Analytical Approaches to Paclitaxel

An Anti Cancer Domain

S. Siddiqui[1], S. Kumar[2], A.A. Siddiqui[2], S. Paliwal[3], K. Kohli[2] and R. Mishra[2]

[1]Subramanium College of Science and Technology,
Holambi Khurd, New Delhi-110082,
[2]Department of Pharmaceutical Chemistry,
Faculty of Pharmacy, Jamia Hamdard (Hamdard University),
New Delhi-110062,
[3]Department of Pharmacy, Banasthali University,
Banasthali Vidyapeeth, Rajasthan-304022,
India.

Cancer is the uncontrolled growth of abnormal cells in the body. Symptoms of cancer depend on the type and location of the cancer. Paclitaxel obtained from *Taxus brevifolia* is an effective natural drug used in cancer treatment, so there arises a need of its estimation in biological fluids for the pharmacokinetic studies in clinical trials and in various pharmaceutical dosage forms including nanoparticles, cremophore based formulations and paclitaxel releasing stents. Literature survey reveals many chromatographic methods for estimation of paclitaxel by HPLC, LC-MS-MS and UPLC to get very accurate, precise and robust method with lower run time and higher recovery. An important step in the development of liposome-based formulations is estimation of the free drug concentration in the aqueous solution surrounding liposomes.

Keywords: Paclitaxel, HPLC, Biological fluids, Liposomes.

Introduction

Paclitaxel is a taxane diterpene amide that was first extracted from the stem bark of western yew (Fig. 1). This natural product is highly effective in treating various human neoplastic diseases, including gastric, ovarian, breast, lung, head and neck cancer and refractory leukemia[1-3]. Paclitaxel acts on the cellular level by promoting the polymerization of tubulin toward stable microtubules, which results in stable tubulin polymers against depolymerization. Its binding to microtubules prevents cell division but does not affect DNA, RNA or protein synthesis. In preclinical and clinical studies, the biological and pharmacological effects of paclitaxel have been shown to be correlated with concentration as well as with duration of exposure[4].

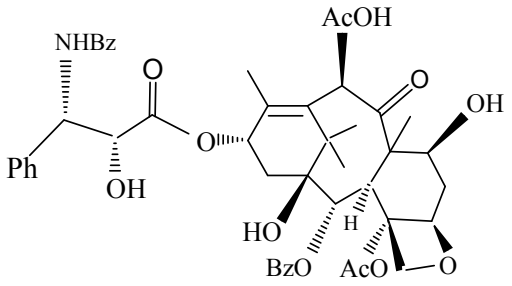
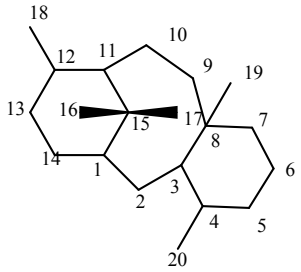


Fig. 1
Paclitaxel chemical structure

Paclitaxel, tax-11-en-9-one,5β,20-epoxy-1,2α,4,7β,10β,13α,hexahydroxy-4,10-diacetate-2-benzoate-13-α-phenylhippurate, a poly-oxygenated naturally occurring diterpene alkaloid, was first isolated by Wall and Wani from the bark of *Taxus brevifolia*. Paclitaxel is one of the broadest spectrum anticancer agent approved by Food and Drug Administration (FDA) for the treatment of advanced ovarian cancer[5]. It is the most effective antitumor agent developed in the past three decades. It has been used for effective treatment of a variety of cancers including refractory ovarian cancer, breast cancer, non-small cell lung cancer, AIDS related Kaposi's sarcoma, head and neck carcinoma and other cancers[6-12]. Paclitaxel is a member of the taxane family of diterpenes, all having unique tri- or tetracyclic 20 carbon skeletons[13].

Paclitaxel has the empirical formula $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9. It consists of a taxane nucleus to which an uncommon four-membered oxetane ring is linked to C_4 and C_5 and an ester is attached at C_{13}.



Taxoid ring system

Mode of Action

Paclitaxel exhibit a unique mechanism of action, it binds to microtubule and causes kinetic suppression (stabilization) of microtubule dynamics. Microtubules are actually cylindrical structures made up of proteins (mainly tubulin) that are involved in various cellular functions such as movement, ingestion of food, controlling the shape of cells, sensory transduction and spindle formation during cell division[14]. In normal case, the tubulin polymerizes to microtubule and again microtubulin converts into tubulin. This whole routine process exists in equilibrium state. But Paclitaxel mainly binds to microtubules rather than to tubulin dimers[15]. The binding site for paclitaxel is the N-terminal 31 amino acids of the β-subunit of tubulin in the microtubule[16], unlike the binding sites of colchicine, vinblastine and podophyllotoxin for GTP. The microtubules formed due to paclitaxel action are not only very stable but are also dysfunctional. The cancerous cells lack a checkpoint to detect the absence of spindle and attempt to continue the cell cycle leading to cell death[17].

Physical Characteristics and Pharmacokinetic Parameters

Paclitaxel is white to off-white crystalline powder. It is highly lipophilic, insoluble in water and melts at around 216-217°C. The generally accepted dose is 200-250 mg m^{-2} and is given as 3 and 24 h infusion. Pharmacokinetics of paclitaxel shows wide variability. Terminal half-life was found to be in the range of 1.3-8.6 h (mean 5 h)[18] and the steady-state volume of distribution was found to be ~87.1 m^{-2}. The drug undergoes an extensive P-450 mediated hepatic metabolism and less than 10% drug in the unchanged form is excreted in the urine[19]. Most of the drug is eliminated in feces. More than 90% of the drug binds rapidly and extensively to plasma proteins[20]. The highest concentration of the paclitaxel following a 6 h infusion in rats was found to be in lung, liver, kidney and spleen and was essentially excluded from brain and testes[21].

Dosage Regimen and Limitations of the Formulations

Paclitaxel has a low therapeutic index and the therapeutic response is always associated with toxic

side-effects[22-23]. It should be only used when the potential benefits of paclitaxel therapy outweigh the possible risks. In the early development of paclitaxel, a high incidence of acute hypersensitivity reaction characterized by respiratory distress, hypotension, angioedema, generalized urticaria and rash were observed. It is generally felt that the vehicle Cremophore EL (Polyoxyethylated castor oil vehicle and dehydrated alcohol) contributes significantly to the hypersensitivity reactions, leading to peripheral neurotoxicity, neutropenia, etc. An additional problem linked to the CrEL solvent is the leaching of plasticizers from PVC bags and infusion sets used routinely in clinical practice. Consequently CrEL formulation need to be prepared and administered in either glass bottles or non-PVC infusion systems with inline filtration. This leads to the need of search of alternative formulations of paclitaxel. The maximum tolerated dose (MTD) of paclitaxel administered by a 3 h infusion to patients with solid tumors was found to be 225-240 mg m^{-2} without any hypersensitivity reactions but resulted in hypotension[24].

Analytical Approaches

Analysis of paclitaxel in biological fluids and in different dosage forms is often performed by reversed-phase high-performance liquid chromatography (RP-HPLC)[25-26] and often represents a challenge due to interferences from the complex matrix like cremophore[27] and tedious extraction procedure from biological fluids. There are several reversed phase HPLC assay methods for paclitaxel reported in the literature but most of them are for determination in plasma and body fluids, very few methods have been developed till now for the determination of paclitaxel in pharmaceutical dosage forms. The different methods utilize different chromatographic conditions to have better accuracy, resolution, efficacy and recovery with reduced run time. Apart from HPLC to get better results, reduced run time and cost effectiveness, UPLC method has also been developed.

Determination of Paclitaxel in Biological Fluids

Contributors	Determination in	Chromatographic conditions	Results	Remarks
Tracy A. Willey[28] *et al.*, 1993	Human Plasma	**Column:** C_8, 5 µm (250×4.6 mm i.d) **Buffer:** 1.0 M ammonium acetate, pH 5 with glacial acetic acid. **MP Composition:** Buffer: CH_3OH:ACN:: 100:20:80 **Flow rate:** 1.0 ml/min **Wavelength:** 227 nm **Run time:** 20 min **Detector:** UV	Recovery: 83% Correlation coefficient-0.007 Range: 10-1000 ng/ml RSD: 10% RT: 10 min	Method is accurate, reproducible and precise with isocratic flow for determination of paclitaxel in Plasma. Suitable for estimation of samples from clinical study of paclitaxel in cancer patients
Huizing, M.T.[29-30] *et al.*, 1995	Human Urine	**Column:** Apex C_8, 5 µm (250×4.6 mm i.d) **Buffer:** 0.02M ammonium acetate, pH 5 **MP Composition:** ACN: CH_3OH: Buffer :: 4:1:5 **Wavelength:** 227 nm **Run time:** 40 min **Detector:** UV	Recovery: 75-79% for SPE & LLE LOQ: 0.01 µg/ml RT: 10 min	Method is accurate, precise, and robust for the estimation of paclitaxel in human urine.

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Contributors	Determination in	Chromatographic conditions	Results	Remarks
Martin, N.[31-33] *et al.*, 1998	Human Plasma and Urine	**Column:** Nucleosil C_{18}, 5 µm (250×4.6 mm i.d), **Buffer:** 35 mM ammonium acetate, pH 5, **MP Composition:** ACN:Buffer:THF :: 45:50:5 **Wavelength:** 227 nm **Flow rate:** 1.8 ml/min **Run time:** 10 min **Internal standard:** Docetaxel **Detector:** UV	Recovery: Plasma-82% Urine -87% LOD: 10 ng/ml LOQ: 25 ng/ml RT: 7.7 min Range: 25-1000 ng/ml	Rapid simple add robust method (with internal standard). Lower run time than earlier developed methods. Suitable for pharmacokinetic studies of children involved in a phase-I clinical trial.
Basileo, G.[34-36] *et al.*, 2003	Human Plasma	**Column:** Zorbax SB C_{18}, 5 µm (150×4.6 mm i.d), **Buffer:** 2mM ammonium acetate, pH 5 **MP Composition:** ACN: Buffer :: 65:35 **Flow rate:** 1.0 ml/min **Run time:** 5 min **Temperature:** 45ºC **Detector:** Tandem mass	LOQ: 1 ng/ml Range: 1000 ng/ml RT: 2.9 Recovery: 70-86%	Simple rapid LC-MS method in plasma with much reduced run time. So proven to be time reductive method.
Sung Chul Kim[37-40] *et al.*, 2005	Biological samples including Plasma and Tissue	**Column:** C_{18}, 5 µm (250×4.6 mm i.d), **MP:** water and acetonitrile **Composition:** H_2O : ACN :: 66 : 34 **Wavelength:** 227 nm **Flow rate:** 1.0 ml/min **Run time:** 30 min **Temperature:** 25ºC **Detector:** UV	LOD: 5 ng/ml LOQ: 10 ng/ml Range: 0.1-20 µg/ml Recovery: 93.7% RT: 18.0 min.	Sensitive specific and reproducible method with very good recovery for estimation of paclitaxel in Biological samples.

Determination of Paclitaxel in Pharmaceutical Dosage Forms

Contributors	Determination in	Chromatographic conditions	Results	Remarks
Renuga T.S.[41-43] *et al.*, 2010	Cremophore and nanoparticle based formulations	**Column:** Phenomenex C_{18}, 5 µm (250×4.6 mm i.d), **Buffer:** 0.02M Potassium dihydrogen phosphate, pH 4.5 with Potassium hydroxide. **MP Composition:** Buffer:ACN:: 60:40 **Flow rate:** 2.0 ml/min **Wavelength:** 230 nm **Run time:** 8 min **Detector:** UV	LOD: 1.68 µg/ml LOQ: 5.09 µg/ml Range: 48-72 µg/ml RT: 4.9 min	Method is simple, linear, accurate and sensitive for estimation of polymeric nanoparticle paclitaxel and solvent based paclitaxel drug which is commercially available.

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Contributors	Determination in	Chromatographic conditions	Results	Remarks
Kumar, V.K[44-47] *et al.*, 1995	Parentrals	**Column:** Unison US C_{18}, 5 µm (250×4.6 mm i.d), **Buffer:** 0.02M Potassium dihydrogen phosphate, pH 2.5 with O-Phosphoric acid. **MP Composition:** CH_3OH: Buffer :: 80:20 **Flow Rate:** 1 ml/min **Wavelength:** 225 nm **Run time:** 12 min **Detector:** UV	Recovery: 101.25% LOD: 0.03 µg/ml LOQ: 0.09 µg/ml Range: 15-180 µg/ml RT: 4.97 min	Simple, accurate, sensitive and reproducible method for estimation of paclitaxel in parentrals. Absence of additional peaks indicates non interference of common excipients. Method shows very good recovery which shows high accuracy of the method.
Khisal, A.[48-52] *et al.*, 1998	Bulk Drugs	**Column:** BEH C_{18}, 1.7 µm (50×2.1 mm i.d), **MP A:** 0.05% formic acid **MP B:** Water:ACN:: 14:86 with 0.035% formic acid **Wavelength:** 190-400 nm **Flow rate:** 0.4 ml/min **Run time:** 5 min **Detector:** PDA	Recovery: 96-98% RT: 4 min Range: 2-405.4 mg/l	Rapid accurate, economical and precise UPLC method for estimation of paclitaxel in bulk drugs. Newer method with reduced flow rate and run time thus saves time and cost reductive.
Rajender, G.[53] *et al.*, 2010	Stents	**Column:** Phenomenax C_{18}, 5 µm (250×4.6 mm i.d), **MP:** ACN:Water:: 80:20+0.1% acetic acid **Flow rate:** 0.8 ml/min **Run time:** 6 min **Temperature:** 45ºC **Detector:** Tandem mass	LOQ: 10 ng/ml Range: 10-1000 ng/ml RT: 4.6 Recovery: 51%	Method is highly sensitive and selective for determination of concentration of paclitaxel coated drug eluting stents.

Determination of Free Concentration of Paclitaxel in Liposome Formulations

Despite the major benefits of these pharmaceutical products based on paclitaxel, patients receiving chemotherapeutic treatment can experience severe life-threatening side effects, primarily myelosuppression and neutropenia. On the other hand, under-dosage might result in suboptimal treatment of cancer[60]. In addition to its narrow therapeutic range, paclitaxel also displays highly variable pharmacokinetics and extremely poor water solubility. Intravenous administration of paclitaxel is associated with multiple side effects related to its pharmaceutical formulation. This problem is sought to be alleviated either by synthesizing more soluble derivatives or by administration of paclitaxel bound to formulation vehicles[61]. Reduced side effects are observed with recently developed micelles and liposome-based formulations[62].

Liposomes, small artificial vesicles formed from one or more layers of lipid are used medicinally to carry a drug, vaccine or enzyme to targeted cells in the body. An important step in the development of liposome-based formulations is estimating the free drug concentration in the aqueous solution surrounding liposomes.

Several methods have been developed to measure the free concentration of drugs and most involve the physical separation of free and bound fractions followed by conventional analysis[63]. Examples of separation techniques include equilibrium dialysis, ultrafiltration, ultracentrifugation and gel filtration. These techniques are usually time consuming, can suffer loss of analyte to membranes, can generate errors due to protein leakage or Donnan effects and can create a shift in concentrations and binding equilibrium during separation[64-65]. Furthermore, when dialysis is used, drug concentration and sample volume have to be determined on both sides of the separation membrane, increasing the complexity of an experiment.

Solid phase microextraction (SPME) has been applied for the determination of free concentrations but only in the case of an interaction between a drug and a specific protein[66-67]. Direct extraction of target analytes from complex matrices is usually hindered by various matrix effects such as fouling and disturbance of uptake kinetics. Interfering compounds or suspended particles can be adsorbed by the fiber coating during direct SPME. Consequently, they cause calibration problems and preclude fiber reusability. To overcome these problems, a hollow membrane is used to form a concentric sheath around a coated SPME fiber.

The resulting method is a simple and sensitive SPME approach for characterizing paclitaxel – liposome interaction, which provides the distribution constant and the free concentration of paclitaxel.

Membrane-protected SPME Protection of SPME fibers with porous membranes prevents the direct interaction between the extraction phase and liposomes, allowing accurate determination of free paclitaxel in liposome formulations[68]. The membranes used for this task had a molecular weight cut-off of 15 kD (for proteins) and 10 nm pore size; according to the manufacturer's instructions, they were soaked in water for 24 h prior to analysis.

Determination of Free Paclitaxel with Membrane-protected SPME

The SPME fiber was placed inside the flat membrane, both sides of the membrane touching the fiber (Fig. 2). Great care was taken to avoid including air in the space between the fiber and membrane (if some air becomes trapped in this space, it must be eliminated by application of slight pressure on the membrane or by introducing the device in a low vacuum vessel). Both ends of the dialysis membrane were kept out of the liquid sample or alternatively, one of the ends was sealed[69].

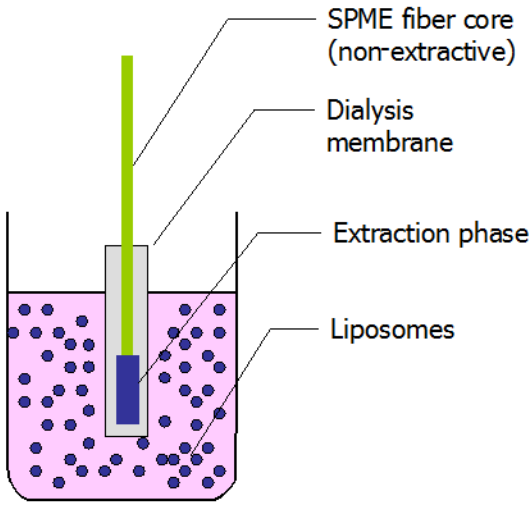

Fig. 2
Experimental setup for determination of free paclitaxel with membrane-protected SPME

Determination of Paclitaxel Distribution Constant

The distribution constant of paclitaxel between liposomes and water was determined as

$$K_D = \frac{\text{concentration of paclitaxel in lipids}}{\text{concentration of paclitaxel in water}}$$

The concentration of paclitaxel in water was considered to be the one determined with SPME in the previous step. The total concentration of paclitaxel in the reconstituted liposome solution was expected to be 10 nm, according to the manufacturer's instructions. This value was verified as follows: the liposomes were diluted 1:100 with methanol, sonicated for 10 minutes (to break the liposomes and release paclitaxel) and further diluted with water. The final solution was analyzed by HPLC and the total concentration of paclitaxel was found to be close to the one indicated by the manufacturer.

Gene Expression Profile Analysis of Paclitaxel-induced Changes in the Mda-MB231 Human Breast Cancer Xenograft Model

In this method, cDNA micro-array and antitumor activity analysis was undertaken to investigate molecular alterations and tumor growth inhibition induced by paclitaxel exposure in the MDA-MB231 mouse xenograft model. Tumor cells (2×10^7 cells/mouse) were subcutaneously implanted into nude mice. Mice were ranked according to tumor volume and randomized to receive either 15 mg/kg paclitaxel or vehicle from day 1 to 5 following randomization. Animals were sacrified and tumor and healthy mouse subcutaneous tissues harvested 6 hr following dosing on days 2 and 5 and immediately frozen in liquid nitrogen. MDA-MB231 cells exposed for 24 hr to 100 nm paclitaxel or vehicle *in-vitro* were also obtained in parallel experiments. Total RNAs were extracted and analyzed on cDNA microarrays containing ca. 9000 genes. Kinetic analysis of paclitaxel-treated tumor tissues revealed strong *in-vivo* antitumor activity (T/C = 6.5%) at relevant pharmacologic doses and transcriptional alterations involving various intracellular pathways or systems, including apoptosis, mitotic regulation, cell cycle control, microtubule regulation and cytoskeletal remodeling. Several differences were identified in paclitaxel-treated tumor tissues compared to drug-exposed cultured cells and may explain discordant phenotypes of response between *in-vitro* and *in-vivo* evaluation. Gene expression profiling of drug effect *in-vivo* may improve preclinical assessment of anti-cancer compounds. In addition, such an approach may identify potential surrogate markers of drug effects that can be monitored in the clinical setting to predict clinical activity[70-71].

Conclusion

Paclitaxel is one of the most important and broad spectrum anticancer drug approved by FDA for the treatment of cancer. This review provides a description of Paclitaxel, mechanism of action, doses with special emphasis on analytical approaches for estimation of paclitaxel in biological fluids and various pharmaceutical dosage forms.

Several publications have been reported for the estimation of paclitaxel in biological fluids as well as in various pharmaceutical dosage forms by HPLC, LC-MS-MS and UPLC. Efforts have been done to have a method which is more rigid, robust, having good recovery and shorter run time.

Estimation of paclitaxel in biological fluids are mainly suitable for Pharmacokinetic study in clinical trials. Baselio *et al.,* have developed a method for estimation of paclitaxel in human plasma with a reduced run time of 5 minutes with good recovery of 70-86%. While Sung Chul Kim *et al.,* developed a method in biological fluids with a recovery of 93.7%.

In Pharmaceutical dosage forms Khisal *et al.,* developed a very good method by UPLC, with reduced flow rate and run time thus proven to be very cost effective method with a very good recovery of 96-98%. While V.K. Kumar developed a simple, precise method for parentrals, with a maximum recovery of 101.25%.

REFERENCES

1. Chunekar, K. and Pandey, G.S., (1990). *Bhavaprakasa Nighantu of Sri Bhavamisra* (Indian Materia Medica), Chaukhambha Bharati Academy, Varanasi, India.
2. Hartwell, J. *et al*., (1951). *J. Am. Chem.,* 73, pp. 2909-2916.
3. Rowinsky, E.K. *et al*., (1992). *Sem. Onc.,* 19, pp. 646-662.
4. Rowinsky, E.K. *et al*., (1997). *Ann. Rev Med.,* 48, pp. 353-374.
5. Kingston, D.G.I., (2001). *Chem. Commun.,* 10, pp. 867-880.
6. McGuire, W.P. *et al*., (1989). *Ann. Int. Med.,* 111, pp. 273-279.
7. Holmes, F.A. *et al*., (1991). *J. Nat. Cancer Inst.,* 83, pp. 1797-1805.
8. Rowinski, E.K. *et al.,* (1991). *Pharmacol. Ther.,* 52, pp. 35-84.
9. Thigpen, T. *et al*., (1990). *Proc Am Soc Clin Oncol.,* 9, p. 156.
10. Einzig, A.I. *et al*., (1992). *J. Clin. Oncol.,* 10, pp. 1748-1753.
11. Seidman, A. *et al.,* (1992). *Proc Am. Soc. Clin. Oncol.,* 11, p. 59.
12. Murphy, W.K.J. *et al*., (1993). *Natl. Cancer Inst.,* 85, pp. 384-388.
13. Srivastava, V. *et al.,* (2005). *Bioorganic and Medicinal Chem.,* 13, pp. 5892-5908.
14. Rowinsky, E. *et al.,* (1990). *J. Natl. Cancer Inst.,* 82, pp. 1247-1259.
15. Parness, J. *et al.,* (1981). *J. Cell. Biol.,* 91, pp. 479-487.
16. Rao, S. *et al.,* (1994). *J. Biol. Chem.,* 269, pp. 3132-3134.
17. Rowinsky, E. *et al.,* (1995). *New Engl. J. Med.,* 332, pp. 1004-1014.
18. Rowinsky, E.K. *et al*., (1993). *Semin Oncol.*, 20, p. 16.
19. Rizzo, R. *et al., J. Pharm. Biomed. Anal.,* 8, p. 159.
20. Wiernik, P.H. *et al.,* (1987). *J. Clin. Oncol.,* 5, p. 1232.
21. Rowinsky, E.K. *et al.,* (1993). *Semin Oncol.*, 20, p. 16.
22. Weiss, R. *et al.,* (1990). *J. Clin. Oncol.,* 8, pp. 1263-1268.
23. Nightingale, S., (1992). *J Am. Med. Assoc.,* 268, pp. 1390-1393.
24. Kramer, I. *et al.,* (1995). *Eur. Hosp. Pharm.,* 1, pp. 37-41.
25. Witherup, K.M. *et al.,* (1989). *J. Liq. Chromatogr.,* 12, pp. 2117-2132.
26. Look, S.A. *et al.,* (1990). *J. Nat. Prod.,* 53, pp. 1249-1255.
27. Friedland, D. *et al*., (1993). *J. Natl. Cancer Inst.,* 85, p. 2036.
28. Tracy, A. Willey *et al.,* (1993). *J. Chrmatogr.,* 621, pp. 231-238.
29. Huizing, M.T. *et al.,* (1995). *J. Chromatogr. B.,* 664, pp. 373-382.
30. Ringel, I. *et al*., (1987). *J. Pharm. Exp. Ther.,* 242, p. 692.
31. Shah, V.P. *et al.,* (1992). *J. Pharm. Sci.,* 81, p. 309.
32. Song, D. *et al*., (1995). *J. Chrmatogr. B.,* 663, p. 337.
33. Martin, N. *et al*., (1998). *J. Chromatogr. B.,* 709, pp. 281-288.
34. Basileo, G. *et al*., (2003). *J. Pharm. Biomed. Anal.,* 32, pp. 591-600.
35. Jamis, C.A. *et al*., (1993). *J. Cancer Chemother. Pharmacol.,* 33, pp. 48-52.
36. Ohtsu, T. *et al*., (1995). *J. Clin. Cancer Res.,* 1, pp. 599-606.
37. Kim, S.C. *et al*., (2005). *J. Pharm. Biomed. Anal.,* 39, pp. 170-176.
38. Lee, S.H. *et al.,* (1999). *J. Chromatogr. B.,* 724, pp. 357-363.
39. Leu, J.G. *et al*., (1993). *Erlanger. J. Cancer Res.,* 53, pp. 1388-1391.
40. Grothaus, P.G. *et al*., (1993). *J. Immunol. Methods,* 158, pp. 5-15.
41. Renuga, T.S. *et al*., (2010). *Der Pharma Chemica.,* 2, pp. 109-115.
42. Gupta, R.K. *et al*., (2009). *Der Pharma Lettre.,* 1, pp. 162-168.
43. Anupama, M. *et al*., (2007). *J. Chromatogr. B.,* 855, pp. 211-219.
44. Kiran, K. *et al.,* (2009). *Asian J. Research Chem.,* 2, p. 1.
45. Marcel, F. *et al*., (2006). *J. Pharm. Pharmaceut. Sci.,* 9, pp. 231-237.
46. Haruo, Y. *et al.,* (2005). *J. Chromatogr.,* 26, pp. 49-50.
47. Panchagnula, R. *et al*., (1999). *Pharmacy and Pharmacol. Commun.,* 5, pp. 587-589.
48. Khisal, A. *et al.,* (2008). *J. Liq. Chromatography and Related Tech.,* 31, pp. 941-949.
49. Wang, L.Z. *et al*., (2003). *J. Pharm. Biomed. Anal.,* 31, pp. 283-289.
50. Theodoridis, G. *et al*., (1998). *J. Chromatogr.,* 802, pp. 297-305.
51. Hoke, S.H. *et al*., (1994). *J. Anal. Chem.,* 57, pp. 277-286.
52. Ricceimer, S.L. *et al.,* (1992). *J. Anal. Chem.,* 64, pp. 2323-2326.
53. Rajendra, G. *et al*., (2010). *Asian J. Exp. Biol. Sci.,* 1, pp. 243-249.
54. Parise, R.A. *et al.,* (2003). *J. Chromatogr.,* 783, pp. 231-236.

55. Wang, L.Z. *et al*., (2000). *Rapid Commun Mass Spectrum.,* 17, pp. 1548-1552.
56. Schellen, A. *et al*., (2000). *Rapid Commun Mass Spectrum.,* 14, pp. 230-233.
57. Alexander, M.S. *et al*., (2003). *J. Chromatogr.,* 785, pp. 253-261.
58. Sottani, C. *et al*., (1998). *Rapid Commun Mass Spectrum.,* 12, pp. 251-255.
59. Terwogt, J.M.M. *et al*., (2000). *J. Liq. Chromatogr. Relat. Technol.,* 23(8), pp. 1233-1251.
60. Wenk, M.R. *et al*., (1996). *J. Pharm. Sci.,* 85(2), pp. 228-231.
61. Marx, V. *et al*., (2005). *Chem. & Eng. News,* 83(30), pp. 25-36.
62. Klotz, I.M. *et al*., (1997). *Ligand-Receptor Energetics: A Guide for the Perplexed,* Ed.; Wiley, New York, p. 170.
63. Oravcova, J. *et al*., (1996). *J. Chrom. B.,* 677(1), pp. 1-28.
64. Cheng, Y. *et al*., (2004). *J. Chrom. B.* 809(1), pp. 67-73.
65. Ostergaard, J. *et al*., (2002). *Electrophoresis,* 23(17), pp. 2842-2853.
66. Wright, J.D. *et al*., (1996). *Clin. Pharmacokinet.,* 30(6), pp. 445-462.
67. Koster, E.H.M. *et al*., (2000). *J. Chrom. B.,* 739(1), pp. 175-182.
68. Krogh, M. *et al*., (1995). *J. Chrom. B.,* 673(2), pp. 299-305.
69. Vaes, W.H.J. *et al*., (1996). *Anal. Chem.,* 68(24), pp. 4463-4467.
70. Musteata, F.M, *et al*., (2005). *J. Proteome. Res.,* 4(3), pp. 789-800.
71. Gonçalves, A. *et al*., (2004). *Genne Journal of Clinical Oncology,* 2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition), Vol. 22, No. 14S (July 15 Supplement), p. 3196.