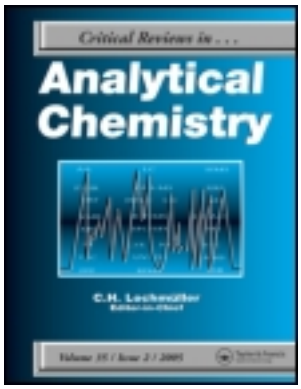


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# Analytical Methods to Determine Anti-Influenza Drugs

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# Analytical Methods to Determine Anti-Influenza Drugs

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**Analytical techniques employed in anti-influenza drugs analysis are summarized. The literature reviewed covers the papers of analytical interest that have appeared in approximately the last 10 years. Specific applications to Amantadine, Rimantadine, Oseltamivir, Zanamivir, and Peramivir are included. Possibilities and limitation of various analytical methods are evaluated.**

**Keywords** anti-influenza drugs analysis, Amantadine, Rimantadine, Oseltamivir, Zanamivir, peramivir



## INTRODUCTION

Influenza is a highly contagious viral infection. It is one of the most severe winter diseases, and it is responsible for epidemics and pandemics (Fedson, 2006). The virus infection causes an acute respiratory disease which results in significant morbidity and mortality throughout the world (Islam and von Ittzstein, 2008). Influenza viruses are divided into three genera, A, B, and C within the family Orthomyxoviridae, based on the antigenic properties of the viral nucleoprotein. Influenza B and C viruses principally infect humans, while the influenza A viruses are maintained in a vast natural reservoir in wild waterfowl and shorebirds, from which they emerge to cause disease in domestic poultry, horses, pigs, and humans. Their ability to infect the human respiratory tract and the periodic emergence of antigenically novel agents through genomic reassortment enable the influenza A viruses to cause worldwide epidemics with high morbidity and mortality (Beigel and Bray, 2008). Two main classes of drugs interfere with the influenza virus infection (Stiver, 2003): a) M2 ion channel inhibitors and b) Neuraminidase (NA) inhibitors. Adamantanes, including amantadine (1-adamantamine hydrochloride) and its analogue, rimantadine ($\alpha$-methyl-1-adamantanemethylamine), exert their antiviral activity by blocking the M2 protein ion channel activity of the type A influenza virus, raise the pH inside the endosome of infected cells to block the uncoating of the virion, and suppress the proliferation of the virus. This M2 protein occurs only in the type A virus, and these drugs are effective for this type A only (Kashiwagi, 2001). It represents the first class of anti-viral licensed for the influenza virus infection. NA inhibitors were introduced into clinical practice in 1999 and the properties of neuraminidase are common to both influenza A and B; its inhibitors are effective against both types. The NA of the influenza virus hydrolyzes the terminal sialic acids of sialoglycans, and it is generally accepted that NA promotes the release of the progeny virus from infected host cells by destroying receptors on the host cell and the virus itself, following the intracellular viral replication cycle. The NA inhibitors interfere with this process and inhibit the release of progeny influenza virus from infected host cells (Cinatl, Jr., 2007). Oseltamivir (Tamiflu®) and Zanamivir (Relenza®) represent two common clinically used NA inhibitors, and most recently, Peramivir has been authorized by the FDA for use in certain adult and pediatric patients with confirmed or suspected influenza infection who are admitted to a hospital (FDA, 2009). New anti-viral agents for influenza treatment have shown preclinical activity and are now being tested in human trials (Hayden, 2009). These drugs include Laninamivir (Kubo et al., 2010), a novel NA inhibitor presented as an alternative treatment against influenza viruses, including oseltamivir-resistant mutants, favipiravir (Kiso et al., 2010) or DAS181 (Triana-Baltzer et al., 2009). The analysis of anti-viral agents poses a significant challenge, and there is an urgent need to develop and validate assays that determine anti-influenza drugs. Therefore, simple, selective, sensitive, and precise methods are still needed for a quantitative determination in pharmaceutical and biological samples (Salem, 2005). In the present work we have reviewed the analytical methods which have appeared in the bibliography from 2000–2010, and are employed in the analysis and control of anti-influenza drugs in biological and pharmaceutical samples. The reviewed drugs include amantadine, rimantadine, oseltamivir, zanamivir and peramivir with chromatographics being the most extensively developed analytical methods. Other analytical techniques such as electrophoresis, optical methods, (spectrophotometry, spectrofluorimety and

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near-infrared spectroscopy) and some electrical methods like potentiometry are reviewed. The bibliography is also grouped by analytical technique. Tables 1–4 summarize the results found.

## ANALYTICAL METHODS WITH LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is the predominant analytical technique for pharmaceutical analysis. A fundamental weakness of LC has been its moderate separation efficiency (or resolution) and speed, limiting its use for very complex mixtures (Dong, 2007), however, it is the most widely used analytical technique in the determination of anti-influenza drugs. Various detection systems including mass spectrometry, UV, fluorescence, or chemiluminescence are employed in the determination of the studied drugs. We have subdivided the consulted bibliography into several groups according to the detections system employed.

### Liquid Chromatography with Mass Spectroscopy as Detection System

Liquid chromatography-mass spectrometry (LC-MS) combines the physical separation capabilities of LC with the mass analysis capabilities of mass spectrometry (MS). LC-MS has gained importance in the past decades for the chemical analysis of anti-influenza drugs since it can greatly improve analytical selectivity and sensitivity. Ro 64-0802 is a potent and highly selective inhibitor of neuraminidase from A and B strains of influenza virus and Oseltamivir phosphate (OP) (Ro 64-0796/002) is a pro-drug of the anti-influenza neuraminidase inhibitor, Ro 64-0802. Wiltshire et al. (2000) have developed a High-performance liquid chromatography-mass spectrometymass spectromety (HPLC-MS-MS) assay for the determination of both compounds in plasma and urine which fulfils all the criteria for a good analytical method. The procedure involves the initial extraction of Ro 64-0796 and Ro 64-0802, together with their respective tri-deuterated internal standards, from plasma or urine using mixed phase cationic extraction discs or catridges. The analytes are eluted from the cartridges, separated by reversed-phase HPLC (RP-HPLC) and detected mass spectrometrically after atmospheric pressure ionization. The analytical method is sensitive with limits of quantification of 1 and 10 ng/mL for the pro-drug and active neuraminidase inhibitor, respectively, in human plasma. It is both accurate and precise with typical coefficients of variation from some 5000 quality control samples of approximately ±3 and ±6%, respectively. In order to provide data that further support the treatment and prophylaxis dosing regimens for zanamivir, Peng et al. (2000) have directly measured zanamivir concentrations in induced sputum and nasal wash samples at 6, 12, and 24 hour following a single, inhaled 10-mg dose in male and female subjects. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) was used to detect total zanamivir levels in sputum and nasal samples. The analytical linear range was 0.5 to 1.0 ng/mL, with coefficients of variation of 3.8 to 11.7% over a linear range. The study demonstrates that zanamivir concentrations are significantly higher than the median viral neuraminidase $IC_{50}$ retained in the respiratory tract following a single 10-mg zanamivir dose. Oo et al. (2003) have studied the metabolic and excretory capacity of oseltamivir and its active carboxylate metabolite in young children (1–5 years old). Plasma and urine concentrations of oseltamivir and carboxylate were determined by means of HPLC-MS-MS using a method previously reported by Wiltshire et al. (2000). The results demonstrate that infants as young as 1 year old can metabolize and excrete oseltamivir efficiently. Arndt et al. (2005) have established a LC-MS/MS method for amantadine analysis in human serum without protein precipitation, centrifugation, extraction, and derivatization steps. The LC-MS/MS system consisted of a Surveyor MS pump and autosampler combined with a triple-quadrupole TSQ Quantum mass spectrometer. The mobile phase was a mixture of water and acetonitrile with formic acid. The mass spectrometer was operated in the electrospray atmospheric pressure ionization, positive ion with a selective reaction monitoring mode. The major ion transitions observed were m/z 152.0→135.1. The method showed a detection limit of 20 mg/L and a linearity of 20–5000 mg/L. The method proved to be specific, sensitive, reproducible, and accurate. Ping et al. (2007) have developed a sensitive liquid chromatography-electrospray ioniozation mass spectrometry (LC-ESI-MS) method for the determination of amantidine in human plasma. The method directly analyzed amantadine without derivatization, and the pretreatment was simple and fast. The compounds were separated by using a Thermo HypersilHyPURITYC18 analytical column. The mobile phase consisted of methanol-acetonitrile ammonium acet ate containing acetic acid with pH 4.0 and was isocratically eluted. Amantadine was measured by an electrospray ion source in the positive selective ion monitoring mode at m/z 152. The good linearity ranged from 3.9 to 1000 ng/mL, and the lowest limit of quantification for the determination of amantadine in human plasma was 3.9 ng/mL. The method was applied to determine the plasma concentration of amantadine after an oral administration (100 mg amantadine) to 20 healthy volunteers. A hydrophilic interaction chromatography (HILIC)/mass spectrometric assay was developed by Baughman et al. (2007) for the determination of zanamivir in rat and monkey plasma. An organic solvent with hydrophilic properties, methanol, was used to precipitate proteins in plasma to assure the hight polar zanamivir of staying in the solution. The method incorporates hydrophilic interaction chromatography silica, a stationary phase capable of retaining very polar compounds and improving sensitivity over reverse phase columns, and multiple reaction monitoring turboionspray positive ion detection. The multiple reaction monitoring (MRM) transitions for zanamivir (333→60) were chosen as these transitions provide the base ion peaks for the (MS-MS) experiments. The lower and upper limits of quantitation were 2 and 10000 ng/mL, respectively. The inter-day precision (relative standard deviation) and accuracy (relative error) in monkey plasma, derived from the analysis of validation samples at five concentrations, ranged from 2 to 8% and –2.3 to 2.1%, respectively. A bioanalytical method for the analysis of

TABLE 1
Chromatographic methods in anti-influenza drugs determination

| Detection system | Analyte | Sample | Minimum working level | Analytical linear range | Reference |
|---|---|---|---|---|---|
| | | LIQUID CHROMATOGRAPHY | | | |
| Mass spectroscopy | Oseltamivir phosphate (Ro 64-0796/002) | Human plasma | LLOQ 1 ng/mL | | (Wiltshire et al., 2000) |
| | | Urine | LLOQ 5 ng/mL | | |
| | Ro 64-0802 | Human plasma | LLOQ 10 ng/mL | | |
| | | Urine | LLOQ 30 ng/mL | | |
| | Zanamivir | Human sputum and nasal wash | | 0.5–1 ng/mL | (Peng et al., 2000) |
| | Oseltamivir | Human plasma and urine | | | (Oo et al., 2003) |
| | Oseltamivir carboxilate | | | | |
| | Amantadine | Human serum | LOD 20 mg/L | 20–5,000 mg/l | (Arndt et al., 2005) |
| | Amantadine | Human plasma | LLOQ 3.9 ng/mL | 3.9–1,000 ng/mL | (Ping et al., 2007) |
| | Zanamivir | Rat and monkey plasma | LLOQ 2 ng/mL | 2–10,000 ng/mL | (Baughman et al., 2007) |
| | Oseltamivir | Human plasma | LLOQ 1 ng/mL | ULOQ 600 ng/mL | (Lindegardh et al., 2007) |
| | | Human saliva | LLOQ1 ng/mL | ULOQ 300 ng/mL | |
| | | Human urine | LLOQ 5 ng/mL | ULOQ 1,500 ng/mL | |
| | Oseltamivir carboxilate | Human plasma | LLOQ 10 ng/mL | ULOQ 10,000 ng/mL | |
| | | Human saliva | LLOQ 10 ng/mL | ULOQ 10,000 ng/mL | |
| | | Human urine | LLOQ 30 ng/mL | ULOQ 30,000 ng/mL | |
| | Oseltamivir phosphate | Human plasma | LOD 0.1 ng/mL | 0.150–200 ng/mL | (Holodniy et al., 2008) |
| | Oseltamivir carboxilate | | LOD 1 ng/mL | 5.0–10,000 ng/mL | |
| | Rimantadine | Rat plasma | LLOQ 2 ng/mL | 2–750 ng/mL | (Xu et al., 2008) |
| | Oseltamivir and oseltamivir carboxilate | Rat plasma, cerebrospinal fluid | LLOQ 0.1 ng/mL | 0.1–500 ng/mL | (Heinig and Bucheli, 2008) |
| | | Rat brain | LLOQ 0.5 ng/g | | |
| | | Human plasma and urine | LLOQ 1 ng/mL | | |
| | Peramivir | Human plasma | LLOQ 1ng/mL | ULOQ 50,000 ng/mL | (Lindegardh et al., 2008) |
| | Oseltamivir and oseltamivir carboxilate | Human serum | | | (Yamazaki et al., 2008) |
| | Oseltamivir | Rat plasma | LOD 1 ng/mL | 2–1,000 ng/mL | (Chang et al., 2009) |
| | Oseltamivir carboxilate | | LOD 3 ng/mL | 20–10,000 ng/mL | |
| | Amantadine hydrochloride | Human plasma | LLOQ 20 ng/mL | 20–2,000 ng/mL | (Feng et al., 2009) |
| | Peramivir | Human plasma | LLOQ 10 ng/mL | 10–10,000 ng/mL | (Li et al., 2009) |
| | Oseltamivir | Sewage works and surface waters | | | (Straub, 2009) |
| | | LIQUID CHROMATOGRAPHY | | | |
| Spectrophotometry | Zanamivir | Spiked human plasma, pharmaceutical preparation | LOD 0.15 ng/mL | 8.0–7,500 ng/mL | (Erk, 2004) |
| | Oseltamivir | Pharmaceutical preparation | | 0.2–0.4 mg/mL | (Lindergärdh et al., 2006) |

*(Continued on next page)*

TABLE 1
Chromatographic methods in anti-influenza drugs determination *(Continued)*

| Detection system | Analyte | Sample | Minimum working level | Analytical linear range | Reference |
|---|---|---|---|---|---|
| | Oseltamivir phosphate | Pharmaceutical preparation | | | (Joseph-Charles et al., 2007) |
| | Amantadine rimantadine | Rat plasma | LOD 20 ng/mL | 0.05–5 $\mu$g/mL | (Shuangjin et al., 2007) |
| | Oseltamivir | Pharmaceutical preparation | | | (Winiarski et al., 2007) |
| | Oseltamivir carboxylic acid | Human serum | LLOD 5 ng/mL | 15–6400 ng/mL | (Bahrami et al., 2008) |
| | Oseltamivir | Pharmaceutical preparation | LOD 0.05 $\mu$g/mL | 70–130 $\mu$g/mL | (Narasimhan et al., 2008) |
| | Oseltamivir | Biological materials | LOD 0.04 $\mu$g/mL | 0.1–3.0 $\mu$g/mL | (Fuke et al., 2007) |
| | Oseltamivir phosphate | Pharmaceutical preparation | LOD 2.2 ng/mL | | (Green et al., 2008) |
| | Oseltamivir phosphate | Human placenta | Minimum sensitivities for detection 30 ng/mL | | (Worley et al., 2008) |
| | Oseltamivir carboxilate | | Minimum sensitivities for detection 2.4 ng/mL | | |
| | Oseltamivir phosphate | Bulk drug Pharmaceutical form | | | (Raghuram et al., 2008) |
| | Oseltamivir | Pharmaceutical preparation | LOD 5 ng/mL | 1.0–11.0 $\mu$g/mL | (Nagarajan and Muralidharan, 2009) |
| Fluorescence | 1-adamantine hydrochloride | Human plasma | LLOD 0.02 $\mu$g/mL | 0.025–2.5 $\mu$g/mL | (Higashi and Fujii, 2004) |
| | 2-adamantine hydrochloride | | LLOD 0.008 $\mu$g/mL | 0.01–2.5 $\mu$g/mL | |
| | Amantadine hydrochloride | Rat plasma | LLOD 0.025 $\mu$g/mL | 0.04–2 $\mu$g/mL | (Higashi et al., 2005) |
| | Rimantadine hydrochloride | | LLOD 0.016 $\mu$g/mL | 0.02–2 $\mu$g/mL | |
| | 1-Amantadine | Biological samples | LOD 0.014 $\mu$M | | (Higashi and Fujii, 2005) |
| | 2-Adamantanamine | | LOD 0.007 $\mu$M | | |
| | Rimantadine | | LOD 0.020 $\mu$M | | |
| | Amantadine | Pharmaceutical preparations | | 2.0–50.0 $\mu$M | (Duh et al., 2005) |
| | | Spiked urine | LOD 0.2 nmol | 1–10 nmol | |
| | Amantadine | Biological samples | LLOD 0.008 $\mu$g/mL | 0.025–2.5 $\mu$g/mL | (Higashi et al., 2006) |
| | Rimantadine | | LLOD 0.0015 $\mu$g/mL | | |
| Chemiluminescence | Amantadine | Human plasma | | 0.1–10,00 pmol/mL | (Yoshida et al., 2001) |
| | | OTHER CHROMATOGRAPHIC METHODS | | | |
| Capillary gas chromatography/mass spectrometry | Amantadine | Sodium chloride injection | LOD 0.2 $\mu$g/mL | 0.1–2.0 mg/mL | (Yao et al., 2006) |
| TLC/Dragendorff's reagent | Amantadine | Bulk and pharmaceutical preparations | LOD 0.72 $\mu$g/spot | 5–40 $\mu$g/spot | (Askal et al., 2008) |

TABLE 2
Electrophoretic methods in anti-influenza drugs analysis

| Procedure | Analyte | Sample | Interest data | Reference |
|---|---|---|---|---|
| Capillary electrophoresis with spectrophotometric detection | Rimantadine | Pharmaceutical preparations | Artificial neural networks is used | (Poláskova et al., 2002) |
| Capillary zone electrophoresis with spectrophotometric detection | Amantadine and rimantadine | Mixture of adamantane aminoderivatives | Cyclodextrins are used | (Reichova et al., 2002) |
| Capillary electrophoresis with fluorimetric detection | Amantadine | Synthetic | Determination of stability constant complex of a cyclodextrin and analyte | (Wang et al., 2003) |
| Capillary zone electrophoresis with spectrophotometric detection | Rimantadine | Pharmaceutical preparations | Procedure validated | (Pazourek et al., 2004) |
| Capillary zone electrophoresis with spectrophotometric detection | Amantadine | Synthetic | Detection limit $<2.5\ \mu$M | (Lin et al., 2008) |
| MEKC with spectrophotometric detection | Oseltamivir | Pharmaceutical preparations | Detection limit 1.7 $\mu$g/mL | (Jabbaribar et al., 2008) |
| MEKC sweeping with spectrophotometric detection | | | Detection limit 0.1 $\mu$g/mL | |
| Capillary zone electrophoresis with spectrophotometric detection | Oseltamivir | Pharmaceutical preparations | Detection limit 0.97 $\mu$g/mL | (Laborde-Kummer et al., 2009) |



oseltamivir and its metabolites oseltamivir carboxilate (OC) in human plasma, saliva, and urine has been developed by Lindegardh et al. (2007) using off-line solid phase extraction and LC coupled to positive MS/MS. Analytes were analyzed on a ZIC-HILIC column (a sulfobetaine type zwitterionic stationary phase covalently attached to silica and it is especially suitable for polar and hydrophilic analytes) using a mobile phase containing an acetonitrile-ammonium acetate buffer (pH 3.5). Neutral and lipophilic compounds in general show poor retention. A triple quadrupole mass spectrometer with an ionization source interface operating in the positive ion mode was used for the multiple reaction monitoring LC-MS/MS analysis. The lower limit of quantification (LLOQ) for oseltamivir was determined to be 1, 1, and 5 ng/mL for plasma, saliva, and urine, respectively, and for oseltamivir carboxilate it was 10, 10, and 30 ng/mL for plasma, saliva, and urine, respectively. The upper limit of quantification for oseltamivir was determined to be 600, 300, and 1500 ng/mL for plasma, saliva, and urine, respectively, and for oseltamivir carboxilate it was 10,000, 10,000, and 30,000 ng/mL for plasma, saliva, and urine, respectively. Alternative dosing strategies for oseltamivir prophylaxis have been explored by Holodniy et al. (2008). Healthy volunteers were randomized to a three-arm, open-label study and given 75 mg oral oseltamivir every 24 hours, 75 mg oseltamivir every 48 hours. combined with 500 mg probenecid four times a day, or 75 mg oseltamivir every 48 hours have combined with 500 mg probenecid twice a day for 15 days. Oseltamivir phosphate and oseltamivir carboxylate were isolated from human plasma by a solid phase extraction method and they were separated using a newly developed HPLC method and were detected by MS-MS with MRM. The separation was performed on a C18 analytical column by using a mobile phase consisting of a mixture of ammonium formate adjusted to pH 3.5 with formic acid and methanol. The detection was carried out by MS with the sample inter-heated with the nebulizer, positive ionization by atmospheric pressure chemical ionization, and mass scanning by MRM analysis. The limits of quantitation (LOQ) were 0.150 and 5.0 ng/mL for oseltamivir phosphate and oseltamivir carboxylate, respectively. The limits of detection (LOD) were 0.10 ng/mL and 1.0 ng/mL, respectively. The study concluded that alternative-day dosing of oseltamivir plus dosing with probenecid four times daily achieved a concentration adequate for neuraminidase inhibition in vitro through oseltamivir carboxylate. Xu et al. (2008) have developed a simple and highly sensitive LC/MS method for the determination of rimantadine in rat plasma, utilizing a single, protein precipitation for treatment, and isocratic elution monitored in the selected ion monitoring mode. Rimantadine was extracted by protein precipitation with methanol, and chromatographic separation was performed on a C18 column with a mobile phase consisting of acetonitrile and deionized water containing formic acid. Rimantadine was monitored in the selected ion monitoring mode at m/z 180.2. The limit of assay quantification was 2 ng/mL and the assay showed a linear dynamic range of 2–750 ng/mL. The developed assay method was applied to a pharmacokinetic study after the intragastric administration of 20 mg/kg of rimantadine hydrochloride to rats. A paper by Heinig and Bucheli (2008) describes the determination of oseltamivir and oseltamivir carboxilate in rat plasma, cerebrospinal fluid, and rat brain and in human plasma and urine using LC coupled to MS-MS. Protein precipitation with perchloric acid

TABLE 3
Optical spectroscopy methods in anti-influenza drugs analysis

| Method | Analyte | Sample | Interest data | | Reference |
|---|---|---|---|---|---|
| Spectrophotometric determination | Amantadine hydrochloride | Pharmaceutical preparations | Tetracyanoethylene as ligand | Beer's law 25–75 $\mu$g/mL | (Rizk et al., 2003) |
| | Amantadine hydrochloride | Pure and pharmaceutical preparations | Reaction with Fe(III) | Determined from 94–949 $\mu$g/mL | (Mustafa et al., 2004) |
| | Amantadine hydrochloride | Pharmaceutical preparations | Oxidation with different inorganic oxidant | Detection limit 0.83–73 $\mu$g/mL | (Darwish et al., 2005) |
| | Amantadine hydrochloride | Pharmaceutical preparations | Oxidation with ammonium molybdate | LOD 1.91 $\mu$g/mL | (Darwish et al., 2006) |
| | | | Iodine complex | LOD 0.17 $\mu$g/mL | |
| | | | Formation of vinylamino-substituted benziquinine | LOD 0.16 $\mu$g/mL | |
| | Oseltamivir | Pharmaceutical preparations | Complex with Congo red or bromochlorophenol blue | | (Green et al., 2008) |
| | Amantadine hydrochloride | Pharmaceutical preparations and plasma | Reaction with 1,2-naphthoquinone-4-sulphonate | LOD 1.39 $\mu$g/mL | (Mahmoud et al., 2009) |
| | Oseltamivir | Pharmaceutical preparations | Detection at 208.5 nm | LOD 0.342 $\mu$g/mL | (Raut et al., 2010) |
| Fluorimetric determination | Zanamivir | Synthetic | Flow-injection analysis method | | (Vannecke et al., 2001; Vannecke et al., 2002) |
| | Amantadine hydrochloride | Pure and pharmaceutical preparations | Oxidation with Ce(IV) | LOD 21 ng/mL | (Darwish et al., 2005) |
| | Oseltamivir phosphate | Pharmaceutical preparations | Reaction with fluorescamine | LOD 1.219 ng/mL | (Aydoğmuş, 2009) |
| Near-infrared spectroscopic determination | Amantadine hydrochloride | Pharmaceutical preparations | Artificial neural networks is used | | (Dou et al., 2005) |
| | Rimantadine hydrochloride | Pharmaceutical preparations | Discriminant analysis was used | | (Titova et al., 2009) |

TABLE 4
Potentiometric methods in anti-influenza drugs analysis

| Electrode | Analyte | Sample | Interest data | Reference |
|---|---|---|---|---|
| Plastic membrane selective | Amantadine | Pure and pharmaceutical preparation | Incorporation of amantadinium tetraphenylborate ion exchanger in membrane | (Abdel-Ghani et al., 2002) |
| Modified carbon paste ion selective | Amantadine hydrochloride | Pharmaceutical and urine | Detection limit 0.19 $\mu$g/mL | (Salem, 2005) |
| Graphite coated ion selective PVC membranes with dibutyl phathalate as plastizer | Amantadine | Pharmaceutical preparation and urine | Practical detection limit $2.5 \times 10^{-5}$ M | (Amorim et al., 2007) |
| PVC membranes with 2-fluoropheyl-2-nitrophenyl ether as plastizer | | | Practical detection limit $2.6 \times 10^{-6}$ M | |
| Chemical modified carbon-paste | Amantadine | Pharmaceutical preparation and blood-serum | Detection limit $6.3 \times 10^{-10}$ M | (Jalali et al., 2009) |



was followed by on-line solid-phase extraction using an in-house assembled column-switching system which provided good selectivity and robustness, removing the need for elaborate off-line sample preparation. After electrospray ionization, the compounds were detected in positive ion selected reaction monitoring mode performed at the transitions m/z 313.1→166.0 for oseltamivir and 285.1→138.0 for oseltamivir carboxilate. The LLOQ were 0.1 ng/mL in rat plasma and cerebrospinal fluid, 0.5 ng/g in rat brain, and 1 ng/mL in human plasma and urine. The recoveries were close to 100% and no significant matrix effect was observed. An assay of the analysis for the quantification of the anti-influenza drug peramivir in human plasma using high-throughput zwitterionic hydrophilic interaction liquid chromatography (HILIC) solid-phase extraction in a 96-wellplate format and LC coupled to positive MS-MS has been developed and validated by Lindegardh et al. (2008). The ZIC-HILIC SPE, together with the LC step, can efficiently remove interferences present using only protein precipitation. A quadrupole mass spectrometer with a turboV ionization source interface operated in the positive ion mode was used for the multiple reactions monitoring (selected for the transitions m/z 329→270 for peramivir) the LC-MS/MS analysis. The LLOQ for peramivir in plasma was 1 ng/mL and the upper LOQ was 50,000 ng/mL. The main benefit of this newly developed high–throughput zwitterionic hydrophilic interaction liquid chromatography solidphase (ZIC-HILIC SPE) method is that it was possible to selectively minimize the co-extraction of lipophilic phospholipids, which reduced total run time and prolonged the life of LC columns. The administration of oseltamivir in humans is suggested to affect the results of the hemagglutinin-inhibition (HI) test. Yamazaki et al. (2008) have investigated this phenomenon; the concentration of oseltamivir and oseltamivir carboxilate in serum obtained from oseltamivir administered to individuals were quantified by the LC-MS-MS system according to a method described previously (Wiltshire et al., 2000). The LC-MS/MS system consisted of a quadrupole MS coupled to a HPLC system with a mobile phase containing methanol-aqueous formic acid (pH 3). After chromatographic separation, the analytes were introduced into the mass spectrometer, and the quantification of oseltamivir carboxilate and phosphate was performed with selected reaction monitoring for the transition of m/z 285.3→179.8 for oseltamivir carboxilate and 313.1→208.0 for oseltamivir phosphate. The study shows that oseltamivir administration increases titers in the HI test. The causative agent of this increase is oseltamivir carboxilate. Chang et al. (2009) have studied the impact of dichlorvos on the rat plasma concentration of oseltamivir and oseltamivir carboxilate and its effect on the pharmacokinetic profile of oseltamivir and oseltamivir carboxilate using an improved LC/MS/MS method. A LC system connected to a mass spectrometer equipped with an electrospray ionization interface was used. The LC procedure uses a mobile phase of methanol containing formic acid. The electrospray ionization in positive mode was performed for the quantification, using the MRM of the protonated molecular ion to predominant product ion pairs at m/z 313→166 for oseltamivir and 285→138 for oseltamivir carboxilate. The results showed that dichlorvos significantly inhibited further hydrolysis of oseltamivir to oseltamivir carboxilate during the period of rat plasma sample treatment. A significant difference in the pharmacokinetic parameters of oseltamivir was found when the plasma samples were treated with dichlorvos. A rapid, simple, and sensitive HPLC with ion electrospray ionization MS-MS has been developed by Feng et al. (2009) for the simultaneous determinations of paracetamol, amantadine, caffeine, and

chlorpheniramine maleate in human plasma using tramadol hydrochloride as an internal standard. The analytes were separed using a mobile phase comprised of methanol-water-0.5% formic acid and analyzed by electrospray ionization MS-MS in the selected reaction monitoring mode with the precursor to product ion transitions 152.3→135.3 for amantadine. The LLOQ was 20 ng/mL for amantadine. The method has been successfully applied to study the pharmacokinetics of paracetamol-amantadine hydrochloride tablets in healthy male volunteers with great precision and selectivity. Li et al. (2009) have developed a hydrophilic interaction chromatography coupled with MS-MS to determine peramivir in human plasma. A HILIC column was used for the LC-MS-MS analysis, under isocratic conditions, with a mobile phase composed of acetonitrile-water containing formic acid. The mass spectrometer was operated using electrospray ionization with an ionspray voltage of +4800v. The positive ion MRM mode analysis was performed using nitrogen as the collision gas. The method was linear over concentration range of 10–10,000 ng/mL. The LLOQ was set at 10 ng/mL for peramivir and it showed enough sensitivity for determining peramivir in human plasma after an intravenous drip of 100-mg dose. In view of a potential influenza pandemic, the environmental safety of the anti-viral oseltamivir that are expected to be used in high amounts over several weeks has been questioned. Oseltamivir has been predicted to reach elevated concentrations in sewage works and surface waters. J.O. Straub (2009) has made an environmental study of oseltamivir for sewage works and surface waters. A LC followed by MS analytical method to detect oseltamivir ethylesther and oseltamivir acid has been employed. A C18 column was used with a mobile phase containing formic acid or acetonitrile. MS was performed on a mass spectrometer with and Turbo IonSpray run in positive ionization mode. Q1→Q3 masses were 285.2→138.1 for oseltamivir acid and 313→166.1 for oseltamivir ethylesther. The author concluded that for both seasonal and worst-case pandemic scenarios, no significant risk to the environment is predicted from the therapeutic and prophylactic use of Tamiflu.

## Liquid Chromatography with UV-Absorption Detection

UV absorption detectors respond to those substances that absorb light in the visible range and they are widely used in drug analyses. A simple, precise, sensitive RP-HPLC technique has been developed for the determination of zanamivir in spiked human plasma and its pharmaceutical formulations by Erk (2004). The procedure uses a simple mobile phase (acetonitrile and water 50:50 v/v) without the need for a buffer and it does not involve a complex procedure to prepare sample solutions. The detection has been realized with a UV detector at 230 nm. The validation of this techniques showed that it is linear in a range of 8–7500 ng/mL (r: 0.9987). The LOD and LOQ is 0.15 (0.5%) and 0.90 ng/mL (1.8%), respectively. Each analysis required no longer than 5 minutes; hence, the method can be used for routine analyses in quality control and development laboratories. A rapid LC assay for the evaluation of potentially counterfeit oseltamivir (Tamiflu capsules) has been developed by Lindergardh et al. (2006). The assay was validated and applied to test Tamiflu purchased over the Internet and in local pharmacies in Thailand and Vietnam. Oseltamivir was analyzed by LC with UV detection (at 220 nm) on a Hypersil Gold column using a mobile phase containing a methanol-phosphate buffer (pH 2.5). The assay was linear in the tested calibration range (0.2–0.4 mg/mL) and the accuracy and precision for the back-calculated calibration standards were less than 0.3% for all series. A simple, fast, and reliable isocratic RP-HPLC method with UV detection ($\lambda_{anal}$ 226 nm) was developed and validated by Joseph-Charles et al. (2007) for the rapid assay of oseltamivir phosphate aimed at the quality control of Tamiflu capsules and a generic version. Isocratic elution at a flow rate of 1.2 ml/min was employed on a Zorbax CN column at ambient temperature. The mobile phase consisted of methanol and 0.04 M formic acid pH 3. The proposed method included a simple sample treatment with the sonication of a small amount of powder sample at ambient temperature, centrifugation, dilution; short analysis time (less than 30 minutes); good precision (R.S.D. less than 5%); and high recovery (greater than 95%). A pre-column derivatization method is described by Shuangjin et al. (2007) for the quantitative determination of amantadine and rimantadine in biological samples by HPLC with UV detection. The derivatization was performed at room temperature using anthraquinone-2-sulfonyl chloride as a reagent for only 10 minutes and without post-derivatization treatment to inactivate excess reagent. The derivatives were analyzed by isocratic HPLC with a UV detector at 256 nm on a Lichrosper C18 column. The linear range for the determination of the drugs spiked in plasma was 0.05–5 $\mu$g/mL for amantadine and rimantadine. The LOD and LOQ were 20 and 50 ng/mL, respectively, for both analytes. The application of the method to the analysis of amantadine and rimantadine in rat plasma and pharmacokinetic studies are demonstrated and proved feasible. From the results obtained, it is hopeful that the method can be applied to determine the analytes in other samples such as pharmaceutical formulations, urine or tissues. Winiarski et al. (2007) have developed a simple, standardized method for the extemporaneous compounding of an oral liquid form of oseltamivir from commercially available Tamiflu 75 mg capsules (Roche Pharmaceuticals) dispersed in either cherry syrup or Ora-Sweet sugar free, and to determine the stability of oseltamivir in these preparations. The chemical composition to indicate the stability of the extemporaneous preparations was determined by HPLC with a UV detector at a wavelength 215 nm. The procedure uses a mobile phase with a potassium phosphate monobasic solution and methanol. The precision of the method was demonstrated by an overall RSD $\leq$2.00% in 30 assay determinations. The study carried out by Bahrami et al. (2008) has developed a fast and sensitive method for the determination of oseltamivir carboxylic acid, the active moiety of anti-influenza agent oseltamivir phosphate, in human serum by a simple RP-HPLC and UV detection (at 215 nm). The analyte and an internal standard (vanillin) were extracted from human serum by a solid phase extraction procedure. Chromatographic separation was achieved using a reverse phase C18 column with a mobile phase

consisting of a phosphate buffer with triethylamine and acetonitrile. The calibration curve was linear over a concentration range of 15–64,00 ng/mL of oseltamivir carboxylic acid in human serum and the lower LOD and LOQ were 5 and 15 ng/mL, respectively. The validated method has been successfully used for the determination of serum concentrations of oseltamivir carboxylic acid in a randomized cross-over bioequivalence study following the single oral administration of two different oseltamivir phosphate preparations in 24 healthy volunteers. Narasimhan et al. (2008) have developed a simple, precise, and rapid analytical reverse HPLC procedure, which would serve as stability indicating an assay method for the analysis of oseltamivir active pharmaceutical ingredient (API). The method uses a Kromasil C18 column, gradient run (using acetonitrile and triethylamine as mobile phase), effluent flow rate (1.0 ml/min), and UV detection at 215 nm. The described method was linear over the range of 70–130 $\mu$g/mL. The precision, ruggedness, and robustness values were also within the prescribed limits (<1% for system precision and <2% for other parameters). The minimum concentration levels at which the analyte can be reliably detected (LOD) and quantified (LOQ) were found to be 0.05 and 0.3 $\mu$g/mL, respectively. Oseltamivir was exposed to acid, basic, oxidative, and thermal stress conditions, and the stressed samples were analyzed by the proposed method. The results demonstrated the specificity of the assay method for the estimation of oseltamivir in the presence of degradation products. An HPLC-UV ($\lambda_{anal} = 230$ nm) method was established by Fuke et al. (2008) for the determination of oseltamivir carboxylate, an active metabolite of oseltamivir, in biological materials. The procedure uses floropipamide as an internal standard, followed by a mixed mode cation exchange extraction. This method has been applied to a case of death from falling after the ingestion of a Tamiflu capsule containing 75 mg of oseltamivir. Chromatographic separation was performed using XTerra® MS C18 analytical column and the mobile phase consisted of an acetonitrile-potassium dihydrogen phosphate buffer. The recovery yields of oseltamivir carboxylate from spiked blood at concentrations 0.1 and 1.0 $\mu$g/mL were 103.8 $\pm$ 16.9% and 95.0 $\pm$ 4.3%, respectively. Linearity was obtained in the concentration from falling 0.1 to 3.0 $\mu$g/mL. The detection limit was 0.04 $\mu$g/mL (S/N = 3). The lower quantification limit of the method was 0.1 $\mu$g/mL in blood. Green et al. (2008) have developed a colorimetric and chromatographic assay for oseltamivir to assess the authenticity of Tamiflu because of a growing concern about counterfeit oseltamivir. The HPLC method described was used to validate the colorimetric test. The HPLC method used a C18 column and a mobile phase with acetonitrile and bicarbonate buffer (pH 10); oseltamivir was detected by UV absorbance at 254 and 220 nm. The LOD for oseltamivir phosphate at 220 and 254 nm detection wavelengths are 2.2 and 4.2 ng, respectively. Oseltamivir phosphate is extensively metabolized in the ex vivo human placenta model, and the transplacental passage of the metabolite oseltamivir carboxylate is incomplete. Worley et al. (2008) have evaluated the metabolism and transfer of oseltamivir in the ex vivo human placenta model using an HPLC method for assaying the prodrug oseltamivir phosphate and the metabolite oseltamivir carboxylate. The HPLC conditions consisted of a mobile phase of acetonitrile and ammonium acetate and UV detection at 230 nm wavelength for oseltamivir phosphate and 220 nm for oseltamivir carboxylate. The recovery of oseltamivir phosphate and carboxilate by HPLC was greater than 80%. The minimum sensitivities for detection were 30 and 2.4 ng/mL, respectively. The results suggest that oseltamivir phosphate is extensively metabolized to oseltamivir carboxilate in the ex vivo human placental model. An isocratic RP-LC assay method was developed by Raghuram et al. (2008) for the determination of oseltamivir phosphate in a bulk drug and in pharmaceutical dosage form. The LC method employs a mixture of phosphate buffer pH 3 metanol-acetonitrile and the spectrophotometric detection was realized at 207 nm. In the developed HPLC method the resolution between oseltamivie phosphate and its potential impurities was found to be greater than 2. The developed RP-LC method was validated with respect to linearity, accuracy, precision, and robustness. A simple, selective, rapid, precise, and economical RP-HPLC method has been developed by Nagarajan and Muralidharan (2009) for the estimation of oseltamivir from pharmaceutical formulations. Chromatographic separation was performed on a LC system equipped with a photo diode array detector. A C18 column was employed with a mobile phase consisting of acetonitrile-potassium dihydrogen ortho phosphate (pH 3.5). Detection was carried out at 254 nm. The LOD was found to be 5.0 ng/mL and the LOQ was 15 ng/mL for oseltamivir. The authors conclude that the RP-HPLC method is suitable for the quality control of the raw materials, formulations, and dissolution studies.

### Liquid Chromatography with Fluorimetric Detection

Higashi and Fujii (2004) have investigated the quantitative analysis of 1-adamantanamide hydrochloride and 2-adamantanamide hydrochloride in human plasma by HPLC after derivatization with o-phthalaldehyde and 1-tio-$\beta$-D-glucose. Extracted human plasma samples were mixed with o-phthalaldehyde and 1-tio-$\beta$-D-glucose and injected onto HPLC. The mobile phase was prepared by the addition of methanol and acetic acid in water at pH = 7 by NaOH. A detection system was employed with a fluorometer operating at an excitation wavelength of 342 nm and an emission wavelength of 410 nm. The LLOD of 1-adamantanamide and 2-adamantanamine were 0.02 and 0.008 $\mu$g/mL, and the LLOQ were 0.025 and 0.01 $\mu$g/mL, respectively. The results obtained indicate that the procedure is simple, rapid, sensitive, and reproducible for determining 1-adamantanamide and 2-adamantanamine in human plasma. Higashi et al. (2005) have investigated the simultaneous HPLC determination of amantadine hydrochloride (AMA) and rimantadine hydrochloride (RIM) levels in rat plasma after fluorescent derivatization with o-phthalaldehyde and 2-mercaptoethanol. The described method is simple, rapid, sensitive, and reproducible. The retention times of AMA and

RIM derivatives were 12.6 and 22.2 minutes and the detection system used was a fluorometer operating at an excitation wavelength of 342 nm and an emission wavelength of 410 nm. The linearity of the standard curves of AMA and RIM was displayed for AMA and RIM concentrations ranging from 0.04 to 2 and 0.02 to 2 $\mu$g/mL, respectively. The LLOD for AMA and RIM using this method were established at 0.025 and 0.016 $\mu$g/mL (signal-to-noise ratio of 3:1), respectively. For the determination of amantadine, 2-adamantanamine, and rimantadine in melanin binding studies, Higashi and Fuji (2005) have investigated a simultaneous determination of these compounds by HPLC assay with dansyl chloride as a fluorescent derivative reagent. The dansyl derivatives were detected with a fluorometer ($\lambda_{ex}$, 370 nm; $\lambda_{em}$, 506 nm). The LOD for 1-amantadine, 2-amantadine, and rimantadine were 0.014, 0.007, and 0,020 $\mu$M, respectively (signal-to-noise ratio of 3:1). The method is simple, sensitive, and reproducible and, also, it is useful to investigate the binding kinetics to melanin. A simple and sensitive LC method is described for the analysis of amantadine by Duh et al. (2005). The method is based on the derivatization of amantadine extracted from alkalified samples with (2-naphtoxy)acetyl chloride under mild conditions. Toluene was used as the solvent for extracting amantadine in samples and the resulting toluene extract was directly subjected to subsequent derivatization without solvent replacement. Resulting derivatives were analyzed by LC with a fluorimetric detector ($\lambda_{ex}$, 277 nm; $\lambda_{em}$, 348 nm). The linear range for the determination of amantadine spiked in urine was 1.0–10.0 nmol with a detection limit of about 0.2 nmol (S/N = 3; injected sample 20 $\mu$L). Quantification of amantadine in tablets or capsules is possible in the linear range of 2.0–50.0 $\mu$M. Higashi et al. (2006) have developed a simultaneous HPLC assay of 1-adamantanamine hydrochloride (amantadine) and four related compounds including 1-(1-adamantyl)ethylamine hydrochloride (rimantadine) in phosphate-buffered saline (pH = 7.4) after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Phosphate-buffered saline samples were mixed with borate buffer and NBD-F solution and injected into HPLC. The detection system involves a fluorometer operating at an excitation wavelength of 470 nm and a emission wavelength of 540 nm. The LLOD of amantadine and rimantadine were 0.008 and 0.0015 $\mu$g/mL, respectively. The results presented demonstrate that NBD-F is a good candidate as a fluorescent reagent for the simultaneous HPLC assay of amantadine and rimantadine after pre-column derivatization. The method was applied to a binding study of these compounds to human $\alpha_1$-acid glycoprotein.

### Liquid Chromatography with Chemiluminescent Detection

Yoshida et al. (2001) have reported a novel luminol-type chemiluminescence derivation reagent, 4-(6,7-Dihydro-5,8-dioxothiazolo [4,5-g]phthalazarin-2-yl)benzoic acid N-hydroxysuccinimide ester (TPB-Suc). TPB-Suc reacts with primary and secondary amines in the presence of triethylamine. The resulting TPB derivatives produce chemiluminescence by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in an alkaline medium. The authors have examined the optimum derivatization, LC separation, and chemiluminescent (CL) reaction conditions and they have developed a selective and sensitive method for the determination of amantadine in human plasma. The TPB derivatives of amines were separated on a RP column by isocratic elution with sodium phosphate buffer-acetonitrile as the mobile phase. The effluent from the LC column was first mixed with a hydrogen peroxide solution and then with a potassium hexacyanoferrate(III) solution in NaOH. The generated CL was monitored by a chemiluminescence detector.

## OTHER CHROMATOGRAPHIC METHODS

Yao et al. (2006) have described a method to determine amantadine sulfate in sodium chloride injection by a capillary GC-MS system. There was a linear relationship between the peak area ratios of the analyte to the internal standard (naphthalene) and the concentration of the analyte over the concentration range of 0.1–2.0 mg/mL. The LOD and LOQ were 0.2 $\mu$g/mL with a signal to noise ratio of 3 and 0.7 $\mu$g/mL with a signal to noise ratio of 10, respectively. A simple and accurate thin-layer chromatographic (TLC) method for the quantitative determination of amantadine hydrochloride in bulk and capsule forms was developed and validated by Askal et al. (2008). From an economic point of view, the method used the most simple and cost effective chromatographic technique. The method employed TLC aluminium plates pre-coated with silica gel as a stationary phase. The solvent system used for the development consisted of n-hexane-methanol-diethylamine. The separated spots were visualized as brown spots after spraying with modified Dragendorff's reagent solution. The optical densities of the separated spots were found to be linear with the amount of amantadine in the range of 5–40 $\mu$g/spot. The LOD and LOQ values were 0.72 and 2.38 $\mu$g/spot, respectively.

## ELECTROPHORETIC METHODS

The main advantages of capillary electrophoresis (CE) methods are speed, resolution, efficiency, analyte solubility and stability, minimal reagent and solvent consumption, compatibility with mass spectrometry, and the availability of several modes which have all made CE a popular analytical technique in the field of pharmaceutical and biomedical analyses (Jabbaribar et al., 2008). Although HPLC is the main analytical method recommended by different pharmacopeia, other techniques are valid. CE is used extensively for drug analysis as an alternative and complementary technique to HPLC with convenient shorter times of analysis. However, the precision of analysis is often insufficient and thus might present limitations for the use of CE. Poláskova et al. (2002) have applied artificial neural networks (ANNs) to the evaluation of calibration graphs and CE analysis is investigated with the aim of treating the random errors in both axes simultaneously to increase the precision in CE. A

comparison of the ANNs approach with ordinary least-squares and bivariate least-squares regression was made. The proposed method was applied to the quantitative analysis of a commercial oral dosage form containing rimantadine. The separation and determination of adamantine derivatives with anti-viral activity, namely amantadine and rimantadine, were examined by capillary zone electrophoresis by Reichova et al. (2002). The authors have developed an electrophoretic system for the rapid separation and determination of adamantine derivatives that could be applied in the purity control of rimantadine after synthesis. An indirect detection method using 4-methylbenzylamine in ethanol/water solution as simultaneously absorbing and buffering background electrolyte with UV-visible detection at 210 nm was found suitable for the determination of the individual compounds. The addition of cyclodextrins allows for the separation of the adamantine amino derivatives of pharmaceutical interest within 6 minutes. The stability of an inclusion complex formed by the ligand of a cyclodextrin and a hydrophobic solute of suitable size is characterized by its stability constant. This constant is an important and widely usable thermodynamic parameter. An indirect CE method was developed by Wang et al. (2003) based on two competitive chemical equilibriums for determining the stability constant of an inclusion complex formed between a cyclodextrin and a solute. The principle of the method was explained using an example for determining the stability constant of an inclusion complex formed between a ligand of hydroxypropyl-$\beta$-cyclodextrin and a solute of amantadine. All CE experiments were carried out using a CE system with a spectrofluorometric detector. Detection was made at 365 nm for excitation and 495 nm for emission. At respective concentrations of $1.5 \times 10^{-3}$ M and $2.0 \times 10^{-3}$ M of amantadine, the apparent stability constants were measured to be $2.2 \times 10^{2}$ $M^{-1}$ and $2.9 \times 10^{2}$ $M^{-1}$ using the described procedure. The number of applications of CE in the field of pharmaceuticals is rapidly growing since CE shows excellent selectivity and efficiency. A capillary zone electrophoresis method with indirect UV-detection (210 nm) for the determination of rimantadine in tablets was validated by Pazourek et al. (2004). The authors have tested the following parameters of the determination methods: instrumental precision, method precision, method accuracy, linearity of calibration curve, selectivity, robustness, and the stability of standards and samples. The analysis is fast (less than 4 minutes) and can also be applied to a dissolution profile test of Rimantadine tablets or used to determine rimantadine in analogous pharmaceuticals. The use of tested internal standards (ammonium nitrite, tetraethyl ammonium iodide, tetrabutyl ammonium hydroxide) did not bring about any improvement in the determination precision. Lin et al. (2008) have found that ofloxacin acyl chloride is a potential chromophoric reagent for labeling amino analytes for CE. Ofloxacin acyl chloride has a tertiary amino function in its structures and the derivatives from ofloxacin acyl chloride reacting with amino analytes can be ionized by an acid treatment and analyzed by simple capillary zone electrophoresis. Ofloxacin acyl chloride was used to derivate amantadine and the resulting derivatives were analyzed by capillary zone electrophoresis with UV detection (300 nm). The results indicate good linearity and a detection limits $<2.5$ $\mu$M (S/N = 3, injection 3s). The precision (RSD) and accuracy relative error (RE) of the method were studied based on the peak-area ratios at three levels, 25, 50, and 125 $\mu$M. The RSD and RE for intra-and inter-day analyses are below 2.6% and 1.9%. A simple and rapid micellar electrokinetic chromatography method (MEKC) with UV/Vis detector (214 nm) was developed by Jabbaribar et al. (2008) for the analysis of oseltamivir and its hydrolyzed product (oseltamivir carboxylate) in Tamiflu capsules. The study has presented an MEKC and MEKC-sweeping method for the analysis of oseltamivir. Background electrolytes consisted of boric acid 10 mM, pH 10, and sodium dodecylsulphate 40 mM. The LOD and LOQ of oseltamivir were 1.7 and 8.0 $\mu$g/mL, respectively. MECK sweeping in a high electroosmotic flow environment for neutral analytes was also used to improve the sensitivity of the assay. In MEKC-sweeping mode, a buffer comprising of 30 mM boric acid, pH 10, and 50 mM sodium dodecylsulphate was used. Using the MEKC-sweeping mode, the LOD and LOQ of oseltamivir were 0.1 and 0.3 $\mu$g/mL, respectively. Both methods were successful in determining oseltamivir concentration in its capsule formulation; however, the hydrolyzed product of oseltamivir, oseltamivir carboxilate, was detectable only with the MEKC method. The capillary zone electrophoresis is now well established as an analytical technique for the assessment of small pharmaceutical molecules. A rapid and reliable capillary zone electrophoresis with the UV detection (226 nm) method was developed and validated for the assay of oseltamivir phosphate in capsules by Laborde-Kummer et al. (2009). A short electrophoretic analysis time (less than 1.5 minute) was obtained using the short end injection mode. The method was validated in terms of specificity, linearity, precision, and accuracy. LOD and LOQ were estimated at 0.97 and 3.24 $\mu$g/mL, respectively. The validated method was successfully applied to the determination of oseltamivir in three commercially available capsules (Tamiflu, Saiflu®, and Flufy®). The results were in good agreement with those obtained by the HPLC method previously developed by the same research group (Joseph-Charles et al., 2007). The method presents advantages including short run time, simple and rapid sample preparation, low cost, and no use of non-aqueous solvent throughout the analysis.

## OPTICAL SPECTROSCOPY METHODS

Spectroscopic methods are a good tool for the identification and quantification of drugs. Spectrophotometric and fluorimetric analysis are considered more convenient alternative techniques in pharmaceutical analysis because of their inherent simplicity, high sensitivity, and availability in most quality control and clinical laboratories. Furthermore, the near-infrared (NIR) spectroscopy is one of the most rapidly growing analytical techniques in pharmaceutical analyses because it offers rapid, non-destructive analyses of pharmaceutical dosage forms (Guo et al., 1999).

## Spectrophotometric Methods

A spectrophotometric method is described by Rizk et al. (2003) for the quantitative determination of some drugs containing a primary amino-group such amantadine hydrochloride. The procedure is based on the formation of a charge-transfer complex between tetracyanoethylene as $\pi$-aceptor and the studied drugs as n-donors in the presence of acetonitrile as solvent. The spectra of the complex with amantadine hydrochloride show a maximum at 330 nm with a molar absorptivity of $2.1 \times 10^3$ l $\times$ mol$^{-1}$ $\times$ cm$^{-1}$, and the absorbances of the formed complex conform with Beer's law in the concentration range of 25–75 $\mu$g/mL. The described method is simple and can be applied to determine amantadine hydrochloride in pharmaceutical dosage forms. A simple spectrophotometric method for the determination of some anti-viral drugs, such as amantadine hydrochloride in pure and dosage forms, has been developed by Mustafa et al. (2004). The method is based on the reaction of the drug with metals. Amantadine hydrochloride was allowed to react with iron (III) at pH 3 and the amantadine hydrochloride-iron (III) complex is formed with a ratio 1:1. The absorbance was measured at 295 nm against an appropriate blank in which the drug is omitted. This method determined from 94–940 $\mu$g/mL with a mean percentage of recovery of 99.49 $\pm$ 0.57. The proposed method can be used for the routine determination of amantadine hydrochloride in pure form and in pharmaceutical formulation. Five direct spectrophotometric methods for the determination of amantadine hydrochloride have been developed and validated by Darwish et al. (2005). These methods were based on the oxidation of the drug by different inorganic oxidants: ceric ammonium sulphate, potassium permanganate, ammonium metavanidate, chromium trioxide, and potassium dichromate. Amantadine hydrochloride was vulnerable to ammonium sulphate and potassium permanganate in acidic solution. This was evident from the decrease in the yellow color of ceric ammonium sulphate at 315 nm, and the violet color of potassium permanganate at 525 nm. Ammonium metavanidate is reduced by amantadine hydrochloride to $VO_3^{-4}$ of $\lambda_{max}$ at 780 nm, and amantadine HCl were also found to be oxidizable by both chromium trioxide and potassium dichromate yielding the green Cr(III) ion of $\lambda_{max}$ 595 nm. Under optimum conditions, linear relationships with good correlation coefficients were found between the reading and the corresponding concentration of the drug in the range of 1–11,00 $\mu$g/mL. The LOD ranged from 0.83–73 $\mu$g/mL for amantadine hydrochloride. The methods involving ceric ammonium sulphate and potassium permanganate gave more sensitive assays than other reagents. The described method gave satisfactory results with the drug in bulk and dosage forms. Three simple and sensitive spectrophotometric methods (A-C) for the determination of amantadine hydrochloride have been developed and validated by Darwish et al. (2006). The first method (A) is based on the oxidation of the drug by ammonium molybdate, which was consequently reduced to the corresponding Mo(V) ions that are blue-colored of $\lambda_{max}$ at 780 nm. The second method (B) was based on the charge-transfer complexation reaction between the amantadine base as an electron donor and iodine as a $\sigma$-acceptor. The different variables affecting the reaction were studied and optimized by measuring at 365 nm. The third method (C) is based on the formation of colored vinylamino-substituted benzoquinone derivative as a result of the condensation of amantadine with acethaldehyde-chloranil combination. The colored vinylamino-substituted benzoquinone exhibits two absorption maximums at 325 and 685 nm. Under optimum conditions, linear relationships with good correlation coefficients were found between the reading and the corresponding concentration of the drug in the range of 2–90 $\mu$g/mL. The LOD ranged from 0.16 to 1.91 $\mu$g/mL. The proposed methods were successfully applied to the analysis of amantadine HCl in its capsules with good accuracy and precision. Green et al. (2008) have developed a colorimetric and chromatographic assay for oseltamivir to assess the authenticity of Tamiflu because of a growing concern about counterfeit oseltamivir. The colorimetric assay is quantitative and relies on an extractable (in ethyl acetate) colored ion-pair complex of oseltamivir with Congo red or bromochlorophenol blue with maximum absorption at 507 and 589 nm, respectively. Greater linearity and lesser variability are observed from the Congo red assay. Colorimetric test are rapid and easy to perform. The reagents and equipment for colorimetric tests are inexpensive, relatively non-toxic, and are ideal for use in field situations. New selective and sensitive spectrophotometric and spectrofluorimetric methods have been developed and validated by Mahmoud et al. (2009) for the determination of amantadine hydrochloride in capsules and plasma. The methods were based on the condensation of amantadine hydrochloride with 1,2-naphthoquinone-4-sulphonate (NQS) in an alkaline medium to form an orange-colored product. The spectrophotometric method involved the measurement of the colored product at 460 nm. Under optimum conditions, linear relationships with good correlation coefficients (0.9972) and low LOD (1.39 $\mu$g/mL) were obtained in the ranges of 5–80 and $\mu$g/mL for the spectrophotometric method. The method was successfully applied to the determination of amantadine hydrochloride in capsules. A simple and rapid UV-spectrophotometer estimation method for the evaluation of Oseltamivir (Fluvir) has been developed and assessed by Raut et al. (2010). The proposed methods were successfully applied for the estimation of Oseltamivir in commercial pharmaceutical preparation with UV detection at 208.5 nm. Linearity for detector response was observed in the concentration range of 4–24 $\mu$g/mL. The LOD and LOQ was found to be 0.342 $\mu$g/mL and 1.0367 $\mu$g/mL, respectively. The developed method was found to be simple, sensitive, accurate, precise, reproducible, and can be used for routine quality control analysis of oseltamivir phosphate in bulk and pharmaceutical formulation.

## Spectrofluorimetric Methods

A generic flow injection analysis (FIA) method to determine compounds with a secondary amine or amide in their

structure was developed by Vannecke et al. (2001) and Vannecke et al. (2002). Twenty-eight pharmaceutical compounds were selected to study, including zamamivir. A chemical reaction with sodium hypochlorite (NaOCl) first converts the secondary amine or the amide to a primary amine. This is followed by a reaction of the primary amine with o-phthalaldehyde (OPA) and thiol (N-acetylcysteine, NAC), to form a derivative which can be measured fluorimetrically. The evaluation of FIA parameters is examined in the first article and the second describes the determination of optimal conditions for the chemical reaction parameters. A simple and sensitive fluorimetric method for determination of the anti-viral drugs, including amantadine hydrochloride, has been developed by Darwish et al. (2005). The method was based on the oxidation of these drugs by cerium(IV) in the presence of perchloric acid and subsequent monitoring of the fluorescence of the induced cerium(III) at $\lambda_{excitation}$ 255 and $\lambda_{emission}$ 355 nm. Under optimum conditions, linear relationships with good correlation coefficient were found between the relative fluorescence intensity and the concentration of the amantadine hydrochloride in the range of 50–12,00 ng/mL. The LOD and LOQ were determined; the LOD and LOQ values were 21 and 62 ng/mL, respectively. The proposed method was successfully applied to the analysis of the investigated drugs in pure and pharmaceutical dosage forms with good accuracy and precision. New selective and sensitive spectrophotometric and spectrofluorimetric methods have been developed and validated by Mahmoud et al. (2009) for the determination of amantadine hydrochloride in capsules and plasma. The spectrofluorometric method involves the reduction of the product with potassium borohydride, and the subsequent measurement of the formed fluorescent reduced AMD-NQS product at 382 nm after excitation at 293 nm. Under optimum conditions, linear relationships with good correlation coefficients (0.9974) and low LOD (0.013$\mu$g/mL) were obtained in the ranges of 0.05–10 $\mu$g/mL for the spectrofluorometric method. The proposed method was successfully applied to the determination of amantadine hydrochloride in capsules and plasma. The results obtained by the proposed methods were comparable with those obtained by the official method. Aydoğmuş (2009) has developed a simple and sensitive spectrofluorometric method for the determination of oseltamivir phosphate in capsules with satisfactory results. The method is based on the reaction between oseltamivir and fluorescamine in a borate buffer solution of pH 8.5 to give highly fluorescent derivatives that are measured at 483 nm using an excitation wavelength of 381 nm. The method was validated with respect to specificity, linearity, precision, and accuracy. The fluorescence intensity concentration plot is rectilinear over the range of 50–450 ng/mL and with a lower detection limit of 1.219 ng/mL and LOQ of 4.064 ng/mL. The developed method may not be applicable for the analysis of oseltamivir phosphate in biological fluids since the other chemicals with primary or secondary amines group in the maintained matrix will interfere with the assay.

### Near-Infrared Spectroscopy Methods

Near-infrared spectroscopy (NIR) y is a non-destructive quantitative analytical technique of samples with the advantages of rapid, simple operation and small samples, particularly the use of solid samples. NIR spectroscopy has proved to be a powerful analytical tool for analyzing a wide variety of samples used in the agricultural, nutritional, petrochemical, textile, and pharmaceutical industries, especially the use of NIR spectroscopy for the quantitative analysis of pharmaceutical samples has significantly increased during the last decade (Dou et al., 2005). The two components (paracetamol and amantadine hydrochloride) were simultaneously determined in combined paracetamol and amantadine hydrochloride tablets by Dou et al. (2005). The ANN models of three pre-treated spectra (first-derivative, second-derivative, and standard normal variate, respectively) were established. In the models, the concentrations of paracetamol and amantadine hydrochloride as the active components were determined simultaneously and compared to the results of their separate determinations. Titova et al. (2009) have studied the ability to use near-IR spectroscopy to analyze substances and tablets of rimantadine hydrochloride, using discriminant analysis, which is a quantitative method. The studies showed that near IR spectroscopy can be used to confirm the authenticity of rimantadine hydrochloride substances.

## POTENTIOMETRIC METHODS

Potentiometry is still one of the analytical tools capable of determining both inorganic and organic substances in medico-biological practices. Applications of the potentiometry techniques in pharmaceutical analysis and clinical chemistry were reviewed (Kulapina and Barinov, 1997). There is a constant increase in the number of electrodes capable of selectively identifying various drugs. In recent years, several sensitive potentiometric methods have been reported for the determination of pharmaceutical and biochemical compounds. Abdel-Ghani et al. (2002) have prepared a plastic membrane selective electrode for the amantadinium ion. It is based on the incorporation of amantadinium tetraphenylborate ion exchange in the PVC membrane plasticized with dioctylphethalate. The electrode was fully characterized under batch conditions and then used for the potentiometric determination of amantadine in its pure state and in pharmaceutical preparations under batch and flow injection conditions. The FIA conditions shortened the time needed for the determination and extended the LOD to higher concentrations of the drug in its pure state or in its pharmaceutical preparation. Electrochemical sensors based on modified carbon paste and graphite coated ion selective electrodes (ISEs) were proposed by Salem (2005) for determining amantadine hydrochloride anti-viral drug. These IESs are simple to prepare, rigid, easy to use, and of low cost. The developed sensors were successfully applied in the determination of the target drugs in pharmaceutical and human urine samples. The

new electrodes have proven sensitive with a detection limit of 0.19–2.08 $\mu$g/mL. The detection limit obtained for amantadine hydrochlorides are far less than pharmacopeial methods. Since about 90% of amantadine is excreted in urine as unchanged, these detection limits are less than amounts excreted in urine. Linear calibration graphs in the range of $10^{-1}$–$10^{-5}$ M were obtained. Amantadine potentiometric detectors were developed and evaluated by Amorin et al. (2007) and, finally, incorporated a sequential injection lab-on-valve procedure (SIA-LOV) in order to accomplish the control of pharmaceutical formulations and urine. The SIA-LOV system is a programmable liquid flow miniaturization concept of the flow detection cell and other manifold components within a monolithic structure mounted on a multi-position valve. The electrodes incorporate $\alpha$-cyclodextrin as inophore, dibutyl phathalate or 2-fluorophenyl 2-nitrophenyl ether as plasticizers, and potassium tetrakis[3,5-bis-(trifluoromethyl)phenyl]borate (KTFPB) as cationic additives. The practical LOD increased from $2.6 \times 10^{-6}$ M to $2.5 \times 10^{-5}$ M when the plasticizer was changed from 2-fluorophenyl 2-nitrophenyl ether to dibutyl phthalate. When incorporated in the flow-manifold the membranes composed by of dibutyl phthalate or with 2-fluorophenyl 2-nitrophenyl ether presented a practical LOD of $1.5 \times 10^{-4}$ M and $5.4 \times 10^{-5}$ M, respectively. The new amantadine potentiometric detector configuration is easy to achieve in common laboratories and allows for the implementation of low volume detection cell, where the electrical noise, frequently present in potentiometric-based procedures, usually requires the resort to a grounding electrode. A simple potentiometric method was applied for the determination of amantadine by Jalali and Maghooli (2009) using a ß-cyclodextrin modified carbon-paste electrode. The electrode was used for the determination of very low concentrations of amantadine in pharmaceutical formulations and blood-serum samples successfully. The LOD of the electrode was $6.3 \times 10^{-10}$ M of amantadine. The electrode responded very rapidly (<60 seconds) to changes in the concentration of amantadine, and its lifetime was more than 3 months. The RSD of measurements for a $2.0 \times 10^{-7}$ M of amantadine was 0.68% ($n = 7$).

## CONCLUSIONS

After the study of the bibliography compiled about analytical methods applied to the determination of drugs used as anti-viral drugs in the treatment of infections with influenza we can conclude that the analytical method most widely applied is the LC with diverse forms of detection, with MS as the procedure more widely studied, especially in the determination of the drugs studied in biological fluids. The spectrophotometric and fluorimetric detection are also widely used both for biological samples and for pharmaceutical preparations. Other analytical methods applied in the determination of the drugs studied in synthetic samples or pharmaceutical forms are the electrophoretic methods, emphasizing the CE with spectrophotometric detection, and optical methods such as uv-visible spectroscopy, fluorimetry, and near-infrared spectrocopy. The potentiometric methods studied only determine amantadine in pharmaceutical and biological fluid samples.

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