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Extraction and LC determination of lysine clonixinate salt in water/oil microemulsions

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Abstract

A new reversed-phase high performance liquid chromatography method has been developed and validated for the quantitative determination of lysine clonixinate salt in water/oil microemulsions. The mobile phase was acetonitrile-buffer phosphate pH 3.3. Detection was UV absorbance at 252 nm. The precision and accurately of the method were excellent. The established linearity range was 5–60 μg ml^{-1} ($r^2 = 0.999$). Microemulsions samples were dispersed with chloroform and extracted lysine clonixinate salt with water. This easy method employing chloroformic extraction has been done three times. The recovery of lysine clonixinate salt from spiked placebo and microemulsion were > 90% over the linear range.

Keywords: Lysine clonixinate salt; Water–oil microemulsions; Extraction of drug from microemulsion; Reversed-phase high performance liquid chromatography

1. Introduction

Lysine clonixinate is a water-soluble non-steroidal analgesic and anti-inflammatory drug (NSAID; Fig. 1). Lysine clonixinate was approved as analgesic for the treatment of moderate to severe pain and as anti-inflammatory in inflammatory events, such as the analgesic action in the treatment of postepisiotomy pain [1], dysmenorrhea [2], etc. and as an anti-inflammatory agent. However, its mechanism of action has yet to be elucidated. Findings to date indicate that lysine clonixinate is a cyclooxygenase inhibitor of moderate potency although it is unclear whether mechanisms other than the inhibition of cyclooxygenase contribute to its analgesic activity[3]. Clinical trials have been prompted by the fact that this agent induces less blood loss by oral intake than acetylsalicylic acid [4]. Further, a novel vasorelaxant effect has been reported which suggests its role as a calcium antagonist [5].

NSAIDs are often detected by high-performance liquid chromatography (HPLC) and gas chromatography (GC)–mass spectrometry, such as phenylbutazone which is commonly quantify by HPLC with UV detection [6,7], naproxen [8,9],

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indomethacin by HPLC [10] and GC [11] and ethacrynic acid by GC [12].

Water-in-oil (W/O) emulsions administered by subcutaneous or intra-muscular routes are usually designed to exert a depot effect or delayed release of drug where the barrier to diffusion is the oil continuous phase. This normal emulsions age by coalescence of droplets and by Ostwald ripening (transfer of material from small droplets to large ones), since these processes lead to a decrease of the interfacial area and hence the free energy system [13].

Microemulsions are fluid, transparent, thermodynamically stable oil and water systems, stabilized by a surfactant usually in conjunction with a cosurfactant. The general advantages of microemulsions cited in the literature are easy of preparation, clarity, stability, ability to be filtered, vehicle for drugs of different lipophilicities in the same system, low viscosity, and no pain upon injection [14].

Few methods have been developed for the extraction of active substances contained in microemulsions, Moreno et al. [15] developed a new extraction method for anmphotericin B in oil/water (O/W) lecithin-based microemulsions. Kenney et al. [16] developed a solid-phase extraction and liquid chromatographic quantitation of the antiarrhythmic drug L-768673 in an O/W microemulsion formulation.

The literature contains no evidence in the formulation of lysine clonixinate as a W/O microemulsion or any method developed and validated for the determination of this drug in such dispersed systems. The objective of this development project was to achieve a highly sensitive and precise method for analyzing lysine clonixinate salt using LC, which can be applied in the determination of lysine clonixinate concentrations in W/O microemulsions. An easy method employing chloroformic–water mixture has been used to extract lysine clonixinate containing in the inner phase of microemulsion system, and then aqueous phase was analyzed without interference of oil phases.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile was obtained from Symta (Madrid, Spain). Reagent-grade chloroform was purchased from Panreac (Barcelona, Spain). Water was purified using a Milli-Q-filter system (Millipore, Madrid, Spain). Phosphate buffer pH 3.3 was made by dissolution of KH_2PO_4 and adjusting the pH with phosphoric acid (Merck). Lysine clonixinate salt was kindly supplied by Roemmers S.A. (Buenos Aires, Argentina).

2.2. Apparatus and chromatographic conditions

A Gilson HPLC was used. The HPLC system consists in a Gilson 306 pump; a Gilson autosampler (Gilson 231XL sampling injector) and Gilson Model 116 diode-array detector set at 252 nm. Retention times were recorded by means of the SP4270 integrator. Compounds were separated on

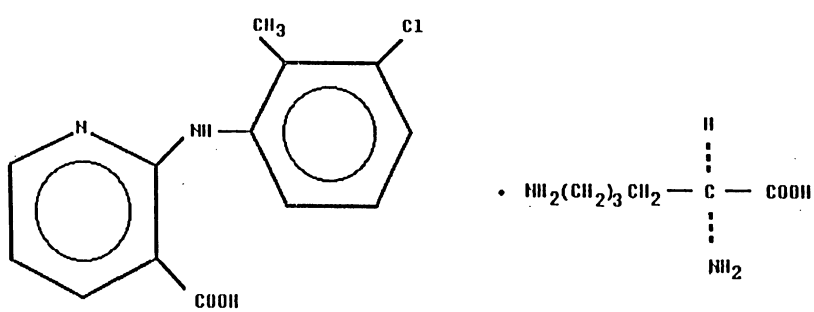

Fig. 1. The chemical structure of lysine clonixinate salt.

a 200×4.6 mm^2 i.d., 5 μm particle size, Spherisorb C18 reversed-phase column (Teknokroma, Madrid, Spain).

The mobile phase was a 70:30 (v/v) mixture of potassium dihydrogen phosphate solution pH 3.3 and acetonitrile passed through a 0.45 μm pore-size filter, degassed before use and pumped at 1.8 ml min^{-1}. The volume injected was 100 μl. All HPLC work was performed in a temperature ambient (≈ 25 °C).

2.3. Standard solutions and samples

A six stock solutions of lysine clonixinate salt, were prepared by dissolving lysine clonixinate salt into a 100 ml volumetric flask and dissolving and diluting to volume with phosphate buffer saline (PBS) pH 7.4. Standard solutions with 5, 10, 20, 30, 40, and 60 μg ml^{-1} were made by further dilution of stock solutions with appropriate volumes of PBS pH 7.4. Peak areas were recorded for all the solutions. Analyses were performed by triplicate.

W/O microemulsion was made with 56.25% isopropyl myristate, 18.75% Span 80, 15% Tween 80, 10% water with lysine clonixinate salt. Clear, stable and transparent microemulsion was obtained independently of addition order of components. Non-dispersability in water was employed to verify that the microemulsion formed was of the W/O type. In addition, microemulsion was examined under polarizing light and found to be non-birefringent as expected from their isotropic nature.

Six W/O microemulsions with different amounts of lysine clonixinate (2.27–5.46 mg) were prepared. A microemulsion without lysine clonixinate was prepared and different amounts of lysine clonixinate (2.6–5.1 mg) were after added, these formulations were called spiked placebos.

2.4. Extraction procedure

The components of the microemulsion exhibit a range of polarities. The strategy was to wash off the non-polar components (oil and surfactant of lower HLB), whose were majority, of the microemulsion using a strong non-polar solvent (chloroform), whilst the polar lysine clonixinate was retained on the aqueous phase. Chloroform was elected because lysine clonixinate was insoluble in this organic solvent, whilst it has a water solubility of $409.78 + 3.41$ mg g^{-1}.

The extraction procedure was realized by three phases, because with only one stage the amount recovery was insufficient, and with two stages too.

Lysine clonixinate microemulsions and *spiked placebo*s ($\cong 100$ mg) were transferred into a tube and diluted with 1 ml of chloroform and 3 ml of water. The samples were vortex mixed for 2 min, and then they were centrifuged during 10 min at $6000 \times g$ until total separation of the phases. Then, the water supernatant was diluted to volume with PBS pH 7.4. A portion of the sample was filtered through 0.45 μm nylon filter membrane and injected.

After first extraction, the second extraction was prepared adding 2 ml of water into the same tube, and the same method was repeated, the second extraction was analyzed in HPLC. The third extraction was prepared by adding 2 ml of water and analyzed too. Every extraction was analyzed by triplicate. Results were expressed as sum of three extractions.

3. Results and discussion

3.1. Chromatography

Preliminary experiments showed that lysine clonixinate was found to be not sensitive in exposures to light, and the pH-stability of the drug was very high over a wide pH range (3–11), and so no special precautions were taken, except concerning to the instrument set-up.

C18 column and this mixed organic-aqueous mobile phase provided adequate good analysis of lysine clonixinate. An isocratic method was preferred over a gradient method. The wavelength detection was adjusted by spectrophotometrically scanning in a Beckman Du-7 spectrophotometer at 252 nm. Good chromatographic profiles were obtained (Fig. 2).

The summary statistics for retention time is average, 6.256 min ($n = 6$); variance, 0.00158;

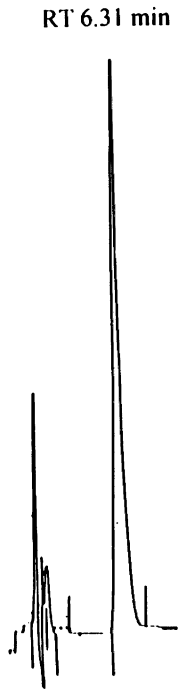

Fig. 2. Chromatogram from analysis of lysine clonixinate standard solution (10 μg ml^{-1}).

S.D., 0.039; standard error, 0.016; standard skewness, 0.168; standard kurtosis, −0.902; and coefficient of variation (CV), 0.636%. Ninety-five percent of confidence interval for mean of retention time: 6.25 ± 0.041. The standardized skewness value is within the range expected for data form a normal distribution. The standardized kurtosis value is within the range expected for data from a normal distribution.

3.2. Linearity

The linearity of the response was determined chromatographing six standard solutions spanning 5–60 μg ml^{-1} three times. The assays exhibited linearity between the response (y) (peak-area ratio of lysine clonixinate) and the corresponding concentration of lysine clonixinate (x), over 5–60 μg ml^{-1} range ($n = 6$) gave the equation $Y = 10225.45X + 64.721$. Table 1 shows statistics parameters of slope and intercept.

The correlation coefficient, $r_x^2 = 0.9997$, indicating strong relationship between the variables, confirmed the linearity of the method over the concentration range analyzed. Table 2 include analysis of variance (ANOVA) with lack-of-fit, who is designed to determine whether the selected model (lineal) is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate

Table 1
Statistics parameters of slope and intercept

	Parameter	Experimental value	Accepted value
Slope parameters	Response factor	10280.52	
	Variation coefficient	3.793	<5%
	Variance of slope	6965.16	
	Standard error	82.45	
	RSD	0.8161%	<2%
	Superior confidence limit	10457.12	
	Inferior confidence limit	9993.77	
	t-Statistic	122.52	>2.776 $(n-2)$ $P = 0.05$
Intercept parameters	Intercept	64.721	
	Variance of intercept	8.41×10^6	
	Standard error	2901.59	
	RSD	4483.17	
	Superior confidence limit	8119.56	}Zero include
	Inferior confidence limit	−7990.11	
	t-Statistic	0.022	P-value 0.9984

Table 2
ANOVA with lack-of-fit

Source	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
Model	6.87×10^{11}	1	687×10^{11}	58679.61	0.00
Residual	1.87×10^{8}	16	1.17×10^{7}		
Lack-of-fit	1.82×10^{8}	4	4.56×10^{7}	110.33	0.00
Pure error	4.96×10^{6}	12	4.13×10^{5}		
Total (Corr.)	6.87×10^{11}	17			

values of the independent variable X (concentration). Since the P-value for lack-of-fit in the ANOVA table is less than 0.01, there is statistically significant lack-of-fit at the 99% confidence level.

3.3. Precision

The system precision was tested by calculating the repeatability intra-day and the reproducibility of the method during three serial days. The precision of a method is expressed as the percentage CV of replicate measurement. To be acceptable, the CV should be lesser than 5%.

3.3.1. Intra-day variability of assay

The intra-day variability of the assay was determined by repeated analysis of standard solutions at low, medium and high concentrations on the same day at time 0, 2 and 4 h. The results are shown in Table 3. The CV < 5% indicate that the assay method has repeatability within the same day.

3.3.2. Inter-day variability of assay

The inter-day variability of the assay was determined by repeated analysis of standard solutions at low, medium and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at ambient temperature until analysis. Results are showed in Table 4. These data indicate that the assay method is reproducible within different days.

3.4. Sensivity of assay

The detection limit and quantitation limit was determined by triplicate analysis of three standard solutions (1, 2 and 5 μg ml^{-1}) using next equations:

$$D_l = (Y_{bl} + 3S_{bl})/b \times 1/\sqrt{n},$$

$$C_l = (Y_{bl} + 10S_{bl})/b \times 1/\sqrt{n},$$

where D_l, detection limit; C_l, quantitation limit; Y_{bl}, intercept of means equation of analysis of the three standard solutions; s_{bl}, intercept of S.D. equation; n, 3; and b, slope of general equation. The estimated limit of detection for lysine clonixinate was 0.183 μg ml^{-1}. The estimated limit of quantitation was 0.215 μg ml^{-1}.

Table 3
Intra-day variability of HPLC method for determining lysine clonixinate salt ($n = 3$)

LC μg ml^{-1}	Mean area ± RSD	CV (%)
10.18	91 620.66 ± 877.2	0.96
30.54	276 310 ± 5977.57	2.16
48.86	450 618.33 ± 8606.09	1.90

Table 4
Inter-day variability of HPLC method for determining lysine clonixinate salt ($n = 3$)

LC μg ml^{-1}	Mean area ± RSD	CV (%)
10.18	93 010.33 ± 3050.93	3.28
30.54	287 642.33 ± 13 376.72	4.65
48.86	481 121.66 ± 13 422.26	2.79

Fig. 3. Chromatogram from analysis of microemulsion without lysine clonixinate salt.

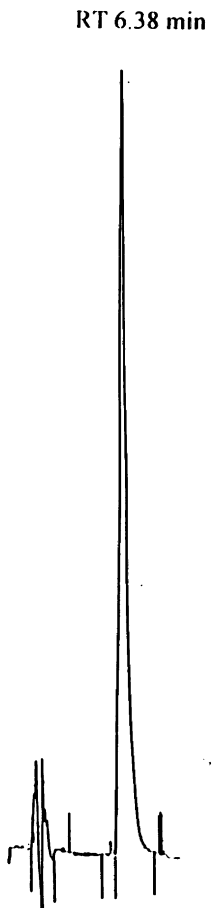

Fig. 4. Chromatogram from analysis of lysine clonixinate spiked placebo with 3.4 mg of lysine clonixinate after first extraction. 2.83 mg lysine clonixinate recovery after first extraction (dilution final 15.11 μg ml^{-1}).

3.5. Selectivity of assay

The selectivity of the assay was determined by analysis of microemulsion without lysine clonixinate salt. Under these chromatographic conditions, no endogenous sources of interference were observed in microemulsion without lysine clonixinate (Fig. 3) compared with a standard solution.

3.6. Accuracy

The recoveries of lysine clonixinate were assessed from spiked placebo formulations and following the extraction procedures used for the dosage form. Placebo was spiked at the same six levels of the amount of lysine clonixinate containing in microemulsions samples. Fig. 4 reveals the chromatograms of spiked placebo with 3.4 mg of lysine clonixinate after first extraction. The mean recovery for the six levels was 101.13% with a CV of 1.74% and relative standard deviation (RSD) of 1.76 (Table 5). This percentage was a little greater than 100, because the aqueous phase with lysine clonixinate was not completely extracted of the chloroformic mixture, but confirmed that this is a good extraction method of lysine clonixinate salt in W/O microemulsions.

3.7. Analysis of lysine clonixinate microemulsions

To establish the validity of the proposed method, six lysine clonixinate microemulsions were assayed. The assay values after three extraction phases are presented in Table 6. The small difference between the amount declared and the amount found could be attributed to the small amounts of drug that cannot be separated with the extraction method. Fig. 5 shows chromatogram of drug in microemulsion (3.472-mg lysine clonixinate declared) after first extraction.

Table 5
Recovery of lysine clonixinate from spiked placebo

Amount added (mg)	Amount recovered (mg)	Recovery (%)
2.6	2.68	103.07
3.4	3.48	102.35
3.8	3.82	100.52
4.2	4.17	99.28
4.7	4.82	102.55
5.1	5.05	99.01

Table 6
Results of assay of lysine clonixinate in microemulsions ($n = 3$)

Amount declared (mg)	Amount recovered (mg) ± RSD	Rec. (%) after 1[a] extr.	Rec. (%) after 2[a] extr.	Rec. (%) after 3[a] extr.	Rec. (%) total
2.27	2.26 ± 0.01	73	21	5.75	99.47
3.00	2.95 ± 0.02	68.74	24.67	6.74	98.42
3.47	3.43 ± 0.01	71.21	23.51	5.45	98.93
3.76	3.82 ± 0.07	74.28	20.02	5.94	101.78
4.66	4.74 ± 0.07	72.47	21.63	5.88	101.60
5.46	5.37 ± 0.01	83.64	12.63	3.74	98.35

Mean, 99.75; RSD, 1.55; and CV, 1.55%.
[a] Rec. (%) after extr – recovery (%) after… extraction.

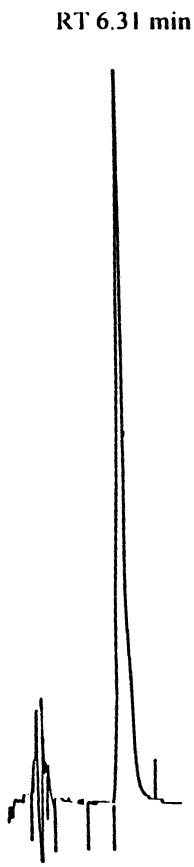

Fig. 5. Chromatogram from analysis of microemulsion with 3.472 mg lysine clonixinate declared after first extraction. 2.44 mg lysine clonixinate recovery after first extraction (dilution final 13.02 μg ml^{-1}).

4. Conclusion

The HPLC assay method presented is linear, rapid, sensitive, precise, specific and robust to analyze lysine clonixinate salt. The extraction method is able to selectively extract and separate lysine clonixinate salt from the microemulsion components with excellent recoveries, and it allows to analyze this drug by HPLC.

Acknowledgements

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References

[1] A.R. De los Santos, M.I. Marti, D. Espinosa, G. Di Girolamo, J.C. Vinacur, A. Casadei, Acta. Physio. Pharmacol. Ther. Latinoam. 48 (1998) 52–58.
[2] G. Di Girolano, R. Zmijanovich, A.R. De los Santos, M.L. Marti, A. Terragno, Acta. Physiol. Pharmacol. Ther. Latinoam. 46 (1996) 223–232.
[3] D. Pallapies, A. Muhs, L. Bertram, G. Rohleder, P. Nagyivanyi, B.A. Peskar, Eur. J. Clin. Pharmacol. 4 (1996) 351–354.
[4] A. Bidlingmaier, A. Hammermaier, P. Nagyivanyi, G. Pabst, J. Waitzinger, Arzneimittelforschung 45 (1995) 491–493.
[5] M.A. Morales, A. Silva, G. Brito, S. Bustamante, H. Ponce, C. Paeile, Gen. Pharmacol. 26 (1995) 430–495.
[6] H. Fabre, B. Mandrou, H. Eddine, J. Pharm. Sci. 71 (1982) 120.

[7] D.F. Gerken, R. Sams, J. Pharmacokin. Biopharm. 13 (1985) 467.
[8] J.W.A. van Loenhout, C.A.A. van Ginneken, H.C.J. Ketelaars, P.M. Kimenai, F.W.J. Gribnau, J. Liq. Chromatogr. 5 (1982) 549.
[9] F. Nielsen-Kuds, Acta. Pharmacol. Toxicol. 47 (1980) 267.
[10] J.N. Moore, H.E. Garner, J.E. Shapland, Equine Vet. J. 13 (1981) 95.
[11] P. Guissou, G. Cuisinaud, J. Sassard, J. Chromatogr. 227 (1983) 368.
[12] W. Stuber, E. Mutschler, D. Steinbach, J. Chromatogr. 227 (1982) 193.
[13] D. Attwood, in: J. Kreuter (Ed.), Colloidal Drug Delivery Systems, Marcel Dekker, New York, 1994, pp. 31–71.
[14] M.J. Lawrence, Eur. J. Drug Metab. Pharmacokinet. (3) (1994) 257–269.
[15] M.A. Moreno, P. Frutos, M.P. Ballesteros, Chromatographia 48 (1998) 11–12.
[16] R.R. Kenney, R.J. Forsyth, H. Jahansouz, J. Pharm. Biomed. Anal. 17 (1998) 679–687.