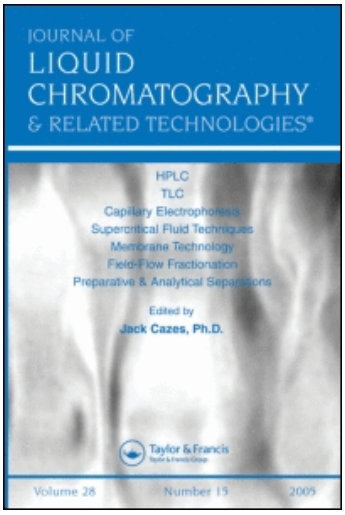

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Abstract: The novel, rapid high performance liquid chromatographic method for the determination of ketoprofen lysine salt was developed and validated. The method can simultaneously assay methyl-*p*-hydroxybenzoate, used as a preservative in OKI spray. The separation was carried out on a C_{18} μBondapack (300 × 3.9 mm i.d., 10 μm particle size) column using acetonitrile—0.05 M KH_2PO_4 buffer (60:40, v/v) as mobile phase (pH value 3.0 was adjusted with orthophosphoric acid) at a flow rate 1.5 mL min^{-1}, temperature of the column 20°C, and UV detection at 254 nm. The method was found to be linear ($r > 0.9999$) in the range of 8–24 μg mL^{-1} for ketoprofen lysine salt, and 7.5–2.5 μg mL^{-1} ($r > 0.9998$) for methyl-*p*-hydroxybenzoate. The developed method was successfully applied to the determination of ketoprofen lysine salt, its investigated preservative in commercial formulation. The recovery for ketoprofen lysine salt and methyl-*p*-hydroxybenzoate were 100.2% and 100.4 with RSD of 0.6 and 0.7%, respectively. The method is rapid and sensitive enough to be used for the analysis of OKI® spray.

Keywords: Ketoprofen lysine salt, Preservative pharmaceutical analysis, HPLC, Spray, Validation

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INTRODUCTION

Ketoprofen lysine salt (KLS), or (RS)-2-(3-benzoylphenyl)propionic(DL)-lysine salt (Figure 1), is an arylpropionic non-steroidal anti-inflammatory drug, prescribed for the treatment of traumatic, orthopaedic, and rheumatic disorders and inflammatory disease.[1,2] The target of this study was developing and validating a new, simple, and fast analytical method by HPLC to quantify KLS and its preservative methyl-*p*-hydroxybenzoate (MP), in a pharmaceutical form (OKI® Spray). This validation study is defined as the process by which it is established, by laboratory studies, that the performance characteristics of the method meet requirements for the intended analytical application.[3,4] This work describes the validation parameters stated either by USP26[5] or by the ICH guidelines[6–8] to achieve an analytical method with acceptable characteristics of suitability, reliability, and feasibility.

EXPERIMENTAL

Chemicals and Reagents

The acetonitrile, methanol, and potassium dihydrogen ortophosphate (HPLC grade) were supplied by Fluka Chemika-BioChemika (Buchs, Switzerland). Phosphoric acid (85%, w/w) and *p*-hydroxybenzoic acid were obtained from Carlo Erba Reagenti (Milan, Italy). Water (HPLC grade) was obtained by passage through the ELIX 3 and Milli-Q Academic water purification system (Millipore, Bedford, MA, USA). Ketoprofen lysine salt (reference material, batch A31295), methyl-*p*-hydroxybenzoate (reference material, batch A20792), OKI® spray Placebo (batch PG063/42/SU01), and OKI® spray (batch PG063/40/SU01), [xilitol, Lutrol 127 (Poloxamer), sodium di-hydrogen orthophosphate monohydrate, methyl-*p*-hydroxybenzoate, glycerin, mint flavour, water] was supplied by Dompé Research and Development Laboratories. Other chemicals and reagents were of analytical grade.

Apparatus and Chromatographic Conditions

The chromatographic system used to develop this technique was a Waters (Waters, Milford, MA, USA) system composed of the following: a Model

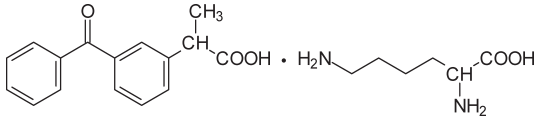

Figure 1. Chemical structure of ketoprofen lysine salt.

2695 sample injector and pump system, a Model 2487 UV-Vis detector, which was set at 254 nm. Data acquisition is performed using chromatography software package Empower (Waters, Milford, MA, USA).

Chromatographic separation was performed on an analytical 300×3.9 mm i.d. reversed-phase μBondapack C_{18} (10 μm particle size) column (Waters, Milford, MA, USA). The mobile phase consists of a mixture of acetonitrile and KH_2PO_4 0.05 M (pH = 3 with H_3PO_4). The mobile phase was prepared daily, degassed using an in-line degasser (Waters, Milford, MA), and delivered at a flow rate of 1.5 mL/min. Phosphoric acid and sodium phosphate dibasic, prior to use, were filtered through a WCN 0.45 μm, while acetonitrile and methanol through WTP 0.5 μm (Whatmann, Ltd, Maidstone, UK). The assay was performed at ambient temperature. The injection volume for the assay and preservative determination was set at 20 μL, while for the purity test was 10 μL. Run time for each test required 15 min. The detector was set at 254 nm.

Stock and Working Solutions

The stock solution of KLS was prepared at a concentration of 0.80 mg/mL, dissolving the appropriate amount of raw material in Milli-Q water. This standard solution will be used to quantify the active component in the final product. A stock solution of methyl-*p*-hydroxybenzoate was prepared by dissolving 75 mg of MP in 100 mL methanol and diluting to 100 mL in the volumetric flask with the same solvent.

Sample Preparation

OKI® spray (2.0 mL) was transferred into a 20 mL volumetric flask and diluted to volume with Milli-Q water. Of this solution, 5.0 mL was transferred into a 50 mL volumetric flask, and the solution was diluted to volume with Milli-Q water. The concentration of KLS was 16 μg/mL and of MP 15 μg/mL for the assay; while for the purity test, KLS concentration was 160 μg/mL.

Preparation of Standard Solution

The standard solution mixture was prepared by pipetting 2.0 mL of KLS stock solution (0.8 mg/mL) and mixing it with 2.0 mL of MP stock solution (0.75 mg/mL) into a 100 mL volumetric flask, to give a solution with a final concentration of 16 μg/mL KLS and 15 μg/mL MP. A sample and standard were kept on the laboratory bench under ambient conditions and light protection for the analyte stability test.

RESULTS AND DISCUSSION

Method Development

In order to effectively and simultaneously separate KLS and MP under isocratic conditions, various chromatographic conditions with different columns (C_8, C_{18}, Phenyl, cyano), pHs, and mobile phase compositions were investigated. A satisfactory separation was obtained using a μBondapack C_{18} column with a mobile phase consisting of acetonitrile:KH_2PO_4 0.05 M (pH = 3 with H_3PO_4) (40:60, v/v). The order of elution was MP ($t_R = 3.9$ min) and KLS ($t_R = 9.5$ min), respectively, at a flow rate of 1.5 mL/min (Figure 2).

Specificity of the Assay

Specificity of the method was investigated by observing the potential interferences between the investigated substances and excipients. A composite placebo solution was prepared in our laboratory according to the excipients listed for OKI® spray. This placebo solution was diluted according to the sample preparation method, and 20 μL was directly injected into the HPLC system. The results with the placebo solutions exhibited no interference of the excipients with the elution of KLS and MP and thus, confirm the specificity of this method (Figure 3).

Forced Degradation Studies

An example chromatogram of the test solution obtained from OKI® Spray Placebo, degraded at 80°C for three days, is given in Figure 4. No peaks

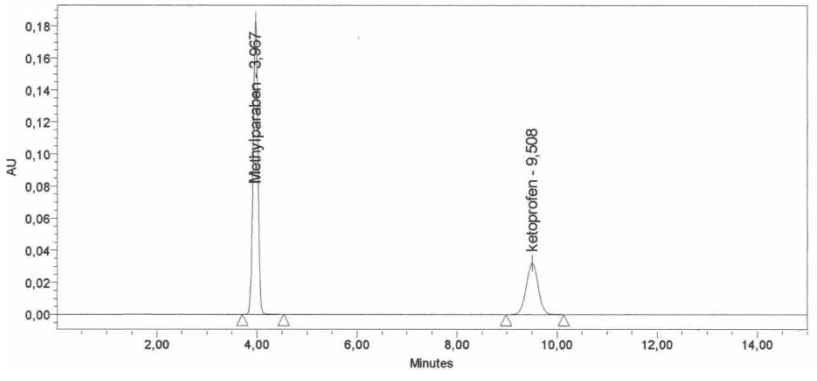

Figure 2. Typical chromatogram of an assay of standards.

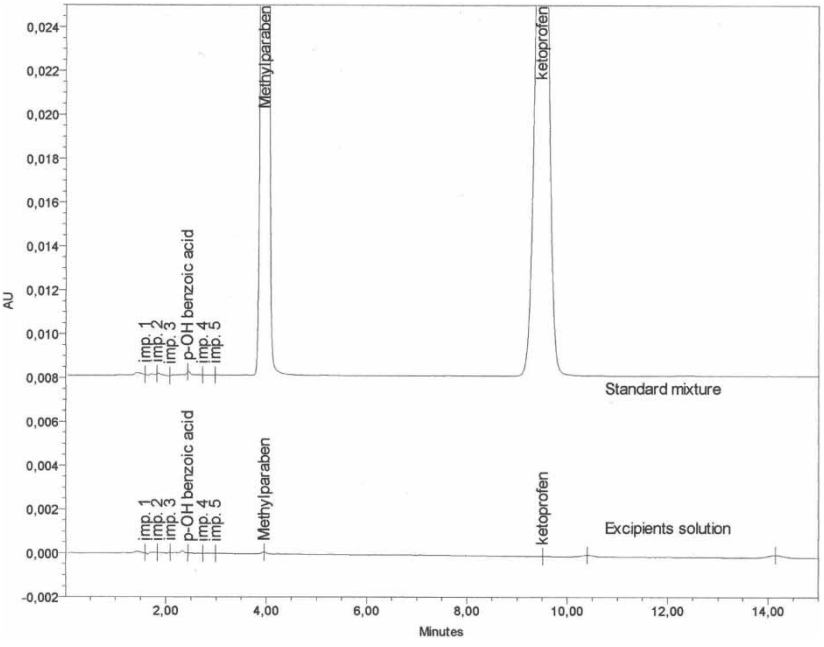

Figure 3. Overlaid chromatograms of assay standard and excipients solutions.

were observed that had similar retention times for KLS and MP in any of the stress studies performed on the standard and placebo solutions.

Linearity of the Assay

Linearity concentration curves for the assay of KLS and MP were obtained by injecting six different concentrations of KLS and MP standard calibration

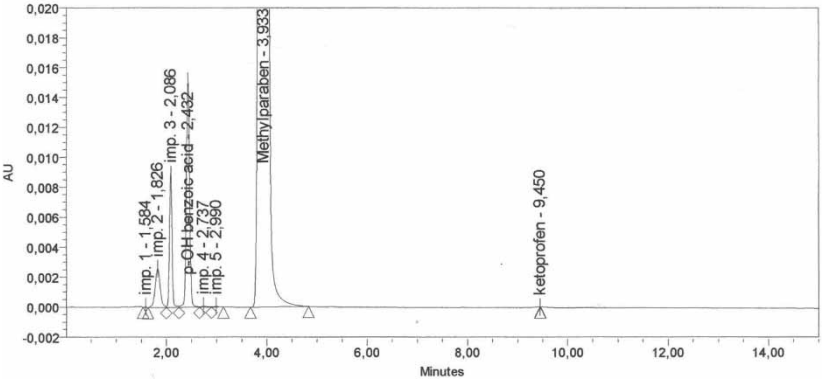

Figure 4. Degraded sample of OKI spray placebo (80°C).

solutions in mobile phase with concentrations ranging from 8.0 to 24.0 μg/mL and 7.5–22.5 μg/mL (corresponding to 50–150% for KLS and MP). Each concentration was analyzed. The linearity of peak area responses versus concentrations was studied from 8 to 24 μg mL for KLS, and from 7.5 μg/mL to 22.5 μg/mL for MP, respectively.

The equations of the regression curves obtained, relating to the tested concentrations and the response obtained, correspond to $y = 33476x + 4887$ for KLS and $y = 79327x + 5756.9$ for MP, where y is the peak area in the arbitrary units of the software Empower used, and x is the analyte concentration (μg/mL), with $r = 1.0000$ and $r = 0.9999$, respectively, between the tested concentration and the response obtained. Both the origins of the curves fall in the 95% of the confidence interval for the intercept. Linearity concentration curves for the purity test of KLS, was obtained by injecting eight different concentrations of KLS standard calibration solutions in Milli-Q water with concentrations ranging from 0.08 μg/mL to 160 μg/mL. The equation of the regression curve $y = 16785x + 624$ obtained, relating to the tested concentrations and the response obtained, correspond to $y = 33476x + 4887$, with $r = 1.0000$. The origin of the curve falls in the 95% of the confidence interval for the intercept.

Precision of the Assay

For the precision study, four different tests were carried out. The instrument precision, was evaluated with a sample corresponding to a concentration of 16 μg/mL for the assay and 0.32 μg/mL for the purity test, that were injected 10 times, consecutively into the chromatograph.

Repeatability

The repeatability test consisted of testing the standard solution precision, where six sample solutions were prepared at 100% of the work concentration for KLS and MP, respectively, and tested against standard solutions, and studying the relative standard deviation (SD). The results obtained for the assay are shown in Table 1.

Intermediate Precision

The intermediate precision was studied by a second analyst doing the same repeatability test in a different day, using a different column batch, equipment, and reagents. All reagents and mobile phases were prepared fresh. The results obtained for the intermediate precision assay are shown in Table 2.

Table 1. Repeatibility for the assay of KSL and MP

Sample	KSL content (μg/mL)	MP content (μg/mL)
1 (Analyst 1)	15,51	14,58
2 (Analyst 1)	15,42	14,47
3 (Analyst 1)	15,47	14,50
4 (Analyst 1)	15,46	14,52
5 (Analyst 1)	15,49	14,48
6 (Analyst 1)	15,39	14,40
Mean	15,46	14,49
RSD (%)	0,3	0,4

KSL = Ketoprofen lysine salt; BP = Methyl-*p*-hydroxybenzoate.

The reproducibility was studied by a second laboratory on a different day, using a different analyst, column batch, equipment, and reagents. Results obtained show a RSD % value below 0.5% for both the analytes.

Accuracy of Assay

The accuracy was determined over the range 50–150% of the nominal working concentrations of KSL and preservative, in the presence of formulation excipients. Three samples of placebo for each point were spiked with known amounts of KLS and MP (50%–100%–150% of nominal concentrations) and analyzed.

Table 2. Intermediate precision for the assay of KSL and MP

Sample	KSL content (μg/mL)	MP content (μg/mL)
1 (Analyst 1)	15,51	14,58
2 (Analyst 1)	15,42	14,47
3 (Analyst 1)	15,47	14,50
4 (Analyst 1)	15,46	14,52
5 (Analyst 1)	15,49	14,48
6 (Analyst 1)	15,39	14,40
1 (Analyst 2)	15,66	14,49
2 (Analyst 2)	15,75	14,62
3 (Analyst 2)	15,68	14,64
4 (Analyst 2)	15,35	14,48
5 (Analyst 2)	15,70	14,57
6 (Analyst 2)	15,57	14,56
Mean	15,54	14,53
RSD (%)	0,8	0,5

KSL= Ketoprofen lysine salt; BP = Methyl-*p*-hydroxybenzoate.

The recovery obtained was 100.1 (±0.2 SD) for 50%, 100.0 (±0.4 SD) for 100%, 101.0 (±0.6 SD) for 150% level for KLS, and 100.3 (±0.1 SD) for 50%, 100.2 (±0.4 SD) for 100%, 101.2 (±0.9 SD) for 150% level for MP, respectively.

Limits of Detection (LOD) and Quantitation (LOQ)

The limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise higher than 10, with precision (CV%) and accuracy (% bias) within ±10%. Thus, the calculated LOD value was 0.016 μg/mL (S/N = 3.35) for KLS, while the LOQ value was 0.08 μg/mL (S/N = 14.87). Figures 5–7 show chromatograms of a blank, a LOD, and a LOQ, respectively for KSL.

Analyte Stability

A sample and standard were kept on the laboratory bench under ambient conditions and light protection and re-assayed against fresh standards after 48 and 72 hours, respectively. Recoveries of the analytes at 48 and 72 hours were: 100.2% and 100.1% for KLS and 99.9% and 99.2% for the MP, respectively.

System Suitability

The chromatographic separation, as explained above, was carried out with a C_{18} column (μBondapack C_{18}, 300 mm × 3.9 mm i.d., 10 μm particle size).

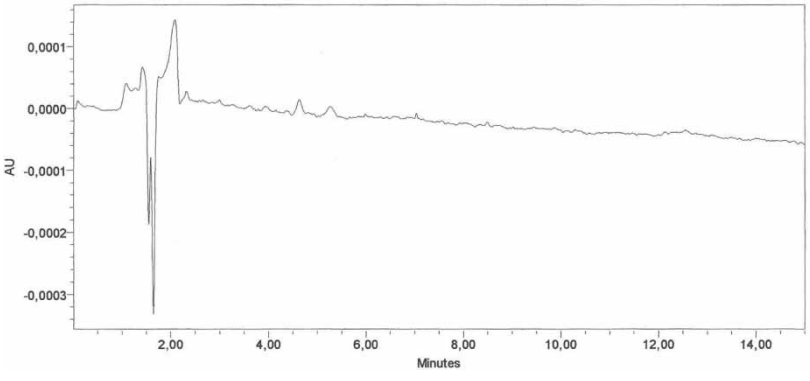

Figure 5. Chromatogram of diluent blank.

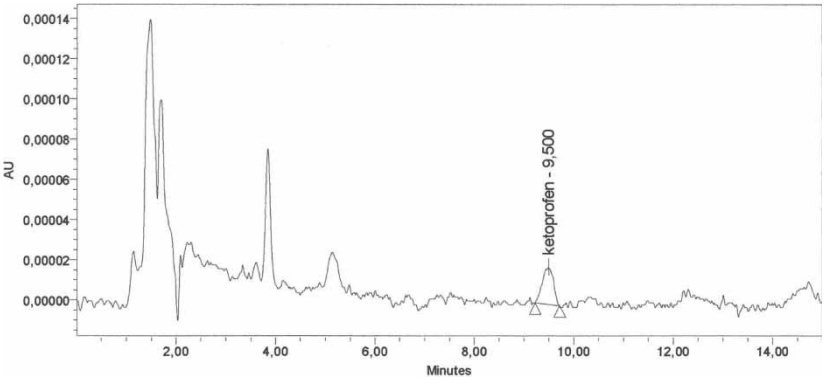

Figure 6. Chromatogram of detection limit solution (0.01%).

To evaluate the chromatographic parameters (capacity factor k, number of theoretical plates, asymmetry of the peaks, tailing factor, and resolution between two consecutive peaks), the chromatogram obtained for the test of a lack of interferences for the impurity provided by the supplier of the raw material KLS, and MP was used. In Table 3 were shown data for SST obtained during the validation study for KLS. The capacity factor obtained is within the accepted values ($2 < k < 10$). The values of the number of theoretical plates were higher than the accepted value of 4000 (minimum value to consider, it is an acceptable method). The tailing factor, another parameter that ICH guidelines consider as a factor to be controlled, was within the limits established by these guidelines. Therefore, this method can be applied to the routine with no problems, its suitability being proven.

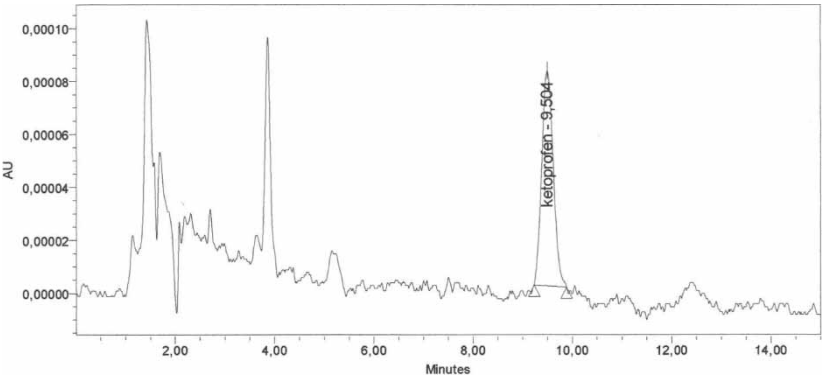

Figure 7. Chromatogram of quantitation limit solution (0.05%).

Table 3. System suitability for the assay of KSL

Determination	Injection reproducibility (RSD%)	Retention time (min)	Symmetry factor (As)	Column efficiency (N)	Capacity factor (*k*)
Limit	≤2.0	N/A	≤2.0	≥4000	6.0–10.0
1	0.1 (n = 6)	9.5	1.00	7554	7.9
2	0.2 (n = 10)	9.6	1.00	7387	8.1
3		9.5	0.99	7508	8.0
4		9.5	0.99	7438	8.0
5		9.6	0.99	7470	8.1
6		9.8	0.98	7525	8.3
7		8.0	1.20	5903	7.0
8	0.02 (n = 5)	9.4	1.00	7790	10.0

Robustness

As defined by ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method

Table 4. Robustness data for the assay of KSL

Condition	Method variable	Symmetry factor	Column efficiency	Capacity factor (*k*)
Assay standard (Ketoprofen peak)				
		≤2.0	≥4000	6.0–10.0
	Normal conditions	0.99	7633	8.0
252 nm	Wavelength[a]	0.99	7523	8.0
256 nm		0.99	7527	8.0
1.4	Flow rate[b]	0.99	7726	8.6
1.6		0.99	7269	7.4
40 mM	Buffer Conc.[c]	0.99	7614	8.0
60 mM		0.99	7650	8.0
38%	Mobile phase composition[d]	0.99	7562	9.6
42%		0.99	7606	6.7
2.8	Mobile phase pH[e]	0.98	7533	8.1
3.2		0.99	7443	8.0
	Normal conditions	0.99	7576	8.2
Mean		0.99	7555	8.0
SD		0.003	116	0.69
3SD		0.01	347	2.1

Normal conditions: [a]Wavelenght = 254 nm; [b]Flow rate = 1.5 mL/min; [c]Buffer Conc. = 50 mM; [d]Mobile phase composition = 40% (organic); [e]Mobile phase pH =3.0; SD = Standard deviation

parameters.[9,10] The robustness with respect to wavelength, flow-rate, pH, buffer strong, and buffer-organic ratio, was evaluated for the assay standard. The symmetry factor, column efficiency, and capacity factor were recorded. The conditions used are summarised in Table 4. The system suitability tests met the acceptance criteria under all conditions. The data indicates the method is robust to most small changes in the system conditions. The mobile phase indicates composition and flow rate being the only parameters which effect separation.

CONCLUSION

This paper describes a developed and validated isocratic reversed-phase HPLC method for the simultaneous determination of KLS and the preservative methyl-*p*-hydroxybenzoate in OKI spray formulations. The proposed RP HPLC method allowed the separation of compounds present in OKI spray due to selectivity of the chromatographic system. Total time of the analysis was about 15 min. Good recoveries and good RSD values confirm that the proposed RP HPLC method is applicable and reliable for the determination of KLS and preservative MP in the pharmaceutical product. The applied method can be used in the quality control and purity testing of OKI spray as sensitive, precise, and accurate.

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