Chapter 4

Analytical methods for estimation of the drugs

4 ANALYTICAL METHODS FOR ESTIMATION OF THE DRUGS

(4.a) Preparation of Simulated Saliva pH 6.8

Phosphate buffer saline solution (2.38 g Na2HPO4, 0.19 g KH2PO4, and 8.00 g NaCl per liter of distilled water adjusted with phosphoric acid to pH 6.8),

(4.b) Preparation of Simulated Gastric Fluid pH 1.2

The gastric fluid simulated (USP, 1995) consists of a mixture of 2.0 g of sodium chloride with or without 3.2 g of pepsin (3200 units per mg protein, in 7.0 ml of hydrochloric acid (37 %) in 1000 ml of water. The final pH of the dissolution medium is 1.2. (http://www.theses.ulaval.ca/2003/20851/ch04.html)

(4.c) Preparation of McIlvaine Buffers of Different pH

The McIlvailne buffers were prepared according to the preparation method given in J Biol Chem 1921, 49, 183. Chem Abs 16-729. Solution A is 0.1 M citric acid (i.e. 21.0 g C6H8O7. H2O per litre), Solution B is 0.2 M disodium hydrogen phosphate (i.e. 35.6 g Na2HPO4. 2H2O per litre). Mix x ml of A and (100-x) ml of B. pH values are quoted at 21 °C.

pH	x	pH	x	pH	x	pH	x	pH	x
2.20	98.00	3.40	71.50	4.60	53.25	5.80	39.55	7.00	18.15
2.40	93.80	3.60	67.80	4.80	50.70	6.00	36.85	7.20	13.05
2.60	89.10	3.80	64.50	5.00	48.50	6.20	33.90	7.40	9.15
2.80	84.15	4.00	61.45	5.20	46.40	6.40	30.75	7.60	6.35
3.00	79.45	4.20	58.60	5.40	44.25	6.60	27.25	7.80	4.30
3.20	75.30	4.40	55.90	5.60	42.00	6.80	22.75	8.00	2.75

The pH of the buffer solution was adjusted with 0.1 M citric acid or 0.1 N NaoH.

4.1 ANALYTICAL METHODS FOR SALBUTAMOL SULPHATE

4.1.1 Preparation of Calibration Curve for Invitro Estimation of Salbutamol Sulphate

Estimation of salbutamol sulphate in dissolution, diffusion media and the dosage form was done by direct UV spectroscopy.

Reagents

- Distilled water
- 0.1 N HCl prepared as per Indian Pharmacopoeia
- Simulated saliva pH 6.8
- Simulated gastrointestinal fluid pH 1.2

In distilled water: 10 mg of salbutamol sulphate was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of distilled water (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with water. Aliquots of 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml, 6.0 ml and 7.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with distilled water in each flask.

In 0.1 N HCl: 10 mg of salbutamol sulphate was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of 0.1 N HCl (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with 0.1 N HCl. Aliquots of 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml, 6.0 ml and 7.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with 0.1 N HCl in each flask.

***In simulated saliva pH 6.8*:** 10 mg of salbutamol sulphate was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of simulated saliva pH 6.8 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated

saliva pH 6.8. Aliquots of 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml, 6.0 ml and 7.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated saliva pH 6.8 in each flask.

In simulated gastric fluid pH 1.2: 10 mg of salbutamol sulphate was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of simulated gastric fluid pH 1.2 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated gastric fluid pH 1.2. Aliquots of 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml, 6.0 ml and 7.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated gastric fluid pH 1.2 in each flask.

4.1.2 Method for Preparation of Calibration Curve of Salbutamol Sulphate

The absorbance maxima λ max was determined by scanning 40 µg/ml standard solution of salbutamol sulphate against solvent blank in each case on Shimadzu UV-1601 UV visible spectrophotometer. The absorbance of the standard solutions of drug was measured at the absorbance maxima (276 nm). The readings were recorded on five consecutive days using freshly prepared stock solutions each time. Mean values along with standard deviation are recorded in table 4.1, 4.2, 4.3 and 4.4. The values of absorbance were plotted graphically against the concentration of the standard solutions as shown in figure 4.1, 4.2, 4.3 and 4.4 respectively.

4.2 ANALYTICAL METHOD FOR ONDANSETRON HYDROCHLORIDE

4.2.1 Preparation of Calibration Curve for Invitro Estimation of Ondansetron Hydrochloride

Estimation of salbutamol sulphate in dissolution, diffusion media and the dosage form was done by direct UV spectroscopy.

Reagents

- Distilled water
- 0.1 N HCl prepared as per Indian Pharmacopoeia
- Simulated saliva pH 6.8
- Simulated gastrointestinal fluid pH 1.2

In distilled water: 10 mg of ondansetron hydrochloride was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of distilled water (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with water. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with distilled water in each flask.

In 0.1 N HCl: 10 mg of ondansetron hydrochloride was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of 0.1 N HCl (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with 0.1 N HCl. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with 0.1 N HCl in each flask.

***In simulated saliva pH 6.8*:** 10 mg of ondansetron hydrochloride was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of simulated saliva pH 6.8 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated saliva pH 6.8. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated saliva pH 6.8 in each flask.

In simulated gastric fluid pH 1.2: 10 mg of ondansetron hydrochloride was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of

simulated gastric fluid pH 1.2 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated gastric fluid pH 1.2. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated gastric fluid pH 1.2 in each flask.

4.2.2 Method for Preparation of Calibration Curve of Ondansetron Hydrochloride

The absorbance maxima λ max was determined by scanning 15 μg/ml standard solution of ondansetron hydrochloride against solvent blank in each case on Shimadzu UV-1601 UV visible spectrophotometer. The absorbance of the standard solutions of drug was measured at the absorbance maxima (249 nm). The readings were recorded on five consecutive days using freshly prepared stock solutions each time. Mean values along with standard deviation are recorded in table 4.5, 4.6, 4.7 and 4.8. The values of absorbance were plotted graphically against the concentration of the standard solutions as shown in figure table 4.5, 4.6, 4.7 and 4.8 respectively.

4.3 ANALYTICAL METHOD FOR LAMOTRIGINE

4.3.1 Preparation of Calibration Curve for Invitro Estimation of Lamotrigine

Estimation of lamotrigine in dissolution, diffusion media and the dosage form was done by direct UV spectroscopy.

Reagents

- Distilled water
- 0.1 N HCl prepared as per Indian Pharmacopoeia
- Simulated saliva pH 6.8
- Simulated gastrointestinal fluid pH 1.2

In distilled water: 10 mg of lamotrigine was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of distilled water (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with water. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with distilled water in each flask.

In 0.1 N HCl: 10 mg of lamotrigine was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of 0.1 N HCl (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with 0.1 N HCl. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with 0.1 N HCl in each flask.

***In simulated saliva pH 6.8*:** 10 mg of lamotrigine was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of simulated saliva pH 6.8 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated saliva pH 6.8. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated saliva pH 6.8 in each flask.

In simulated gastric fluid pH 1.2: 10 mg of lamotrigine was accurately weighed and transferred to a clean and dry 100 ml volumetric flask. Sufficient quantity of simulated gastric fluid pH 1.2 (about 70 ml) was added and the flask was gently skaken by circular motion till all the powder dossolved. The volume was made up to 100 ml with simulated gastric fluid pH 1.2. Aliquots of 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml were transferred to clean and dry 10 ml volumetric flasks and volume was made up to 10 ml with simulated gastric fluid pH 1.2 in each flask.

4.3.2 Method for Preparation of Calibration Curve of Lamotrigine

The absorbance maxima λ max was determined by scanning 20 µg/ml standard solution of lamotrigine against solvent blank in each case on Shimadzu UV-1601 UV visible spectrophotometer. The absorbance of the standard solutions of drug was measured at the absorbance maxima (305 nm). The readings were recorded on five consecutive days using freshly prepared stock solutions each time. Mean values along with standard deviation are recorded in Table 4.9, 4.10, 4.11 and 4.12. The values of absorbance were plotted graphically against the concentration of the standard solutions as shown in figure 4.9, 4.10, 4.11 and 4.12, respectively.

4.4 RESULTS OF THE ANALYTICAL METHODS

4.4.1 Analytical Methods for Salbutamol Sulphate

Table 4.1. Calibration plot of salbutamol sulphate in distilled water.

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	10	0.055 ± 0.016
2	20	0.119 ± 0.035
3	30	0.163 ± 0.012
4	40	0.219 ± 0.009
5	50	0.264 ± 0.027
6	60	0.306 ± 0.008
7	70	0.374 ± 0.009

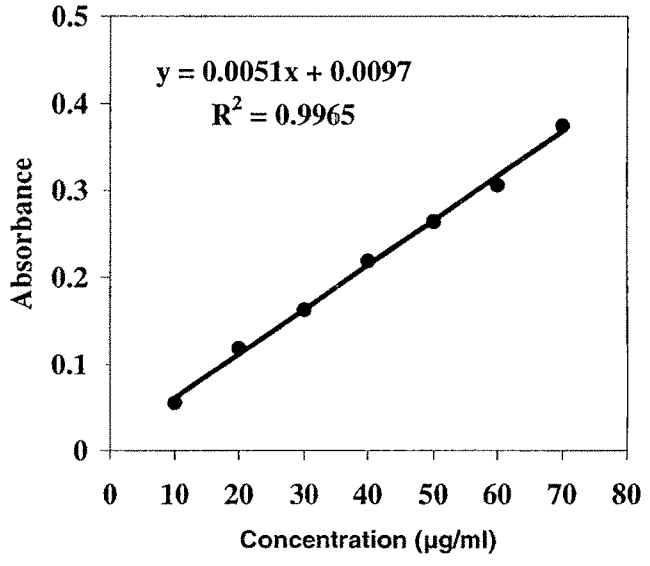


Figure 4.1. Calibration curve of salbutamol sulphate in distilled water.

Table 4.2. Calibration plot of salbutamol sulphate in 0.1 N HCl.

Sr. No.	Concentration (µg/ml)	Absorbance ± S. D. (n=5)
1	10	0.063 ± 0.026
2	20	0.116 ± 0.047
3	30	0.175 ± 0.026
4	40	0.25 ± 0.007
5	50	0.268 ± 0.011
6	60	0.312 ± 0.05
7	70	0.384 ± 0.015

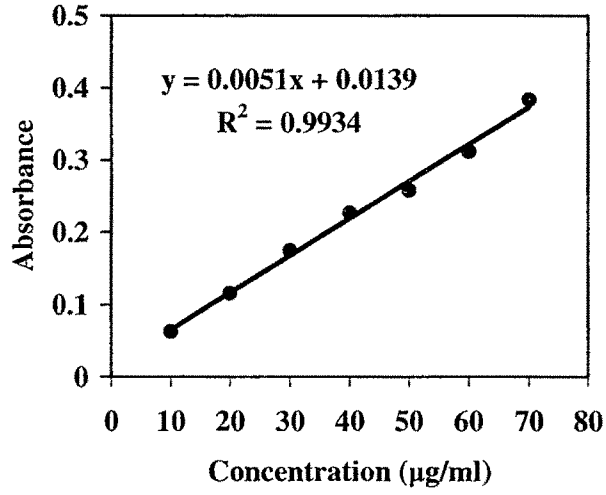


Figure 4.2. Calibration curve of salbutamol sulphate in 0.1 N HCl.

Table 4.3. Calibration plot of salbutamol sulphate in simulated saliva (pH 6.8).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	10	0.057 ± 0.021
2	20	0.135 ± 0.025
3	30	0.174 ± 0.008
4	40	0.226 ± 0.004
5	50	0.274 ± 0.013
6	60	0.314 ± 0.037
7	70	0.379 ± 0.042

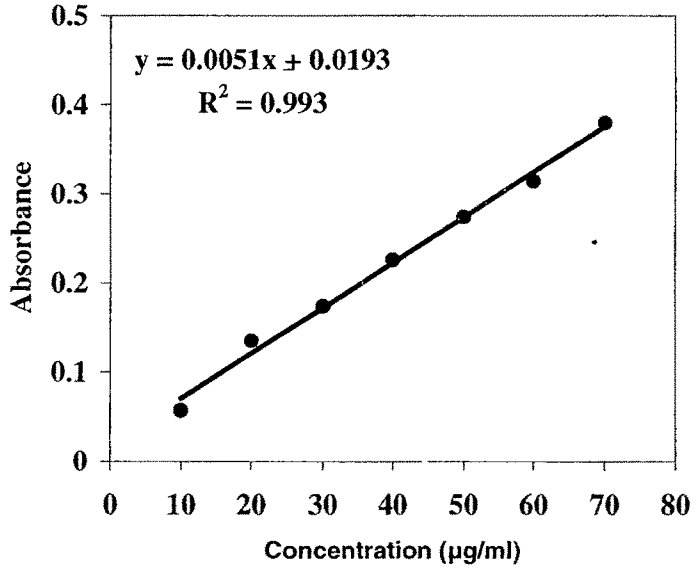


Figure 4.3. Calibration curve of salbutamol sulphate in simulated saliva (pH 6.8).

Table 4.4. Calibration plot of salbutamol sulphate in simulated gastrointestinal fluid (pH 1.2).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	10	0.067 ± 0.012
2	20	0.124 ± 0.006
3	30	0.178 ± 0.008
4	40	0.223 ± 0.063
5	50	0.278 ± 0.029
6	60	0.325 ± 0.052
7	70	0.392 ± 0.043

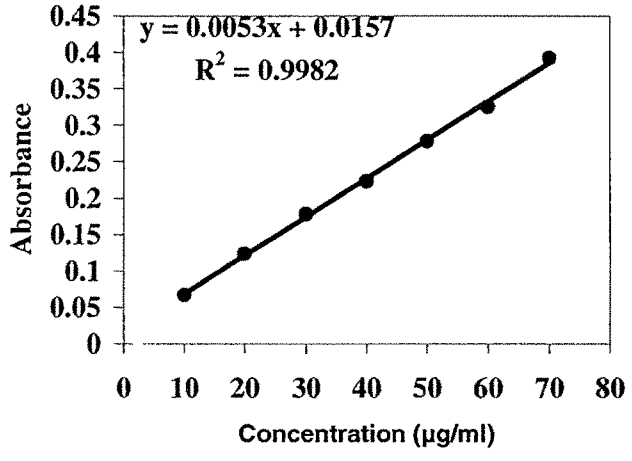


Figure 4.4. Calibration curve of salbutamol sulphate in simulated gastrointestinal fluid (pH 1.2).

4.4.2 Analytical Methods for Ondansetron Hydrochloride

Table 4.5. Calibration plot of ondansetron hydrochloride in distilled water.

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.227 ± 0.004
2	10	0.397 ± 0.008
3	15	0.587 ± 0.011
4	20	0.744 ± 0.07
5	25	0.921 ± 0.014
6	30	1.049 ± 0.009

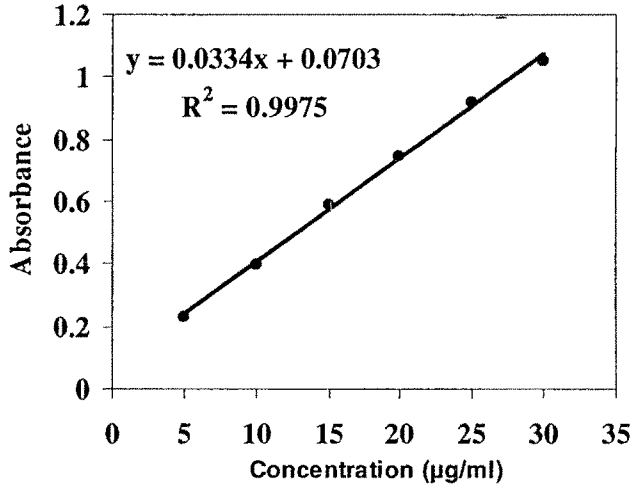


Figure 4.5. Calibration curve of ondansetron hydrochloride in distilled water.

Table 4.6. Calibration plot of ondansetron hydrochloride in 0.1 N HCl.

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.266 ± 0.012
2	10	0.492 ± 0.008
3	15	0.715 ± 0.005
4	20	0.905 ± 0.017
5	25	1.036 ± 0.024
6	30	1.241 ± 0.007

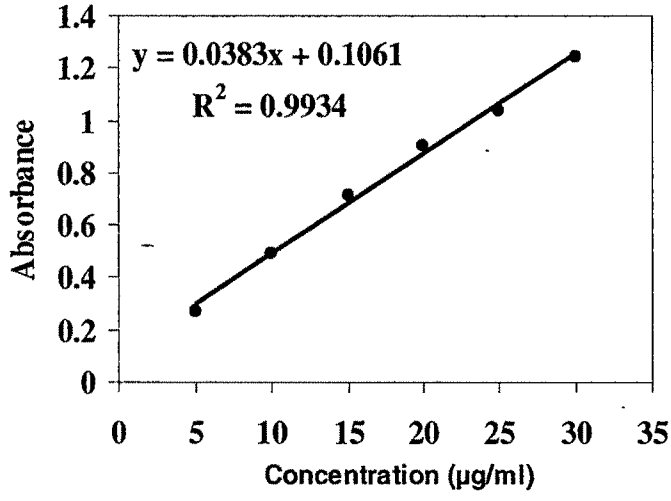


Figure 4.6. Calibration curve of ondansetron hydrochloride in 0.1 N HCl.

Table 4.7. Calibration plot of ondansetron hydrochloride in simulated saliva (pH 6.8).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.216 ± 0.009
2	10	0.402 ± 0.011
3	15	0.553 ± 0.007
4	20	0.737 ± 0.008
5	25	0.878 ± 0.012
6	30	1.065 ± 0.015

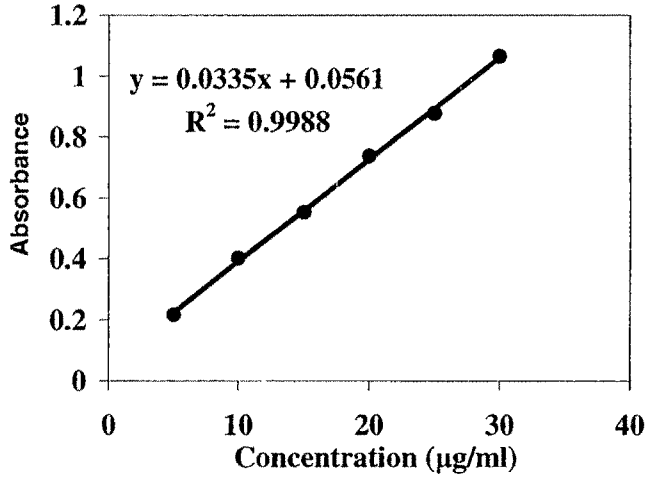


Figure 4.7. Calibration curve of ondansetron hydrochloride in simulated saliva (pH 6.8).

Table 4.8. Calibration plot of ondansetron hydrochloride in gastrointestinal fluid (pH 1.2).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.272 ± 0.007
2	10	0.496 ± 0.014
3	15	0.718 ± 0.015
4	20	0.904 ± 0.009
5	25	1.036 ± 0.008
6	30	1.375 ± 0.018

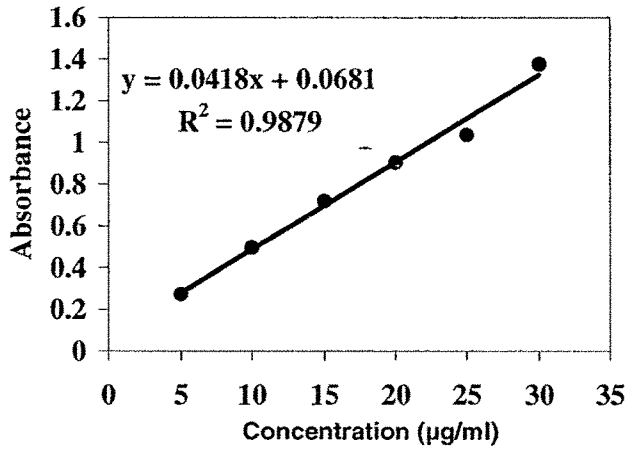


Figure 4.8. Calibration curve of ondansetron hydrochloride in gastrointestinal fluid (pH 1.2).

4.4.3 Analytical Methods for Lamotrigine

Table 4.9. Calibration plot of lamotrigine in distilled water.

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.115
2	10	0.243
3	15	0.326
4	20	0.447
5	25	0.538
6	30	0.645
7	35	0.734
8	40	0.839

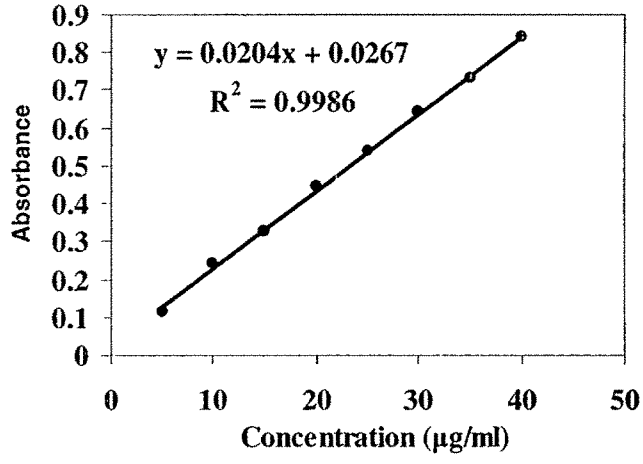


Figure 4.9. Calibration curve of lamotrigine in distilled water.

Table 4.10. Calibration plot of lamotrigine in 0.1 N HCl.

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.117
2	10	0.247
3	15	0.328
4	20	0.458
5	25	0.547
6	30	0.658
7	35	0.7568
8	40	0.908

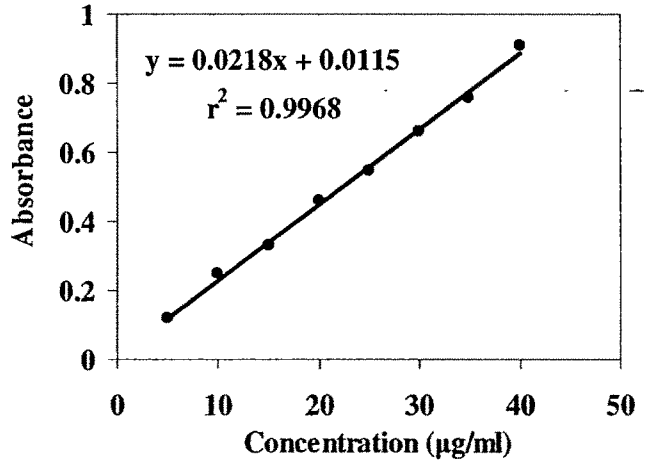


Figure 4.10. Calibration curve of lamotrigine in 0.1N HCl.

Table 4.11. Calibration plot of lamotrigine in simulated saliva (pH 6.8).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.124
2	10	0.253
3	15	0.345
4	20	0.463
5	25	0.568
6	30	0.673
7	35	0.762
8	40	0.914

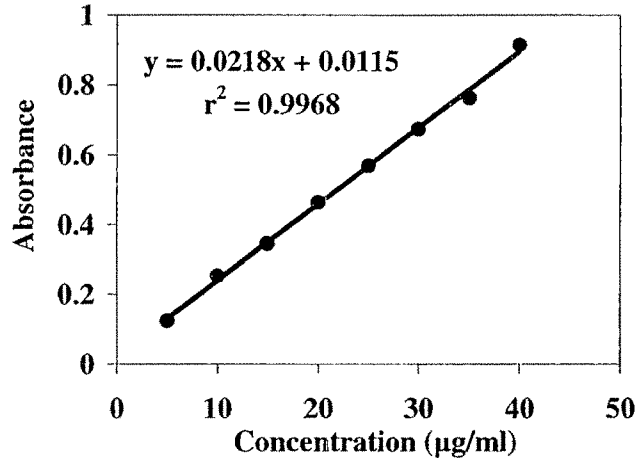


Figure 4.11. Calibration curve of lamotrigine in simulated saliva (pH 6.8).

Table 4.12. Calibration plot of lamotrigine in gastrointestinal fluid (pH 1.2).

Sr. No.	Concentration (µg/ml)	Absorbance ± S.D. (n=5)
1	5	0.136
2	10	0.264
3	15	0.348
4	20	0.467
5	25	0.563
6	30	0.637
7	35	0.783
8	40	0.917

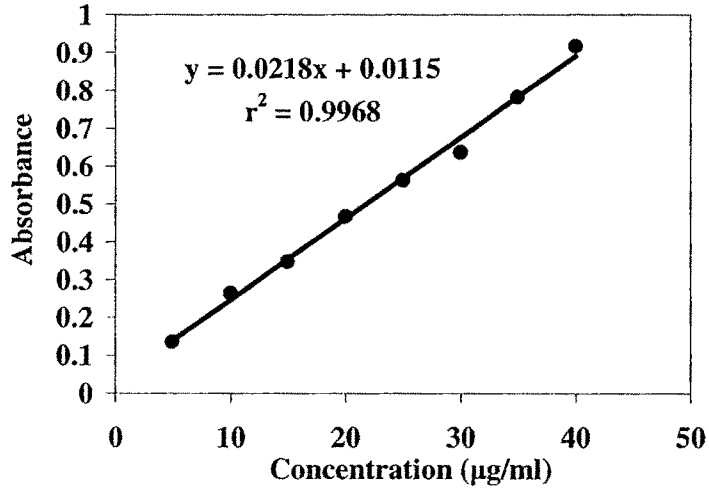


Figure 4.12. Calibration curve of lamotrigine gastrointestinal fluid (pH 1.2).

Standard solutions of salbutamol sulphate were prepared in distilled water, 0.1 N HCl, simulatd saliva pH 6.8, simulated gastrointestinal fliud pH 1.2, McIlvaine buffer pH 4.0, McIlvaine buffer pH 6.0, McIlvaine buffer pH 6.8, McIlvaine buffer pH 7.4, McIlvaine buffer pH 8.0 and McIlvaine buffer pH 9.0. The aborbance maxima determined in each case by scanning a standard solution of concentration 40 μg/ml on Shimadzu UV-1601 UV visible spectrophotometer was found to be 276 nm. The results are corded in Table 4.1 to table 4.4 and shown graphically in figure 4.1 to figure 4.4. Regression analysis proved the linearity of data ($r > 0.992$) in the concentratio range of 10-70 μg/ml.

Standard solutions of ondansetron hydrochloride were prepared in distilled water, 0.1 N HCl, simulatd saliva pH 6.8, simulated gastrointestinal fliud pH 1.2, McIlvaine buffer pH 4.0, McIlvaine buffer pH 6.0, McIlvaine buffer pH 6.8, McIlvaine buffer pH 7.4, McIlvaine buffer pH 8.0 and McIlvaine buffer pH 9.0. The aborbance maxima determined in each case by scanning a standard solution of concentration 15 μg/ml on Shimadzu UV-1601 UV visible spectrophotometer was found to be 249 nm. The results are corded in Table 4.5 to table 4.8 and shown graphically in figure 4.5 to figure 4.8. Regression analysis proved the linearity of data ($r > 0.98$) in the concentratio range of 5-30 μg/ml.

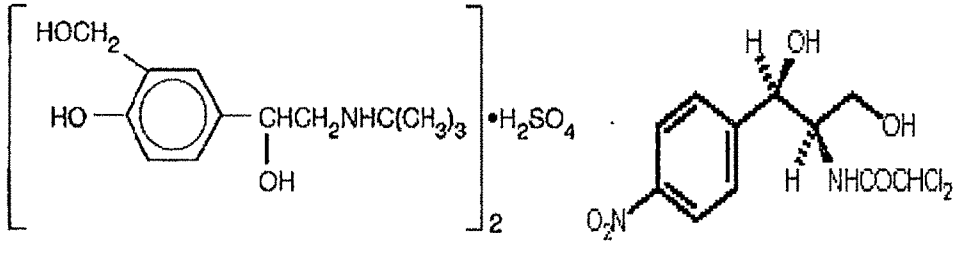
Standard solutions of lamotrigien were prepared in distilled water, 0.1 N HCl, simulatd saliva pH 6.8, simulated gastrointestinal fliud pH 1.2, McIlvaine buffer pH 4.0, McIlvaine buffer pH 6.0, McIlvaine buffer pH 6.8, McIlvaine buffer pH 7.4, McIlvaine buffer pH 8.0 and McIlvaine buffer pH 9.0. The aborbance maxima determined in each case by scanning a standard solution of concentration 20 μg/ml on Shimadzu UV-1601 UV visible spectrophotometer was found to be 305 nm. The results are corded in Table 4.9 to table 4.12 and shown graphically in figure 4.9 to figure 4.12. Regression analysis proved the linearity of data ($r > 0.995$) in the concentratio range of 5-40 μg/ml.

4.5 DETERMINATION OF SALBUTAMOL SULPHATE IN RABBIT PLASMA BY HPLC METHOD

4.5.1 Introduction

Salbutamol sulphate, (RS)-1-(4-hydroxy-3-hydroxy-methyl phenyl)-2-(tert-butylamino) ethanol sulphate, a β_2 receptor agonist is most widely used sympathomimetics for the treatment of acute as well as chronic asthma. Generally, it is given through inhalation route but is also effective after oral administration. Despite the fact that salbutamol is well absorbed, its systemic bioavailability is only 50% due to extensive presystemic metabolism in the gastrointestinal tact (GIT) and liver.

Several high performance liquid chromatographic methods with fluorescence detector have been reported for the determination of salbutamol sulphate in plasma[1-10], in urine[11] and in serum[12]. A simple HPLC with UV detection for the determination of salbutmaol sulphate in rabbit plasma after liquid liquid extraction is developed and it is applied to a preliminary pharmacokinetic study in rabbit. The chemical structure of salbutmaol sulphate and internal standard is given in figure 4.13.



(A) (B)

Figure 4.13. Chemical structure of (A) salbutamol sulphate and (B) internal standard (chloramphenicol).

4.5.2 Experimental

4.5.2.1 *Materials*

Salbutamol sulphate and chloramphenicol (internal standard) were received as gift samples from Relax Pharmaceutical Pvt. Ltd. (Baroda, India). HPLC grade acetonitrile, methanol, chloroform and hydrochloric acid (analytical grade) were purchased from S. D. Fine Chemicals (Mumbai, India). Analytical grade di-2-ethyl hexyl phosphate and Phosphoric acid were purchased from Merck Ltd. (Mumbai, India). In all experiments double distilled water was used.

4.5.2.2 *Chromatographic conditions*

The analysis was performed on a Perkin Elmer chromatographic system (Tokyo Japan) equipped with a LC 200 pump, PE Nelson 1020S integrator and UV detector. The mobile phase involved a mixture of water, methanol and acetonitrile (70:20:10 v/v) pH adjusted to 2.5 by 10% phoaphoric acid, pump at a flow rate of 1.2 ml/min through the column (Thermohypersil, 5µm RP C_{18}, 250 × 4.6 mm) at a room temperature (29 ± 2 °C).). The samples were injected manually using a Rheodyne 7123 injector (Rheodyne, Cotai, CA) with a 100-µl loop. Peaks were monitored by UV absorbance at 276 nm. Under this condition, the retention time observed for salbutamol sulphate and chloramphenicol were 3.0 and 15.4 minutes, respectively. Quantification of salbutamol sulphate was obtained by plotting drug to internal standard peak area ratio as a function of concentration.

4.5.2.3 *Standard solutions*

A stock standard solution was prepared in methanol and contained 1.00 mg/ml of salbutamol sulphate. Working standard solutions were prepared by dilution of the stock standard solution of with methanol to concentration of 1, 2, 3, 5, 7, 10 and 15 µg/ml.

An internal standard stock solution was prepared in methanol and contained 1 mg/ml. A working solution was made by dilution of the stock solution with methanol to100 µg/ml.

4.5.2.4 *Calibration standard solution*

Rabbit blood containing heparin as an anti-coagulant was used for the preparation of calibration standard. Calibration standard solution samples were freshly prepared in rabbit plasma by adding to 0.5 ml of plasma appropriate aliquots of working standard solutions to yield concentration of 100, 200, 300, 500, 700, 1000, 1500 ng/ml.

4.5.2.5 *Plasma extraction procedure*

To 0.5 ml of plasma sample, 10 µl of internal standard (100 µg/ml) was added. The solution was mixed for a few seconds. Five ml of 0.1 M bis-(2 ethyl hexyl) phosphate in chloroform which was subsequently shaken on a cyclomixer for 10 min. followed by centrifugation at 2000 rpm for 10 min. The separated organic layer was mixed with 1 ml 0.5 M hydrochloric acid on a cyclo-mixer for 10 min. and was centrifuged at 2000 rpm for 10 min. The aqueous layer was separated and 100 µl was injected into the HPLC system.

4.5.2.6 *Extraction efficiency*

The extraction efficiency was calculated by adding known amount of salbutamol sulphate (200, 500 and 1000 ng/ml; n = 5 per concentration) to 0.5 ml of blank rabbit plasma. The salbutamol sulphate was extracted into 5 ml of chloroform containing 0.10 M bis-(2 ethyl hexyl) phosphate. The chloroform layer was back extracted into 1 ml 0.1M hydrochloric acid solution by agitation for 1 minute and centrifuged for 10 minutes. The known amount of aqueous layer was injected into the chromatographic system.

The peak area was compared to those obtained from equivalent volumes of standard solution of salbutamol sulphate in 0.1 M hydrochloric acid solution directly injected into the HPLC system. The determination of unextracted samples was performed in quadruplicate for each concentration.

4.5.2.7 *Specificity*

The specificity of the method was determined by comparing the chromatograms obtained from the samples containing salbutamol sulphate and internal standard with those obtained from blank plasma. Five blank plasma samples from six lots of rabbit plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

4.5.2.8 *Linearity and range*

The linear detector response for the assay was tested as follows. These determination (n=5) from minimum of five concentration levels (100, 200, 300, 500, 700, 1000, 1500 ng/ml) of the analyte were made. Detector response was correlated against analyte concentration by least squares regression.

4.5.2.9 *Accuracy and precision*

For the determination of intra-day and inter-day accuracy and precision of the assay, aliquots of 0.5 ml rabbit plasma were 20 µl of 100 µg/ml of internal standard and various quantities of salbutamol sulphate to yield 100, 200, 300, 500, 700, 1000, 1500 ng/ml. Accuracy was expressed as the mean % [(mean measured concentration)/(expected concentration)] × 100 (3). Precision was calculated as inter and intra-day coefficient of variation [% C.V. = (S.D./mean) × 100].

4.5.3 Results and Discussion

System reproducibility, expressed as % R.S.D. and based on absolute peak height, was 4.1 and 2.7 % (n=8) for salbutamol sulphate at a concentration of 50 and 700 ng/ml, respectively. Mean reproducibility of the internal standard was 0.83 % (n=10) at 1000 ng/ml concentration in rabbit plasma.

A mobile phase composed of water: methanol: acetonitrile (70:20:10 v/v) gave well resolved, sharp peaks for salbutamol sulphate and internal standard (chloramphenicol) with a retention time of 3.0 minutes and 15.4 minutes, respectively. The chromatograph of rabbit blank plasma and plasma spiked with salbutamol sulphate and internal standard is given in figure 4.14 and figure 4.15.

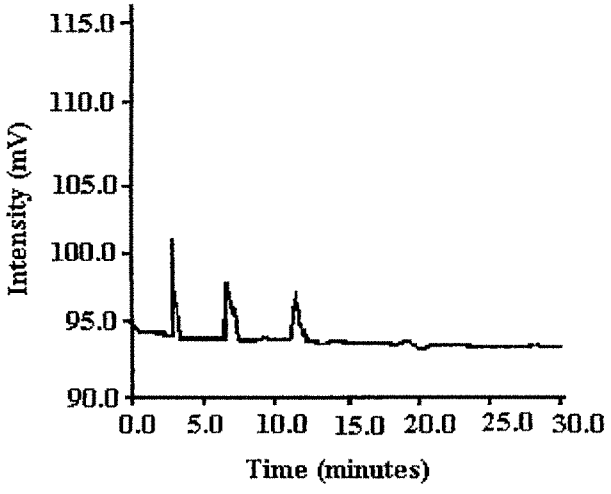


Figure 4.14. Representative chromatogram of blank rabbit plasma.

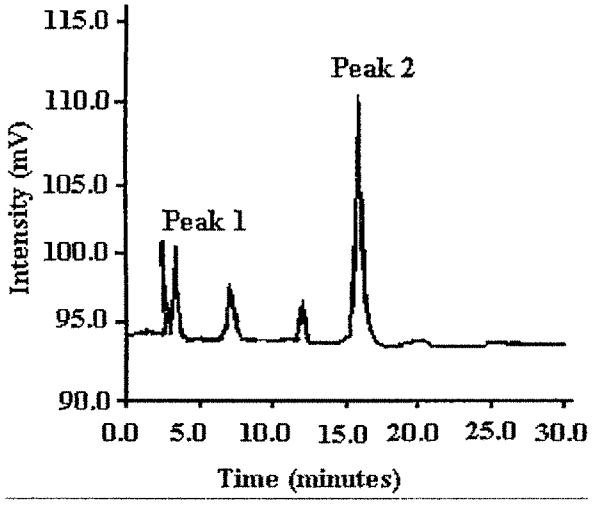


Figure 4.15. Representative chromatogram of plasma spiked with 300 ng/ml salbutamol sulphate (peak 1) and 2000 ng/ml chloramphenicol (peak 2).

They showed that there was no any interference from blank plasma peaks to peaks of drug and internal standard. The quantification of the chromatogram was performed using the ratio of the area of salbutamol sulphate peak to area of internal standard peak. The ratio of peak area versus concentration was plotted.

The peak area ratio of drug to internal standard was plotted versus drug concentration. It was linear (r^2 = 0.9920) over 100-1500 ng/ml. The extraction efficiency was greater than 80.00 % which is shown in table 4.13.

Table 4.13. Extraction efficiency of salbutamol sulphate from rabbit plasma at various concentration. (n=5).

Concentration (ng/ml)	Extraction efficiency ± S. D.
200	83.64 ± 1.84
500	78.72 ± 2.86
1000	80.28 ± 0.23
Average extraction efficiency	80.88 ± 2.92

Limit of quantification and limit of detection were 100 ng/ml and 30 ng/ml, respectively based on 500 µl of plasma. The intraday coefficient of variation (C.V.) varied from 1.9 to 5.2 % and interday varied from 1.1 to 5.9 % which are shown in table 4.14 and table 4.15.

Table 4.14. Intraday precision and accuracy for the salbutamol sulphate assay in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b] (%) ± S.D.
Actual	Observed ± S.D.		
100	99.52 ± 4.6	4.7	99.52 ± 4.60
200	198.64 ± 4.9	2.5	99.32 ± 2.45
300	304.19 ± 1.3	0.43	101.39 ± 0.43
500	492.29 ± 5.4	1.1	98.45 ± 0.22
1000	997.83 ± 8.7	0.88	99.78 ± 0.09
1500	1507.18 ± 5.2	0.35	100.47 ± 0.34

[a] Expressed as % relative standard deviation.

[b] Expressed as [(mean observed concentration/actual concentration)×100]

Table 4.15. Interday variability of the salbutamol sulphate assay over five consecutive days in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b] (%) ± S.D.
Actual	Observed ± S.D.		
100	99.52 ± 4.91	4.8	102.41 ± 4.91
200	198.64 ± 5.29	2.6	101.92 ± 2.64
300	304.19 ± 5.04	1.7	98.97 ± 1.68
500	492.29 ± 4.18	0.84	99.62 ± 0.83
1000	997.83 ± 7.74	0.77	100.64 ± 0.77
1500	1507.18 ± 7.38	0.49	100.44 ± 0.49

[a] Expressed as % relative standard deviation.

[b] Expressed as [(mean observed concentration/actual concentration)×100]

The % recovery of salbutamol sulphate in plasma ranged from 98.97 to 102.41 % which indicated excellent recovery. The assay was simple, sensitive, precise and accurate for the determination of salbutamol sulphate in the rabbit plasma.

This assay is convenient, simple and there is no need of utilizing the fluorescence or electrochemical detector. The described method is simple, sensitive, precise and accurate for the analysis of salbutamol sulphate from biological samples as simple extraction procedure is required. This proposed method can be recommended for routine patient monitoring and for pharmacokinetic studies.

4.5.4 Conclusions

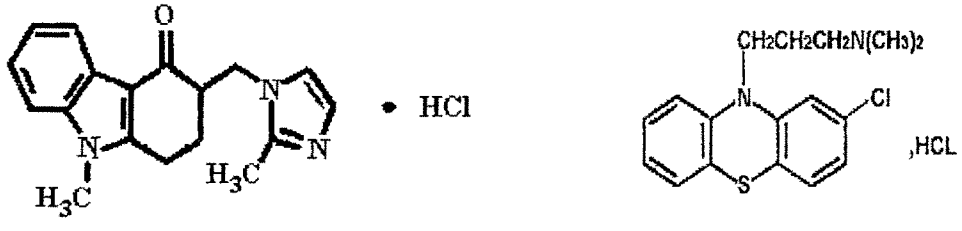
The proposed HPLC assay for the determination of salbutamol sulphate is suitable for pharmacokinetic study of it in rabbits. This assay is convenient, simple, precise and accurate and there is no need of utilizing the fluorescence detector.

4.6 HPLC METHOD FOR DETERMINATION OF ONDANSETRON HYDRO-CHLORIDE IN PLASMA

4.6.1 Introduction

Methods such as capillary electrophoresis[13], radio immuno assay[14] and high performance liquid chromatographic methods[15-21] have been reported for the determination of ondansetron hydrochloride in biological fluid.

A rapid, specific and cost effective high performance liquid chromatographic (HPLC) method with ultraviolet detector is developed with simple extraction procedure for the assay of ondansetron hydrochloride in rabbit plasma. The applicability of this method was demonstrated by the analysis of rabbit plasma containing 300 ng/ml of ondansetron hydrochloride. Figure 4.16 shows the structures of ondansetron hydrochloride and internal standarad.



A B

Figure 4.16. Chemical structures of (A) ondansetron hydrochloride and (B) chlorpromazine hydrochloride (Internal standard).

4.6.2 Experimental

4.6.2.1 *Materials*

Ondansetron hydrochloride and chlorpromazine hydrochloride (Internal standard) were received as gift samples from Relax Pharmaceutical Pvt. Ltd. (Baroda, India). HPLC grade acetonitrile, methanol, dichloromethane were purchased from S. D. Fine Chemicals (Mumbai, India). In all experiments double-distilled water was used.

4.6.2.2 *Instrumentation*

The chromatography system consisted of an LC 200 pump (Perkin-Elmer, Japan) with PE Nelson model 1050 integrator. The samples were injected manually using a Rheodyne 7123 injector (Rheodyne, Cotai, CA) with a 100-µl loop. HPLC column (Thermohypersil, 5µm RP C_{18}, 250 × 4.6 mm) was held at a room temperature (29 ± 2 °C) with UV detection at 249 nm. The mobile phase consisted of water: methanol: acetonitrile (70:20:10 v/v) and was filtered through a 0.45-micron whatman filter paper before use. The chromatography was performed isocratically at a flow rate of 1 ml/min at room temperature. Total run time was 18 min for each injection.

4.6.2.3 *Standard solutions*

A stock standard solution was prepared in methanol and contained 1.00 mg/ml of ondansetron hydrochloride. Working standard solutions were prepared by dilution of the stock standard solution with methanol to concentration of 0.25, 0.5, 1, 2, 3, 5 and 10 µg/ml. An internal standard stock solution was prepared in methanol and contained 1.00 mg/ml. A working solution was made by dilution of the stock solution with methanol to10 µg/ml.

4.6.2.4 *Calibration standard solution*

Rabbit blood containing heparin as an anti-coagulant was used for the preparation of calibration standard. Calibration standard solution samples were freshly prepared in rabbit plasma by adding to 0.5 ml of plasma appropriate aliquots of working standard solutions to yield concentration of 25, 50, 100, 200, 300, 500, 1000 ng/ml.

4.6.2.5 *Plasma extraction procedure*

Rabbit plasma sample (0.5 ml) was shaken with 50 µl of chlorpromazine hydrochloride (internal standard, 10 µg/ml) and 10 ml of dichloromethane for 1 hour. Then it was centrifuged at 2000 rpm for 20 min. The organic layer was separated and was evaporated to dryness. The residue was dissolved in 200 µl of mobile phase and aliquot of 100 µl was injected into the HPLC system.

4.6.2.6 *Extraction efficiency*

The extraction efficiency was calculated by adding known amount of ondansetron hydrochloride (50, 100 and 500 ng/ml, n = 5 per concentration) or internal standard (1000 ng/ml, n = 5) to 0.5 ml of blank rabbit plasma. The ondansetron hydrochloride was extracted into 10 ml of dichloromethane. The separated organic layer was evaporated to dryness and residue was reconstituted with mobile phase. The known amount of aqueous layer was injected into the chromatographic system. The peak area was compared to those obtained from equivalent volumes of standard solution of ondansetron hydrochloride in mobile phase directly injected into the HPLC system. The determination of unextracted samples was performed in quadruplicate for each concentration.

4.6.2.7 *Specificity*

The specificity of the method was determined by comparing the chromatograms obtained from the samples containing ondansetron hydrochloride and internal standard with those obtained from blank plasma. Five blank plasma samples from six lots of rabbit plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

4.6.2.8 *Linearity and range*

The linear detector response for the assay was tested as follows. These determination (n=5) from minimum of five concentration levels (25, 50, 100, 200, 300, 500, 1000 ng/

ml) of the analyte were made. Detector response was correlated against analyte concentration by least squares regression. A weight of 1/y was used to determine slopes, intercept and correlation coefficients.

4.6.2.9 *Precision and accuracy*

For the determination of intra-day and inter-day accuracy and precision of the assay, different aliquots of 0.5 ml rabbit plasma with 50 µl of 10 µg/ml of internal standard and various quantities of ondansetron hydrochloride to yield 25, 100, 200, 300, 500, 1000 ng/ml. Precision was calculated as inter and intra-day coefficient of variation [% C.V. = (S.D./mean) × 100]. Assay percentage recovery for ondansetron hydrochloride in plasma versus water was measured by comparing the peak heights. Spiked samples in both plasma and water were prepared as described above after centrifugation and the percentage recovery was determined.

4.6.3 Results and Discussion

System reproducibility, expressed as % R.S.D. and based on absolute peak height, was 4.2 and 1.6 % (n=8) for ondansetron hydrochloride at a concentration of 25 and 500 ng/ml, respectively. Mean reproducibility of the internal standard was 0.7% (n=10) at 1000 ng/ml concentration in rabbit plasma.

The chromatograph of rabbit blank plasma and plasma spiked with ondansetron hydrochloride and internal standard is given in figure 4.17 and figure 4.18.

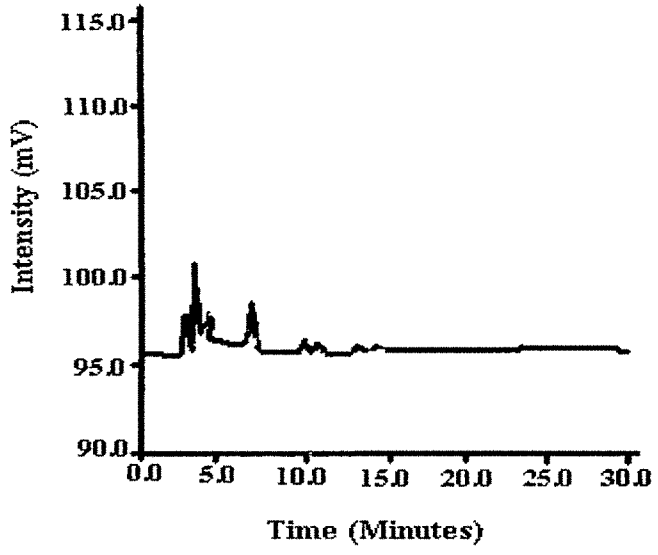


Figure 4.17. Chromatogram of blank rabbit plasma.

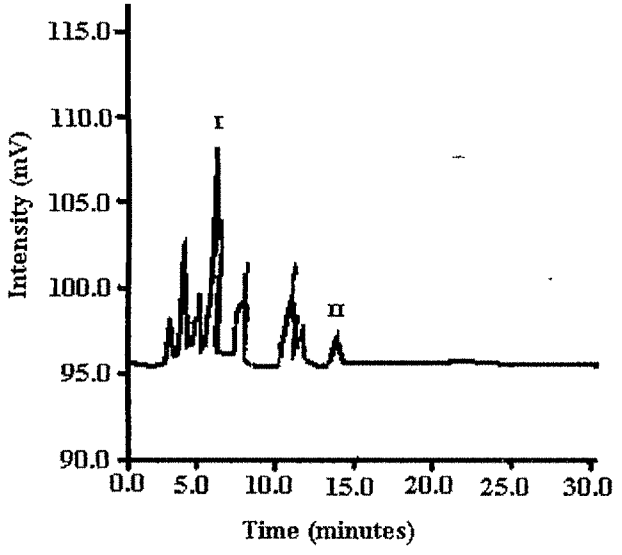


Figure 4.18. Chromatogram of extracted rabbit blood spiked with 100 ng/ml ondansetron hydrochloride (peak 1) and 1000 ng/ml internal standard (peak 2).

They showed that that there was no any interference from blank plasma peaks to peaks of drug and internal standard. The peaks of ondansetron hydrochloride and the internal standard were sufficiently separated with typical retention times of 4.96 minutes for

ondansetron hydrochloride and 13.23 minutes for internal standard. Usual running time for the sample was 18 minutes. The extraction recovery for ondansetron hydrochloride was 94.52 ± 6.1 %, 96.75 ± 4.73% and 96.18 ± 3.667 % at 50, 100 and 500 ng/ml concentrations, respectively. Mean extraction recovery of internal standard at 1000 ng/ml was 91.63 ± 2.38.

The calibration curve was linear over the concentration range 25-1000 ng/ml of ondnasetron hydrochloride in rabbit plasma with correlation coefficient $r^2 \geq 0.99$. Residuals were randomly distributed when plated against concentration. Table 4.16 shows linearity of calibration obtained on five separate days in one week.

Table 4.16. Linearity of calibration curve plots obtained in five separate days within 1 week.

Day	Slope	Intercept	r^2
1	0.001527	0.01334	0.9994
2	0.002184	0.00631	0.9952
3	0.002372	0.00948	0.9991
4	0.001942	0.01427	0.9983
5	0.001853	0.00825	0.9995
Mean	0.01976	0.01033	0.9983
Standard deviation	0.000323	0.003384	0.001606

Using regression analysis, the following equation was obtained from five calibration plots prepared three separate days within one week:

$$R = 0.01976 \times C + 0.01033$$

Where C (ng/ml) is the concentration of ondansetron hydrochloride in plasma, and R is the peak area ratio (ondansetron hydrochloride /internal standard). The intercept and slope were mean values.

The limit of detection (LOD) for this assay was 10 ng/ml. The limit of quantification (LOQ) of the method was 25 ng/ml. Intraday precision was determined by calculating the % RSD for five determinations at each concentration of four samples and was found to be between 2.52-4.71 %. Intraday accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 96.92 % (table 4.17).

Table 4.17. Intraday precision and accuracy for the ondansetron hydrochloride assay in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b] (%) ± S.D.
Actual	Observed ± S.D		
25	24.23 ± 4.82	4.71	96.92 ± 4.82
100	98.42 ± 2.52	1.24	98.42 ± 2.53
300	297.83 ± 7.81	2.63	99.27 ± 2.60
500	504.74 ± 5.27	1.06	100.94 ± 1.05
1000	1007.62 ± 8.35	0.83	100.76 ± 0.8

[a] Expressed as relative standard deviation

[b] Expressed as [(mean observed concentration/actual concentration) × 100]

Interday precision and accuracy were assessed by assaying four samples of different concentrations on five consecutive days. Interday precision for ondansetron hydrochloride ranged between 3.12-7.22 %. Interday accuracy, assessed by calculating the estimated concentrations as a percent of the nominal concentrations, was better than 96.04 % (table 4.18).

Table 4.18. Interday precision and accuracy for the ondansetron hydrochloride assay in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b] (%) ± S.D.
Actual	Observed ± S.D.		
25	24.01 ± 1.25	5.22	96.04 ± 0.37
100	100.64 ± 3.63	3.61	100.64 ± 3.63
300	298.73 ± 4.95	1.66	99.57 ± 1.65
500	494.63 ± 6.03	1.22	98.92 ± 1.21
1000	1003.42 ± 4.21	0.42	100.34 ± 0.42

[a] Expressed as relative standard deviation

[b]Expressed as [(mean observed concentration/actual concentration) × 100]

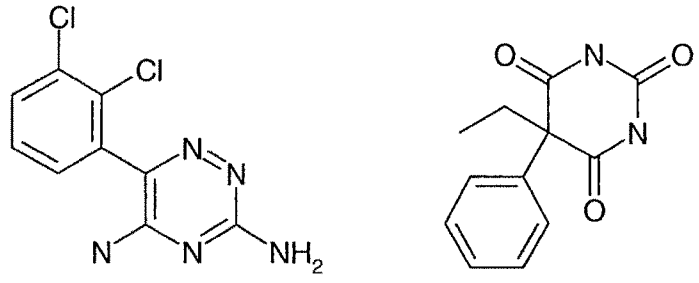
4.6.4 Conclusions

The described method is simple and rapid for the analysis of ondansetron hydrochloride from biological samples as simple extraction procedure is required. This proposed method can be recommended for pharmacokinetic studies in rabbits.

4.7 DETERMINATION OF LAMOTRIGINE IN PLASMA BY HPLC METHOD

4.7.1 Introduction

Rapid and automatic methods, widely used like immunoassay, are available for plasma determination of many antiepileptic drugs, such as phenobarbitone, phenytoin, and valproic acid which is not the case of lamotrigine. Several methods of assay have been reported for lamotrigine in biological fluids. These include HPLC[22-27], gas chromatography[28,29], spectrophotometry[30] and combinations of these techniques. The HPLC methods for determination of lamotrigine in plasma required adjustment of pH of mobile phase or complex extraction procedure or florescence or electrochemical detector. This paper describes a HPLC method with ultraviolet detector and simple extraction procedure for the assay of lamotrigine in rabbit plasma. A new, specific, rapid, and cost effective HPLC method has been developed for the determination of lamotrigine in rabbit plasma. The applicability of this method was demonstrated by the analysis of rabbit plasma containing 500 ng/ml of lamotrigine. Chemical structures of lamotrigine and internal standard are shown in figure 4.19.



Lamotrigine Phenobarbitone

Figure 4.19. Chemical structures of lamotrigine and phenobarbitone (internal standard).

4.7.2 Experimental

4.7.2.1 *Materials*

Lamotrigine and phenobarbitone (Internal standard) were received as gift samples from Relax Pharmaceutical Pvt. Ltd. (Baroda, India). HPLC grade acetonitrile, methanol, ether (analytical grade) were purchased from S. D. Fine Chemicals (Mumbai, India). In all experiments doubly-distilled water was used.

4.7.2.2 *Instrumentation*

The analysis was performed on a Perkin Elmer chromatographic system (Tokyo Japan) equipped with a LC 200 pump, PE Nelson 1020S integrator and UV detector. The mobile phase involved a mixture of water, methanol and acetonitrile (70:18:12 % v/v), pump at a flow rate of 1.0 ml/min through the column (Thermohypersil, 5µm RP C_{18}, 250 × 4.6 mm) at a room temperature (29 ± 2 °C). The samples were injected manually using a Rheodyne 7123 injector (Rheodyne, Cotai, CA) with a 100-µl loop. Peaks were monitored by UV absorbance at 220 nm.

4.7.2.3 *Standard solutions*

A stock standard solution was prepared in methanol and contained 1.00 mg/ml of lamotrigine. Working standard solutions were prepared by dilution of the stock standard solution with methanol to concentration of 1, 2, 3, 5, 7, 10 and 15 µg/ml. An internal standard stock solution was prepared in methanol and contained 1.00 mg/ml of phenobarbitone. A working solution was made by dilution of the stock solution with methanol to10 µg/ml.

4.7.2.4 *Calibration standard solution*

Rabbit blood containing heparin as an anti-coagulant was used for the preparation of calibration standard. Calibration standard solutions were freshly prepared in rabbit

plasma by adding appropriate aliquots of working standard solutions to the 0.5 ml of plasma to yield concentration of 100, 200, 300, 500, 700, 1000, 1500 ng/ml.

4.7.2.6 *Plasma extraction procedure*

To 0.5 ml of plasma sample, 20 µl of internal standard (100µg/ml) was added. The solution was mixed for 2 minutes and was extracted with 5 ml ether. The separated organic layer was evaporated to dryness and was reconstituted with mobile phase. The mobile phase of 100 µl was injected into the HPLC system.

4.7.2.7 *Extraction efficiency*

The extraction efficiency was calculated by adding known amount of lamotrigine (200, 500 and 1000 ng/ml; n = 5 per concentration) to 0.5 ml of blank rabbit plasma. The lamotrigine was extracted with 5 ml ether. The organic layer was evaporated to dryness and was reconstituted with mobile phase. The known amount of mobile phase was injected into the chromatographic system. The peak area was compared to those obtained from equivalent volumes of standard solution of lamotrigine in mobile phase solution directly injected into the HPLC system. The determination of unextracted samples was performed in quadruplicate for each concentration.

4.7.2.8 *Specificity*

The specificity of the method was determined by comparing the chromatograms obtained from the samples containing lamotrigine and internal standard with those obtained from blank plasma. Five blank plasma samples from six lots of rabbit plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

4.7.2.9 *Linearity and range*

The linear detector response for the assay was tested as follows. These determination (n = 5) from minimum of five concentration levels (100, 200, 300, 500, 700, 1000, 1500

ng/ml) of the analyte were made. Detector response was correlated against analyte concentration by least squares regression. A weight of 1/y was used to determine slope, intercept and correlation coefficient.

4.7.3.10 *Accuracy and precision*

For the determination of intra-day and inter-day accuracy and precision of the assay, different aliquots of 0.5 ml rabbit plasma with 20 µl of 100 µg/ml of internal standard and various quantities of lamotrigine to yield 100, 200, 300, 500, 700, 1000, 1500 ng/ml. Precision was calculated as inter and intra-day coefficient of variation [%CV = (SD/mean)×100]. Assay percentage recovery for lamotrigine in plasma versus water was measured by comparing the peak heights. Spiked samples in both plasma and water were prepared as described above after centrifugation and the percentage recovery was determined.

4.7.4 Results and Discussion

The present assay is sensitive, reproducible and convenient. The liquid-liquid extraction method used is more convenient and cost-effective than the solid-phase extraction procedure. System reproducibility, expressed as % R.S.D. and based on absolute peak height, was 4.86 and 1.39 % (n=8) for lamotrigine at a concentration of 50 and 700 ng/ml, respectively. Mean reproducibility of the internal standard was 0.92 % (n=10) at 1000 ng/ml concentration in rabbit plasma.

A mobile phase composed of 0.01M phosphate buffer (70%), methanol (18%) and acetonitrile (12%) gave well resolved, sharp peaks for lamotrigine and internal standard (phenobarbitone) with a retention time of 13.24 minute and 18.55 minutes, respectively. All peaks were baseline resolved and clear of interference from endogenous components in rabbit plasma. The analytical run time was found to be 25 minutes. The chromatograph of rabbit blank plasma and plasma spiked with lamotrigine and internal standard is given in figure 4.20 and figure 4.21.

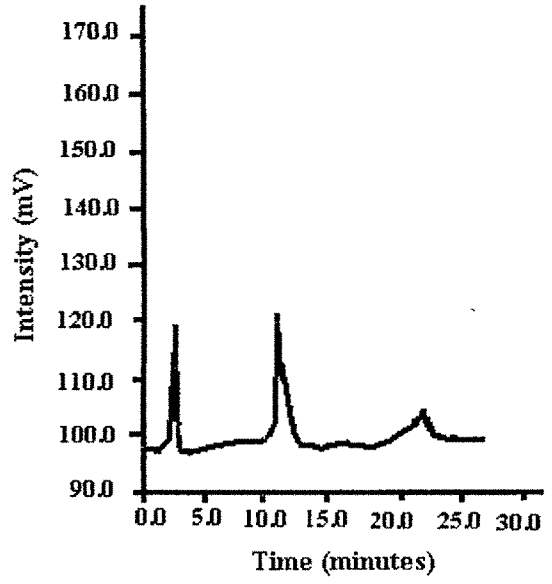


Figure 4.20. Chromatogram of blank rabbit plasma.

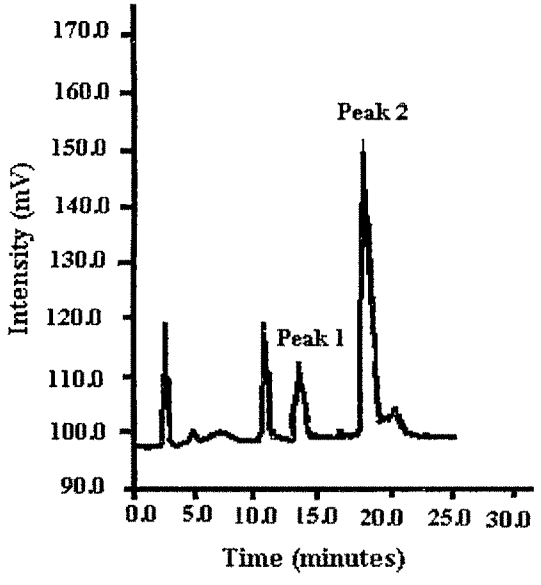


Figure 4.21. Chromatogram of 1000 ng/ml lamotrigine (peak 1) and 10 µg/ml internal standard (peak 2).

They showed that that there was no any interference from blank plasma peak to peak of drug and internal standard. The quantification of the chromatogram was performed using the ratio of the area of lamotrigine peak to area of internal standard peak.

The peak area ratio of drug to internal standard was plotted versus drug concentration. It was linear over 100-1500 ng/ml. The mean (n=5) calibration curve for lamotrigine was y = 0.0003x – 0.0256, r^2 = 0.9947 where, y and x are the peak height ratio and concentration (ng/ml), respectively. The extraction efficiency was greater than 80.00% which is shown in table 4.19.

Table 4.19. Extraction efficiency of lamotrigine from rabbit plasma at various concentration, (n=5).

Concentration (ng/ ml)	Extraction Efficiency ± S.D.
200	83.64 ± 5.8
500	78.72 ± 2.8
1000	80.28 ± 9.3
Average Extraction efficiency	80.88 ± 5.9

Limit of quantification and limit of detection were 100 ng/ml and 30 ng/ml, respectively based on 500µl of plasma. The intraday coefficient of variation (CV) ranged from 1.87 to 5.73 % and interday varied from 2.62 to 6.48 % which are shown in table 4.20 and table 4.21.

Table 4.20. Intraday precision and accuracy for the lamotrigine assay in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a] (%)	Accuracy[b](%)± S.D.
Actual	Observed ± S.D.		
100	100.18 ± 5.74	5.73	100.18 ± 5.74
200	201.15 ± 4.44	2.19	100.57 ± 4.40
300	298.20 ± 3.63	1.22	99.44 ± 1.21
500	503.72 ± 4.18	0.83	100.74 ± 0.83
1000	1005.61 ± 5.73	0.57	100.56 ± 0.57
1500	1511.87 ± 6.50	0.43	100.79 ± 0.43

[a] Expressed as % relative standard deviation

[b]Expressed as [(mean observed concentration/actual concentration) × 100].

Table 4.21. Interday variability of the lamotrigine assay over five consecutive days in rabbit plasma (n=5).

Standard concentration (ng/ml)		Precision[a](%)	Accuracy[b](%)
Actual	Observed		
100	98.33 ± 4.48	4.48	98.33 ± 4.48
200	202.72 ± 6.65	3.31	1.1.36 ± 6.65
300	301.51 ± 5.24	1.76	100.50 ± 1.74
500	510.67 ± 6.59	1.31	102.13 ± 1.31
1000	992.81 ± 7.84	0.78	99.28 ± 0.78
1500	1492.53 ± 3.77	0.25	99.50 ± 0.25

[a] Expressed as relative standard deviation

[b]Expressed as [(mean observed concentration/actual concentration) × 100]

The accuracy of assay was > 90 % and did not exceed 10 %. The lamotrigine stock solution was stable at least for 3 months when the solution was protected from light and kept at - 4°C. Assay percentage recovery for lamotrigine in plasma versus water was measured by comparing the peak heights. The results are shown in table 4.22.

Table 4.22. Recovery of lamotrigine from rabbit plasma (n=5).

Concentration (ng/ml)	Peak height ratio		Recovery (%) ± S.D.
	Plasma	Water	
100	0.097	0.13	74.61 ± 2.53
200	0.14	0.17	82.35 ± 3.48
300	0.15	0.18	83.33 ± 1.99
500	0.23	0.27	85.18 ± 0.63
1000	0.32	0.36	88.88 ± 0.47
1500	0.41	0.48	85.41 ± 0.08
Overall recovery of lamotrigine			83.29 ± 0.27

The Spiked samples in both plasma and water were prepared as described above after centrifugation. The percentage recovery was determined as follows

$$\% \text{ Recovery} = \frac{(\text{Peak height ratio of lamotrigine/phenobarbitone in plasma})}{(\text{Peak height ratio of lamotrigine/phenobarbitone in water})} \times 100$$

The % recovery of lamotrigine in plasma ranged from 74.61 % to 88.88 % which is satisfactory. The assay was simple, sensitive, precise and accurate for the determination of lamotrigine in the rabbit plasma.

4.7.5 Conclusions

The described method is simple, accurate, precise and rapid for the analysis of lamotrigine from biological samples as simple extraction procedure is required. There is

no need of utilizing the fluorescence detector. This proposed method can be recommended for routine patient monitoring and for pharmacokinetic studies.

4.8 REFERENCES

1. Tamisier-Karolak L, Delhotal-Landes B, Jolliet-Riant P, Milliez J, Jannet D, Barre J, Flouvat B, Plasma assay of salbutamol suphate by means of high performance liquid chromatography with amperometric determination using a loop column for injection of plasma extract. Application to the evaluation of subcutaneous administration of salbutamol, The Drug Minot, 14, 243-248, 1992.

2. Bland RE, Tanner RJ, Chen WH, Lang JR, Powell JR, Determination of albuterol concentration in human plasma using solid-phase extraction and high-performance liquid chromatography with fluorescence detection, J Pharm Biomed Anal, 8, 591-596, 1990.

3. Miller LG,Greenblatt DJ, Determination of albuterol in human plasma by high performance liquid chromatography with fluorescence detection, J Chromatogr, 381, 205-208, 1986.

4. Oosterhui B, Van Boxtel CJ, Determination of salbutamol sulphate in human plasma with bimodal high performance liquid chromatography and a rotated disc amperometric detector, J Chromatogr 32, 327-334, 1982.

5. McCarthy PT, Atwal S, Sykes AP, Ayres JG, Measurement of terbutaline and salbutamol sulphate in plasma by high performance liquid chromatography with fluorescence detection, Biomed Chromatogr, 7, 25-28, 1993.

6. Ong H, Adam A, Perreault S, Marleau S, Bellemare M, Du Souich P, Beaulieu N, Analysis of albuterol in human plasma based on immunoaffinity chromatographic clean-up combined with high-performance liquid chromatography with flourimetric detection, J Chromatogr, 497, 213-221, 1989.

7. Sagar KA, Kelly MT, Smuth MR, Simultaneous determination of salbutamol sulphate and terbutaline at overdose levels in human plasma by high performance

liquid chromatography with electrochemical detection, Biomed Chromatogr, 7, 29-33, 1993.

8. Hutchings MJ, Pauu JD, Morgan DJ, Determination of salbutamol sulphate in plasma by high performance liquid chromatography with fluorescence detection, J Chromatogr Biomed Appl, 28, 423-426, 1983.

9. Chen X, Zhao XL, Simple and rapid High performance liquid chromatographic determination of salbutamol concentration in human plasma with fluorescence detection, Yaowu Fenxi Zazhi, 18, 98-101, 1998.

10. Li. K, Yuan YS, Zhang JH, Tu X, Lu WL, High performance liquid chromatographic determination of albuterol in plasma and its pharmacokinetic parameters, Yaowu Fenxi Zazhi, 13, 389-392, 1993.

11. Boulton DW, Fawett JP, Determination of salbutamol sulphate in human plasma and urine by chiral high-performance liquid chromatography, J Chromatogr B Biomed Appl, 672, 103-109, 1995.

12. Tan YK, Soldin SJ, Determination of salbutamol in human serum by reversed phase high performance liquid chromatography with fluorescence detection, J Chromatogr, 311, 311-317, 1984.

13. Siluveru M, Stewart JT, Enantioselective determination of S-(+)- and R-(-)-ondansetron in human serum using derivatized cyclodextrin-odified capillary electrophoresis and solid extraction, J Chromatgr B: Biomed. Appl, 671, 217-222, 1997.

14. Wring SA, Rooney RM, Goddard CP, Waterhouse I, Jenner WN, A sensitive radioimmunoassay, combined with solid phase extraction, for the subnanogram per ml determination of ondansetron in human plasma, J Pharm Biomed Anal, 12, 361-371, 1994.

15. Bauer S, Stormet E, Kaiser R, tremblay PB, Brockmoller J, Roots I, Simultaneous determination of ondansetron and tropisetron in human plasma using HPLC with UV detection, Biomed Chromatogr, 16, 187-190, 2002.

16. Depot M, Leroux S, Caille G, High-resolution liquid chromatographic method using ultraviolet detection for determination of ondansetron in human plasma, J Chromatogr, 694, 399-406, 1997.

17. Colthup PV, Felgate CC, Palmer JL, Scully NL, Determination of ondansetron in plasma and its pharmacokinetics in the young and elderly, J Pharm Sci, 80, 868-871, 1991.

18. Clothup PV, Palmer JL, The determination in plasma and pharmacokinetics of ondansetron, Eur J Clin Oncol, 25, S71-74, 1989.

19. Hsyu PH, Pritchard JF, Bozigian HP, Lloyd TL, Griffin RH, Shamburek R, Krishna G, Barr WH., Comparison of the pharmacokinetics of an ondansetron solution (8 mg) when administered intravenously, orally, to the colon, and to the rectum, Pharm Res, 11, 156-159, 1994.

20. Rojanasthien N, Manorot M, Kumsorn B, Nawoot S, Teekachunhatean S, Sangdee C, Apisariyakul A, Pharmacokinetics and bioavailability studies of generic ondansetron, and the innovator preparation, in healthy Thai male volunteers, J Med Assoc Thai, 82, 713-720, 1999.

21. Rojanasthien N, Manorot M, Kumsorn B, Pharmacokinetics and bioequivalence testing of generic ondansetron preparations in healthy Thai male volunteers, Int J Clin Pharmacol Ther, 37, 548-554, 1999.

22. Barbosa NR, Midio AF, Validated high-performance liquid chromatographic method for the determination of lamotrigine in human plasma, J Chromatogr B Biomed Sci Appl, 741, 289-293, 2002.

23. Ren S, Scheuer ML, Zheng W, Determination of lamotrigine in biologic materials by a simple and rapid chromatographic method, Ther Drug Monit, 20, 209-214, 1998.

24. Matar KM, Nicholls PJ, Bawazir SA, Al-Hassan MI, Tekle AJ, A rapid liquid chromatographic method for the determination of lamotrigine in plasma, Pharm Biomed Anal, 17, 525-531, 1998.

25. Angelis-Stoforidis P, Morgan DJ, O'Brien TJ, Vajda FJ, Determination of lamotrigine in human plasma by high-performance liquid chromatography, Chromatogr B Biomed Sci Appl, 727, 113-118, 1999.

26. Croci D, Salmaggi A, de Grazia U, Bernardi G, New high-performance liquid chromatographic method for plasma/serum analysis of lamotrigine, Ther Drug Monit, 23, 665-668, 2001.

27. Castel-Branco MM, Almeida AM, Falcao AC, Macedo TA, Caramona MM, Lopez FG, Lamotrigine analysis in blood and brain by high-performance liquid chromatography, J Chromatogr B Biomed Sci Appl 755, 119-127, 2001.

28. Hallbach J, Vogel H, Guder WG, Determination of lamotrigine, carbamazepine and carbamazepine epoxide in human serum by gas chromatography mass spectrometry, Eur J Clin Chem Clin Biochem, 35, 755-759, 1997.

29. Watelle M, Demedts P, Franck F, De Deyn PP, Wauters A, Neels H, Analysis of the antiepileptic phenyltriazine compound lamotrigine using gas chromatography with nitrogen phosphorous detection, Ther Drug Monit, 19, 460-464, 1997.

30. Talekar RS, Dhake AS, Sonaje DB, Mourya VK, Spectrophotometric determination of lamotrigine. Ind J Pharm Sci, 62, 51-52, 2000.