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Simultaneous high-performance liquid chromatographic assay of furosemide and propranolol HCL and its application in a pharmacokinetic study

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Abstract

A practical, sensitive, selective and efficient reversed-phase high-performance liquid chromatographic (HPLC) method is reported for the determination of two commonly used antihypertensive drugs, furosemide and propranolol hydrochloride. The drugs were eluted through a Nucleosil C_{18} column with a mobile phase composed of 0.02 M potassium dihydrogen phosphate and acetonitrile (80:20, v/v) adjusted to pH 4.5 and the effluent from the column was monitored at 235 nm. The present method enabled simple and isocratic HPLC with UV detection of these drugs in raw materials and in pharmaceutical formulations. These procedures were also applied for the assay of furosemide in rabbits' plasma, using propranolol hydrochloride as an internal standard. The linear concentration range of the assay was 0.1–200 and 5–200 μg ml^{-1} for furosemide and propranolol hydrochloride, respectively. The inter and intra-day assay precision and accuracy showed reproducibility and good linearity ($r^2 > 0.99$). The method retained its accuracy and precision when applying the standard addition technique. The results obtained by applying the proposed method was statistically analysed and compared with those obtained by the reported methods.

Keywords: Simultaneous; Assay; Furosemide; Propranolol HCl; Pharmacokinetic; HPLC

1. Introduction

Furosemide is a potent and widely used diuretic in the treatment of edematous states associated with cardiac, chronic renal failure [1,2], hypertension, congestive heart failure [3,4] and cirrhosis of the liver [5], with structural formula shown in Fig. 1.

Various methods have been developed for the determination of furosemide [6], including titrimetric methods [7–9], spectrophotometric methods [10–12], colorimetric methods [13,14], and nuclear magnetic resonance method [15]. British Pharmacopoeia [7] and USP [8] described a titrimetric method for the assay of the drug in raw material, while spectrophotometric and high-performance liquid chromatographic (HPLC) methods for determination of the dosage forms, respectively.

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Several analytical methods for the determination of furosemide in plasma and urine have been published. These are spectrophotometric [16–18] and spectrofluorometric methods [19–22]. These methods suffer from lack of sensitivity and selectivity. Although gas chromatographic methods [23,24] are more sensitive, they require a laborious extraction procedure and derivatisation of the drug prior to analysis. Thin-layer chromatography was reported that allows the determination of furosemide in plasma [25]. A variety of HPLC techniques [26–32] were recently developed.

Propranolol hydrochloride, a non-selective β-blocker used in the treatment of hypertension, angina pectoris and cardiac arrthmias [33], has the structural formula shown in Fig. 1.

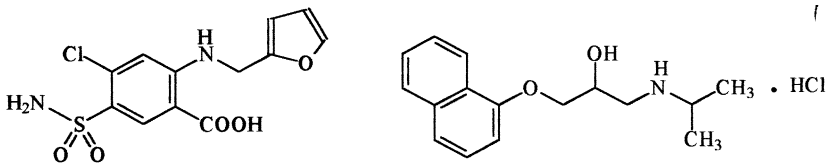
The BP [7] and USP [8] methods adopted an aqueous titrimetric method for the determination of the drug. Different methods have been developed for the determination of propranolol HCl, including spectrophotometric methods [34] and colorimetric methods [35–37]. Many chromatographic procedures are described for the assay of propranolol HCl. These include TLC [38], GC [39], HPLC [40–43] and capillary electrophoresis [44]. Conductometry [45] and osillopolarographic [46] methods were also reported.

The beta-adrenergic blocking drug, propranolol hydrochloride, and the diuretic, furosemide are frequently administered antihypertensive drugs. The methods available in the literature for the simultaneous determination of these drugs are either densitometry [47], or laborious HPLC [48,49]. In this work a rapid and specific improved high performance liquid chromatographic for determination of furosemide and propranolol HCl in one step that is applicable to pharmacokinetic studies is described.

2. Experimental

2.1. Samples

1) Furosemide batch no 213 was kindly supplied by Arab Co. for Pharmaceutical and Chemical industry, Cairo, Egypt. Its purity was found to be 100.38 ± 0.35 according to the acid base titrimetric BP method [7].
2) Propranolol HCl was obtained from El Kahira Pharmaceutical and Chemical industrial company, Cairo, Egypt. Its purity was found to be 99.78 ± 0.47 according to acid base titrimetric BP method [7].
3) Lasix tablets batch no. 10E13, Hoechst Orient, S.A.E., Cairo, Egypt, under licence from Hoechst AG Frankfurt (Main), Germany. It was labelled to contain 40 mg furosemide per tablet as the active ingredient and the following inactive materials: lactose, magnesium stearate, starch and talc.
4) Lasix ampoules batch no. 11E22, Hoechst Orient, S.A.E., Cairo, Egypt, under licence from Hoechst AG Frankfurt (Main), Germany. It was labelled to contain 40 mg furosemide per 4 ml ampoule.
5) Inderal 10 and 40 mg tablets batch no. 0011181 and 810089, respectively, El Kahira Pharmaceutical and Chemical industrial company, under licence from Zennica Pharmaceuticals, Cairo, Egypt. It was labelled to contain 10 and 40 mg propranolol HCl per tablet, respectively, plus lactose, magnesium stearate,



Furosemide,
mol. wt. = 330.7

Propranolol HCl,
mol. wt. = 295.8

Fig. 1. Furosemide, mol. wt. = 330.7. Propranolol HCl, mol. wt. = 295.8.

microcrystalline cellulose and stearic acid as inactive ingredients.

2.2. Materials

Acetonitrile, ethanol, methanol, ethyl acetate and ether (HPLC grade), Merck, Darmstadt, Germany.

Hydrochloric acid, potassium dihydrogen phosphate, Prolabo, France.

Sodium hydroxide, BDH, UK.

2.3. Animals

Adult male albino rabbits, weighing 2–2.5 kg, were used in this study.

2.4. Equipment

The HPLC system consisted of a Shimadzu LC-10 AD HPLC pump and a model SPD-10A Shimadzu UV-Visible detector connected to a Shimadzu C-R6A chromatographic integrator. The analytical column was a Nucleosil C18 (250×4.6 mm I.D., particle size 10 μm) from Phenomenex, Torrance, USA. The system was operated at ambient temperature, and the detector was operating at 235 nm and the sensitivity was set at 0.001 AUFS. The flow rate was isocratic at 3 ml min^{-1}.

2.5. Prepared solutions

The mobile phase was prepared by mixing 0.02 M KH_2PO_4 with acetonitrile, 80:20 v/v and the pH was adjusted to 4.5, using phosphoric acid.

2.6. Stock solution of propranolol HCl

1) Stock solution of propranolol HCl in distilled water at a concentration of 1 mg ml^{-1} was prepared.
2) Stock solution of propranolol HCl, as internal standard, was prepared in distilled water at a concentration of 10 mg ml^{-1}. This was diluted to contain 2500 μg ml^{-1} by distilled water.

2.7. Stock solution of furosemide

Stock solution of furosemide in ethanol at a concentration of 1 mg ml^{-1} was prepared. It should be protected from light. The solution should be discarded if it showed any discoloration.

Spiking solutions containing 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 μg of furosemide per 100 μl of ethanol, were prepared by serial dilution of the stock solution.

2.8. Procedure

2.8.1. Analysis of furosemide

2.8.1.1. Construction of in vitro calibration curve. Aliquot portions of the furosemide stock solutions (0.001, 0.005, ..., 2 ml) were transferred into 10-ml volumetric flasks. Ethanol was added to complete the volume to give final concentrations ranging from 0.1 to 200 μg ml^{-1}. A 20-μl aliquot from each of the previous solutions was analysed by HPLC and the corresponding area was determined. The linear regression equation was calculated between the area under the curve and the concentration of the drug in μg ml^{-1}.

2.8.1.2. Determination of furosemide in Lasix tablet. The contents of 20 tablets of Lasix were accurately weighed and powdered. The amount of powder equivalent to 40 mg of furosemide (equals to the content of one tablet) was taken, extracted with successive portions (3×10 ml) of ethanol and filtered in 100 ml volumetric flask. The extraction process was repeated until the volume was completed to the mark; then proceed as under construction of calibration curve.

2.8.1.3. Determination of furosemide in Lasix ampoule. The contents of three ampoules were thoroughly mixed, from which 4 ml were taken into 100 ml volumetric flask. The volume was completed to the mark with ethanol after complete mixing; then proceed as under construction of calibration curve.

2.8.2. Analysis of propranolol HCl

2.8.2.1. Construction of in vitro calibration curve. Aliquot portions of propranolol HCl stock solutions (0.05, 0.1, ..., 2 ml) were transferred into 10-ml volumetric flasks. Ethanol was added to complete the volume to give final concentrations ranging from 5 to 200 μg ml^{-1}. A 20-μl aliquot from each of the previous solutions was analysed by HPLC and the corresponding area was determined. The linear regression equation was calculated between the area under the curve and the concentration of the drug in μg ml^{-1}.

2.8.2.2. Determination of propranolol HCl in Inderal tablet. The contents of 20 tablets of Inderal (10 and 40 mg) were accurately weighed and powdered. The amount of powder equivalent to 10–40 mg of propranolol HCl (equals to the content of one tablet) was taken, extracted with successive portions (3×10 ml) of ethanol and filtered in 100 ml volumetric flask. The extraction process was repeated until the volume was completed to the mark. A 20 μl aliquot was analysed by HPLC and the concentration was determined as under construction of calibration curve.

2.8.3. Determination of furosemide and propranolol HCl in laboratory prepared mixtures

Transfer aliquot portions 0.1, 0.2, ..., 0.5 ml of furosemide from its stock solution (1 mg ml^{-1}) into series of 10 ml measuring flasks, add 0.5, 0.4, ..., 0.1 ml of propranolol HCl using its prepared stock solution (1 mg ml^{-1}) was added to the same flasks and complete to the mark with distilled water. Calculate the concentration of furosemide and propranolol HCl from the corresponding regression equations.

2.8.4. Determination of furosemide in rabbits plasma

2.8.4.1. Administration of the drug formula to rabbits. Furosemide as Lasix tablets was administered orally to six rabbits in its solid form by tube feeding, in a dose of 58.5 mg kg^{-1} body weight [27].

2.8.4.2. Blood sampling. Blood samples (3 ml) were withdrawn from the ear vein of the animals, prior to dosing and at 0.5, 1, 2, 3, 4, 6, 8 and 12 h after drug administration. The blood samples were taken into tubes washed with dilute heparin solution to guard against coagulation of blood. The blood samples were then centrifuged and the clear plasma was extracted and analysed.

2.8.4.3. Preparation of plasma samples for determination of furosemide. The extraction procedure of Straughen et al. [50] was adapted as follows:

1) 1 ml of rabbit plasma was transferred into a 15-ml culture tube (16×125 mm) fitted with a teflon-lined screw cap. 100 μl of propranolol HCl solution in distilled water as an internal standard (250 μg per 100 μl), 1.5 ml of acetonitrile and 2 ml of ethyl acetate were added to the sample.
2) For the calibration graphs, different amounts (0.1, 0.5, ..., 200 μg) of furosemide, as a methanol solution, were added at the beginning of the procedure to blank plasma. The calibration curve was obtained by plotting the ratio of the peak area of the analyte to that of the internal standard against the amount of the analyte added.
3) The sample mixture was agitated on a vortex mixer for 1 min after each addition, and then centrifuged for 10 min at $2370 \times g$.
4) The supernatant was collected and evaporated to dryness at 45 °C.
5) The residue was immediately dissolved in 100 μl of HPLC grade methanol, and 20 μl of this final solution was injected onto the liquid chromatograph for analysis. UV-detection at 235 nm was interpreted in the form of reported peak areas.
6) Concentrations of furosemide in unknown samples were calculated with reference to the prepared calibration curve.

2.9. Method validation

The limit of quantification (LOQ), recovery, day-to-day variation, within-day variation, accu-

racy and precision were calculated from the calibration curves. On three different days, three aqueous and three plasma calibration curves were made.

For determination of LOD plasma was spiked in triplicate with 2, 4, 6, 8 and 10 ng ml^{-1} of furosemide. The LOD was defined as the concentration at which the relative standard deviation (R.S.D.) of repeated analyses ($n = 3$) amounts to 20%.

3. Results and discussion

To optimise the HPLC conditions for the separation of furosemide, the effects of pH, mobile phase composition, the type of column and its dimension, the choice of an internal standard and wavelength of detection were investigated. The first factor examined was the type of the stationary phase. C_8 and C_{18} columns of different dimensions and particle sizes were used. It was found that C_{18} column (250 × 4.6 mm I.D.) with particle size 10 μm gave the most suitable resolution.

The mobile phase compositions were prepared with appropriate ratios of either phosphate or acetate buffer and methanol or acetonitrile. The adjustment of pH was performed using drops of acetic or phosphoric acid, respectively, to achieve a pH range of 3–5.5. It was shown that the best resolution of furosemide was achieved with a mobile phase composed of 0.02 M potassium dihydrogen phosphate and acetonitrile (80:20, v/v) with pH 4.5 at a flow rate of 3 ml min^{-1}. The high buffer concentration was found to be necessary to ensure a good separation between the drug and the internal standard. At a lower buffer concentration (0.01 M KH_2PO_4), the separation of these compounds and even their order of elution was found to be varied.

Increase of pH resulted in shorten the retention time and vice versa. This is understandable since the carboxylic acid of furosemide has a pK_a of about 3.6 [26] and approximately at pH of 4.5, the ionisation of carboxylic acid group would be sufficient. The use of acetate buffer and methanol or acetonitrile showed an elevated base line and worsens the resolution of the drug.

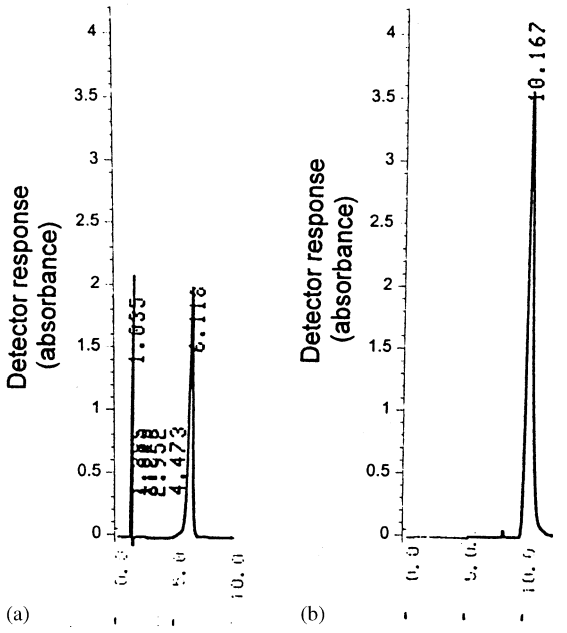


Fig. 2. HPLC chromatograms of (a) furosemide (b) propranolol HCL

Several internal standards have been tried. Propyl paraben and caffeine citrate showed peaks adjacent to plasma peaks. Amitreptyline HCl and nicardipine HCl did not separate at all. Ceftazidime gave bad resolution. Butenamide and chlorophenaramine maleate separated very close to the drug with no base line separation. Propranolol HCl was eluted with optimum resolution factor, about 9.9.

In an attempt to increase the sensitivity of the assay, different wavelengths, 235, 254 and 280 nm, for detection were utilised. The highest sensitivity with minimum interference for furosemide was accomplished at 235 nm. While there was no reasonable difference between these wavelengths for propranolol HCl, adjustment of the detector at 235 nm made the simultaneous determination of propranolol HCl and furosemide possible.

Literature survey revealed that two methods for precipitation of proteins can be utilised, using either 6 M hydrochloric acid [27] or acetonitrile [31]. The use of acetonitrile was preferred as hydrochloric acid extract showed abnormal brown

Table 1
HPLC determination of pure samples of furosemide

Experiment number	Taken (μg ml^{-1})	Found (μg ml^{-1})[a]	Accuracy (%)	R.S.D. (%)	
				Intra-day	Inter-day
1	10	9.95	99.50	2.49	2.21
2	30	30.12	100.04	1.79	1.91
3	50	50.16	100.32	2.42	2.30
4	80	80.36	100.45	2.12	2.41
5	100	100.35	100.35	1.77	1.81
6	150	151.45	100.97	1.82	2.11
Mean ± S.D.			100.27 ± 0.49		

[a] Average of three determinations.

Table 2
HPLC determination of pure samples of propranolol HCl.

Experiment number	Taken (μg ml^{-1})	Found (μg ml^{-1})[a]	Accuracy (%)	R.S.D. (%)	
				Intra-day	Inter-day
1	10	10.05	100.50	2.33	2.22
2	20	20.11	100.55	2.61	2.09
3	50	50.17	100.34	2.44	2.16
4	70	70.26	100.37	1.91	2.70
5	100	100.46	100.46	2.19	1.97
6	150	149.51	99.67	1.43	2.07
Mean ± S.D.			100.32 ± 0.33		

[a] Average of three determinations.

Table 3
Determination of furosemide and propranolol HCl in laboratory-prepared mixtures by the suggested HPLC method

Mixture number	Furosemide			Propranolol HCl		
	Claimed taken (μg ml^{-1})	Found[a] (μg ml^{-1})	Found (%)	Claimed taken (μg ml^{-1})	Found[a] (μg ml^{-1})	Found (%)
1	10	10.07	100.70	50	49.72	99.44
2	20	20.13	100.65	40	39.79	99.48
3	30	30.18	100.60	30	30.13	100.43
4	40	40.11	100.28	20	20.17	100.85
5	50	50.31	100.62	10	9.92	99.20
Mean ± S.D.			100.57 ± 0.17			99.88 ± 0.72

[a] Average of four determinations.

discoloration, which could be understand in the basis of unstability of the drug in acid media [51]. Ether or ethyl acetate can accomplish drug extraction from plasma. The high boiling point and clean chromatogram of ethyl acetate was in the side of using this solvent.

Fig. 2 shows the chromatograms of pure furosemide (a) as well as propranolol HCl (b). The

Table 4
Application of standard addition technique to the analysis of furosemide and propranolol HCl by the suggested HPLC method

Product	Found[a] (%)	Pure added (μg ml^{-1})	Found[a] (μg ml^{-1})	Recovery (%)
Lasix tablet (40 mg) (batch no. 10E13)	100.35±0.64	10	10.01	100.10
		20	20.03	100.15
		30	30.11	100.37
		40	40.27	100.68
Mean±S.D.				100.33±0.26
Lasix ampule (10 mg per 4 ml) (batch no. 11E22)	100.18±0.66	10	9.97	99.70
		20	19.85	99.25
		30	30.09	100.30
		40	40.22	100.55
Mean±S.D.				99.95±0.59
Inderal tablet (10 mg) (batch no. 0011181)	99.91±0.47	10	10.04	100.40
		20	20.13	100.65
		30	30.19	100.63
		40	40.31	100.87
Mean±S.D.				100.64±0.19
Inderal tablet (40 mg) (batch no. 810089)	99.56±0.33	10	10.11	101.10
		20	20.13	100.65
		30	30.08	100.27
		40	40.19	100.48
Mean±S.D.				100.63±0.35

[a] Average of four determinations.

Table 5
Statistical analysis of the results obtained by applying the proposed and reference methods [7] for the analysis of pure furosemide and propranolol HCl

Values	The proposed methods		Reference methods [7]	
	Furosemide	Propranolol HCl	Furosemide	Propranolol HCl
Range	0.1–200 μg ml^{-1}	5–200 μg ml^{-1}	250 mg	250 mg
LOQ	0.1 μg ml^{-1}	5 μg ml^{-1}	–	–
LOD	0.04 μg ml^{-1}	1 μg ml^{-1}	–	–
Mean±S.D.	100.27±0.485	100.32±0.326	100.38±0.353	99.78±0.465
N	6	6	4	4
Variance	0.235	0.106	0.125	0.216
t (2.306)[a]	0.201	0.143	–	–
F (9.01)[a]	1.880	2.038	–	–

[a] The values in parenthesis are corresponding to the theoretical values of *t* and F at ($P = 0.05$).

quantitative determination of furosemide and propranolol HCl by applying the suggested HPLC procedure, Fig. 2, is valid in concentration range from 0.1 to 200 and 5 to 200 μg ml^{-1}, respectively. The linear regression equations were found to be

$y = 24.8100x + 0.4600$ for furosemide

$y = 5.0497x + 3.6203$ for propranolol HCl

Where "y" is the area under the curve and "x" is the concentration of the drug in μg ml^{-1}, with correlation coefficients 0.9997 and 0.9995, respectively. Different blind authentic samples were determined using the suggested HPLC procedures.

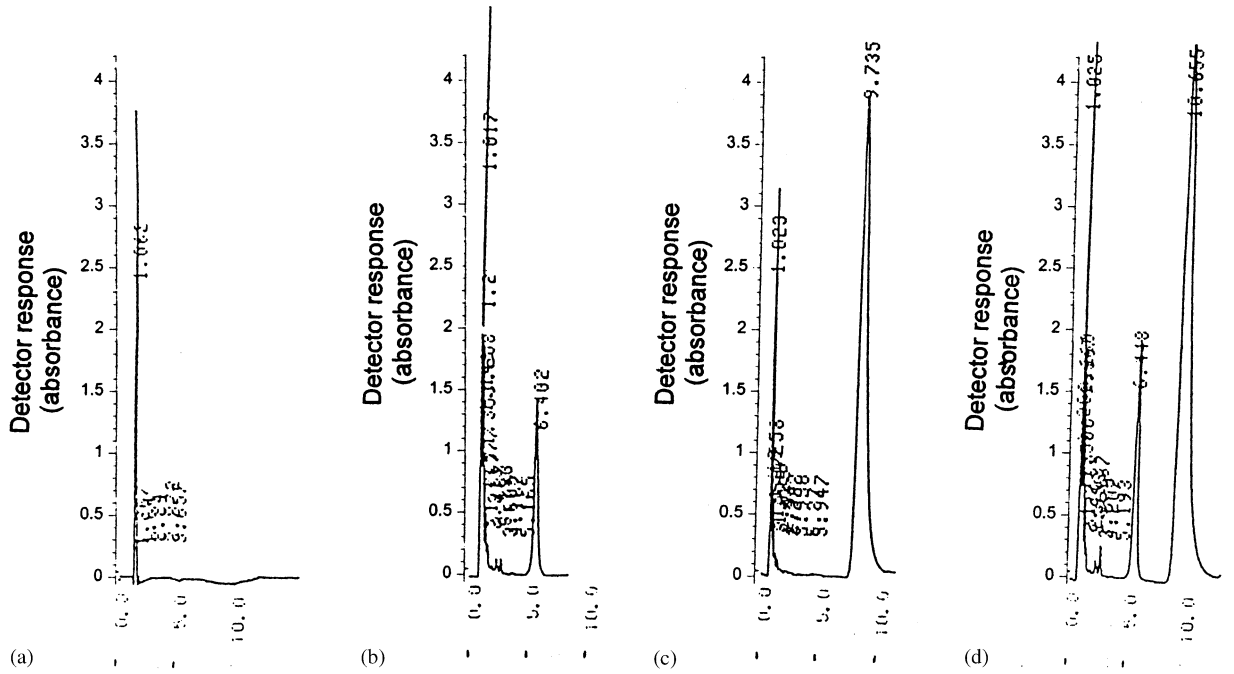


Fig. 3. HPLC chromatograms of (a) blank plasma (b) blank plasma spiked with furosemide (c) blank plasma spiked with propranolol HCL as internal standard (d) furosemide in rabbit plasma spiked with propranolol HCL as internal standard.

Table 6
Recovery of furosemide from spiked plasma samples

Taken (ng ml^{-1})	Mean found (μg ml^{-1})	Recovery (%)	S.D.	C.V. (%)
5	4.92	98.40	0.326	4.31
10	9.83	98.30	0.911	4.12
20	19.45	97.25	1.241	4.91
50	50.97	101.94	1.903	4.51
100	102.89	102.89	1.149	1.32
150	148.32	98.88	1.732	1.67

Mean ± S.D. 99.61 ± 2.25.

Results are found to be reproducible, as shown in Tables 1 and 2, with mean percentage recoveries of 100.27 ± 0.49 and 100.32 ± 0.33 for furosemide and propranolol HCl, respectively.

Results obtained in Table 3 showed that the proposed method is valid and applicable for determination of furosemide and propranolol HCl simultaneously in different laboratory prepared mixtures with mean percentage recoveries of 100.57 ± 0.17 and 99.88 ± 0.72, respectively.

The validity of the HPLC method was further assessed by applying standard addition technique for the analysis of Lasix and Inderal tablets, Table 4.

Statistical analysis of the obtained results was carried out comparing the results of the suggested procedure to those of the official BP titrimetric method [7]. No significant difference was observed as regards accuracy and precision, as shown in Table 5. In addition, ruggedness of the HPLC

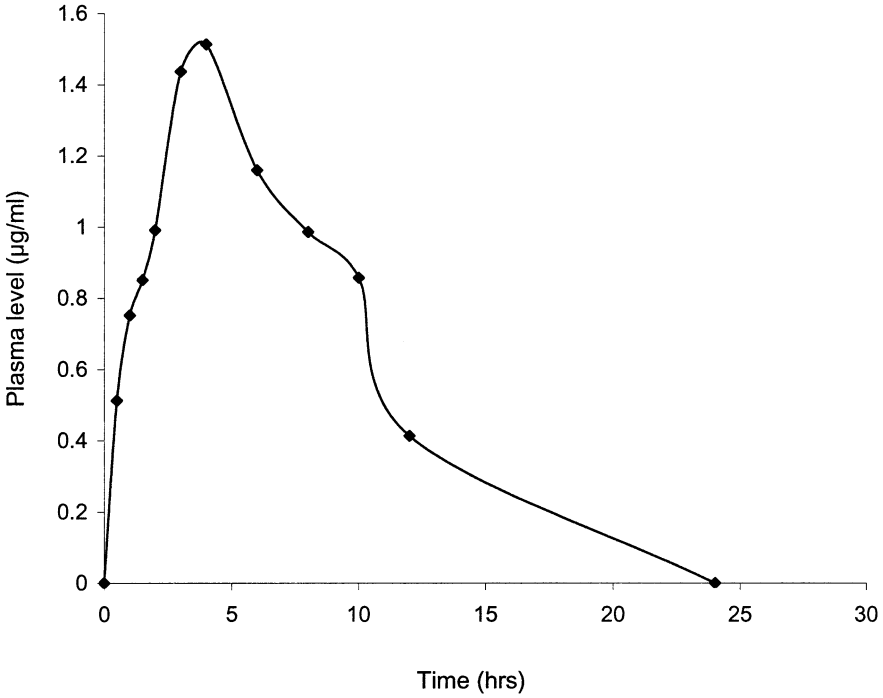


Fig. 4. Average plasma level of furosemide after administration of Lasix tablets to rabbits

method as a function of time, at room temperature, is carried out for different concentrations of the drug Tables 1 and 2. The assay was precise, as the R.S.D. was less than 3.00%.

A typical chromatogram for 1 ml of blank rabbit plasma is shown in Fig. 3. Representative chromatogram for 1 ml of blank rabbit plasma after 0.5 h from administration of furosemide spiked with propranolol HCl (internal standard) at level of 100 ng and processed by this procedure is shown in Fig. 3. Good separation of furosemide and internal standard from each other and from other components was obtained.

The quantitative determination of furosemide in rabbit plasma using propranolol HCl as internal standard by applying the suggested HPLC procedure, Fig. 3, is valid in concentration range from 0.1 to 200 μg ml^{-1}. The linear regression equations were found to be

$$y = 3.7500x + 0.0700$$

Where "y" is the ratio of the peak area of the analyte to that of the internal standard and "x" is the concentration of the drug in μg ml^{-1}, with a correlation coefficient 0.9997. Data for the recovery of furosemide from samples of plasma fortified with the drug in the range of 0.1–200 μg ml^{-1} are shown in Table 6. The low coefficient of variation ($\leq 5\%$) provides evidence of the reproducibility of the method. Fig. 4 shows the time course of plasma concentrations of furosemide after administration of Lasix tablet.

Samples were usually analysed immediately after preparation (same day), therefore, an explicit study of sample stability was not carried out. Aliquots of few samples were stored in dark at −5 °C for not more than 3 days and reanalysed did not show any deterioration, as far as their chromatographic profile is concerned.

The analytical validation of the method was performed concurrently to sample analysis. The assay parameters LOQ and LOD are given in Table 5. The intra-assay accuracy and precision was evaluated by preparing and analysing three

series of calibrators in the range. The quantitative determination of furosemide and propranolol HCl by applying the suggested HPLC procedure, Fig. 2, is valid in concentration range from 0.1 to 200 and 5 to 200 μg ml^{-1}, respectively. Inter-assay accuracy and precision was evaluated from the three calibration curves, each associated with many study samples, prepared and analysed within a 3 days period, Tables 1 and 2.

In Summary, numerous methods have been described for the analysis of furosemide and propranolol HCl, but only few are reported for the simultaneous analysis of both drugs. These procedures are either in accurate TLC procedures or HPLC one, which required tedious and time consuming sample preparation. The suggested procedures are more simple and accurate. It could be considered for routine analysis of furosemide and propranolol HCl in raw materials, pharmaceutical formulations and biological assay.

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