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# Development and validation of an LC–MS/MS method for the determination of adapalene in pharmaceutical forms for skin application

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*Abstract*. The development and validation of a liquid chromatography– tandem mass spectrometry (LC–MS/MS) method for the determination of adapalene in pharmaceutical forms for skin application were presented in this study. The MS/MS analysis of adapalene was performed by use of three mobile phases, consisted of acetonitrile and a) 0.1 % formic acid, b) 0.1 % trifluoroacetic acid and c) 20 mM ammonium acetate. The strongest signals of parent ion and dominant product ion were obtained in negative mode by use of the mobile phase c). The validation of this method was performed according to the ICH guidelines. Small variations of selected chromatographic parameters (concentration of ammonium acetate, mobile phase composition, column temperature and flow rate) did not affect significantly the qualitative and quantitative system responses, which proved the method's robustness. The method is specific for the determination of adapalene. The linearity was proved in the concentration range of 6.7–700.0 ng $mL^{-1}$ ($r = 0.9990$), with limits of detection and quantification of 2.0 and 6.7 ng $mL^{-1}$, respectively. The accuracy was confirmed by calculated recoveries (98.4–101.5 %). The precision was tested at three levels: injection repeatability, analysis repeatability and intermediate precision. The calculated relative standard deviations were less than 1, 2 and 3 %, respectively.

*Keywords*: adapalene; MS/MS analysis; LC–MS/MS determination; pharmaceutical forms for skin application.

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## INTRODUCTION

Adapalene (Fig. S-1 of the Supplementary material to this paper), chemically designated as 6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid, is a third generation synthetic retinoid used in the topical treatment of acne vulgaris, psoriasis and photo aging.[1] The adapalene was quantified by measuring the radioactivity in the plasma as well as in other biological samples (urine, faeces and skin strips). The measurements showed that its concentrations were below the limits of quantification.[2] Additionally, no evidence of teratogenicity has been reported.[3]

Pharmaceutical forms of adapelene are creams, gels, emulsions, microemulsions, lotions, soy bean oil-ciclodextrin complexes and solid lipid microparticle dispersion.[4–6] These forms contain complex matrices and specific and sensitive analytical methods are required for adapalene analysis.

According to the European Pharmacopoeia 7$^{th}$ edition, high performance liquid chromatography (HPLC) determination of adapalene is performed by using a phenyl column (column length: 250 mm; column diameter: 4.6 mm; particle size: 5 μm) and mobile phase consisting of glacial acetic acid, water, acetonitrile and tetrahydrofuran.[7] Several HPLC methods with UV detection for the determination of adapalene in gel topical formulations,[8–11] suspensions of nanocapsules[12] and *in vitro* release studies of adapalene from cream formulation[13] have been reported so far. Rühl and Nau presented a method for the determination of adapalene and retinol in biological samples that combines solid-phase extraction and HPLC with UV and fluorescence detection.[14] Brenna *et al.* used mass spectrometry (MS) and gas chromatography–mass spectrometry (GC–MS) for the characterization of three adapalene impurities, but these methods were not applied to the analysis of adapelene.[15] MS has not been applied to the quantification of adapalene so far and there are no liquid chromatography–mass spectrometry (LC–MS) or liquid chromatography–tandem mass spectrometry (LC––MS/MS) methods for the determination of this drug in pharmaceutical forms, biological samples, biopharmaceutical and formulation studies. LC–MS and LC––MS/MS are usually used for the analysis of biological samples. However, in the last years these methods have been used more often for the analysis of pharmaceutical and cosmetic forms (*e.g.*, tablets and creams).[16–19]

The goal of this study was to develop and validate a simple, specific and sensitive method for the determination of adapalene in pharmaceutical forms for skin application (cream, gel and microemulsion). For this purpose, tandem mass spectrometry (MS/MS) analysis of adapalene under different ionization conditions was performed and, on the basis of these results, an LC–MS/MS method was developed and validated. This method could be used as a starting point for the development of a bioanalytical method for the determination of adapalene in

biopharmaceutical and pharmacokinetic studies (*e.g.*, estimation of the adapalene potential to penetrate the skin using the tape stripping testing technique).

## EXPERIMENTAL

Details related to chemicals and apparatus used and method optimization are given in Supplementary material to this paper.

### *Method validation*

The validation of this method was performed according to the ICH guidelines.[20] A standard stock solution was prepared by dissolving 2 mg of adapalene in 100 mL of ethanol (20 μg $mL^{-1}$). Subsequently, this solution was diluted with ethanol to obtain working standard solutions. Solutions of adapalene formulations (microemulsion APG-1, Sona® 0.1 % gel and Sona® 0.1 % cream) were prepared by dissolving these formulations in ethanol (the concentration of adapalene corresponding to 100 % accuracy and precision of the testing was 400 ng $mL^{-1}$). Solutions of placebos were prepared in accordance with the preparation of adapalene formulation solutions, which contain adapalene in concentration of 400 ng $mL^{-1}$. The surfactants and solvents used in the formulation studies were diluted with ethanol prior to injecting.

The robustness was tested by small variations of selected chromatographic parameters: column temperature (23 and 25 °C), flow rate (0.75 and 0.85 mL $min^{-1}$), concentration of ammonium acetate solution (18 and 22 mM) and mobile phase composition (69 and 71 % acetonitrile). For this purpose, a mixture of adapalene (400 ng $mL^{-1}$) and uracil (200 ng $mL^{-1}$) was used. The uracil (LC–MS, negative mode, $m/z$ = 111.0) was used as an unretained compound for the calculation of the retention factors ($k$). The changes in the adapalene peak shape, $k$ and peak area ($A$) were monitored.

The specificity was tested by injecting an adapalene standard solution (400 ng $mL^{-1}$), placebos, as well as solvents and surfactants used in formulation studies. The chromatograms were examined at retention time corresponding to adapalene.

The limit of detection (*LOD*) and limit of quantification (*LOQ*) were concentrations of adapalene standard solutions whose signal-to-noise ratios were 3:1 and 10:1, respectively.

The linearity of the method was tested in the range *LOQ* – 700 ng $mL^{-1}$ (*LOQ*, 67, 250, 400, 500 and 700 ng $mL^{-1}$). The influence of different weighting factors ($1/x$, $1/x^2$, $1/y$ and $1/y^2$) on the sum of percentage relative error was evaluated and the results were compared with an unweighted calibration curve.

The accuracy was tested by spiking placebo or formulation with standard solution of the tested substance to obtain three concentration levels: 80, 100 and 120 % of the target concentration (in this study the target concentration of adapalene was 400 ng $mL^{-1}$). The accuracy of this method was tested by spiking microemulsion APG-1 placebo, Sona® 0.1% gel and Sona® 0.1 % cream with adapalene standard stock solution (20 μg $mL^{-1}$) to obtain three concentrations (around 320, 400 and 480 ng $mL^{-1}$) and was evaluated on the basis of calculated recovery values.

Three levels of method precision (injection repeatability, analysis repeatability and intermediate precision) were tested by analysis of an adapalene microemulsion APG-1, Sona® 0.1 % gel and Sona® 0.1 % cream at concentration level 100 % (400 ng $mL^{-1}$). The injection repeatability was determined after ten injections of the same sample solution of each formulation. The analysis repeatability was tested by preparing and injecting six sample solutions of each formulation, whereas the intermediate precision was tested in the same manner by another analyst, another day, with different set of reagents. The precision of the method was evaluated by calculating the relative standard deviations of adapalene determinations.

## RESULTS AND DISCUSSION

The MS/MS spectra of adapalene, obtained by use of three mobile phases (in negative and positive mode) are presented in Figs. 1–3. The applied collision energies were 25 (Figs. 3a, 4a and 4b), 30 (Figs. 1b and 2b) and 35 eV (Fig. 1a).

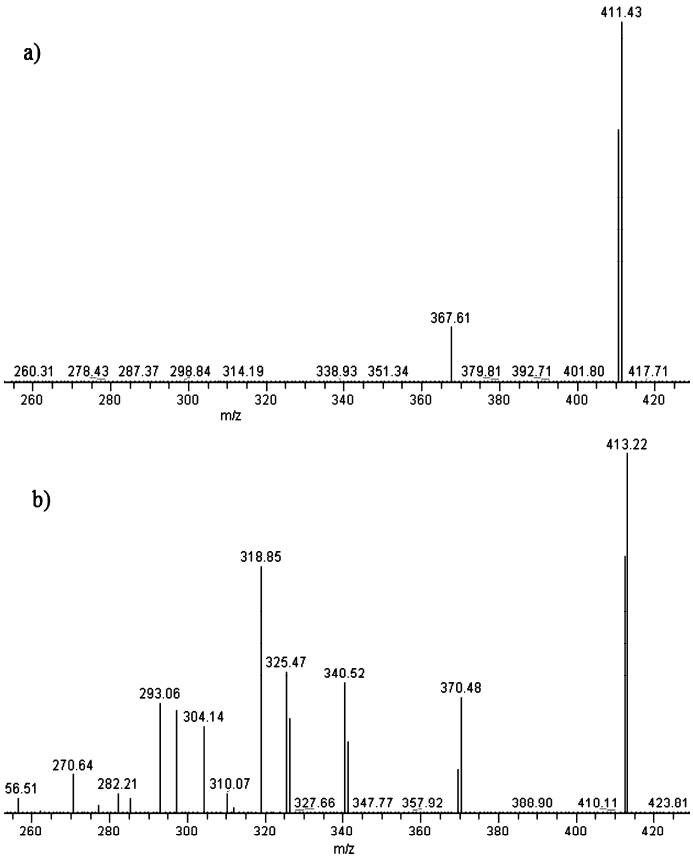


Fig. 1. MS/MS spectra of adapalene, obtained by use of mobile phase acetonitrile/0.1 % trifluoroacetic acid (1:1 volume ratio), flow rate: 500 µl $min^{-1}$, in: a) negative and b) positive mode.

The most dominant fragment ion in negative mode is a product of the adapalene decarboxilation (Fig. 4).

An elimination of both carboxyl and methoxy group could be observed in negative mode (*m/z* 336.7 in Fig. 2a and 337.1 in Fig. 3a), whereas elimination of hydroxyl moiety from the carboxyl group was observed in positive mode (*m/z* 394.3, Fig. 2b).

In both negative and positive mode, the fragmentation of adapalene depends on the mobile phase used in MS/MS analysis. Additionally, the increase in aceto-

nitrile content and the decrease in flow rate improved the signal strength of parent ion and product ions.

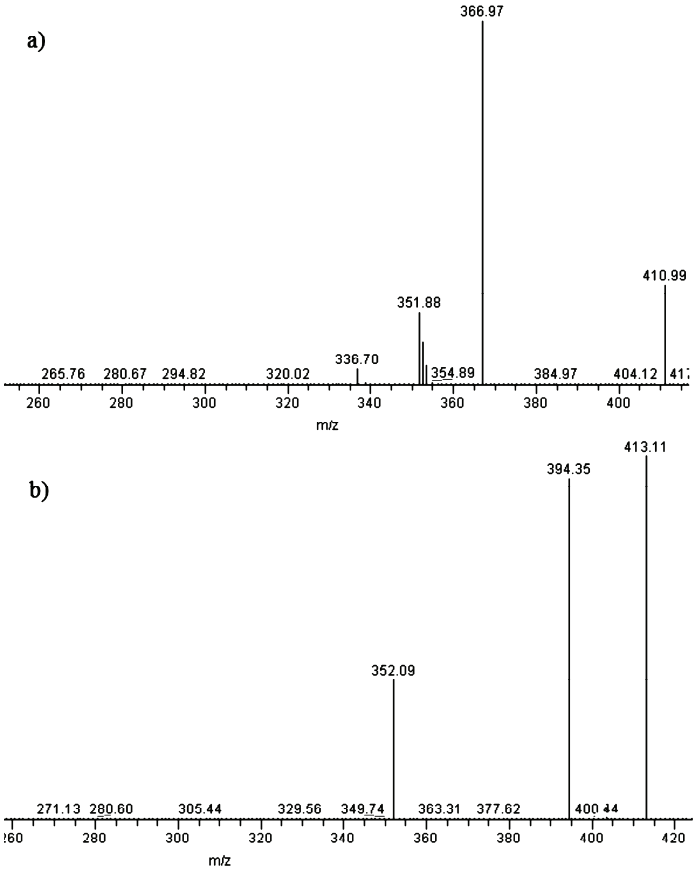


Fig. 2. MS/MS spectra of adapalene obtained by use of mobile phase acetonitrile/0.1 % formic acid (1:1 volume ratio), flow rate: 500 µl $min^{-1}$, in: a) negative and b) positive mode.

The strongest signals of parent ion and product ions in positive mode were observed when a mobile phase with volume composition acetonitrile/0.1 % trifluoroacetic acid = 50:50 was used. This mobile phase has the lowest pH value (pH 2.1) and an efficient protonation of adapalene in positive mode could be expected, resulting in the strongest signals of parent ion and product ions. In comparison with the negative mode, the signal strength of parent ion and product ions in positive mode was significantly lower for all the tested mobile phases.

The strongest signals of parent ion and product ions in negative mode were observed for a mobile phase with volume composition acetonitrile/20 mM ammonium acetate = 50:50. This mobile phase has the highest pH value (pH 7.2). Therefore, it could be expected that the production of an adapalene anion in

negative mode would be the most efficient with this mobile phase, thus resulting in the strongest signals of the parent ion and product ions.

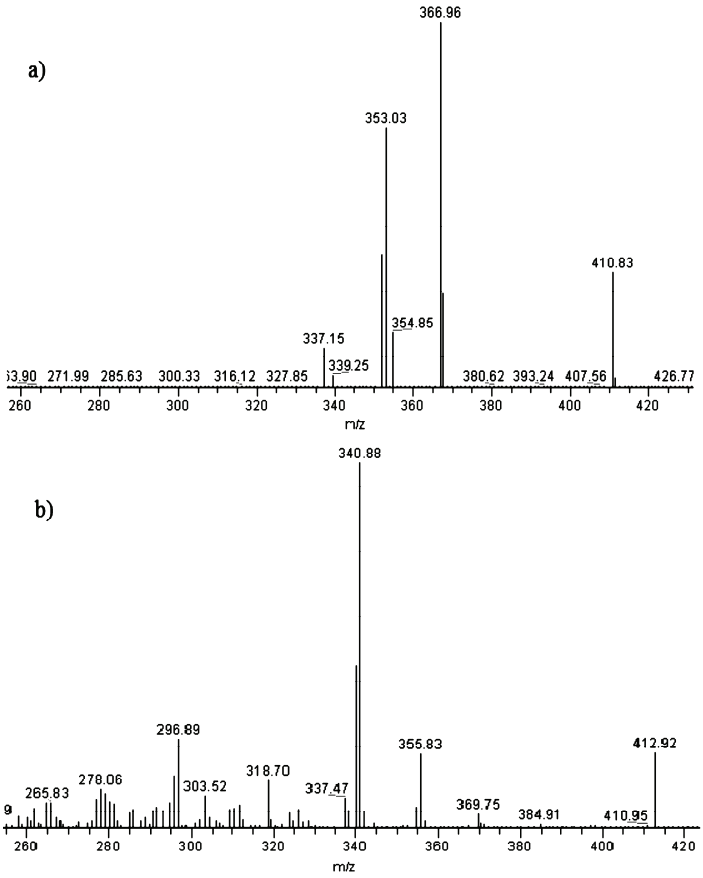


Fig. 3. MS/MS spectra of adapalene obtained by use of mobile phase acetonitrile/20 mM ammonium acetate (1:1 volume ratio), flow rate: 500 μl $min^{-1}$, in: a) negative and b) positive mode.

Among all the tested mobile phases and ionization modes, the strongest signals of parent ion and most dominant product ion were observed when a mobile phase with acetonitrile/20 mM ammonium acetate volume composition of 50:50 (negative mode, selected reaction monitoring SRM transition: 410.8→367.0) was used. Therefore, this mobile phase was selected for further development of the method.

In the next step, the composition of the mobile phase was optimized. A simultaneous monitoring of ion 410.8 (LC–MS, negative mode) and SRM transition 410.8→367.0 (LC–MS/MS, negative mode) was performed and the peak parameters (retention time, peak area and peak shape) were analyzed. The inc-

rease in the percentage of acetonitrile above 70 % led to slight decrease in the adapalene retention time, whereas the use of a mobile phase with volume composition acetonitrile/20 mM ammonium acetate = 90:10 resulted in a peak shape deformation. The decrease in the acetonitrile content below 70 % resulted in adapalene retention times higher than 10 min, which significantly lengthened the analysis. Therefore, the mobile phase with volume composition acetonitrile/20 mM ammonium acetate = 70:30 and flow rate 0.8 mL $min^{-1}$ was selected as the optimal one.

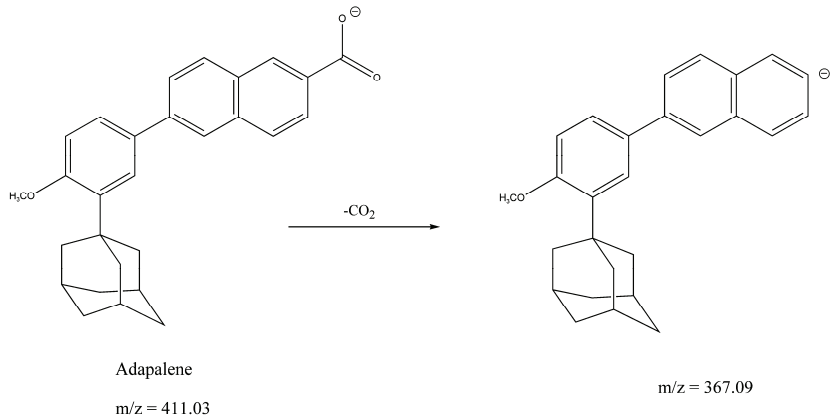


Fig. 4. Fragmentation of adapalene (negative mode; mobile phase: acetonitrile/20 mM ammonium acetate).

The area of the adapalene peak in LC–MS/MS mode was approximately 20 % higher than the one obtained by LC-MS and there were no other peaks apart from those corresponding to adapalene (Fig. 5).

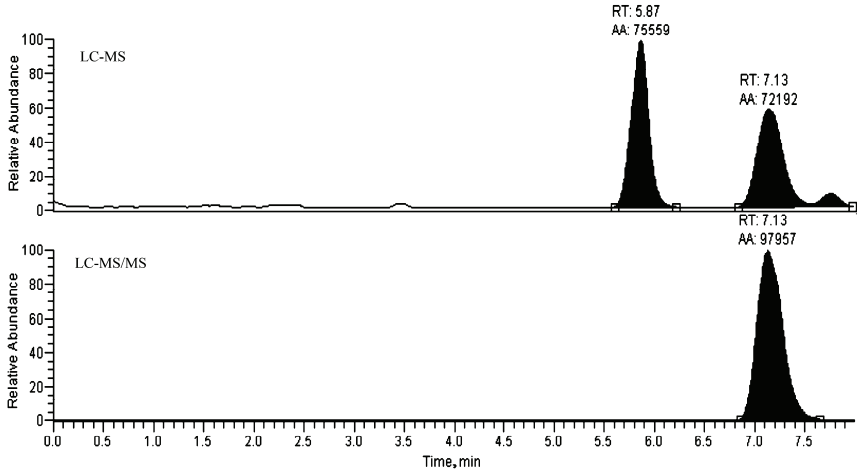


Fig. 5. LC–MS and LC–MS/MS chromatograms of microemulsion APG-1.

This indicated the higher sensitivity and specificity of the LC–MS/MS method.

Finally, the LC–MS/MS method (negative mode, SRM transition 410.8→ →367.0) with mobile phase volume composition acetonitrile/20 mM ammonium acetate = 70:30, mobile phase flow rate of 0.8 mL $min^{-1}$ and column temperature 25 °C was selected as optimal and validated according to the ICH guidelines.[20]

*Method validation*

*Robustness.* Small and deliberate changes in the column temperature, flow rate, concentration of ammonium acetate solution and mobile phase composition affected the retention factors and peak areas with less than 4.80 % (without any significant peak shape alterations), which proved the method's robustness.

*Specificity.* The method is specific for the determination of adapalene as no interference was observed at retention time corresponding to this substance. Overlaid sample chromatograms are presented in Fig. S-2 of the Supplementary material.

Since the specificity was proven by the use of different solvents and excipients, the application of this method to the analysis of adapalene in formulation studies (*e.g.*, solubility experiments in tested solvents and excipients, optimization of formulation composition and stability evaluation of adapalene topical forms) could be considered.

*Limits of detection and quantification.* The limits of detection and quantification were 2.0 and 6.7 ng $mL^{-1}$, respectively. The *LOQ* value was used as a first point in the linearity testing.

*Linearity.* Due to the wide range of expected concentrations in formulation studies, the linearity of the method was tested and proved in the range of 6.7 (*LOQ*)–700.0 ng $mL^{-1}$. The selected weighting factor was $1/y^2$, because its application resulted in the lowest sum of percentage relative error (17.63) in comparison to the other weighting factors (18.53–30.20) and unweighted calibration curve (115.44). The regression equation was $y = 144236x + 526.31$ and correlation coefficient ($r$) was 0.9990.

*Accuracy.* The recoveries of adapalene in tested formulations (microemulsion APG-1, Sona® 0.1 % gel and Sona® 0.1 % cream) at three concentration levels (80, 100 and 120 % of the target concentration) are presented in Table I. The recovery at each level is presented as a mean value of three determinations.

The recoveries were within the specified limits for active pharmaceutical ingredients (98–102 %), which proved the method to be accurate for the determination of adapalene in tested pharmaceutical forms.[21]

*Precision*. The precision was tested at three levels: injection repeatability, analysis repeatability and intermediate precision. The results are expressed as relative standard deviations (*RSD*, Table II).

TABLE I. Recovery values ($n$ = 3)

| Formulation | Level % | Total concentration, ng mL$^{-1}$ | Found concentration, ng mL$^{-1}$ | Recovery % | *RDS* % |
|---|---|---|---|---|---|
| Microemulsion | 80 | 328.00 | 330.69 | 100.82 | 1.43 |
| APG-1 | 100 | 410.00 | 404.89 | 98.75 | 1.31 |
| | 120 | 492.00 | 499.30 | 101.48 | 1.49 |
| Sona® 0.1% gel | 80 | 320.00 | 324.67 | 101.46 | 1.81 |
| | 100 | 400.00 | 405.92 | 101.48 | 0.78 |
| | 120 | 480.00 | 484.61 | 100.96 | 1.32 |
| Sona® 0.1 % | 80 | 340.00 | 341.81 | 100.53 | 1.11 |
| cream | 100 | 412.00 | 411.32 | 99.83 | 1.86 |
| | 120 | 484.00 | 476.16 | 98.38 | 1.64 |

TABLE II. Precision of the method

| Formulation | Level | Found concentration, mg g$^{-1}$ | *RSD* / % |
|---|---|---|---|
| Microemulsion | Injection repeatability | 1.04 | 0.86 |
| APG-1 | Analysis repeatability | 1.19 | 1.83 |
| | Intermediate precision | 1.06 | 2.58 |
| Sona® 0.1% gel | Injection repeatability | 0.99 | 0.81 |
| | Analysis repeatability | 1.02 | 1.19 |
| | Intermediate precision | 1.01 | 2.80 |
| Sona® 0.1% | Injection repeatability | 1.04 | 0.91 |
| cream | Analysis repeatability | 0.98 | 1.83 |
| | Intermediate precision | 0.96 | 2.05 |

The relative standard deviations were within the specified limits for injection repeatability (<1 %), analysis repeatability (<2 %) and intermediate precision (<3 %), which proved the precision of the method.[21]

## CONCLUSIONS

The development and validation of a LC–MS/MS method for the determination of adapalene in pharmaceutical forms for skin application were presented. The MS/MS analysis of adapalene was performed by use of three mobile phases (acetonitrile and 0.1 % formic acid; acetonitrile and 0.1 % trifluoroacetic acid; acetonitrile and 20 mM ammonium acetate). The strongest signals of parent ion and dominant product ion were obtained in negative mode, by use of the mobile phase consisting of acetonitrile and 20 mM ammonium acetate. Subsequently, optimization of the mobile phase composition and flow rate was performed. The method was validated according to the ICH guidelines, which confirmed its robustness, specificity, linearity, accuracy and precision. Due to its specificity tested by using different solvents and excipients and low *LOD* and *LOQ* values, application of this method to the analysis of adapalene in formulation studies (*e.g.*, solubility experiments in tested solvents and excipients, optimization of formulation composition and stability evaluation of adapalene topical forms) could be considered.

## SUPPLEMENTARY MATERIAL

Details about adapalene, chemicals and apparatus used and metod optimization and specificity are available electronically at the pages of journal website: http://www.shd.org.rs/ /JSCS/, or from the corresponding author on request.

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И З В О Д

## РАЗВОЈ И ВАЛИДАЦИЈА LC–MS/MS МЕТОДЕ ЗА ОДРЕЂИВАЊЕ АДАПАЛЕНА У ФАРМАЦЕУТСКИМ ПРЕПАРАТИМА ЗА ПРИМЕНУ НА КОЖИ

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Приказани су развој и валидација методе течне хроматографије са тандем масеном спектрометријом (LC–MS/MS) за одређивање адапалена у фармацеутским препаратима за примену на кожи. Извршена је MS/MS анализа адапалена употребом три мобилне фазе, које су се састоје из ацетонитрила и: a) 0,1 % раствора мрављe киселине, b) 0,1 % раствора трифлуоросирћетне киселине и c) 20 mM амонијум-ацетата. Најјачи сигнал полазног јона и главног фрагмента је добијен у негативном моду употребом мобилне фазе c). Валидација методе је извршена према смерницама ICH. Мале промене одабраних хроматографских параметара (концентрација амонијум-ацетата, састав мобилне фазе, температура колоне и проток) нису значајно утицале на квалитативне и квантитативне одговоре система, што доказује робусност методе. Метода је специфична за одређивање адапалена. Линеарност је доказана у опсегу 6,7–700,0 ng $mL^{-1}$ ($r$ = 0,9990), са границама детекције и квантификације од 2,0 и 6,7 ng $mL^{-1}$, редом. Тачност методе је потврђена израчунатим „recovery“ вредностима (98,4–101,5 %). Прецизност је испитана на три нивоа: поновљивост инјектовања, поновљивост анализе и средња прецизност. Израчунате релативне стандардне девијације су мање од 1, 2 и 3 %, редом.

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