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Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 624–630
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous determination of naproxen, ketoprofen and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation

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Received 18 October 2004; received in revised form 10 April 2005; accepted 11 April 2005
Available online 17 May 2005

Abstract

A simple reversed-phase high performance liquid chromatographic method with UV detection at 270 nm was developed for simultaneous quantitation of ketoprofen and naproxen sodium along with phenol red as a non-absorbable marker for in situ permeability studies. The mobile phase was a mixture of 20% methanol, 28% of acetonitrile, 52% water and 0.4 ml triethylamine (adjusted to pH 3.2 using orthophosphoric acid). Analysis was run at a flow of 1.5 ml/min with a 20 min run time. The calibration curves were linear for all three compounds ($r > 0.999$) across the concentration range of 15.6–250 μg/ml with a limit of quantitation of 0.3, 0.25 and 0.2 ng/ml for naproxen, ketoprofen and phenol red, respectively. The coefficient of variation for intra-assay and inter-assay precision was less than or equal to 5.3% and the accuracy was between 95.36 and 101.6%. Using the SPIP technique and the suggested HPLC method for sample analysis, the mean values of $1.17e^{-4}$ (± 0.28) cm/s and $0.97e^{-4}$ (± 0.2) cm/s were obtained for naproxen and ketoprofen, respectively.

Keywords: Ketoprofen; Naproxen; Phenol red; Liquid chromatography; HPLC; Permeability; Single pass intestinal perfusion; Regional in situ perfusion

1. Introduction

Naproxen and Ketoprofen are well-known non-steroidal anti-inflammatory drugs which are clinically used in treatment of rheumatoid arthritis and other painful musculoskeletal disorders. Oral administration is the most convenient and useful route for drug delivery and prediction of drug absorption is therefore, very important for the design of an oral preparation. Human in vivo studies using earlier intestinal perfusion techniques have been applied for several decades to investigate absorption and secretion processes, motor activity and gastric emptying. The reproducibility of the data obtained using these techniques are reduced by several factors; therefore, new experimental techniques using a multichannel tube with two inflatable balloons have been developed [1–4]. In 1992, Lennernas et al. evaluated the potential of the new perfusion technique as a tool for investigation of drug absorption mechanisms in the human intestine [3]. Moreover, a segmental steady-state perfusion technique has been carried out by Gramatte and Richter in 1993 using a triple lumen tube to investigate the site-specific absorption of paracetamol in human small intestine [5]. Although human in vivo studies would be the most desirable, they seem difficult and rather time consuming. Therefore, some experimental methods, such as animal in vivo and ex vivo models have so far been evolved to estimate gastrointestinal absorption of drugs [6–11]. One of the most used classic techniques employed in the study of intestinal absorption of compounds has been the single-pass intestinal perfusion (SPIP) model

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doi:10.1016/j.jpba.2005.04.008

[12,13], which provides experimental conditions closer to what is faced following oral administration. This technique has lower sensitivity to pH variations because of a preserved microclimate above the epithelial cells and it maintains an intact blood supply to the intestine [14,15]. Because water absorption and secretion during the perfusion may cause errors in the calculated P_{eff} values, a non-absorbable marker to correct water flux is needed [13]. For this purpose phenol red is co-perfused with drug compounds. It was first introduced as a non-absorbable marker by Gorham in 1923 [16].

Since the membrane permeability for passively absorbed compounds, such as NSAIDs [17] is a function of the partition coefficient and pK_a of the compound [6], the combination of naproxen and ketoprofen together with phenol red as a marker can be perfused through the intestinal segment in an experiment to determine intestinal permeabilities at the same time. Therefore, simultaneous determination of these two drugs and phenol red is meaningful. Various HPLC methods have been reported to determine NSAIDs using different mobile phases and wavelengths for each drug [18–21]. In addition there are several methods available to separate NSAIDs by HPLC simultaneously [22–28] which some of them lack suitable sensitivity. However, there is still no method available for simultaneous determination of a NSAID and a non-absorbable marker, such as phenol red in biologic and non-biologic samples.

The objective of this study was to develop and validate a simple RP-HPLC method for simultaneous quantitation of naproxen and ketoprofen in the presence of phenol red.

2. Experimental

2.1. Chemicals

Naproxen, ketoprofen and Phenol red were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were HPLC grade and obtained from Merck (Darmstadt, Germany). KH_2PO_4, NaH_2PO_4, Na_2HPO_4, orthophosphoric acid, NaOH, NaCl and triethylamine were purchased from Merck (Darmstadt, Germany). Double distilled water was used during the entire HPLC procedure.

2.2. Apparatus

A liquid chromatographic system (Beckman, USA) comprising of 126 gold solvent delivery module equipped with a Rheodyne (Cotati, CA) injector and a variable wavelength ultraviolet spectrophotometric detector (166 gold) set at 270 nm. Analytical column used for chromatographic separations was Shimpack VP-ODS 5 μm 4.6 mm × 250 mm with a Shimpack VP-ODS 5 μm 4.6 mm × 50 mm precolumn guard column. System Gold software was used for data acquisition and System Gold nouveau software was used for data reporting and analysis.

2.3. Chromatographic conditions

The mobile phase was a mixture of 20% methanol, 28% of acetonitrile, 52% water and 0.4 ml triethylamine (adjusted to pH 3.2 using orthophosphoric acid). The mobile phase filtered through sintered glass filter P5 (1–1.6 μm) (Winteg, Germany) and degassed in sonicator (Liarre, Italy) under vacuum. The mobile phase was pumped in isocratic mode at a flow rate of 1.5 ml/min at ambient temperature. The UV detection was accomplished at 270 nm and samples of 20 μl were injected using Hamilton injector syringe onto column.

2.4. Composition of perfusion solution

The perfusion buffer consisted of 5.77 g/L Na_2HPO_4 (anhydrous), 4.085 g/L $NaH_2PO_4{\cdot}2H_2O$ and 7g/L NaCl. The pH of buffer was 7.2. Preliminary experiments showed that no considerable adsorption of the compounds on the tubing and syringe took place. Samples from perfusion study were filtered and directly injected onto HPLC column and required no sample preparation prior to analysis.

2.5. Preparation of standard solutions

Primary stock solution of all three compounds was prepared in phosphate buffered saline (PBS) to obtain a concentration of 2.5 mg/ml of naproxen and phenol red and 2 mg/ml of ketoprofen. Then it was diluted ten times to make a secondary stock solution and standards for calibration curves and quality control samples were prepared using serial dilution of the secondary stock solution in PBS. The concentration range for working standard solutions was 15.6–250 μg/ml for naproxen and phenol red and 12.5–200 μg/ml for ketoprofen. Preliminary studies showed that there is no chemical interactions and stability problem in the solution for all components.

2.6. In situ permeation studies

In situ permeation studies were performed using established methods adapted from the literature [29,30]. Briefly, male Wistar rats (weight, 250–300 g; age, 7–9 weeks) were maintained on 12 h light–dark cycle and fasted 12–18 h before experiment. However, drinking water was readily accessible. The rats were anaesthetized using an intraperitoneal injection of pentobarbital (60 mg/kg) and placed on a heated pad to keep normal body temperature. By making a midline abdominal incision, a 10 cm section of the proximal rat jejunum was located gently rinsed with saline (37 °C) and attached to the perfusion assembly. Care was taken to handle the small intestine gently and to minimize the surgery in order to maintain an intact blood supply. The entire surgical area was then covered with Parafilm to reduce evaporation Blank perfusion buffer was infused for 10 min by a syringe pump (Palmer, UK) followed by perfusion of compounds (0.19 and 0.99 mM for ketoprofen and naproxen, respectively)

at a flow rate of 0.2 ml/min for 90 min. The perfusate was collected every 10 min in microtubes. The length of segment was measured at the end and finally the animal was euthanitized with a cardiac injection of saturated solution of KCl. Samples were stored at −20 °C until analysis. In all animal studies "Guide to the care and use of experimental animals" by Canadian Council on Animal Care, was followed [31].

3. Results and discussion

3.1. Chromatography and specificity

In Fig. 1 the representative chromatogram of a sample containing phenol red, ketoprofen and naproxen is presented. The retention times were 3.9, 17.2 and 19 min for phenol red, ketoprofen and naproxen, respectively. The chromatographic run time of 20 min was sufficient for sample analysis that allows to analyze large number of samples in a short period of time. Injection of blank PBS buffer onto HPLC column represented that no peak could be seen on chromatogram.

3.2. Linearity

The six-point calibration curves for phenol red and naproxen were prepared in the range of 15.6–250 μg/ml. The respected range was 12.5–200 μg/ml for ketoprofen. These concentration ranges were selected based on drug concentration used in permeability studies. Since in each experiment we have already an inlet solution with defined concentration of compounds, therefore the external standard method has been used. The concentration-peak area relationships were described by a simple regression analysis. The minimum correlation coefficient of the calibration curves for three substances was 0.999. Standard curves were prepared on four consecutive days and regression parameters are listed in Table 1.

3.3. Accuracy and precision

Four quality control samples with concentrations within calibration range were used in triplicates ($n = 3$) to determine the accuracy and precision of the method. The samples were prepared in PBS. The mean observed concentrations for all three compounds from the prepared samples were calculated. The repeatability (intra-assay precision) and the intermediate (between-assay) precision were calculated from data obtained during 4 day validation. Results are shown in Tables 2 and 3, respectively.

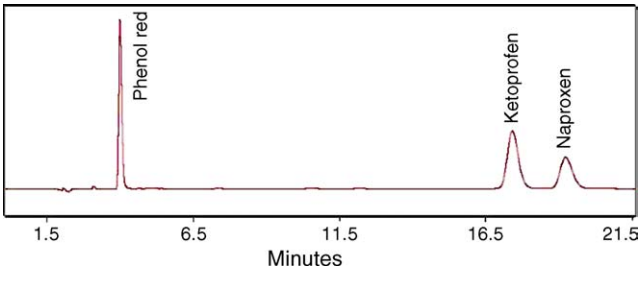

Fig. 1. Representative chromatogram of a sample from intestinal perfusion containing naproxen, ketoprofen and phenol red. For chromatographic conditions refer to Sections 2.2 and 2.3.

3.4. Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method; LOQ is the minimum quantifiable concentration. The signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ, respectively, which were calculated using Gold nouveau software, and then confirmed by taking dilutions from the secondary stock solution. The peaks were clearly identifiable and discrete and had acceptable precision. In the present study the LOQ values for phenol red, ketoprofen and naproxen were 0.2, 0.25 and 0.3 ng/ml, respectively, and the detection limits were found to be maximum 0.05 ng/ml.

3.5. System suitability tests

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. Parameters which are typically used in suitability evaluations are mentioned below [32]:

3.5.1. Capacity factor (k')

Capacity factor (retention factor) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column. It is defined as $k'_{(A)} = (t_A - t_0)/t_0$ in which t_A is the retention time of the compound and t_0 refers to retention time for an unretained compound. In the present work, t_0 was 2.38 min. For an optimum separation, retention factor should be in the range of $0.5 < k' < 10$. Calculated k' values were 0.64, 6.22 and 6.98 for phenol red, ketoprofen and naproxen, respectively.

3.5.2. Selectivity factor (α)

Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. For two components A and B it is defined as $\alpha = k'_A/k'_B$ (k' is the respective capacity factor). Therefore, it is the ratio of the relative retentions of the two compounds. In the present study the calculated selectivity parameter for separation of ketoprofen and phenol red was 9.7. The value of 1.12 was obtained for separation of phenol red and naproxen as well.

3.5.3. Resolution (R)

Resolution is a measure of the degree of separation between adjacent peaks. For two compounds A and B in a chromatographic run it is expressed as $R = 2(t_A - t_B)/(w_A + w_B)$ in which t_A and t_B are the retention times and w_A and w_B refers to the width of the base of the component peaks. A value of 1.5 for resolution implies a complete separation of

Table 1
Analytical parameters of calibration curves of ketoprofen, naproxen and phenol red

Regression equation[a]	Ketoprofen	Naproxen	Phenol red
Range	12.5–200 μg/ml	15.6–250 μg/ml	15.6–250 μg/ml
Slope ($b \pm$ S.D.)[b]	24211.6 (± 117)	17091 (± 469.3)	3247.8 (± 158.4)
Intercept ($a \pm$ S.D.)[b]	17842.6 (± 4580)	52512.6 (± 5732.7)	6442.6 (± 2740)
r (n)[c]	0.9998 (5)	0.9994 (5)	0.9997 (5)

The chromatographic conditions: Shimpack VP-ODS 5 μm 4.6 mm × 250 mm column, mobile phase mixture of 20% methanol, 28% of acetonitrile, 52% water and 0.4 ml triethylamine (adjusted to pH 3.2 using orthophosphoric acid), flow rate 1.5 ml/min, detection wavelength 270 nm and ambient temperature.

[a] Linear regression analysis with a regression equation of $y = a + bx$, in which x is the concentration in μg/ml and y is the peak area.

[b] S.D. is the standard deviation of intercept and slope.

[c] r is the correlation coefficient and n is the number of points in calibration curves. Each point is the mean of four measurements.

Table 2
Intra-assay precision obtained from four calibration curves with four levels of QC samples

Added concentration (μg/ml)	Mean measured concentration (μg/ml)	S.D. (μg/ml)	C.V. (%)	Accuracy (%)
Phenol red				
200	201.09	1.18	0.58	100.5
125	123.65	1.91	1.54	98.9
62.5	61.91	0.77	1.24	99.1
31.25	30.80	1.63	5.30	98.6
Ketoprofen				
160	160.85	0.77	0.48	100.5
100	99.10	1.13	1.14	99.1
50	49.58	0.57	1.15	99.2
25	23.84	0.48	2.01	95.4
Naproxen				
200	203.31	1.43	0.70	101.7
125	119.86	2.80	2.33	95.9
62.5	62.56	1.01	1.62	100.1
31.25	30.15	0.24	0.82	96.5

two compounds. In this work the resolution value for separation of ketoprofen and phenol red was 18.09. For separation of ketoprofen and naproxen the value was 1.63.

3.5.4. Column efficiency (number of theoretical plates)

In a particular separation, column efficiency refers to the performance of the stationary phase. It means how well the column is packed. There are several methods to measure the column efficiency which may or may not be affected by chromatographic anomalies, such as tailing or fronting. In the present study the number of theoretical plates was calculated using the following equation: $N = 16(t_R/w)^2$. For the column used in this study the N values of 1777, 3912 and 4773 obtained for phenol red, ketoprofen and naproxen, respectively. The height equivalent to theoretical plate (HETP) was found to be 0.14 mm, 0.064 mm and 0.052 mm, respectively.

Table 3
Inter-assay precision obtained from from calibration curves with four levels of QC samples

Added concentration (μg/ml)	Mean measured concentration (μg/ml)	S.D. (μg/ml)	C.V. (%)	Accuracy (%)
Phenol red				
200	200.19	0.78	0.39	100.1
125	124.70	1.13	0.90	99.8
62.5	63.00	0.42	0.67	100.8
31.25	30.18	0.40	1.33	96.6
Ketoprofen				
160	160.80	0.28	0.17	100.5
100	98.94	0.22	0.22	98.9
50	49.79	0.15	0.31	99.6
25	24.09	0.07	0.29	96.4
Naproxen				
200	201.96	0.61	0.30	100.1
125	121.91	1.25	1.03	97.5
62.5	62.46	0.31	0.49	100.3
31.25	30.16	0.09	0.32	96.7

3.5.5. Tailing factor (T)

Tailing factor refers to peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. Therefore, tailing factor should be calculated using the following equation for chromatographic peaks: $T = w_{0.05}/2f$ in which $w_{0.05}$ is the distance from the leading edge to the tailing edge of the peak measured at a point 5% of the peak height from the baseline and f is the time from width start point at 5% of peak height to retention time. A tailing factor of 1 refers to a symmetric peak. The calculated values of 1.03, 1.14 and 1 were obtained for phenol red, ketoprofen and naproxen peaks, respectively which are in the acceptable range of $0.5 \leq T \leq 2$.

3.6. Data analysis

Effective permeability coefficient (P_{eff}) was calculated from the steady-state concentrations of compounds in the perfusate collected. It was reached about 40 min after the beginning of the perfusion which is confirmed by plotting the ratio of the outlet to inlet concentrations (corrected for water transport) versus time. Representative results are plotted in Fig. 2. Permeability values are calculated using following equation according to the parallel tube model [33,34]:

$$P_{eff} = \frac{-Q \ln\{C_{out(corrected)}/C_{in}\}}{2\pi r l}$$

In which C_{in} is the inlet concentration and C_{out} is outlet concentration of compound which is corrected for volume change in segment using phenol red concentration in inlet and outlet tubing. Q is the flow rate (0.2 ml/min), r is the rat intestinal radius (0.18 cm) [33] and l is the length of the segment. It has been demonstrated that in humans at a Q_{in} of 2–3 ml/min, P_{eff} is membrane-controlled. In the rat model the Q_{in} is scaled to 0.2 ml/min, since the radius of the rat intestine is about 10 times less than that of human. The intestinal net water flux (NWF, μl/min/cm) was calculated according to:

$$\text{NWF} = \frac{(1 - [\text{Ph.red}_{(out)}/\text{Ph.red}_{(in)}])Q_{in}}{l}$$

where [Ph.red$_{(in)}$] and [Ph.red$_{(out)}$] are the inlet and outlet concentrations of the non-absorbable, water flux marker phenol red. A negative net water flux indicates loss of fluid from the mucosal side (lumen) to the serosal side (blood). A positive net water flux indicates secretion of fluid into the segment [17]. The determined P_{eff} and NWF values and concentrations used for compounds tested in the single pass intestinal perfusion are listed in Table 4. As it is seen the overall mean for NWF is low for both series of experiments. The stable water fluxes and P_{eff} values, with time, for tested compounds indicated that intestinal barrier function was maintained in all experiments. Any un-physiological leakage across the jejunal mucosa makes changes in the intestinal barrier function which is probably one of the main reasons for several of the contradictory results obtained by the in situ and in vitro models. In fact it is known that non-steroidal anti-inflammatory drugs increase intestinal permeability in a concentration-dependent manner; however, the concentrations used in the present study are lower than those of previous studies which have been reported to make permeability changes in rat intestine [35,36] which is confirmed by the low values of NWF (Table 4).

On the other hand, as an important aspect, the age-dependency of rat intestinal permeability should be noticed.

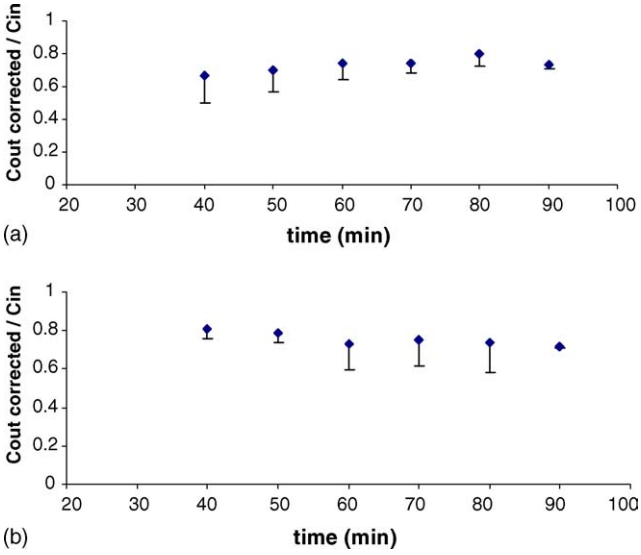

Fig. 2. Plot of concentration ratio of outlet and inlet concentrations vs. time for ketoprofen (a) and naproxen (b).

Table 4
Concentrations used for compounds tested in SPIP method and Intestinal permeability coefficients determined in rats

Compound	C_{in} (mM)	Rat no.	P_{eff} ($\times 10^4$) cm/s	Mean P_{eff} ($\times 10^4$) cm/s	Mean NWF (μl/min/cm)
Ketoprofen	0.19	Rat 1	0.77	0.97 (± 0.16)	1.12 (± 1.0)
		Rat 2	1.17		
		Rat 3	0.99		
		Rat 4	0.95		
Naproxen	0.99	Rat 1	0.89	1.17 (± 0.23)	0.91 (± 1.5)
		Rat 2	1.45		
		Rat 3	1.17		
		Rat 4	1.2		

Although an age-dependent intestinal permeability might be valid for very young and very old rats, there is not any influence of age on the jejunal permeability in the rat within the age interval of 5–30 weeks [37].

Previous studies have examined the permeability of these compounds using single-pass intestinal perfusion in rats. Salphati et al. reported the effective intestinal permeability coefficients of $1.09e^{-4}$ (± 0.71) cm/s and $1.67e^{-4}$ (± 0.82) cm/s for ketoprofen and naproxen, respectively [10]. The respected values according to Fagerholm et al. were $2.4e^{-4}$ (± 0.55) and $2.1e^{-4}$ (± 0.41) cm/s [17]. However, we determined the P_{eff} values of $0.97e^{-4}(\pm 0.16)$ and $1.17e^{-4}(\pm 0.23)$ cm/s for ketoprofen and naproxen, respectively. Both of these compounds belong to Class II of Biopharmaceutics Classification System (BCS). That means both of them are low soluble and high permeable drugs which are rapidly transported across the apical cell membrane of the human intestine. According to BCS, the human jejunal P_{eff} values for high-permeable drugs are considered to be within $1–10e^{-4}$ cm/s. The reported values by Lennernas et al. for ketoprofen and naproxen are $8.5e^{-4}$ and $8.0e^{-4}$ cm/s, respectively [17,38], and the fraction (%) of dose absorbed in human as reported in literature is 100% for both of compounds [17]. Moreover, compounds with an average P_{eff} of approximately $<0.3e^{-4}$ cm/s in human intestine are considered to be low-permeable compounds. Our preliminary studies indicate that corresponding estimates in rats for high and low-permeability are $>0.3e^{-4}$ and $<0.2e^{-4}$ cm/s, respectively. Determination of P_{eff} values using SPIP method in the rat intestine for large number of compounds with different physicochemical properties, which is in process in our lab, could provide a prediction of oral dose absorbed in human. Since human in vivo studies are difficult and time consuming, this prediction is of considerable interest at an early stage of drug development.

4. Conclusion

In permeability studies we need to quantitate samples obtained from experimental models, such as rat intestinal perfusion model in which a non-absorbable marker like phenol red is also used. A simple and reliable RP-HPLC method with acceptable precision, accuracy and linearity has been developed and validated for simultaneous determination of ketoprofen, naproxen and phenol red. The method also has a relatively short run time (20 min) that allows to quantify a large number of samples daily for all three compounds at the same time rather than separate analysis of three components.

Acknowledgment

The authors would like to thank Zahravi Co. Tabriz, Iran for their interest and supply of some consumables.

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