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ORIGINAL RESEARCH PAPER

DEVELOPMENT AND VALIDATION OF A DISSOLUTION TEST FOR URSODEOXYCHOLIC ACID AND TAURINE FROM COMBINED FORMULATION

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Abstract: The objective of the work was to establish experimental conditions for the dissolution test for ursodeoxycholic acid (UDCA) and taurine in tablet dosage form (artichoke leaf extract, UDCA, taurine, and *Angelica sinensis* roots extract) and to validate the dissolution test for specificity, precision, linearity and range, accuracy, and robustness. A fast and robust method applicable for quantification of the four compounds was developed and validated according to International Council for Harmonisation (ICH) guidelines. A dissolution test for the UDCA-taurine combined formulation was developed and validated, using a suitable High-Performance Liquid Chromatography (HPLC) and titration method for simultaneously quantitation both dissolved substances. The optimized conditions include the use of USP apparatus 2 at a paddle rotation rate of 75 rpm and 900 mL of water as dissolution medium, at 37.0 ± 0.5 °C. The HPLC quantitation method for UDCA and titration for taurine was also adapted and validated. The both methods developed in this study showed specificity and selectivity with linearity (r = 0.9995) in the working range (50 - 130 %) and good precision and accuracy.

Keywords: *dissolution test, HPLC, ursodeoxycholic acid, taurine, validation*

INTRODUCTION

Chronic diseases of the hepatobiliary system are some of the most common human diseases and inferior to atherosclerosis only. According to World Health Organization, there are more than 2 billion people in the world who suffer from liver disease, which is 100 times the prevalence of HIV infection. Over the past 20 years, there has been a clear tendency in the world to increase the number of hepatobiliary diseases. There is an increase in the frequency of pathology of the hepatobiliary system at a young age, in women 4 - 7 times more often than in men. The fact that cholecystectomy is the most common surgical operation in the abdominal organs is evidenced by the widespread distribution of the pathology of the biliary system. Anxiety is caused by the fact that the number of patients with cholelithiasis increases in young and infant. Diseases of the biliary system are closely related to violations of the functional state of the liver. Synthesis of cholesterol supplemented bile with reduced bile acid content significantly increases the risk of gallstones, as well as gallbladder cholesterol [1 – 2].

As part of previous work the scientific substantiation of the safety of the pharmaceutical combination of artichoke leaf extract, ursodeoxycholic acid, taurine, and *Angelica sinensis* roots extract was conducted. The following preparation is designed for the treatment of dyspeptic disorders with functional disorders of the biliary system, biliary dyskinesia of the hypokinetic type, and gastritis with reflux of bile [2]. It should be noted that there is no such fixed combination in the drug market that is being discussed. However, components of the proposed combined formulation are used in the complex treatment of different diseases of the hepatobiliary system [2]. Recently, special attention is paid to the issues of pharmacological safety, including for combined preparations [3]. However, as we showed earlier [2], the proposed combination has a favorable safety profile.

The development of new and optimization of existing methods of drug analysis, as well as their validation is an important task of pharmaceutical science [4]. The dissolution test is intended to control the quality of solid dosage medications to demonstrate the conformity of the production process with the final product to pre-released series. Also, this test is an important tool for characterizing the biopharmaceutical quality of the product at its various stages.

Validation of dissolution test involves several aspects: validation of specifications, validation of dissolution conditions and validation of the analysis method. The United States Pharmacopeia (USP) and the European Pharmacopoeia (Ph. Eur.) as well as harmonized State Pharmacopoeia of Ukraine (SPU) provides guidelines on development and validation of dissolution procedures. Validation of a dissolution method typically involves validation of the end analysis method for specificity, precision, linearity, accuracy, and range [5].

The functional properties of the preparation are due to the physiological activity of ursodeoxycholic acid, as well as the complex of biologically active substances contained in the leaves of artichoke leaves and taurine.

Ursodeoxycholic acid (UDCA), also known as ursodiol is 3α, 7β-dihydroxy-5β-cholan-24-oic acid (Figure 1). It belongs to class 2 of the biopharmaceutical classification system (BCS) and is indicated for the management of cholestatic liver diseases. Ursodeoxycholic acid has membrane-stabilizing and hepatoprotective properties, helps to reduce the concentration of toxic bile acid for hepatocytes (cholic, lithocholic,

deoxycholic, etc.), has immunomodulatory properties; reduces the absorption of lipophilic bile acids in the intestine and dissolving cholesterol gallstones, prevents their formation [6 – 7].

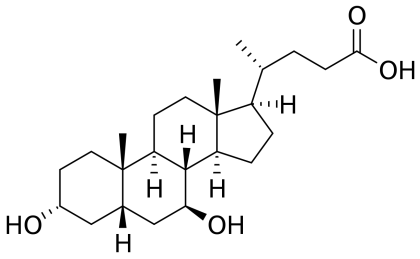

Figure 1. *Chemical structure of ursodeoxycholic acid*

Taurine (Figure 2), or 2-aminoethanesulfonic acid, reduces the risk of liver damage when bile stagnation. It is an irreplaceable amino acid that binds to ursodeoxycholic acid and other free bile acids to form a tauroconjugates, which increases their biological function [8].

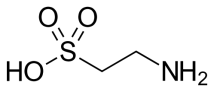

Figure 2. *Chemical structure of taurine*

A herbal components - artichoke leaves extract (*Cynara cardunculus* var. *scolymus*) with choleretic effect and *Angelica sinensis* roots extract with anti-arthrosclerotic, anti-hypertensive, antioxidant, anti-inflammatory activities are multicomponent herbal drugs [2]. The quantity of artichoke extract is determined by the amount of the sum of hydroxycinnamic acids by the UV-spectrophotometry method at 327 ± 2 nm, using the optical absorption index of chlorogenic acid. But content of hydroxycinnamic acids is too small and is about 5 mg per tablet. So it complicates determination of dissolution test for artichoke leaves extract which has been transferred to the solution from combined tablet formulation.

Many analytical methods for analysis of taurine have been reported: UV-spectrophotometric methods; HPLC using a pre- and post column derivatization with different types of detection; HILIC-chromatography with UV and ELSD detection, etc. [9 – 11, 22]. There are a lot of quantification methods for UDCA such as HPLC, LC/MS, but they are not adapted for its determination in dissolution media [12 – 15]. Also those methods are not suitable for simultaneous identification and quantitation of UDCA and taurine.

The aim of the present work was to establish experimental conditions for the dissolution test for UDCA and taurine in tablet dosage forms and to validate the dissolution test for specificity, precision, linearity and range, accuracy, and robustness.

MATERIALS AND METHODS

Materials and reagents

Ursodeoxycholic acid reference standard was purchased from EDQM and raw material was donated by Dr. Gustav Klein GmbH & Co. KG, Germany. Taurine reference standard was purchased from Qianjiang Yongan Pharmaceutical Co, Ltd, China, and raw material was donated by Dr. Gustav Klein GmbH & Co. KG, Germany. Dry extract of artichoke leaves (*Cynara scolymus* L.) was purchased from Dr. Müller Pharma, Czech Republic. Microcrystalline Cellulose VIVAPUR® MCC 102 Spheres (JRS Pharma, Germany) and plasdone povidone (Ashland) were used as binders. The other excipients were hydrophilic fumed silica with a specific surface area of 200 $m^2 \cdot g^{-1}$ Aerosil 200 (Evonik Ind., Germany), talc (Ferdunand Kreutzer Sabamuhle GmbH, Germany), magnesium stearate (Magnesia, Germany), Explotab® (JRS Pharma, Germany) and Opadry® II green (Colorcon) as colorant.
Ultrapure water was obtained from a Milli-Q® apparatus, methanol and acetonitrile HPLC grade were purchased from Sigma-Aldrich (USA), sodium dihydrogen phosphate and phosphoric acid was of analytical reagent grade. Formaldehyde, sodium hydroxide and phenolphthalein were obtained from Chimlaborreactiv (China). Buffer solutions were prepared according to Ph. Eur. 9.0.
All dilutions were performed in standard volumetric flasks. Solvents and solutions were filtered through 0.45 μm nylon filters before use.
All measurements are carried out in areas which meet the requirements of the measurement methods and requirements of the operational documents for the measuring equipment used during the measurements (climatic conditions, lighting, sound and vibration isolation, power supply parameters, availability of water supply and sewage, equipment with grounding, etc.): stable temperature (18 - 25 °C) and relative humidity (70 ± 5 %).
Filter validation was performed to determine if the filter effectively removes undissolved API from the dissolution media and stops the dissolution process of the sample. For this validation a sample solution of about 50 % of nominal analytical concentration was prepared. Three aliquots through separate filters filtered and dispense each into a separate test tube. One sample was analyzed immediately, one sample after 5 min of ultrasonication, and one sample after 10 min of sonication. Sonicated samples showed less than a 2 % increase in dissolved sample than the non-sonicated sample. Also an adsorbance test was performed to ensure that filters do not adsorb dissolved API and artificially lower results.
Standard and solution stability was performed for determination of the length of time which the samples and standards yield equivalent results as when prepared fresh.

Optimization of the dissolution test protocol

In the preliminary study of the combined tablets, it was found that the *p*H of the dissolution medium (artificial intestinal juice, *p*H 8.0, without pancreatin) given in the USP monograph for tablets with ursodeoxycholic acid [17] is not suitable for its determination in the presence of other active substances and concomitant substances (the effect of the physicochemical properties of the substance and the auxiliary

substances) – there is no peak of the ursodeoxycholic acid on chromatogram of the test solution. The same is true for the dissolution medium – artificial intestinal juice, *p*H 6.8, without pancreatin. However, taurine is reliably determined in these conditions.
To optimize the procedure for the "dissolution" section and simultaneously determine the ursodeoxycholic acid and taurine in the preparation, it is proposed to use water as the solvent medium. In this case, dissolved ursodeoxycholic acid and taurine are reliably determined, as confirmed by validation data and experimental data obtained at the pharmaceutical development stage and for the series used for stability studies and dissolution profiles.
The dissolution experiments were performed in a VAN KEL 7000 dissolution Test Station, configured as USP apparatus 2 (paddles).
Optimization was carried out employing 900 mL of water as dissolution medium per vessel, thermostatized at 37.0 ± 0.5 °C. The effect of the paddle rotation speed was examined at 50, 60 and 75 rpm. Sink conditions were verified through the analysis of dissolution samples taken from a vessel, where amounts of the drug equivalent to three times those in the tablet (150 mg of UDCA and 300 mg of taurine) were added.
Dissolution tests for determination of UDCA and taurine were performed on six tablets in accordance with the requirements of SPU 2.9.3, with the use of a paddle device (instrument 2).
For the combined tablet formulation, it is suggested to use one point of study for the release of active substances (taurine and ursodeoxycholic acid), as it relates to normal release drugs containing active substances that are rapidly soluble.
The drug can withstand the test if the amount of ursodeoxycholic acid and taurine that has been transferred to the solution in 45 minutes is at least 80 % (Q + 5 %) of the amount specified in the section "Composition per tablet".
This regulation is proposed on the basis of experimental data and SPU requirements (§ 2.9.3) for solid dosage forms with traditional release.
The dissolution samples were analyzed using HPLC for UDCA and titration for taurine.

HPLC-RID analysis of ursodeoxycholic acid

HPLC was performed on Shimadzu LC-20 Prominence module system equipped with a LC-20AD quaternary pump, a CTO-20A column oven, a SIL-20A autosampler, a Refractive Index Detector - RID-10A and LC-20 chemstation for data analysis was used. Chromatographic separations were carried out using an XBridge C18 (4.6 mm × 150 mm, i.d. 5 µm) column from Waters. Mobile phase consists of acetonitrile: 0.005 M sodium dihydrogen phosphate *p*H 3.0: methanol (30 : 37 : 40). Total run time is 15 min. The column was equilibrated for 10 min prior to each analysis at 40 ºC. RID temperature was 40 °C. Flow rate was 1.0 $mL \cdot min^{-1}$ and injection volume was 50 µL.
The stock solution of standard of ursodeoxycholic acid corresponding to 15.0 mg was prepared in a 50 mL volumetric flask by dissolving an accurately weighed amount of UDCA in a dissolution medium, heated up to 37 °C and, if necessary, placed in an ultrasound bath.
System suitability: the relative standard deviation (RSD, %) of the areas of the ursodeoxycholic acid peak at 6 re-injections of the standard solution should not exceed 2.0 %. The convergence between the two prepared standard solutions should be 98 - 102 %.

The amount of ursodeoxycholic acid transferred to the solution is calculated as a percentage of the content in the tablet by the following formula (equation 1):

$$X = \frac{A_{\text{samp}}}{A_{\text{std}}} \times \frac{W_{\text{std}}}{50} \times \frac{900}{\text{a}} \times \frac{P_{std}}{100} \times 100 \quad (1)$$

where A_{samp} - the peak area of the ursodeoxycholic acid on the chromatogram of the test solution; A_{std} - the peak area of the ursodeoxycholic acid on the chromatogram of the standard solution; W_{std} - weight of working standard ursodeoxycholic acid used for preparation of standard solution, mg; P_{std} - purity of the working standard of ursodeoxycholic acid, %; a - the claimed amount of ursodeoxycholic acid, mg / tablet (150 mg).

Titration analysis of taurine

The content of taurine is determined by the acid-base titration method. Taurine, due to the amphoteric nature, cannot be directly titrated with alkaline solution. Titration is possible if the amino group is blocked by the formaldehyde. The formed compound can be titrated alkalimetrically in a presence of phenolphthalein indicator.
In order to determine the content of taurine in dissolution media 50.0 mL of the solution are placed in a titration flask, 5 mL of formaldehyde are added and titration was performed with 0.1 M sodium hydroxide solution until pale pink color appears (indicator - 60 µL of phenolphthalein). Blank determination was performed.
1 mL of a 0.1 M solution of sodium hydroxide corresponds to 0.01252 g of $C_2H_2NO_3S$ (taurine).
The amount of taurine that has been transferred to the solution from combined tablet formulation is calculated as a percentage of the content in the tablet by the formula from equation (2):

$$X = \frac{T \times (V - V_0) \times K \times 900 \times 100}{a \times 1 \times 50} \quad (2)$$

where T - titre for titrated sodium hydroxide solution by taurine; V - volume of 0,1 M solution of sodium hydroxide, used on titration, mL; V_0 - volume of 0.1 M solution of sodium hydroxide used for titration in the control test, mL; K - coefficient of correction of 0.1 M solution of sodium hydroxide; a - the content of taurine in one tablet, indicated in the section “Composition” in mg.

RESULTS AND DISCUSSION

The experimental results revealed that, regarding of the paddle rotation speed, the dissolution rate of UDCA and taurine is increased with increasing of rpm. It was also observed that the dissolution specification for conventional-release oral dosage forms of the Ph. Eur. (no less than 75 % of the labeled amount in 45 min) are more confidently at 75 rpm.
According to EU-guidelines, validation is called to prove that any procedure, process, equipment, material, activity or system actually leads to the expected results. Various

guidance documents regulated validation process are ICH Q2A, ICH Q2B, FDA and Pharmacopoeias (USP and Ph. Eur.) [16 – 18].
The HPLC and titration methods were validated by the analysis of specificity, linearity, accuracy, precision, and robustness to demonstrate reproducibility and reliability, uncertainty [5].

Validation of HPLC-RID method

Specificity of the method

This parameter was determined by comparing the chromatograms of the UDCA standard, drug-loaded model combined tablet formulation and the last one without UDCA (placebo), and the chromatograms are shown in Figures 3, 4 and 5.

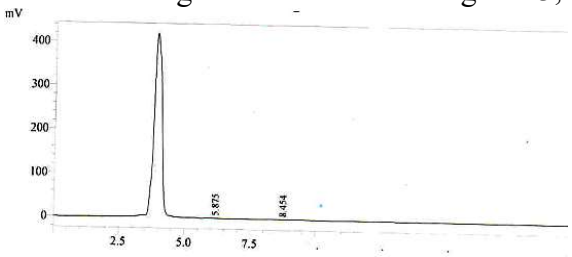

Figure 3. *Typical chromatogram of placebo*

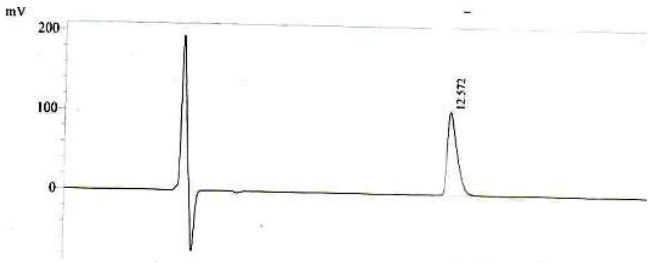

Figure 4. *Typical chromatogram of UDCA standard*

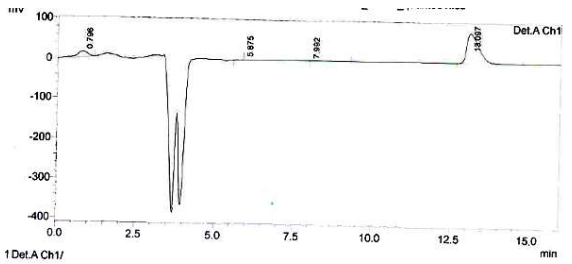

Figure 5. *Typical chromatogram of UDCA drug-loaded model combined tablet formulation*

Specificity of the test is confirmed by: the retention time of the UDCA peak on the test solution chromatogram coincides with the retention of this peak on the chromatogram of the drug-loaded model combined tablet formulation solution; the chromatographic conditions chosen allow us to separate the peak of the ursodeoxycholic acid from the peaks of the auxiliary substances (placebo) and from systemic peaks (blank solution) (Table 1).

Thus, the absence of the interference of the peak of the ursodeoxycholic acid, which is determined by the HPLC method, with the peaks of any component of the matrix solution and the solvent is shown.

Table 1. *Specificity parameters of the HPLC method*

Analyte - UDCA		
Retention time [min]	Blank	–
	UDCA standard	12.6
	Drug-loaded model combined tablet formulation	13.0
	Placebo	–
Number of theoretical plates	Blank	–
	UDCA standard	6768
	Drug-loaded model combined tablet formulation	5212
	Placebo	–
Tailing factor	Blank	–
	UDCA standard	1.9
	Drug-loaded model combined tablet formulation	1.8
	Placebo	–

Accuracy, linearity, range and recovery

Linearity, recovery, accuracy and range of titration method were defined on model mixtures with known content of active ingredients in the range of 50 to 130 % of the UDCA nominal value.

One of linearity indexes is residual standard deviation s_0 – the confidence interval of the points scattered around the line Y_i = a+b×X_i is t(95 %, g-2)×RSD$_o$ and is a confidence interval of the uncertainty of the analysis method (Δ_{AS}).

Reporting statistical parameters like the intersection of the calibration curve to the origin a characterizes the systematic error in the analysis and have to be statistically insignificantly different from zero: the value of a must be less than the confidence interval of its uncertainty (equations 3 and 4).

$$|a| \le t(95\%, g-2) \times s_b \tag{3}$$

$$|a| \le \left| \frac{0.32 \times \Delta_{AS,r}}{1-(X_{\min}/100)} \right| \tag{4}$$

Since g = 9 (the number of solutions used to build a regression line) critical values for linearity, precision, accuracy and uncertainty testing was calculated (Tables 2 and 3).

Table 2. *Critical values for linearity, precision, accuracy and uncertainty testing of the HPLC and titration method*

Range	**S_v [%]**	**max Δ_{AS} [%]**	**max δ [%]**	**max S_0 [%]**	**min r**	**max a [%]**
50 - 130 %, step 10 %	30.43	3.0	0.96	1.58	0.9987	9.6* 1.92*

*criterion of statistical insignificance

Table 3. *Accuracy and precision of the HPLC method*

Model solution	**Weight of UDCA [mg]**	**Theoretical concentration [%]**	**Mean peak area**	**Relative value of peak area [%]**	**Calculated concentration [%]**	**Found in % to the theoretical concentration [%]**
MP	m_0	$X = \frac{C_i}{C_{st}} \times 100\%$	A	RA	$Y = \frac{A_i}{A_{st}} \times 100\%$	$Z = \frac{Y_i}{X_i} \times 100\%$
lin50	75	50.00	930087	55.80	50.28	100.57
lin60	90	60.00	1096939	65.81	59.30	98.84
lin70	105	70.00	1314175	78.84	71.05	101.50
lin80	120	80.00	1478863	88.72	79.95	99.94
lin90	135	90.00	1670725	100.23	90.32	100.36
lin100	150	100.00	1849975	110.98	100.02	100.02
lin110	165	110.00	2025752	121.53	109.52	99.56
lin120	180	120.00	2248654	134.90	121.57	101.31
lin130	195	130.00	2386812	143.19	129.04	99.26
Value $\overline{Z}$ [%]						100.15
SD_z [%]						0.89
Relative confidence interval $\Delta_Z = t(95\%,g-1) \times SDz/\sqrt{3} = 1.8595 \times s_z \leq 1{,}07$ [%]						1.65
Critical value to the convergence of results Δ_{AS} [%]						3.00
Systematic error $\delta = \lvert \overline{Z} - 100 \rvert$ [%]						0.15
Criterion of insignificance of systematic error 1) $\delta \leq \Delta_Z / 3 = 0.29$ %						TRUE
2) if not true 1), than $\delta \leq 2 \times \max\Delta_{AS}/3 = 2.13$ %						TRUE
Conclusion						COMPLIES

Calibration Curves, the Limit of Detection (LOD) and Quantification (LOQ)

Regression line was calculated by the method of least squares (Table 4, Figure 6).

Table 4. *Parameters of linear regression line for HPLC method*

Index	**Value**	**Criterion (50 - 130 %), g = 9**	**Result**
b	0.998029	–	–
s_b	0.011174	–	–
a	0.002941	1) ≤ 9.6. 2) ≤ 1.92	TRUE
s_a	0.010462	–	–
s_r	0.008655	≤ max S_0	TRUE
r	0.999562	≥ min r	TRUE

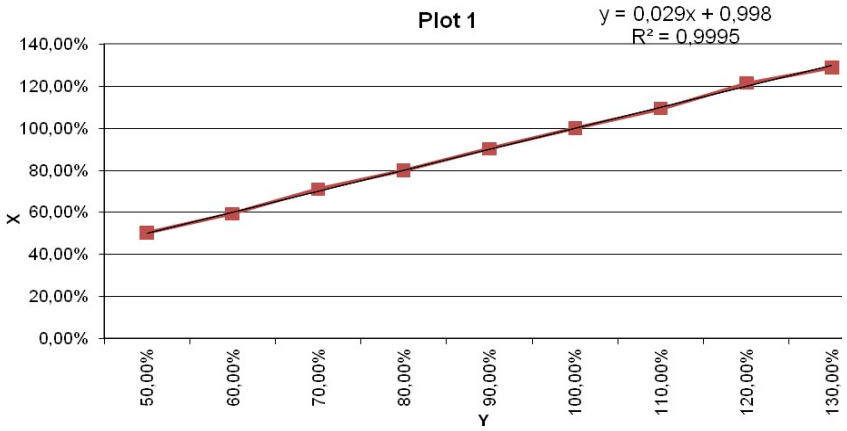

Figure 6. *Linear dependence of the theoretical concentration of UDCA on the concentration found in normalized coordinates*

The linearity of the method is confirmed throughout the range of concentrations (50 - 130 %).
Limit of detection (LOD) and limit of quantitation (LOQ) of UDCA:
LOD = 3.3 × s_a / b = 0.034 % to the concentration of the standard solution;
LOQ = 10 × s_a / b = 0.104 % to the concentration of the standard solution.
The values of LOD and LOQ are significantly lower than the lower limit of the concentration range (50 %) and can not affect the accuracy of the test.

Precision
The precision of an analytical procedure is expressed as the standard deviation (SD) or relative standard deviation (RSD %). To assess precision, two replicates at 150 mg of UDCA were analyzed by different analytics and relative standard deviation for each level was calculated (Table 5).

Table 5. *Calculated results of intra-laboratory precision*

No of solution	Value Zi	
	Analyst 1	Analyst 2
MS1	102.59 %	97.78 %
MS2	100.25 %	101.15 %
MS3	99.47 %	99.13 %
MS4	99.83 %	101.48 %
MS5	99.89 %	99.96 %
MS6	102.04 %	100.00 %
$\overline{Z}$	100.68 %	99.92 %
Combined average value Z_{intra} [%]	100.30 %	
S_z	1.30 %	1.35 %
RSD_{intra} $\overline{Z}$	1.33 %	
Δ_{intra}	2.38 %	

$\Delta_{intra} = 2{,}38\% \le \max \Delta_{AS} = 3{,}0\%$. Thus, the method is characterized by high intra-laboratory precision throughout the range of concentrations close to the nominal concentration.

Stability and robustness

Standards are typically considered stable as long as they have > 98 % recovery against a fresh standard. The 100 % LC solution from linearity can also act as the Day 0 Standard Stability.

Aliquots of this solution was split and stored at lab bench in clear volumetric flask, lab bench in amber volumetric flask and in cold room at ~4 ºC. These aliquots were compared against fresh standards at various intervals to determine standard stability (Table 6).

Table 6. *UDCA solution stability at different conditions*

Conditions	**Solution stability [%]**								
	Days								
	0	**1**	**2**	**3**	**4**	**Mean**	**RSD_t**	**Δ_t**	**max δ**
Clear flask r. t.	100	99.7	99.1	98.2	96.3	98.66	0.015	0.0321	0.96
Clear flask 4 ºC	100	99.8	99.6	98.5	98.0	99.18	0.009	0.0189	
Amber flask r. t.	100	99.6	99.0	98.3	96.8	98.74	0.013	0.0272	

r. t. – room temperature

Thus, it is recommended to perform all experiments during as a maximum 2 - 3 days and store stock and standard solutions in a refrigerator.

The calculated results of robustness investigation are shown in Table 7.

Table 7. *HPLC method robustness*

Factor	**Parameters**	**UDCA peak area**	
		Drug-loaded model combined tablet formulation	**UDCA standard**
Column temperature [°C]	36	1889950	1938805
	40	1847699	1956127
	44	1866821	1951431
	RSD [%]	0.011	0.005
Flow of the mobile phase [mL·min^{-1}]	0.8	1899609	1984566
	1.0	1847699	1956127
	1.2	1995132	1994858
	RSD [%]	0.039	0.010
RID detector temperature [°C]	36	1986021	19689395
	40	1847699	1956127
	44	1891872	1947502
	RSD [%]	0.037	1.302

The column temperature, the mobile phase flow and the detector temperature within ± 10 % does not significantly affect the test results.

Uncertainty of the HPLC-RID Method

For the case of the dissolution test, the maximum relative absolute uncertainty of the analysis method (Δ_{AS} (%)) is 3.0 %.

$$\Delta_{AS} \leq \max\Delta_{AS} = 3.0\% \quad (5)$$

The complete uncertainty of the test results consists of the uncertainty of the sample preparation Δ_{SP} and the uncertainty of the final analytical procedure Δ_{FAO}:

$$\Delta_{AS} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} \quad (6)$$

Δ_{SP} = 0.26 %, as the error of analytical balances is 0.2 % and the uncertainty of 50 mL flask for dilution of standard solution is 0.17 %.

The greatest uncertainty in sample preparation makes the weighing of the standard sample.

The uncertainty of the final analytical operation for the HLPC method is 0.16 %, this value follows from the requirements of the SPU for RSD_{max}.

$$\Delta_{FAO} = 0.16\% \quad (7)$$

$$\Delta_{AS} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{0.26^2 + 0.16^2} = 0.31\% \quad (8)$$

$$\Delta_{AS} = 0.31\% \leq \max\Delta_{AS} = 3\% \quad (9)$$

Validation of titration method

Specificity of the method

To assess the specificity of the method it was necessary to check insignity of the solvent and the effects of placebo. Suitable blank solution and placebo solution was prepared. Results of specificity check showed that volume of titrant was 4 µL for placebo solution and 0.75 mL for model mixture solution of UDCA, taurine, artichoke leaves extract.

Accuracy, linearity, range and recovery

Linearity, recovery, accuracy and range of titration method were defined on model mixtures with known content of active ingredients in the range of 50 to 130 % of the taurine nominal value.

One of linearity indexes is residual standard deviation s_0 – the confidence interval of the points scattered around the line $Y_i = a + b \times X_i$ is $t(95\%, g\text{-}2) \times RSD_o$ and is a confidence interval of the uncertainty of the analysis method (Δ_{AS}).

$$s_0 \leq \frac{\sqrt{k} \times \Delta_{AS}}{t(95\%, g-2) \times 3} \quad (10)$$

Reporting statistical parameters like the slope of the calibration curve, b and its intersection to the origin a characterizes the systematic error in the analysis and have to be statistically insignificantly different from zero: the value of a must be less than the confidence interval of its uncertainty.

$$|a| \leq t(95\%, g-2) \times s_a \quad (11)$$

$$|b-1| \leq t(95\%, g-2) \times s_b \quad (12)$$

Systematic error δ within the range of 50 - 130 % of the method of titration:

$$\delta 50 = |a + b \times 50 - 50| \leq \frac{2}{3} \times \Delta_{AS} \quad (13)$$

$$\delta 130 = |a + b \times 130 - 130| \leq \frac{2}{3} \times \Delta_{AS} \quad (14)$$

Since $g = 9$ (the number of solutions used to build a regression line) values for linearity, precision, accuracy and uncertainty testing was calculated (Table 8).

Table 8. *Accuracy and precision of the titrimetric method*

Model solution	**Weight of taurine [mg]**	**Theoretical concentration [%]**	**Mean volume of titrant**	**Relative value of titrant volume**	**Calculated concentration [%]**	**Found in % to the theoretical concentration [%]**
MS	m	$X = \frac{m_i}{m_{st}} \times 100\%$	V	RV	$Y = \frac{V_i}{V_{st}} \times 100\%$	$Z = \frac{Y_i}{X_i} \times 100\%$
lin50	241	80.33	0.6	80.72 %	81.55	101.51
lin60	255	85.00	0.63	84.75 %	85.62	100.73
lin70	267	89.00	0.66	88.79 %	89.70	100.79
lin80	285	95.00	0.7	94.17 %	95.14	100.14
lin90	303	101.00	0.75	100.90 %	101.93	100.92
lin100	318	106.00	0.78	104.93 %	106.01	100.01
lin110	333	111.00	0.82	110.31 %	111.45	100.40
lin120	348	116.00	0.85	114.35 %	115.52	99.59
lin130	365	121.67	0.9	121.08 %	122.32	100.54
Value $\overline{Z}$ [%]						100.52
SD_z [%]						0.56
Relative confidence interval $\Delta_Z = t(95\%,g\text{-}1) \times SDz/\sqrt{3} = 1.8595 \times s_z \leq 1{,}07\%$						0.60
Critical value to the convergence of results Δ_{AS}						3.20
Systematic error $\delta = \lvert \overline{Z} - 100 \rvert$						0.52
Criterion of insignificance of systematic error 1) $\delta \leq \Delta_Z / 3 = 0.187$ %						FALSE
2) if not true 1), than $\delta \leq 2 \times \max\Delta_{AS}/3 = 2.13$ %						TRUE
Conclusion						COMPLIES

Calibration Curves, the Limit of Detection (LOD) and Quantification (LOQ)

Regression line was calculated by the method of least squares (Table 9, Figure 7).

The linearity of the method is confirmed throughout the range of concentrations (50 - 130 %).

Limit of detection (LOD) and limit of quantitation (LOQ) of taurine:

LOD = $3.3 \times s_a / b$ = 0.04 % to the concentration of the standard solution;

LOQ = $10 \times s_a / b$ = 0.11 % to the concentration of the standard solution.

The values of LOD and LOQ are significantly lower than the lower limit of the concentration range (50 %) and cannot affect the accuracy of the test.

Table 9. *Parameters of linear regression line for titration method*

Index	Value	Criterion (50 - 130 %), g = 9	Result
b	0.980757	–	–
s_b	0.011389	–	–
a	0.024063	1) ≤ 1.92 2) ≤ 9.6	TRUE
s_a	0.011554	–	–
s_r	0.00461	≤ max S_0	TRUE
r	0.999528	≥ min r	TRUE

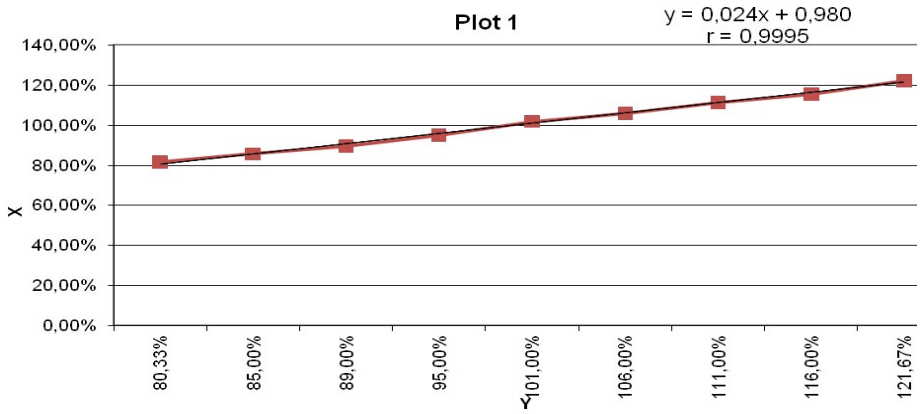

Figure 7. *Linear dependence of the theoretical concentration of taurine on the concentration found in normalized coordinates*

Precision

The precision of an analytical procedure is expressed as the standard deviation (SD) or relative standard deviation (RSD %). To assess precision, two replicates at 300 mg of taurine were analyzed by different analytics and relative standard deviation for each level was calculated (Table 10).

Table 10. *Calculated results of intra-laboratory precision of taurine analysis*

No. of solution	Value Zi	
	Analyst 1	Analyst 2
MS1	101.59 %	100.57 %
MS2	101.26 %	100.71 %
MS3	100.57 %	100.91 %
MS4	99.21 %	100.57 %
MS5	100.57 %	100.57 %
MS6	100.92 %	101.06 %
$\overline{Z}$	100.69 %	100.73 %
Combined average value Z_{intra}, %	100.71 %	
S_z	0.82 %	0.21%
RSD_{intra} $\overline{Z}$	0.57 %	
Δ_{intra}	1.03 %	

$\Delta_{intra} = 1.03\% \leq \max \Delta_{AS} = 3.0\%$. Thus, the method is characterized by high intra-laboratory precision throughout the range of concentrations close to the nominal concentration.

Stability and robustness

In the procedure for assay of taurine, no time is given for titration; therefore, it was tested for its stability over time. Titration of the investigated solutions was carried out with a time interval of 15 min for 1 h for the model solution and the standard solution of taurine (Table 11).

Table 11. *Taurine solution stability*

Solution	Time [min]					Mean [mL]	RSD_t [%]	Δ_t [%]	max δ [%]
	0	15	30	45	60				
Model solution	0.74	0.74	0.74	0.75	0.75	0.744	0.007	0.015	0.96
Standard solution of taurine	15.4	15.4	15.4	15.5	15.5	15.44	0.004	0.008	

Since Δ_t for taurine is less than max δ (0.96 %), the method is stable over investigated time.

Titration result does not depend on the acidity of the solution since buffer solutions are not used in the preparation of solutions.

Uncertainty of the titration method

For the case of the dissolution test, the maximum relative absolute uncertainty of the analysis method (Δ_{AS} (%)) is 3.0 %.

The complete uncertainty of the test results consists of the uncertainty of the sample preparation Δ_{SP} and the uncertainty of the final analytical procedure $\Delta_{FAO} \cdot \Delta_{SP}$ = 0.2 %, as the error of analytical balances is 0.2 %.

The uncertainty of the final analytical operation is related to measuring the volume of titrated solution (burette) and determining the float coefficient to the titrant solution.

$$\Delta_{FAO} = 0.001\% \tag{15}$$

$$\Delta_{AS} = \sqrt{\Delta_{SP}^2 + \Delta_{FAO}^2} = \sqrt{0.2^2 + 0.001^2} = 0.2\% \tag{16}$$

$$\Delta_{AS} = 0.2\% \leq \max \Delta_{AS} = 3.0\% \tag{17}$$

An analytical method used in formulation screening, dissolution test and quality control of products with a fixed dosage should be reliable, effective and reliable. During the development of prototypes of fixed-dose formulations containing ursodeoxycholic acid, taurine, extract of *Angelica sinensis* and *Cynara scolymus,* and it became apparent that the method that fulfills the above criteria and which can be applied for simultaneous quantification of ursodeoxycholic acid and taurine has not yet been described in the literature. A detailed literature study has shown that over the past decades several reliable and effective HPLC methods have been published for the analysis of ursodeoxycholic acid in raw material and pharmaceutical formulations with a single API [12 – 14, 19, 20], as well as simultaneous detection of UDCA and other substances

(*e.g.* chenodeoxycholic acid [21]. Several methods have been published regarding taurine analysis in biological samples [10] and in foods [9, 11, 22]. These methods are mostly distinguished by the detection method used, but in principle they would be applicable for our purpose.

The initial screening of these methods in our laboratory showed that the methods were not directly applicable to the detection of ursodeoxycholic acid in the presence of taurine and extracts of *Angelica sinensis* and *Cynara scolymus*. Selective and reliable HPLC methods for the detection of more than one compound within one run have been published [15]. However, the above methods cannot be used directly in our case. The method of simultaneous determination of ursodeoxycholic acid and chenodeoxycholic acid in a pharmaceutical dosage form, published by Khairy *et al.* [21] for example, uses a C18 column and mobile phase consisting of an acetonitrile-phosphate buffer mixture (*p*H 2.3, 100 mM, 50 + 50, v/v) at a flow rate of 2.0 mL·min^{-1} with UV detection at 210 nm. Another work was devoted to HPLC analysis of ursodeoxycholic acid in raw material and pharmaceutical formulations [19]. Cromatographic conditions were the following: symmetry-C18 column (150 mm × 4.6 mm, id; particle size 5 μm), 40 °C, 100 μL injection volume and UV detection at 200 nm. The flow rate was 1 mL·min^{-i} using acetonitrile - phosphoric acid (*p*H 3.0; 0.15 mM) (48 : 52) as mobile phase. Our preliminary studies showed that this techniques was not suitable for analyzing UDCA and taurine in the fixed combination in the form of tablets. Consequently, we had to develop a novel method.

The preconditions for optimizing the dissolution test protocol were the results of our previous studies on the *p*H of the dissolution medium (artificial intestinal juice, *p*H 8.0, without pancreatin), according to the USP monograph for tablets with UDCA [17]. Under these conditions, UDCA peak on the chromatogram of the test solution was absent. Consequently, such conditions are not suitable for the determination of UDCA in the presence of other active substances and excipients due to their physical and chemical properties. Similar results were obtained with the use of another dilution medium – artificial intestinal juice, *p*H 6.8, without pancreatin. It should be noted that in such conditions, the determination of taurine was quite reliable. Optimization of the dissolution test procedure and the simultaneous determination of ursodeoxycholic acid and taurine in the developed tablet preparation were to use water as a dissolution medium. In our opinion, the content of taurine is better determined by the simple method of acid-base titration. It should be noted that the amphoteric substance taurine cannot be directly titrated with an alkaline solution of titrant. For this purpose, the amino group of taurine was previously blocked by formaldehyde, and the formed compound is already possible to titrate alkalimetrically in the presence of an appropriate indicator (phenolphthalein). UDCA has an acidic group but it doesn't interfere with the titration of taurine because sulfo group of taurine has strongest acidic property than UDCA (*p*Ka taurine 1.5 < *p*Ka UDCA 6.0).

CONCLUSIONS

First suggested use of tablet dosage form containing following active pharmaceutical ingredients: artichoke leaf extract, UDCA, taurine, and *Angelica sinensis* roots extract. We were able to develop a simple titration method for the determination of taurine

content and HPLC method for the determination of ursodeoxycholic acid in combined tablet formulation and the methods was found accurate, precise, rugged and specific and can be used as alternative to the current pharmacopeial methods with confidence (Table 12). The conducted studies are an element of pharmaceutical development of the corresponding dosage form in accordance with ICH guideline Q8(R2).

Table 12. *Validation parameters for developed analytical methods*

Parameters	**Results**	
Specificity	HPLC analytical procedure is specific	Titration analytical procedure is specific
Range	50 - 130 %	50 - 130 %
Linearity	r = 0.9995 $Y_i = 0.003 + 0.9980X_i$	r = 0.9995 $Y_i = 0.024 + 0.9807X_i$
Accuracy	$\delta\% = 0.15\ \% < 0.29\ \%$	$\delta\% = 0.52\ \% < 2.13\ \%$
Precision: Repeatability	$\Delta_Z = 1.65\ \% < 3.0\ \%$	$\Delta_Z = 0.60\ \% < 3.0\ \%$
Intra-laboratory precision	$\Delta_{intra} = 2.38\ \% < 3.0\ \%$	$\Delta_{intra} = 1.03\ \% < 3.0\ \%$
Stability	$\Delta_t = 0.032\ \% < \max\ \delta = 0.96\ \%$	$\Delta_t = 0.015\ \% < \max\ \delta = 0.96\ \%$
LOQ	0.10 %	0.11 %
LOD	0.03 %	0.04 %

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REFERENCES

1. Chhabra, S.K., Ahmed, Z., Massey, A., Agarwal, S., Vij, V., Agarwal, B., Kankaria, J., Jenaw, R.K.: Laparoscopic cholecystectomy in a 2 year old male child with choleilithiasis and recurrent right hypochondrial pain: Case report and review of literature, *International Journal of Surgery Case Reports*, **2016**, **26**, 142-145;
2. Bondarenko, L.B., Gorchakova, N.O., Golembiovska, O.I., Galkin, O.Y.: Promising new fixed combination for the treatment of diseases of the hepatobiliar system: Substantiation of pharmacotherapeutic properties and pharmaceutical quality profile, *Regulatory Mechanisms in Biosystems*, **2018**, **9** (1), 23-40;
3. Gorchakova, N., Heimuller, E., Galkin, A.: Current safety data of the complex herbal medicine with sedative and cardioprotective actions, *Innovative Biosystems and Bioengineering*, **2018**, **2** (3), 163-174;
4. Lutsenko, T.N., Kovalenko, M.V., Galkin, O.Y.: Validation of biological activity testing procedure of recombinant human interleukin-7, *Ukrainian Biochemical Journal*, **2017**, **89** (1), 82-89;
5. The State Pharmacopoeia of Ukraine, The 2nd edition, Vol. 1, Ukrainian Scientific Pharmacopoeial Center for Medicines Quality, **2015**;
6. Larghi, A., Crosignani, A., Battezzati, P.M., De Valle, G., Allocca, M., Invernizzi, P., Zuin, M., Podda, M.: Ursodeoxycholic and tauro-ursodeoxycholic acids for the treatment of primary biliary cirrhosis: a pilot crossover study, *Alimentary Pharmacology & Therapeutics*, **1997**, **11**, 409-414;

7. Lazaridis, K.N., Gores, G.J., Lindor, K.D.: Ursodeoxycholic acid mechanisms of action and clinical use in hepatobiliary disorders, *Journal of Hepatology*, **2001**, **35** (1), 134-146;
8. Chesney, R.W.: Taurine: its biological role and clinical implications, *Advances in Pediatrics,* **1985**, **32**, 1-42;
9. McConn, B.: Determination of taurine in energy drinks by high-performance liquid chromatography, *Journal of Analytical Chemistry*, **2012**, **3**, 47-52;
10. Wang, X.F., Chi, D.F., Su, G.M., Li, L., Shao, L.H.: Determination of taurine in biological samples by high-performance liquid chromatography using 4-fluoro-7-nitrobenzofurazan as a derivatizing agent, *Biomedical and Environmental Sciences*, **2011**, **24** (5), 537-542;
11. Catharino, R., Haddad, R., Godoy, H., Eberlin, M., Santos, L.: Fast analysis of taurine in energetic drinks by electrospray ionization mass spectrometry, *Journal of the Brazilian Chemical Society*, **2011**, **22** (4), 801-806;
12. El-Kafrawy, D.S., Belal, T.S., Mahrous, M.S., Abdel-Khalek, M.M., Abo-Gharam, A.H.: Validated spectrophotometric and RP-HPLC–DAD methods for the determination of ursodeoxycholic acid based on derivatization with 2-nitrophenylhydrazine, *Journal of AOAC International*, **2017**, **100** (3), 677-685;
13. Mukherjee, J., Pal, T.K.: Development and validation of RP-HPLC method to determine ursodeoxycholic acid in pharmaceutical dosage forms, *International Journal of Pharmaceutical Sciences and Research*, **2010**, **2** (1), 73-78;
14. Hasan, N., Chaiharn, M., Abbas, T., Sherwani, S.K., Mukayeva, S., Shah, S.N., Raheem, A., Shahid, A.: Development and validation of RP-LC-UV method for determination of ursodeoxycholic acid in capsule and human serum, *World Applied Sciences Journal*, **2014**, **32** (4), 560-566;
15. Voelker, T., Wang, H., Irish, M., Wang, J., Harrison, S., Reuschel, S., Bennett, P., Meng, M.: Method development and validation of six bile acids for regulated bioanalysis: improving selectivity and sensitivity, *Bioanalysis*, **2013**, **5** (10), 1229-1248;
16. International conference on harmonization (ICH), Validation of analytical procedures: Methodology, Q2B (CPMP/ICH/281/95), **1996**;
17. United States Pharmacopeia Convention, USP 39, Rockville MD, USA: United States Pharmacopeia Convention, Inc., **2017**;
18. European Pharmacopoeia (Ph. Eur.), The 9th edition, European Directorate for Quality Medicines, **2017**;
19. Boscolo, O., Flor, S., Dobrecky, C., Salvo, L., Tripodi, V., Lucangioli, S.: Development and validation of a LC-UV method applied to the quality control of ursodeoxycholic acid in raw material and pharmaceutical formulations, *IOSR Journal of Pharmacy*, **2017**, **7** (2), 111-116;
20. Varinder, S., Kumar, P., Gurjeet, S., Shah, G., Dhawan, R.K.: Analytical method development and validation for the estimation of ursodeoxycholic acid using RP-HPLC, *Journal of Pharmacy Research*, **2015**, **9** (1), 46-53;
21. Khairy, M.A., Mansour, F.R.: Simultaneous determination of ursodeoxycholic acid and chenodeoxycholic acid in pharmaceutical dosage form by HPLC-UV detection, *Journal of AOAC International*, **2017**, **100** (1), 59-64;
22. Zheng, G., Lin, S.: Determination of taurine in foods by high performance liquid chromatography, *Wei Sheng Yan Jiu*, **1998**, **27** (4), 266-268.