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Determination of loperamide in rat plasma and bovine serum albumin by LC☆

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Abstract

A rapid, isocratic liquid chromatographic (LC) method was developed for the determination of loperamide (Lop) in solutions of bovine serum albumin (BSA) and rat plasma. Prior to LC analysis, BSA solutions or rat plasma samples were treated with metaphosphoric acid to precipitate protein. Supernatant was directly injected onto a C_{18} reverse phase column and loperamide was monitored by a UV detector set at 195 nm. The concentrations of Lop in both rat plasma and BSA solution samples were determined by comparison with their calibration curves, which were generated from the peak area ratio of Lop to internal standard, clomipramine versus loperamide concentration. The calibration curves were linear in the range 0–3.0 μg ml^{-1} of Lop for the BSA solution sample and 0–1.0 μg ml^{-1} for the rat plasma sample. Overall recoveries of loperamide added to BSA and rat plasma samples were 101.4 and 95.5%, respectively. The method is simple (no extraction), rapid (22 min separation time), sensitive (the detection limit of loperamide is 50 ng ml^{-1} for the BSA solution sample and 100 ng ml^{-1} for the rat plasma sample), reproducible (within-day R.S.D. of 2.59–7.11%, among-day R.S.D. of 1.25–5.97%), and suitable for routine analysis of loperamide in rat plasma and BSA solution samples.

Keywords: Liquid chromatography (LC); Isocratic; Loperamide; Clomipramine; Rat plasma

1. Introduction

Since it was introduced in 1973, loperamide (Lop, Fig. 1) hydrochloride has been widely used as an effective drug for the control and symptomatic relief of acute non-specific diarrhea and chronic diarrhea associated with inflammatory bowel diseases [1]. Recently, Lop has received attention as an antihyperalgesic agent that reduces pain without any central nervous system (CNS) side effects [2]. Lop (designated ADL 2-1294) is now being developed by Adolor Corporation as a peripheral opiate agonist with selectivity for the μ subtype of the opioid receptor.

The determination of Lop in pharmaceutical products and biological fluids has been described and summarized in a review [3]. The most widely

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used analytical assays include HPLC [4–6] and spectrophotometry [4,7,8]. The spectrophotometric assay can be used only if Lop is the only component to form a colored complex during the measurement, or if Lop is in a simple matrix. However, in most situations solvent extraction is needed before quantitation of Lop. Although HPLC methods reported in literature give reasonable sensitivity, all have some limitations such as high volumes of plasma samples required, a time consuming, cumbersome and costly extraction procedure needed during the sample pretreatment, and gradient or long running time required for a satisfactory separation. The HPLC method presented in this paper provides a simple and rapid extraction pretreatment for determining Lop in rat plasma and in solutions of bovine serum albumin (BSA). The isocratic HPLC method with reasonable separation time gives a clean separation, free of interference from complicated matrices. Reliable analytical results are obtained by using an internal standard to compensate for experimental errors.

2. Experimental

2.1. Reagents

Loperamide (Lop) hydrochloride was purchased from Research Biochemicals (Natick, MA). Clomipramine (Clo), used as the internal standard (IS), was purchased from Sigma (St Louis, MO). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher (Atlanta, GA). Phosphoric acid (99.99%), sodium hydroxide, and diethylamine (DEA, 99.5%) were obtained from Aldrich (Milwaukee, WI).

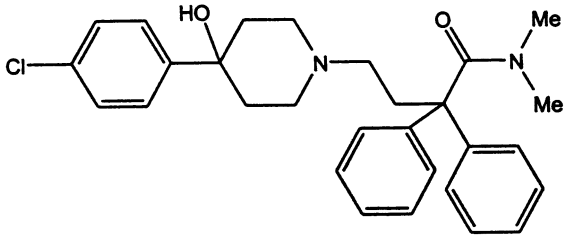

Fig. 1. Structure of loperamide.

Metaphosphoric acid (MPA) was purchased from Sigma (St Louis, MO). Sodium phosphate monobasic monohydrate was purchased from EM Science (Gibbstown, NJ).

2.2. Sample preparation

Aliquots (150 μl) taken from rat plasma samples (from a pharmacokinetic study with Lop) or from 4% BSA solutions (from a Franz cell skin penetration study with Lop) and their corresponding blanks were placed in disposable plastic tubes with a cap. Following the addition of 15 μl of internal standard (Clo) solution (10 μg ml^{-1} for BSA solutions or 20 μg ml^{-1} for rat plasma) in a solvent mixture (ACN/water = 1:1) to each tube, 15 μl of the solvent mixture was added to the unknown samples and 15 μl of Lop standard solutions in the solvent mixture was added to the blanks for calibration curves and quality control samples. All the tubes were vortexed and incubated at room temperature for 5 min. MPA (120 μl of 10%) was added to each tube for precipitating the protein in the sample, tubes were vortexed again, and then centrifuged (12 000 rev. min^{-1}, 21–22°C, 10 min). Supernatant (200 μl) was mixed with 8 μl of 2.0 M NaOH in injector vials to bring the pH of the supernatant to around 3 (to prolong the column life). All the treated samples had a dilution factor of two.

2.3. Chromatography

All chromatographic separations were carried out using a HP 1050 liquid chromatography system (Hewlett-Packard, Palo Alto, CA) with an autosampler and a UV/VIS variable wavelength detector set at 195 nm. An aliquot of sample (20 μl) was injected onto a Luna 250 × 4.6 mm 5 μm particle C18(2) column with a Securityguard guard cartridge (Phenomenex, Torrance, CA) at room temperature (20–22°C). The flow rate was set at 1.0 ml min^{-1} during all the separations with the mobile phase composed of acetonitrile–sodium phosphate buffer (pH 2.30; 20 mM)–diethylamine (40:60:0.08, v/v/v) and the elution time was 22 min.

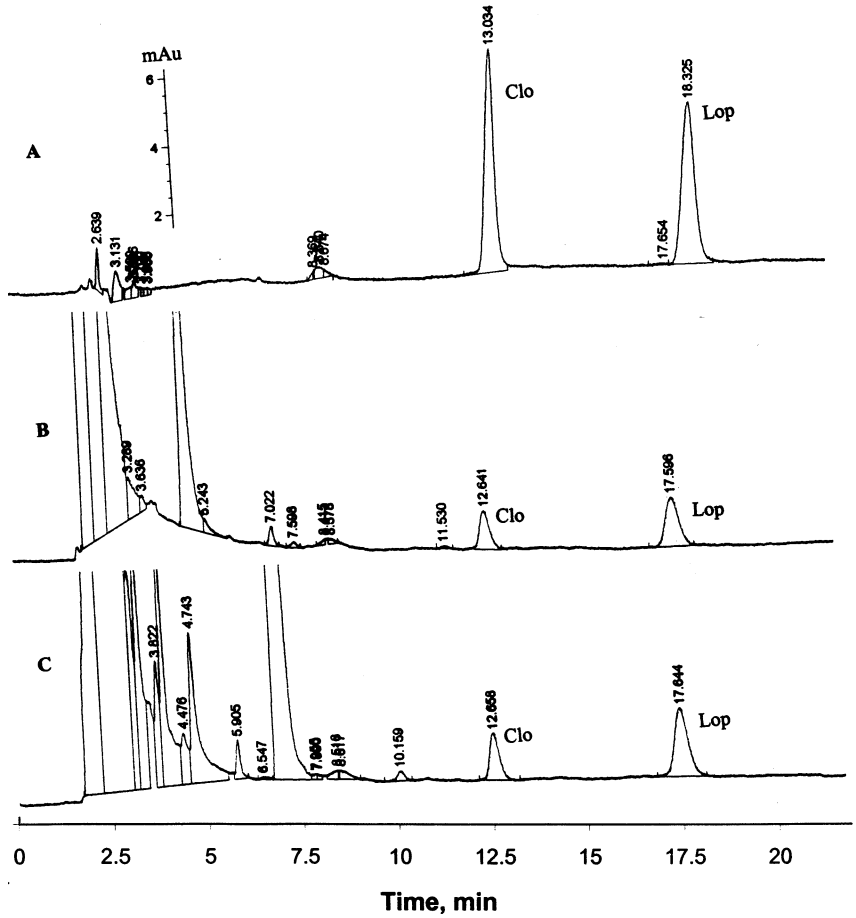

Fig. 2. Chromatograms of rat plasma and BSA samples. (A) standards (Clo: 1.0 μg ml^{-1}, Lop: 1.0 μg ml^{-1}) in mobile phase; (B) standards (Clo: 1.0 μg ml^{-1}, Lop: 1.0 μg ml^{-1}) in BSA solution; (C) BSA sample (Clo: 1.0 μg ml^{-1}); (D) blank rat plasma sample (Clo: 2.0 μg ml^{-1}); (E) standards (Clo: 2.0 μg ml^{-1}, Lop: 1.0 μg ml^{-1}) in rat plasma; (F) rat plasma sample (Clo: 2.0 μg ml^{-1}). Injection volume was 20 μl. B–F had a dilution factor of two.

2.4. Data analysis

Data acquisition and calculations were performed using Chemstation Software from Hewlett-Packard. Lop concentrations in rat plasma and BSA samples were calculated relative to peak areas of the internal standard, Clo, to correct for the Lop loss during sample preparation and for volumetric error during sample injection.

3. Results and discussion

The method described here gave complete separation of Lop or the internal standard (Clo) in BSA solution and rat plasma samples (Fig. 2). The retention time for Lop was 17.5–18.3 min, and for Clo was 12.5–13.0 min. No peaks were found at the retention times of Lop and internal standard (Clo) in the chromatograms of blank BSA solution and rat plasma samples (not

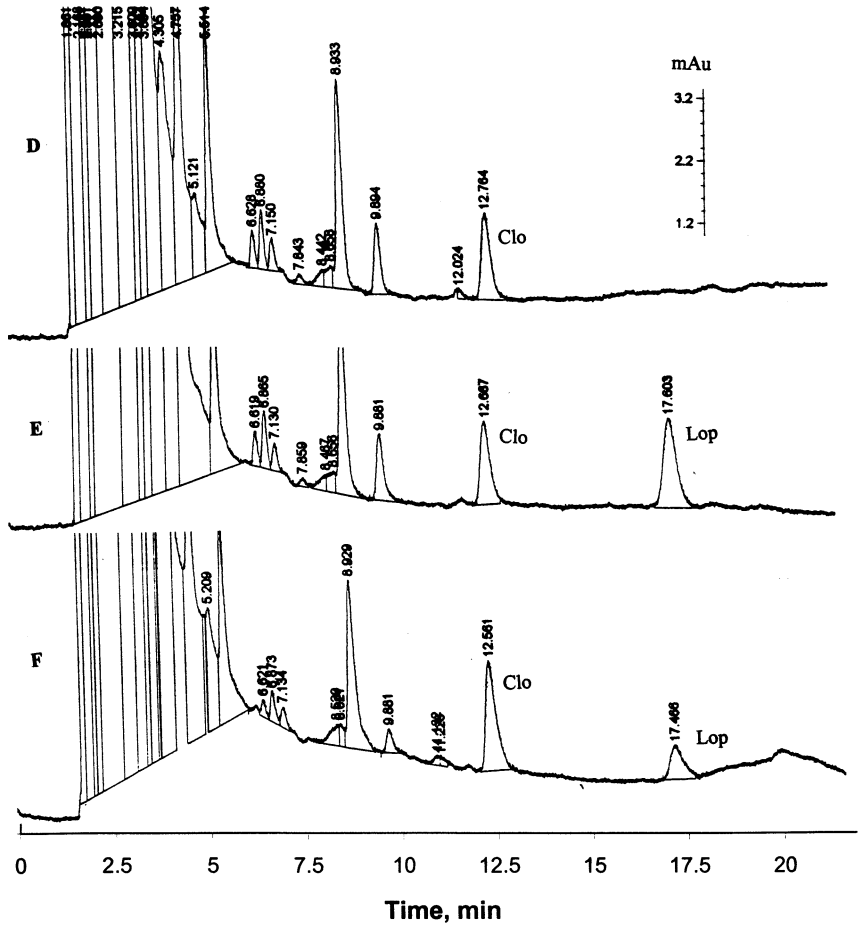

Fig. 2. (*Continued*)

shown). Therefore, there were no unknown components from BSA solution or rat plasma that interfered with the Lop and Clo peaks. Clo was chosen as an internal standard because its has a structure comparable to Lop and its retention time was similar to that of Lop.

Alteration of the mobile phase by increasing the concentration of ACN from 40 to 45% shortened the separation time for Lop (from 17.42 to 10.12 min) and Clo (12.52 to 7.783 min). However, an unknown peak from the BSA solution or the rat plasma matrix interfered with the Clo peak, which made the integration of the Clo peak area difficult. To achieve the complete separation of Lop and Clo from the rest of the unknown peaks, 40% ACN in mobile phase was chosen.

The effect of varying the pH of the phosphate buffer solution from 2.10 to 2.93 on the retention time of Clo and Lop was minimal (12.34–13.11 min for Clo, 17.05–18.26 min for Lop). However, higher pH (2.62–2.93) dramatically worsened the separation of Lop and Clo in BSA and rat plasma samples. There was an unknown peak that appeared at about 10 min, and the baseline also shifted during the separation when pH 2.62 (or higher) mobile phase was used, which disturbed the integration of the Clo and Lop peaks. The column life was dramatically decreased when the mobile phase with lower pH (2.10) was used during the separation. The best baseline separation for Lop and Clo in BSA and rat plasma samples was achieved using pH 2.3 mobile phase.

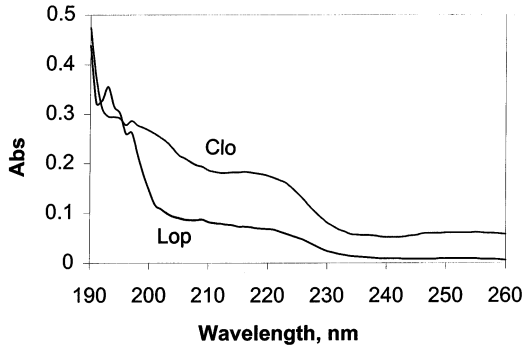

Fig. 3. UV absorption spectra of 1.25 μg ml^{-1} of Lop and 2.50 μg ml^{-1} Clo in mobile phase.

A trace amount of DEA was used in the mobile phase to decrease the tailing of Lop and Clo peaks.

Although huge unknown peaks appeared in the early time point (before 12 min) in the chromatograms of BSA solution and rat plasma samples (Fig. 2C,F) when detection wavelength was set 195 nm, the assay sensitivity for Lop detection was maximized without any interference from those unknown peaks. Fig. 3 shows that the UV absorbance of Lop at 195 nm is 4.4-fold higher than that at 220 nm (220 nm was used in most published HPLC assays). A mixture of phosphate buffer (rather than acetate buffer) and ACN (rather than MeOH) was chosen as the mobile phase to minimize the noise level of the baseline at 195 nm.

The regression equations of Lop calibration curves in rat plasma and BSA solutions were $y = 0.015x - 0.0604$ (range: 0–1.0 μg ml^{-1}) and $y = 0.0017x - 0.0035$ (range: 0–1.0 μg ml^{-1}), respectively, where y is the peak area ratio (Lop/Clo) and x is the concentration of Lop in rat plasma and BSA solutions. The resulting correlations were highly linear, yielding R^2-values between 0.9926 and 0.9971. Limits of detection (signal/noise = 3) to determine Lop in rat plasma and BSA solutions were 100 and 50 ng ml^{-1}, respectively, for 20-μl sample injection. The concentration range of Lop in the analyzed rat plasma samples collected from pharmacokinetic study was 191.0–317.1 ng ml^{-1}. The analyzed BSA solution samples collected from an in vitro

Table 1
Within-day precision (triple injections for each sample on the same day)

Sample	Measured (ng ml^{-1})	Mean (ng ml^{-1})	S.D.	R.S.D. (%)
BSA solution	1782.8; 1814.1; 1704.8	1767.3	56.3	3.18
Spiked BSA[a]	2140.0; 2317.3, 2092.7	2183.3	118.4	5.42
Rat plasma	309.6; 268.9; 285.4	288.0	20.5	7.11
Spiked plasma[a]	736.0; 762.9; 774.2	757.7	19.6	2.59

[a] The spiked samples was prepared by adding 500 ng ml^{-1} Lop to the BSA and rat plasma samples.

Table 2
Among-day precision[a]

Samples	Measured[b] (ng ml^{-1})	Mean (ng ml^{-1})	S.D.	R.S.D. (%)
BSA solution	Day 1: 750.1 Day 2: 782.8 Day 3: 695.1	742.6	44.3	5.97
Rat plasma	Day 1: 281.0 Day 2: 285.4 Day 3: 288.0	284.8	3.56	1.25

[a] Each injection was given over three consecutive days.
[b] All the measured values were based on the day one BSA solution or rat plasma calibration curve. The samples were kept at 4°C after each injection.

Table 3
Assay recovery

	Added Lop (ng ml^{-1})	Measured Lop (ng ml^{-1})	Recovery[a] (%)	Mean (%)	S.D.	R.S.D. (%)
BSA solution	500.0	541.7	108.3	101.4	5.60	5.91
	500.0	486.5	97.30			
	500.0	493.8	98.76			
Rat plasma	500.0	472.0	94.40	95.18	1.20	1.26
	500.0	472.9	34.58			
	500.0	482.8	96.56			

[a] Recoveries were calculated as: % Recovery = (measured Lop × 100)/added Lop.

Franz cell skin penetration study had Lop concentration ranging from 45.4 to 1642 ng ml^{-1}.

The MPA-treated BSA solution and rat plasma samples were stable. Tables 1 and 2 show that R.S.D.s of the concentration of Lop measured within-day and among-day were small. Within-day precision was analyzed using BSA solutions, rat plasma samples and their corresponding spiked samples containing varying concentrations of Lop from 288.0 to 2183.3 ng ml^{-1}. The samples were then run on three separate occasions over the course of the day. The within-day variation of this method was minimal, with R.S.D. of 2.59–7.11%. Among-day precision involved assessing the same BSA solution or rat plasma samples each day over three consecutive days. The samples were sealed and kept at 4°C after each injection. Among-day precision was also good, with R.S.D. of 1.25–5.97%.

The assay precision in BSA solution was determined by evaluating three sample preparations from each of three different BSA samples in which concentrations of Lop were from 784.7 to 1596.4 ng ml^{-1}. The R.S.D.s of the assay precision for three different samples were between 0.777 and 4.78%.

Assay recovery was evaluated in BSA solution and rat plasma (Table 3). Three unspiked and three spiked samples from each species were extracted with 10% MPA and analyzed. Recoveries were calculated as

$$\text{Recovery} = (S_I - U_m) \times 100/\text{added Lop}$$
$$= \text{measured Lop} \times 100/\text{added Lop}$$

where S_I is analyzed Lop in the spiked sample and U_m is the mean of analyzed Lop in the three unspiked BSA or rat plasma samples. Overall recoveries of Lop from BSA and rat plasma samples at concentration 500 ng ml^{-1} were 101.4 and 95.5%, respectively.

4. Conclusions

A method using high performance liquid chromatography (HPLC) for the quantitation of Lop in rat plasma and in BSA solutions was developed and validated. The samples were prepared for analysis by simply adding metaphosphoric acid to precipitate proteins. Chromatography was performed isocratically on a C_{18} reverse phase column using UV detection at 195 nm. The peak area of Lop for each sample was compared to that of a known amount of internal standard (Clo) to determine the amount of Lop in the sample. The limit of detection was 50 ng ml^{-1} for BSA solution samples and 100 ng ml^{-1} for rat plasma samples. Overall recoveries of Lop added to BSA solutions or rat plasma were 101.4 and 95.5%, respectively. The discussed isocratic HPLC method with a simple, rapid pretreatment and minimal sample handling is sensitive and reproducible for analysis of Lop in rat plasma and BSA solutions.

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