dissimilar substances get adsorbed to different extent and give rise to colored bands at different positions on the column. Tswett named this system of colored bands as the Chromatogram and the method as Chromatography after the Greek words chroma and graphos meaning "color" and "writing" respectively. In 1930's chromatography in the form of TLC and Ion Exchange chromatography was introduced as separation techniques. In 1941, Martin and Synge introduced Partition and Paper chromatography. They introduce Gas chromatography in 1952.

Chromatography is a non-destructive method from which multi component can be derived and separated chromatography is the most important single analytical technique used today and will most expected continue to be so far for the predictable future. It is the foundation stone of molecular and pharmaceutical analytical chemistry and recently it is coupled with atomic absorption and Mass spectroscopy which has extended its application in the world pharmaceutical analysis. Various chromatographic techniques have been used for simultaneous estimation of API's or pharmaceutical dosage forms such as (i) Paper chromatography; (ii) High performance liquid chromatography (HPLC); (iii) Thin layer chromatography

Table 6: Simultaneous estimation of drugs by normal phase HPLC.

Drugs	Mobile phase	References
Drotaverine hydrochloride and Omperazole	n-heptane: Dichloromethane: Methanolic Ammonia (5%) : MeOH(50:25:1:4) v/v/v	[45]
Benzoyl peroxide and Benzoic Acid	MeOH : water (65:35) v/v	[46]
Tocopherols and Tocotrienols	Hexane : 1,4-dioxane (95.5:4.5) v/v	[47]

Table 7: List of few drugs estimated simultaneously by RP-HPLC.

Drugs	Mobile phase	References
Aceclofenac and Paracetamol	MeOH and water (70:30) v/v	[49]
Losartan potassium and Amlodipine besylate	0.02% Triethylamine in water: ACN (60:40)v/v	[50]
Naproxen and Esomeprazole	Part A: Mixing buffer, ACN and MeOH in the ratio of (70:20:10) v/v/v Part B: Mixing buffer, ACN in the ratio of (20:80) v/v Buffer is prepared by dissolving 0.71 g (0.005M) of sodium perchlorate in 1000 mL of water, added with 5 mL of N-butyl amine. The pH of the buffer is adjusted to pH to 8.7 using diluted solution of Perchloric acid.	[51]
Paracetamol and Etroricoxib	MeOH : ACN: Phosphate buffer (40:20:40)v/v	[52]
Cefixime and Cloxacilin	Phosphate buffer : ACN: MeOH (80:17:3)v/v	[53]
Metoprolol and Hydrochlorothiazide	Di-sodium hydrogen phosphate: MeOH: ACN (525:225:250) v/v	[54]
Chlorpheniramine Maleate and Phenylephrine	ACN: Phosphate buffer (55:45) v/v	[55]
Lansoprazole and Domperidone	ACN and MeOH (81:19) v/v	[56]
Ambroxal hydrochloride and Loratidine	ACN and Ammonium acetate (50:50) v/v	[57]

(TLC); (iv) High performance liquid chromatography (HPTLC); (v) Gas chromatography (GC); (vi) Ultra-high performance liquid chromatography (UPLC); (vii) Column chromatography.

High Performance Liquid Chromatography (HPLC)

Chromatography is the most commonly used analytical technique in pharmaceutical analysis. The technique is used by the chemists to separate and determine species in variety of organic, inorganic, and biological materials [26]. HPLC has been around for about 35years and it is the major separation technique use. HPLC is one of the best method of choice for analyzing various varieties of natural and synthetic compounds [43]. Various types of HPLC's have been used for simultaneous estimations e.g. normal phase HPLC, reversed phase HPLC, size exclusion HPLC, ion-exchange HPLC, bio-affinity HPLC.

In case of normal phase HPLC, the stationary phase is polar and mobile phase is non polar. Adsorption extends with increase in polarity, and the interaction with polar analyte and polar stationary phase increases the elution time [44]. Few drugs enlisted in Table 6 which is simultaneously estimated by normal phase HPLC.

Furthermore, RP-HPLC is opposite to normal phase HPLC. The polarities of the mobile and stationary phase are reversed. It is the most popular mode of liquid chromatography. Almost 90% of all analysis of low molecular weight sample is carried out using RP-HPLC [48] (Table 7).

Size Exclusion HPLC

Size exclusion or Gel chromatography is one of the latest types of the liquid chromatographic procedures. It is the most potent technique that is particularly applicable to high molecular-weight species [58]. It separates biomolecules on the basis of true size difference; small molecules of analyte can enter the pores of gel without difficulty and therefore spend more time in these pores escalating their retention time. On the other hand, large analyte spend little time in the pores and elute quickly [59].

Ion-exchange HPLC

In ion exchange chromatography, the stationary phase is ion exchange resin and ions of the opposite charge are electro statically attached to the surface of the resin. When the mobile phase is passed through resin, the electro statically bound ions are free as other ions which are bounded preferentially. This technique involves the exchange equilibria between ions in solution and ions of like sign on the resin [60] (Table 8).

Bio Affinity HPLC

Affinity chromatography is a precious tool in areas such as biochemistry, pharmaceutical science, clinical chemistry, and environmental testing, where it has been used for both the purification and analysis of compounds in complex sample mixtures. Affinity chromatography is a liquid chromatographic technique that uses a "biologically related" agent as a stationary phase for the purification or analysis of pharmaceutical components. The method of analysis is usually based on the same types of specific, reversible interactions and reactions that are initiated in biological system, such as the binding of an enzyme with a substrate or an antibody with an antigen. These interactions are exploited in affinity chromatography by immobilizing (or adsorbing) one of a pair of molecules on to a solid support which is called as a stationary phase. This immobilized molecule provides the site for binding to particular compounds in a sample [68].

Thin Layer Chromatography

In 1958, Stahl demonstrated purpose and use of TLC in analysis, a method based on adsorption chromatography. Presently TLC is an important analytical tool for the qualitative and quantitative analysis, of number of natural as well as synthetic products. The TLC technique is very important tool in analysis of alkaloids, glycosides, isoprenoids,

Table 8: Pharmaceutical compounds estimated by Ion-exchange chromatography.

Analyte	Column	Eluent	Detection	Method	References
Amikacin	Anion Exchanger	0.115 N NaOH	PAD	Assay	[61]
Bethinicol chloride injection	Weak cation Exchanger	20 mM CH3SO3H	NS,CD	Assay	[61]
Erythromycin	Cation Exchanger	Mixture of ACN, NaOH and water	PAD	Assay	[61]
Kanamycin sulfate	Anion Exchanger	0.115 N NaOH	PAD	Assay	[61]
Fenoldopam	Anion Exchanger	2.8mM NaHCO3 + 2.2mM Na2CO3+ 0.8mM 4-cyanophenol+2% ACN	NS,CD	Assay	[61]
Paracetamol	Waters IC-PAK A HR	5 mM LiOH in 5% ACN at 1mL/min	UV at 300nm	Quantitation in solid dosage form	[62]
Oxytetracycline, tetracycline, chlortetracycline, doxycycline.	Dionex OmniPac PCX 100 (250 × 4mm)	0.2M HCL in ~28% ACN at 1 mL/min	Direct UV at 300 nm	Method developed for primarily residual testing	[63]
Caffeine, Theobromine, Theophylline	DionexHPLC-CS3(cationic coloumn) Dionex OmniPac PAX-100(anionic)	100mM HCL at1mL/min(cation) 15mM KOH in 1% ACN at 1mL/min	Direct UV at 274nm	Quantitation in injections and tablets .	[64]
Flucloxacillin and Amoxicillin	Zorbax 300-SCX (Agilent) 250×4.6 mm, 5µm particles	0.025M ammonium dihydrogen phosphate (pH 2.6)-ACN (95/5) at 1.5mL/min	UV at 225nm	Used in QC test in pharmaceutical injection products.	[65]
Methenamine, Methenamine mandelate, Methenamine hippurate	Zorbax SCX-300(Agilent), 150×4.6 mm, 5µm	ACN-sodium perchlorate monohydrate (0.1M, pH05.8), 1mL/min	UV at 212nm	Assay of pharmaceutical tablets	[66]
Simultaneous determination of chloride, bromide and iodide in food stuff .	Anion-Exchange resin column (0.6 cm × 13 cm)	4.0 mM Na2CO3 with flow rate of 0.70mL/min	Optical detector	Simultaneous Estimation	[67]

Table 9: List of some drugs estimated by TLC.

Drug examined	Stationary phase	Mobile phase	Visualization reagent	Reference
Polymyxin B, Framycetin, and Dexamethasone	Silica gel 60 and F254 silica gel 60 plates	MeOH and MeOH–n-butanol–Ammonia (25%)–Chloroform (14:4:9:12) v/v/v/v	0.3% Ninhydrin solution	[71]
Oxo-steroids	Silicagel GF254	Chloroform: MeOH(97:3) v/v/	Dansylhydrazine was used as a prelabeling reagent.	[72]
Aceclofenac, Paracetamol, and Chlorzoxazone	Silica gel 60 F 254	Toluene:2-Propanol: Ammonia (4:4:0.4)v/v/v	Detection at 274nm	[73]
Levamisole	Silica gel 60F254	MeOH:Toluene:Chloroform (14:36:50)v/v/v	UV light 223nm	[74]

Table 10: Impurity profiling by TLC.

Drugs	Impurity	Reference
Ethambutol	2-amino butanol	[75]
Framycetin sulphate	Neamine	[75]
Norgestrel	3,17α-diethinyl-13-ethyl-3,5-gonadiene-17-ol	[75]
Clopidogrel	(+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno3,2-c.pyridine-5(4H)-acetic acid	[76]

lipid components, sugars and derivatives and practically all bio constituents. In TLC sample is spotted on the plate with micropipette and the chromatogram is developed by placing the bottom of the plate or strip. The solvent or the mobile phase is drawn up by the phenomenon of capillary action, and the sample components move up the plate at different rates, depending on their solubility and their degree of affinity towards mobile phase. The spots will generally move at certain fraction of the rate at which the solvent moves, and they are characterized by the R_f value: $R_{f=}$ distance solute moves /distance solvent from moves [69] TLC plays important role in the analysis of different groups of drugs. The last few years shows that interest in TLC application in pharmaceutical analysis has increased with improvements in TLC instrumentation such as TLC combined with densitometry or with MS and IR. As TLC has some advantages as comparison to HPLC and GC methods are absence of UV activity or when absence of volatility in compounds, TLC is cheap equipment and easy to work in comparison to HPLC and GC and allows parallel separation and quantitative determination of many samples at the same time [70]. The application of TLC has been limited due to low sensitivity and does not appropriate for volatile compounds. Mainly, TLC is frequently used by BP monographs as part of number of identity tests performed on pure substances or used to identify the marker compounds. There are some examples of TLC based identity tests described in pharmacopoeial monographs as shown in Table 9. Various impurities present in the pharmaceutical API's can be detected by TLC. Some drugs with impurities which can be detected by TLC can be detected (Table 10).

Simultaneous identification of drugs: TLC provides the identification of drugs simultaneously in various pharmaceutical dosage forms which is very reliable. Some examples of the drugs which are identified by TLC method are described in Table 11.

Determination of the amino acids: Various amino acids can be detected by application of TLC. Various amino acids which are

Table 11: Simultaneous Identification of Drugs by TLC.

Drug	Dosage Form	Stationary phase	Mobile phase	Identification	References
Clotrimazole, Miconazole, and Ketoconazole	Creams and Ointments	Silica gel F254	n- hexane: Chloroform: MeOH: Diethylamine (50:40:10:1) v/v	By UV at 220nm	[77]
Neomycin sulfate, Polymixin B sulfate, Zinc bacytracin and Methyl and Propyl hydroxybenzoates	Opthalmic Ointment	Silica gel	MeOH:n-butanol: Ammonia 25%–Chloroform (14:4:9:12)v/v/v/v for determination of antibiotics and n-pentane:Glacial Acetic Acid (66:9)v/v for methyl and propyl hydroxybenzoates.	Antibiotic were identified by using ninhydrin ethanol solution, while densitometric measurements were made at λ=550nm. Hydroxy benzoates were identified by UV measurements at λ=260 nm	[78]
Atorvastatin calcium and Fenofibrate	Pharmaceutical Dosage form	Silica gel 60 F254	Toluene:MeOH:Triethylamine (7:3:0.2 v/v	densitometrically at 258 nm	[79]

Table 12: Determination of various important constituents of food used on daily basis.

Food sample	Analyte determined	Stationary phase	Mobile phase	Determination	Refernces
Green salad	Ascorbic Acid	Silica Gel F254	Ethanol:1.0% acetic Acid(9:1) v/v	By UV at 254 nm	[81]
Potatoes	Vitamin C	Kiesel gel-G	Oxalic acid, MeOH, Chloroform (2gm:20:60)	UV radiation	[82]
Fish Liver	Vitamin A	Alumina	Cyclohexane	Molybdophosphoric acid	[82]
Vegetable oils	VitaminE	Kiesel gel-G	Dichloromethane, trichloroethylene	sbCl2 2,2-bipyridyl Fecl3	[82]
Coca butter	Triglyceride	Silica impergnated by silver nitrate	Carbon tetrachloride: Chloroform: Acetic Acid and small volumes of ethanol (60:40:0.5) v/v/v	0.2% Ethanolic solution of dibromo-R-fluorescein	[83]
Ziziphusmauritiana	Sugar	Silica gel	Butanol: Water : Acetic Acid (55:30:15) v/v/v	Visulaized under UV the sprayed with 80% Folin –C phenyl reagent	[84]

Table 13: Simultaneous estimation of drugs by HPTLC.

Drugs	Stationary phase	Mobile phase	Detection	Reference
Valsartan and Hydrochlorothiazide	Silica gel 60F254	Chloroform : MeOH : Toluene : Glacial acetic acid (6:2:1:0.1) v/v/v/v	By UV at 260 nm.	[88]
Emtricitabine and Tenofovir	Silica gel 60F254	Chloroform: MeOH (9:1) v/v	By UV at 265nm.	[89]
Telmisartan and Ramipril	Silica gel 60F254	Acetone: Benzene : Ethyl acetate : Glacial acetic acid in the proportion of (5:3:2:0.03) v/v/v/v	Densitometrically using a UV detector at 210 & 296 nm.	[90]
Diosgenin and Levodopa	Silica gel 60F254	Toluene : Ethyl acetate: Formic acid : GAA in the ratio (2:1:1: 0.75) v/v	194 nm for Diosgenin and 280 nm for Levodopa using absorbance reflectance mode.	[91]
Diclofenac Sodium and Misoprostol	Silica gel 60F254	Toluene : Ethyl Acetate : Ethanol : Glacial Acetic acid (8:2:1:0.1) v/v/v/v	Densitometric evaluation of the separated zones was performed at 220 nm	[92]
Pseudoephedrine and Cetirizine	Silica gel 60F254	Ethyl Acetate : MeOH : Ammonia (7:1.5:1) v/v/v	Spectrodensitometric scanning at a wavelength of 240nm	[93]
Telmisartan and Amlodipine	Silica gel 60F254	Ethyl acetate : 1, 4 Dioxane : MeOH : 25% Ammonia (15:1.5:3:1.5) v/v/v/v	By UV detection at 323 nm.	[94]
Perindopril Erbumine and Indapamide	Silica gel 60F254	Dichloromethane : MeOH : Glacial acetic acid (9.5:0.5:0.1) v/v/v	By UV detection at 215 nm.	[95]
Atorvastatin calcium and Ezetimibe	Silica gel 60F254	Toluene: MeOH (8:2) v/v	Densitometric evaluation was performed at 240 nm	[96]

detected by TLC are Glycine, Alanine, Valine, Leucine, Isoleucine, Serine, Threonine, Aspartic acid, Aspargine, Glutamic acid, Glutamine, Lysine, Histidine, Arginine, Phenylalanine, Tyrosine, Tryptophan, Cysteine, cystine, Methionine, Proline and Hydroxy Proline [80] and analysis of essential constituents of food by TLC (Table 12)

High Performance Thin Layer Chromatography (HPTLC)

Among the significant modern analytical tools HPTLC is a powerful analytical method equally appropriate for qualitative and quantitative analytical tasks (Table 13). HPTLC is playing important role in today pharmaceutical analytical world, not in competition to HPLC [85]. Separation may result due to adsorption or partition or by both phenomenons's depending upon the nature of adsorbents used on plates and solvents system used for development. Different aspects on HPTLC fundamentals such as qualitative and quantitative analysis have been reported such as phytochemical analysis, biomedical analysis, herbal drug quantification, analytical analysis, finger print analysis and potential for hyphenation (HPTLC-MS, HPTLC- FTIR and HPTLC-Scanning Diode Laser) have been reported [86].

There are several advantages of using HPTLC for the analysis of compounds as compared to other techniques, like HPLC, spectrophotometry, titrimetry, etc. Some of the advantages of HPTLC are:

- Capability to analyze crude samples containing multi-components.
- In this process, it is easy to separate colored compounds.
- Several samples can be divided parallel to each other on the same plate resulting in a high output, time saving, and a rapid low-cost analysis.
- Two dimensional separations of the natural as well as pharmaceutical compounds are easy to perform.

- HPTLC can combine and it can be used for different modes of evaluation, allowing identification of compounds having different light absorption characteristics or different colours.
- HPTLC method may help to minimizes exposure risk of toxic organic effluents and significantly reduces its disposal problems as a result reducing environment pollution [87] (Table13).

Gas Chromatography

Gas chromatography is a technique used for separation of mixtures into single entities by a process which lies on the redistribution of components between a stationary phase or supporting material in the form of a liquid-solid or combination of both and gaseous mobile phase. Mechanism of the GC is based on adsorption, mass distribution or size exclusion [97].

Application of GC in quantitative analysis

Assay of pharmaceuticals products: B.P., U.S pharmacopoeia and the European pharmacopoeia are based on the GC as well as on other estimation techniques for the assay of various pharmaceutical products. Some of the pharmaceutical product which are assayed or determination of related substances by the GC are mentioned below:

1. Analysis of methyl testosterone and related substances in API and Tablet.
2. Assay of atropine in eye drops.
3. Quantification of ethanol in a formulation.

Estimation of degradation product in the pharmaceuticals or impurity profiling: GC is the only technique used for the estimation of volatile degradation products in the pharmaceutical preparations and in impurity profiling of the drugs For example

1. Estimation of pivalic acid in dipivefrin.
2. Dimethylanaline in bupivacaine injection.
3. Determination of residual glutaraldehyde in polymeric form [26].
4. Impurity profiling of cocaine [98].

Use of GC in bio analysis: Use of GC in the bio analysis of the drugs in the plasma and its metabolism increase the efficacy of this type of chromatography.

1. Determination of Valproic acid in the plasma can be determined by this method.
2. Quantification of bupivacaine in plasma [26].
3. Measurement of isoprene solubility in water, human blood and plasma [99].
4. Simultaneous determination of 3,4-Dihyroxyphenylglycol, Catecholamines and 3,4-Dihydroxyphenylalanine in plasma [100].

Environment analysis: Use of GC-GC played important role in investigation of Oil-Sand Napthenic acid which are waste products formed during the the Oil-sand digestion and extraction processes [101].

Simultaneous estimation by GC: Simultaneous estimation of the pharmaceutical formulations can be done by this technique which provides the use of less time and increase in the sensitivity. For example:

Simultaneous estimation Miconazole Nitrate and Metronidazole in Different Pharmaceutical Dosage Forms [102].

Simultaneous estimation of the amino acids in the food suppliments [103].

Simultaneous estimation of Turpentine oil, Chlorbutol, Para-Dichlorobenezene and Benzocaine [104].

Ultra-High Performance Liquid Chromatography

Ultra–high performance liquid chromatography (UPLC) has marked a radical and outstanding change through opening new doors intended for analyst to fetch rapid analytical separation techniques without sacrificing high-quality results obtain former from high performance liquid chromatography (HPLC). The principle is based on the principal of use of stationary phase consisting of particles less than 2μm while HPLC columns are typically filled with particles of 3 to 5μm. The underlying principles of this evaluation are governed by the Van Deemter equation, which is an empirical formula that describes the relationship between linear velocities (flow rate) and plate height (HETP or column efficiency) [105].

The potential of UPLC is to get better analysis of the samples that are encountered during pharmaceutical development and manufacturing. UPLC generated higher separating efficiencies through the make use of smaller diameter particle packing and higher operating pressures. A commercial system competent of generating much higher pressures than used in standard HPLC has been evaluated to determine its potential in routine analysis. UPLC has been shown to generate high peak capacities in short times and this is found to be quite beneficial in analyzing the complex mixtures that comprise metabolism samples. The application of UPLC resulted in the detection of additional drug metabolites, improved the spectrum quality and separation efficiency [106]. UPLC is one of the advantageous analytical techniques in less run time, high sensitivity, high resolution; faster analysis due to fine particle size, cost effective, less solvent consuming and real time analysis is possible with the manufacturing processes in pharmaceutical industries as well as in research programs. But it has some restrictions also such as increased pressure requires for high maintenance, less column life [107] and non regenerable phases of size 1.7μm [108] (Table 14).

Hyphenated Techniques

A hyphenated technique is combination or coupling of two different analytical techniques with the help of proper or various interfaces. The word Hyphenated technique comes from the combination of separation-separation, separation-identification and identification-identification techniques. The term "hyphenation" was first adapted by Hirschfield in 1980 to describe a possible combination of two or more instrumental analytical techniques in a single run. The main objective of coupling of different analytical techniques to obtain an information-rich detection for both identification and quantification compared to that with a single analytical technique [118].

Table 14: Application of UPLC in simultaneous estimation.

Drugs	Mobile phase	Detection	Reference
Abacavir Sulphate and Lamivivudine	Triethylamine phosphate buffer (pH 2.5) and MeOH (50:50%) v/v	UV detection at 230 nm	[109]
Aspirin and Dipyidamole	Triethylamine phosphate buffer (pH 2.5) and MeOH (50:50) v/v	UV detection at 230 nm	[110]
Losartan potassium, Atenolol and Hydrochlorothiazide	Water : ACN : Triethyl amine: Ortho phosphoric Acid (60:40:0.1:0.1) v/v/v/v	UV detection at 225 nm	[111]
Ceftriaxone and sulbactum injection	0.05M Sodium di-hydrogen ortho-phosphate dehydrate : ACN (86:14) v/v	PDA detection at 254 nm and 195 nm	[112]
Bambuterol Hydrochloride and Montelukast	0.025 % TFA in water as aqueous and 0.025% TFA in ACN as organic solvent	PDA detection at 210 nm	[113]
Impurities in Telmisartan and Chlorthalidone	The mobile phase A consists pH 4.5 buffer & ACN in the ratio 90:10 (v/v). Mobile phase B consists pH 4.5 buffers & ACN in the ratio 20:80 v/v	Detection at 290 nm	[114]
Metformin and pioglitazone	0.2% Triethylamine in water: ACN (70:30) v/v ph adjusted with orthophosphoric acid .	Detection at 243 nm	[115]
Thiocolchicoside and Aceclofenac	5% ammonium acetate buffer and MeOH in the ratio of (40:60) v/v	PDA detection at 276 nm	[116]
Vitamins, caffeine and Preservatives	The mobile phase consisting of A : buffer (0.1% Trifluoro acetic acid in water) and B : a mixture of 50% ACN and 50% MeOH with a timed gradient programme	Detection at 200, 254, and 290 nm	[117]

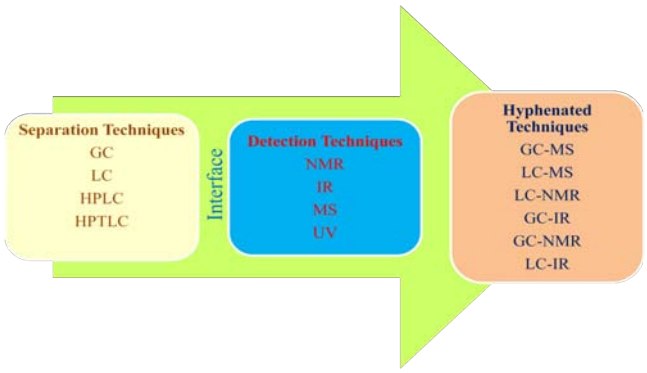

Figure 2: Diagram representing coupling of two techniques using different interfaces.

Hyphenated technique is usually comprised of separation and detection technique which are coupled to give a new technique. The use of hyphenated technique provides the high degree of assurance for the pharmaceutical estimation and impurity profiling (Figure 2).

GC-MS

This hyphenated technique developed from the coupling of GC and MS, was the first of its type to become functional for research and development purposes. Mass spectra obtained from this hyphenated technique presents more structural information based on the interpretation of fragmentations. Compounds that can be easily volatile, small, and stable in high temperature in GC conditions can be easily analyzed by GC-MS. The most common derivatization technique is the conversion of the analyte to its trimethylsilyl derivative which provides the accurate and precise results.

LC-IR

The hyphenated technique is a coupling of an LC and IR or FTIR and is known as LC-IR or HPLC-IR. While HPLC is one of the most dominant separation techniques available today, the IR or FTIR is a useful spectroscopic technique for the identification of organic compounds, because the mid-IR region provides maximum possibilities of functional groups e.g., –OH, –COOH, and so on. However, combination of HPLC and IR is difficult and the evolution in this hyphenated technique is extremely slow because of large number of bands of the mobile phases thus it is difficult to understand the small signal generated by the sample components [119].

LC-MS

Hyphenated techniques LC-MS or HPLC-MS is composed of an LC (Liquid chromatography) with a mass spectrometer (MS). The information obtained from a single LC-MS run, on the structure of the compound is limited in use because the ionization techniques used in this technique are soft ionization techniques which present the molecular ion species with only a few fragment ion. Hyphenated techniques such as HPLC coupled with UV and Mass spectrometry (LC-UV-MS) has been proved to be more useful in biological screening for rapid survey of natural products [120].

LC-NMR

The on-line coupling of high-performance liquid chromatography (HPLC) principles to high-resolution NMR spectrometers offers a powerful tool for analyzing and characterizing complex chemical mixtures without the need of chemical separation. LC-NMR promises to be of great value in the analysis of complex compounds of all types, particularly the analysis of natural products and drug-related metabolites in biofluids. The major advancement have been made in LC-NMR technology, a strong case can be made that HPLC purification of metabolites followed by conventional tube NMR is equally useful and may provide results in very less time and show very high sensitivity [121]. Hyphenated techniques provide the high degree of word for the compounds purity and impurity (Table 15).

Simultaneous Estimation by Hyphenated Techniques

Use of hyphenated techniques in the pharmaceuticals, environmental sciences and study of pharmacokinetic parameters by simultaneous estimation played an important role. Few examples of the various hyphenated techniques which are reported and their role in different fields are enlisted below.

Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromatography/mass spectrometry (GC/MS) [129].

Differentiation of the regioisomeric 2-, 3-, and 4-trifluoromethyl phenylpiperazines (TFMPP) by GC–IRD and GC–MS [130].

Simultaneous determination of melamine, ammelide, ammeline,

Table 15: Impurity profiling of some compounds by hyphenated techniques.

Drug	Impurity	Techniques	Reference
Quetiapine	Degredation impurities	LC-MS/MS	[122]
Lopinavir	Methanesulfonate and Ethyl Methanesulfonate	LC-MS/MS	[123]
Acyclovir	Drug associated impurity	LC-MS	[124]
Irbesartan	Degredation impurity	LC-NMR	[125]
N-acetylcysteine	Cysteine, cystine, N,N-diacytylcysteine	LC-UV-MS	[126]
Triton	1,4-dioxane	GC-MS	[127]
Selenium	Elemental impurities	ICP-MS	[128]

Table 16: Analytical tools used in toxicology and medical diagnosis.

Medical conditions	Analytical Techniques	Reference
Coronory heart diseases	MRI	[140]
Overdose of Barbiturate Amphetamine,Cocaine Heroine and Cannabis.	LC-MS, EIA, GC-MS, LC-MS/MS,LC-NMR, ELISA	[141-145]
Detection of cadmium, cobalt, chromium, iron, molybdenum, nickel, selenium, titanium, vanadium and zinc in blood	Neutron activation Analysis	[146]
Asphyxia	Gas chromatography-thermal conductivity	[147]

and cyanuric acid in milk and milk products by gas chromatography-tandem mass spectrometry [131].

Rapid sensitive speciation analysis of butyl- and phenyltin compounds in water by capillary gas chromatography atomic emission spectrometry (GC-AES) after in-situ ethylation and in-liner preconcentration [132].

Investigation of the composition of *Pinus peuce* needle oil by GC-MS and GC-GC-MS [133].

Simultaneous determination of rosuvastatin and fenofibric acid in human plasma by LC–MS/MS with electrospray ionization [134].

Identification of the major constituents of Hypericum perforatum by LC/SPE/NMR and/or LC/MS [135].

Simultaneous determination of pioglitazone and its two active metabolites in human plasma by LC/MS/MS [136].

Simultaneous Bioanalytical Techniques used in the Medical Diagnosis

The application of analytical tools in the clinical as well as in diagnostic fields is up to the mark. The bioanalytical techniques like GC-MS, HS-GC/MS, Gas chromatography-thermal conductivity are used in the diagnosis of the diseases. All these technique are non-invasive techniques and have a great potential in medical as well as in the development of medical biomedical analytical method. The various approaches of bioanalytical tools like chromatography, spectroscopy, and immunoassay have advantages in the field of narcotics and toxicology. Diseases like AIDS, Hepatitis, Acute respiratory disorders can be diagnosed by the use of analytical tools [137-139]. In future these hybrid techniques like LC-NMR, LC-MS/MS, GC-MS/MS etc can be used in field of medical and forensic science (Table 16).

Conclusion

From the above studies, it has been concluded that analytical techniques can be used successfully for the simultaneous estimation of many drug combinations as medicines which are given to the patients should be free from impurity and other interferences which can affect the therapeutic index and may show harmful effects to the patients. Above techniques are also used to elucidate and determine the structure of natural compounds. The advantages of the simultaneous estimation are fast, simple, less time consuming, accurate and sensitive for research purpose where no new method of estimation and analysis has been reported yet. This review also represents the advancement of chromatographic and spectroscopic techniques to hyphenated techniques. Hence, the simultaneous estimation of chemical entities using various analytical techniques are very much valuable for the future needs in pharmaceutical as well as other fields of investigation. Nevertheless, more expansion and advancement is needed in these techniques which will be beneficial to the analytical and bioanalytical researchers in developing strategies for new analytical method and high output results in the laboratories.

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