JURNAL ILMU KEFARMASIAN INDONESIA, September 2016, hlm. 147-157 Vol. 14, No. 2
ISSN 1693-1831

# Validated TLC Method for Determination of Curcumin Concentrations in Dissolution Samples Containing *Curcuma longa* Extract

# (Validasi Metode Kromatografi Lapis Tipis untuk Penentuan Konsentrasi Kurkumin dalam Sampel Disolusi yang Mengandung Ektrak *Curcuma longa*)

DEWI SETYANINGSIH[1,2A] , YOSI BAYU MURTI[2B], ACHMAD FUDHOLI[2A], WOUTER L.J. HINRICHS[3], ROCHMAT MUDJAHID[4], SUDIBYO MARTONO[2C], TRIANA HERTIANI[2B]

**[1]Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia.**
**[2a]Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Gadjah Mada University, Indonesia.**
**[2b]Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Indonesia.**
**[2c]Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Indonesia.**
**[3]Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, the Netherlands.**
**[4]Medicinal Plant and Traditional Medicine Research and Development (B2P2TOOT), Tawangmangu, Jawa Tengah, Indonesia.**

**Diterima 23 November 2015, Disetujui 30 Juli 2016**

**Abstract:** Curcumin is a lipophilic compound which suffers from the poor bioavailability after oral administration. Increasing its dissolution rate can be a successful strategy to improve the bioavailability. Along with the formulation developments, a rapid and simple analytical method to determine curcumin concentrations in the dissolution medium is required. The aim of this study was to develop and to validate an analytical method based on thin layer chromatography (TLC) to determine curcumin concentrations in a dissolution medium containing 0.5% w/v sodium lauryl sulfate (SLS) and 20 mM sodium phosphate buffer. A polyvinylpyrrolidone K30 based solid dispersion of *Curcuma longa* extract and its corresponding physical mixture were dissolved in a medium containing 0.5% w/v SLS and 20 mM sodium phosphate buffer (pH 6.0). Dissolution samples were spotted on a normal TLC plate and eluents of various compositions were evaluated. The retardation factor (Rf), resolution (Rs), and asymmetry factor (As0.05) of the optimized method were determined. Using the optimized eluent, proper separation of curcumin peak was achieved with an Rf of 0.50, Rs of 2.62 and As 0.05of 0.87. Linearity (5-30 µg/mL) was demonstrated by r value of 0.9965. The TLC method provided precision with RSD ≤3.50 and accuracy with recovery value of 94-105%.

**Keywords:** *Curcuma longa*, curcumin, solid dispersion, dissolution, thin layer chromatography.

**Abstract:** Kurkumin merupakan senyawa lipofilik dengan permasalahan bioavailabilitas setelah pemberian oral. Meningkatkan laju disolusi menjadi strategi utama untuk meningkatkan bioavailabilitas kurkumin. Seiring dengan perkembangan formulasi, metode analisis yang cepat dan sederhana diperlukan untuk menentukan konsentrasi kurkumin dalam medium disolusi. Tujuan dari penelitian ini adalah untuk mengembangkan dan memvalidasi metode kromatografi lapis tipis (KLT) untuk analisis kurkumin dalam medium disolusi yang mengandung 0,5% b/v sodium lauryl sulfate (SLS) dalam 20 mM dapar fosfat. Formulasi dispersi padat ekstrak *Curcuma longa* dalam matriks polyvinylpyrrolidone K30 dan campuran fisiknya dilarutkan dalam media yang mengandung 0,5% b/v SLS dalam 20 mM buffer fosfat (pH 6,0). Sampel disolusi yang telah ditotolkan pada lempeng KLT dikembangkan dalam beberapa komposisi eluen. Faktor retardasi (Rf), resolusi (Rs), dan faktor asimetri (As0.05) dievaluasi. Komposisi eluen yang optimal dari kloroform, etanol, dan 2% asam asetat memberikan pemisahan sempurna puncak kurkumin dari puncak senyawa derivat kurkumin dengan Rf 0,50, Rs 2,62 dan As0,05of 0,87. Linearitas diperoleh pada 5-30 µg/ mL yang ditunjukkan oleh nilai r 0,9965. Metode KLT ini memberikan presisi dan akurasi sesuai dengan regulasi dengan RSD ≤ 3,50 dan perolehan kembali 94-105%.

**Kata kunci:** *Curcuma longa,* kurkumin, dispersi padat, disolusi, kromatografi lapis tipis.

* Penulis korespondensi,Hp. 082135993494
e-mail: dewisetya03@yahoo.com

## INTRODUCTION

*CURCUMA longa L.* (*C. longa*) or Curcuma domestica Val (turmeric), belonging to *Zingiberaceae* family, is an herbaceous plant native to tropical South-eastern Asia. Turmeric is an important ingredient of JAMU, a traditional Indonesia medicine from plants[1]. Traditionally, it has been used for the prevention of diseases and therapy for many illnesses such as, liver disease, digestion problem, and dysmenorrhoea.

Curcumin [1.7-bis(4-hydroxy-3-methoxyphenyl)-1. 6-heptadiene-3.5-dione] as presented in Figure 1 is a naturally occurring phytoconstituent of C. longa and other curcuma species. In the plant of C.longa, curcumin[1] exists together with the two curcumin derivatives, demethoxycurcumin[2] and bis-demethoxycurcumin[3], which the three compounds are named as curcuminoids. Curcumin is considered to be tolerable to human and is already granted as a Generally Recognized as Safe (GRAS) compound. Phase I 7clinical studies has indicated that oral administration of high curcumin doses up to 8 g/day can be well tolerated[2]. In addition to its safety (or in other words the efficacy dose is much lower than the toxic dose), the potential of curcumin as a drug substance is highlighted by its several biological activities such as anti-inflammatory, antioxidant, antibacterial, antifungal, hepatoprotective, and anticancer[3].

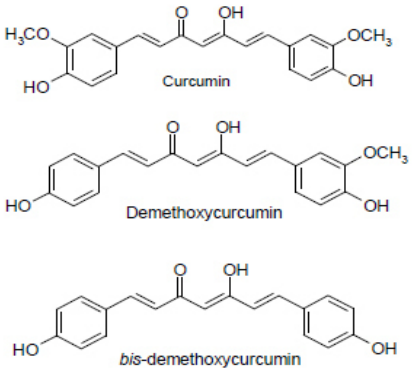


**Figure 1. Curcuminoids molecular structures**

Despite its large varieties of biological activities, clinical studies always point out at the limitation of the use of curcumin because of its poor bioavailability upon oral administration. Its lipophilic nature has attributed to its poor bioavailability. Since curcumin is a Biopharmaceutical Classification System class II compound, as its solubility is only 11 ng/mL[4] and a calculated log P of 3.2, enhancing its dissolution rate in aqueous medium is being the best strategy to overcome its inefficient bioavailability. Numerous formulations have been developed to improve the dissolution characteristics of curcumin such as, nanoparticles[5, 6], micelles[7] and solid dispersions[8, 9], and complex formation using cyclodextrins[10].

In the formulation research, curcumin was used either as pure curcumin or as a mixture of curcumin and other curcuminoids. Commercially available curcumin extract typically contains curcuminoids compounds which refers to about 75-78% curcumin, 15-17% demethoxycurcumin and 2-3% bisdemethoxycurcumin[11]. In a formulation development study, dissolution has being the most useful parameters in a screening of a formula candidate. Sampling procedures may end up with large number of dissolution samples and those need quick analysis due to instability issues. Various methods to measure curcumin concentrations have been reported in literature. Spectrophotometry is the most reported method[5, 7, 8, 12-15], probably due to its ease of operation. However, when C. longa extract is used in the formulation studies, spectrophotometry alone is not suitable to selectively determine curcumin from the other two curcuminoids, because there is an overlapping absorption spectra of the three curcuminoids at the wavelength used for curcumin determination[16].

A satisfactory separation of curcuminoids has been performed by chromatography methods. High performance liquid chromatography methods (HPLC) based on a reverse phase (RP) system were reported as selective, sensitive, precise, and accurate for quantification of curcumin concentrations in the formulation studies[17, 18]. Even though the RP-HPLC provides excellence in curcumin analysis, it can be quite inefficient when quick analysis of a large number of samples is needed. Samples as obtained, thus without pre-treatment procedures, may not be directly charged onto the RP-HPLC column due to possible deleterious effects to the column. In our formulation studies, we used a dissolution medium containing 0.5% w/v sodium lauryl sulfate (SLS) and 20 mM sodium phosphate buffer (pH 6.0). SLS is an anionic surfactant which is used to maintain sink conditions during the dissolution study, while 20 mM sodium phosphate buffer is required to keep the pH at 6.0, at which curcumin is chemically most stable[19]. The use of SLS can cause specificity problems when directly charged onto the HPLC column[20]. Therefore, extensive sample preparation, which may involve multiple clean up steps followed by extraction procedures, is required. However, besides being laborious and time consuming, according to Tonnesen and Karlson (1986), extensive sample extraction can also lead to the chemical changes of curcumin, because curcumin may be exposed to the condition

susceptible to its instability for a longer time. e.g. they suggested that when using buffers and ethyl acetate for extraction, polymerization of curcumin can occur at the interface of the two phases[21].

Thin layer chromatography (TLC) has been widely used particularly in phytochemistry. Compared to HPLC, this method is relatively efficient and cheaper. TLC provides an opportunity to simultaneously analyse a large number of samples. The separation of curcuminoids by TLC/high performance thin layer chromatography (HPTLC) has been discussed in several publications[22-25]. However, none of the studies determined curcumin concentrations in the presence of the other two curcuminoids in aqueous dissolution media. In order to tackle poor curcumin bioavailability, the availability of an efficient and validated method for curcumin quantification is crucial during early screening of formulations, which can serve to optimize the dissolution behavior.

In the current study, a TLC method for curcumin quantification from samples collected directly from the dissolution experiment, thus containing SLS and sodium phosphate buffer, was developed and validated. An established TLC method for curcumin quantification[24] was used as a starting point. The presence of dissolution media containing SLS and sodium phosphate buffer in the dissolution samples, however, reduced the separation efficiency of curcumin from the other two curcuminoids. Therefore, the aim of this study was to develop and validate a TLC based method for the determination of curcumin concentraion in a dissolution medium containing 0.5% w/v of SLS in 20 mM sodium phosphate buffer.

## MATERIAL AND METHOD

**MATERIAL.** Curcumin standard was purchased from Nacalai, Japan. *C.longa* extract (98.3% curcuminoids as mentioned in the Certificate of Analysis) was kindly given by PT. Phytochemindo Reksa, Bogor, Indonesia. The extract contained 67.9% curcumin as quantified in our lab using a validated HPLC method[26]. Polyvinylpyrrolidone K30 (PVP K30) was a gift from Zenith Pharmaceuticals, Semarang, Indonesia. De-ionized water was prepared in our laboratory. SLS was obtained from Merck, Darmstadt, Germany. Precoated silica gel aluminium plate 60F254 (TLC plate) (20x20 cm and a thickness of 0.2 mm; were purchased from Merck, Darmstadt, Germany. All solvents were analytical grade and obtained from Merck, Darmstadt, Germany.

**METHOD. TLC Instrument and Conditions.** The chromatographic separation was based on the method described by Tonnesen and Karlson[24]. The method was first employed to analyze pure curcumin/curcuminoids dissolved in methanol. Next, the method was applied to dissolution samples containing 0.5% w/v SLS and 20 mM sodium phosphate buffer. As controls, a solution of *C. longa* extract and SLS in mixture of methanol and water at a volume ratio of 92/8 and a solution of *C. longa* extract and sodium phosphate in mixture of methanol at a volume ratio of 80/20 was analyzed. The weight ratios of curcumin/SLS and curcumin/sodium phosphate in these solutions were adjusted to 1/31 and 1/19.5, respectively, which is identical to the ratios found in dissolution samples when all curcumin would have been dissolved. The separation was performed on a precoated silica gel aluminium plate $60F_{254}$ (TLC plate) which was activated at 90 °C for 5 minutes prior to analysis. In brief, 10 µL of the dissolution samples were spotted in the form of bands of width 6 mm with a 100 µL CAMAG microliter syringe on the TLC plate. On the same plate, 10 µL of a 15 µg/mL curcumin standard solution was spotted. Camag Linomat V (CAMAG, Muttenz, Switzerland) connected to nitrogen gas was used for the spot application at a constant rate of 30 nL/s, with pre-dosage volume of 0.2 µL, and distance between the bands were 10 mm. The settings were maintained throughout the analysis. The TLC plates were developed in ascending order in a closed CAMAG 20 x 20 cm glass flat bottom chamber which was pre-saturated with mobile phase for at least 30 minutes. To maintain the chamber saturated with vapour of the mobile phase, the wall of inner side of the chamber was lined with filter paper. The TLC plate, which was spotted with samples, was developed to a distance of 10 cm from the spotting zone. The developed plate was dried at room temperature after which the TLC bands were visually inspected using UV light (366 nm; CAMAG) to evaluate the peak separation. The spots densities were densitometrically scanned sequentially using a CAMAG TLC Scanner 3 at 424 nm. The scanning speed was 20 mm/s with the slit dimension of 8 mm x 0.2 mm. All the TLC analysis was carried out under ambient conditions, i.e. 20±2 °C and a relative humidity of 55%. The data were collected and the obtained peaks were integrated with filter factor of Savitsky-Golay 7 using winCATS software V1.4.4.

**Mobile Phase Optimization.** In the TLC method described previously[24], a mobile phase consisting of chloroform/ethanol/water (at a volume ratio of 50:1.92:0.08) was used. Although the TLC system was suitable for solutions of pure curcumin/curcuminoids in methanol, it failed for curcumin determination in aqueous solutions containing 0.5% w/v SLS and 20 mM sodium phosphate buffer (pH 6.0). To be able

to accurately quantify curcumin concentrations in the dissolution medium by TLC, the composition of the mobile phase was modified by replacing water by various amounts of aqueous acetic acid solutions. Dissolution samples taken 15 minutes after initiating the dissolution experiment were employed to optimize the mobile phase composition. A volume of 10 µL sample was spotted on a TLC plate and was processed using the described method in "TLC instrument and conditions". After optimization, the mobile phase was applied to dissolution samples taken at different time points (5, 45, 90, 120 min). A solution of curcumin standard spiked in blank sample (dissolution medium) at 15 µg/mL was used as a reference.

**Calibration Curve for Curcumin**. A stock solution of curcumin was prepared in methanol (950 µg/mL). To prepare the calibration curve, blank samples were spiked with curcumin standard solution to obtained series of calibration solutions of 5, 10, 15, 20, 25, 30 and 35 µg/mL. The blank samples consisted of dissolution medium which contained 0.5% w/v SLS and 20 mM sodium phosphate buffer (pH 6.0). The series of calibration samples were spotted in 10 µL each and further analyzed using the method as described in section "TLC instrument and conditions". The calibration studies were performed in triplicate. A calibration curve was derived by plotting the peak area against the curcumin concentration. Furthermore, the data were subjected to a least-squares analysis to obtain the regression parameters. Linearity was derived from the calibration curve.The data were subjected to the least-square regression analysis[27, 28].

**Method Validation**. The developed method was validated according to Q2R1 ICH guidelines[29]. The validation parameters analyzed in this study were specificity, linearity, range, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Specificity was determined by evaluation of retardation factor (Rf), peak resolution (Rs) and asymmetry factor (A0.05)[30, 31]. Rf is the ratio between the distance of the centre of the spot (peak) resulting from the migration of analyte through the TLC plate from its initial spot and the length of elution. Rs is calculated by 1.18 x distance between 2 adjacent peaks/sum of the 2 peaks width at half height. A0.05 is determined by calculation of width ratio of the 2 halves of the peak measured at 5% height[30-32]. To assess method specificity, TLC experiments of 1) a blank sample consisted of dissolution medium, 2) a blank sample (dissolution medium) spiked by curcumin standard solution (5 µg/mL), and 3) a dissolution sample taken after 120 minutes followed by 40 times dilution with the dissolution medium were conducted and followed by determination of Rf, Rs, and A0.05. Additionally, identity of curcumin peak was confirmed by comparing the UV-Vis spectra (200-700 nm) and the Rf value of the TLC bands of the dissolution sample with that of standard curcumin peak using a CAMAG TLC Scanner 3.

Intraday and interday precision and accuracy were carried out on three different curcumin concentrations. Blank samples were spiked with curcumin standard solution to achieve 10, 20 and 30 µg/mL, which were considered as low, medium, and high concentration levels. Each solution was spotted on TLC plate as described in section "TLC instrument and conditions". All experiments were performed in triplicate. Precision was expressed as RSD (relative standard deviation) or CV (coefficient of variation), and accuracy was determined by recovery. RSD was calculated by dividing the standard deviation by the mean of obtained concentrations x 100%. Recovery is the ratio between measured concentrations to the accordingly nominal concentration x 100%.

LOD and LOQ were determined following the procedure as described previously[28]. LOD and LOQ were determined based on standard deviation of response and slope. LOD and LOQ were defined as 3.3 σ / S and 10σ / S, respectively, where σ is the standard error of intercept and S represents the mean of slopes as determined by the least-square analysis. To test the optimized method, solid dispersion (SD) of *C. longa* extract and the physical mixture (PM) were prepared and the curcumin concentrations in dissolution samples were determined. A PVP K30 based solid dispersion was prepared by the vacuum rotary evaporation method according to a method described previously[8], with a slight modification. In brief, *C. longa* extract and PVP K30 were dissolved in ethanol with the help of a sonicator for 15 minutes and the resulting solution was subsequently dried using a vacuum rotary evaporator. The rotary evaporator (IKA RV 05 basic, Staufen, Germany) was set at 95 rpm. The solution was dried at a reduced pressure of 175 mbar (IKAVAC® VC2 vacuum controller, Staufen, Germany). The temperature of the heating bath (IKA HB 4 basic, Staufen, Germany) was initially set at 50 °C. After 4 hours, the temperature of heating bath was increased to 75 °C and drying was continued for another 30 minutes. The concentrations of *C. longa* extract and PVP K30 in ethanol were 7.36 mg/mL and 17.64 mg/mL, respectively, which thus resulted in the formation of a solid dispersion containing 20 wt-% curcumin. During the whole production process, the sample was shielded from light. The powder was sieved using a mesh size 60 and then stored in a desiccator for at least 1 day.

A PM formulation of curcumin of *C. longa* extract

and *PVP* K30 was used as a control. The PM was prepared by gently mixing *C. longa* extract with PVP K30 in a mortar following sieving using a mesh size 60. The curcumin content in PM formulation was 20 wt-%. The powder was kept in a desiccator for at least one day.

For dissolution studies, gelatine capsules of size 00 were filled with approximately 400 mg of either SD or PM. The dissolution studies were carried out on a SOTAX AT-7 dissolution tester apparatus II (Sotax AG, Basel). The medium consisted of 0.5% w/v SLS and 20 mM sodium phosphate buffer (pH 6.0). The experiment was conducted at the temperature of 37±0.5 °C and a paddle speed of 75 rpm in 500 mL medium. An aliquot of 1 mL samples were withdrawn at predetermined time intervals (5, 10, 15, 20, 30, 45, 60, 90, 120 minutes) and was replaced with the fresh medium at identical volume and of the same temperature (37±0.5 °C). During the experiment, the capsules were kept on the bottom of the dissolution vessels using inert metal jackets. The experiments were performed in triplicate.

Dissolution samples were centrifuged at 2000 rpm for 2 minutes prior to spotting on the TLC plate. If necessary, the samples were diluted to such an extent that no overload in the spotting zone of the TLC plate occurred, while the spots after development could still be analyzed well. An aliquot of 10 µL of supernatant was spotted on the TLC plate using the method as described in section "TLC instrument and conditions". The dissolution behavior was assessed as follows. Dissolution profiles of different formulations were evaluated on percentage of dissolution efficiency (DE) according to the equation below[33].

$$DE = \frac{\int_{t1}^{t2} y.\,dt}{y_{100}.t}) \times 100\%$$

DE : dissolution efficiency
y : area under the curve of dissolved drug at time t
$y_{100}xt$ : rectangle area where 100% of drug dissolved at time

Area under the curve (the integral part) is calculated by trapezoidal method:

$$AUC = \sum_{i=1}^{i=n} \frac{(t_1 - t_{i-1})(y_{i-1} + y_i)}{2}$$

## RESULT AND DISCUSSION

**Optimization of Mobile Phase**. The goal of this study was to find a suitable TLC system which provides an appropriate separation of the curcuminoids in dissolution samples of solid dispersions or physical mixtures containing C.longa extract. The mobile phase composition employed by Tonnesen and Karlson[24], which was chloroform/ethanol/water at a volume ratio of 50:1.92:0.08, was used as a starting point. The dissolution samples were spotted without prior extraction of the curcumin.

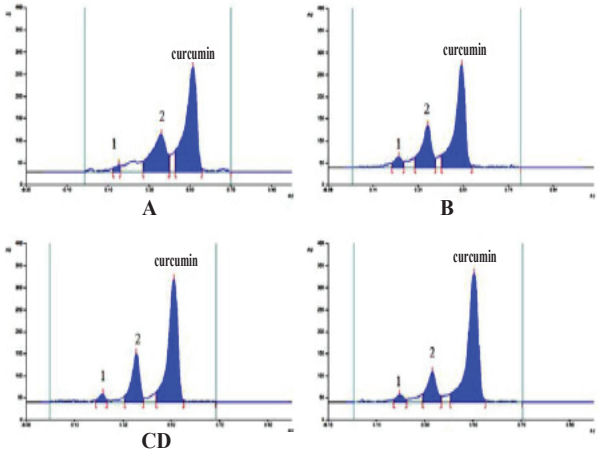


**Figure 2. Effect of acetic acid addition on peaks separation of curcuminoids.**
Chromatographic separation was performed using chloroform/ethanol/water at 50:1.92:0.08 mL as eluent (Fig.2A). The other chromatogram were obtained using eluent of the same composition but with water was replaced by 0.12 mL (Fig. 2B), 0.15mL (Fig. 2C) and 0.18 mL (Fig. 2D) of 2% acetic acid.

Figure 2A shows chromatogram of dissolution sample of SD *C. longa* extract after TLC development using chloroform/ethanol/water (50:1.92:0.08 mL) as eluent. Application of this mobile phase in the analysis of our dissolution sample resulted in chromatogram with a typical curcuminoids peaks of *C. longa* extract as also found by Tonnesen and Karlson (1986). The peak with the highest Rf was ascribed to curcumin as confirmed by the fact that a peak of curcumin standard spotted on the same TLC plate had an identical Rf (0.50). The Rx values (i.e. Rf values of identified spots divided by the Rf value of curcumin of the same TLC plate) of the two other peaks were identical to those find by Govindarajan and Stahl who used a similar TLC system for *C. longa* extract determination[16] indicating that these two peaks are associated with demethoxycurcumin and bis-demethoxycurcumin.
Although the system was able to emerge typical curcuminoid peaks in the chromatogram from the dissolution samples containing SD *C. longa* extract, peak separation was poor, which will jeopardize accurate quantification of the curcumin concentration (Figure 2A). The poor resolution of the curcumin peak from the adjacent peak might be ascribed to the

medium used in these experiments (aqueous solution containing 0.5% SLS and 20 mM sodium phosphate buffer) which was different from the one used by Tonnesen and Karlson (methanol). When water in the mobile phase was replaced by 0.08 mL of 1% acetic acid, the peak separation of curcuminoids from the dissolution sample of SD *C.longa* extract was slightly improved (data not shown). However, when water was replaced by 2% acetic acid at various volumes (i.e. 0.12 - 0.18 mL), the quality of peak separation in the chromatograms was much better (Figure 2B-D). The best peak separation was achieved using 0.15 mL of 2% acetic acid (see Figure 2C). Using this mobile phase, which thus consisted of chloroform/ethanol/2% acetic acid of 50:1.92:0.15 mL, the shape of the curcumin peak was sharper and less diffused than in the other chromatograms. The resulted Rs value was 2.52, which is considered to be acceptable(30). Further increase of the amount of 2% acetic acid to 0.18 mL did not provide any better peak separation as shown in Figure 2D. Thus, further experiments were conducted by using chloroform/ethanol/2% acetic acid of 50:1.92:0.15 mL as mobile phase.

Separation of compounds by a TLC system is influenced by the combination of 1) the mobile phase composition that transports the compounds to be separated and 2) the physical and chemical interaction between the compound with the stationary phase[30]. Too strong interaction of compounds with the stationary phase relative to the eluent can cause a tailing effect, which may lead to a poor peaks separation. Tonnesen and Karlson (1986) studied the interaction of curcumin with (soluble) silicic acid which was used to mimic the (solid) stationary phase, silica gel, as both contain silanol groups. They found that the 1-3 diketone groups of curcuminoids can interact with silicic acid: using UV-Vis spectra, a bathochromic shift and an increase of □ value was observed when a silicic acid solution was added to 1-3 diketone compounds including curcuminoids, indicating intermolecular H bonding.

Although intermolecular interaction of curcuminoids at the 1-3 diketone sites with silanol of the stationary phase is important, a successful chromatographic separation of curcuminoids also depends on their interaction with the mobile phase. There is a difference in polarity among curcuminoids compound which causes a specific interaction to a certain mobile phase system and leads to peaks separation. In addition to that, it is noted that peaks separation is also influenced by other component(s), which might present in the TLC spotting zone e.g. the sample matrix[34].

Therefore, we hypothesized that the poor chromatographic separation of curcuminoids from our dissolution samples using chloroform/ethanol/water at 50:1.92:0.08 mL as mobile phase can be ascribed to the dissimilarity of the used solvent (matrix) of this dissolution samples with the one used by Tonnesen and Karlson (1986). Since the dissolution medium contained 0.5% w/v SLS and 20 mM sodium phosphate buffer, it can be calculated that 500 mL medium contains 2.5 g SLS and 1.56 g sodium phosphate buffer. As approximately 400 mg of C. longa extract powder with 20 wt-% drug load was used in the dissolution study, maximally 80 mg of curcumin dissolved in the bulk medium. Thus, the minimal weight ratio of SLS and sodium phosphate buffer to curcumin in sample were 31:1 and 19.5:1, respectively, implying that the samples spotted onto a TLC plate contained a substantial amount of SLS and sodium phosphate buffer. Contrary to that, in the study of Tonnesen and Karlson (1986), only the curcuminoids were present in spotting zone as the solvent, methanol, was fully evaporated. Therefore, we conducted additional TLC analysis to clarify the effect of the sample matrix.

**The Influence of Dissolution Matrix on The Separation of Curcuminoids Peaks.** To investigate the effect of 0.5 w/v SLS and 20 mM sodium phosphate buffer on the peak separation of curcuminoids, TLC analysis of C. longa extract dissolved in either 0.5% w/v SLS in methanol or 20 mM sodium phosphate buffer in methanol was carried out. Due to their limited solubility in methanol, SLS or sodium phosphate buffer were first dissolved in small amount of water and then added to an excess of methanol. The volume ratios of water/methanol required to prepared clear solutions of 0.5% SLS and 20 mM sodium phosphate buffer were 8/92 and 20/80, respectively.

Two mobile phase systems which consisted of chloroform/ethanol/water of 50:1.92:0.08 mL and chloroform/ethanol/2% acetic acid of 50:1.92:0.15 mL were used to develop the TLC plate. The samples contained SLS-curcumin and sodium phosphate-curcumin at identical ratios to those found in the dissolution samples when the formulations were fully dissolved.

In Figure 3, it is demonstrated that elution of the sample containing SLS-curcumin and sodium phosphate-curcumin, with two mobile phase systems, generated different chromatogram profiles. Chloroform/ethanol/water elution resulted in a poor peak separation of curcumin/curcuminoids (Figure 3A, B). In contrast, similar C. longa extract sample eluted with chloroform/ethanol/2% acetic acid (50:1.92:0.15 mL) resulted in an excellent separation of the three curcuminoids peaks (Figure 3C,D).

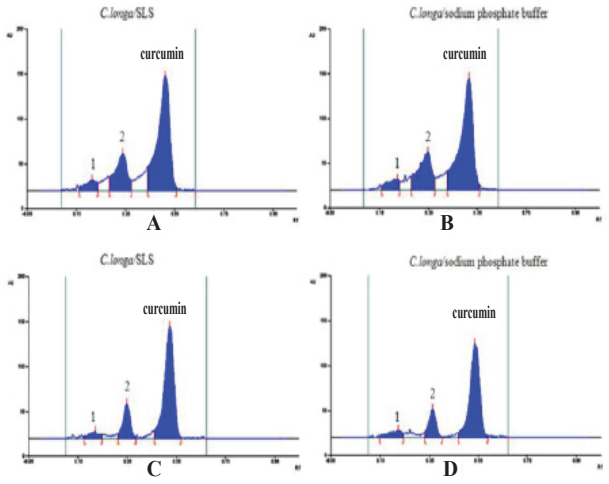


**Figure 3. The influence of 20 mM sodium phosphate buffer and 0.5% w/v SLS in chromatographic separation of curcuminoids from *C. longa* extract.**

TLC of C. longa/SLS (Fig. 3A, 3C) and C. longa/sodium phosphate (Fig. 3B, 3D) were developed in two mobile phase systems: chloroform/ethanol/water at 50;1.92:0.08 mL (Fig. 3A, 3B); chloroform/ethanol/2% acetic acid at 50:1.92:0.15 mL (Fig. 3C, 3D).

From this phenomenon, it is clear that by substitution of water with 2% acetic acid, the detrimental effects of SLS and sodium phosphate buffer on peak separation of curcuminoids in the dissolution samples are avoided. It is likely that compared to water, 0.15 µL of 2% acetic acid plays an important role during competitive interaction between the eluent and silanol groups of stationary phase to each curcuminoids compound in the co-existence of sample matrixes i.e. SLS and sodium phosphate. The role of acetic acid in the chromatographic separation of curcuminoids in this study (Figure 3C,D) seems to facilitate a specific interaction of each curcuminoids compounds with chloroform being the major component in the mobile phase, leading to a much better separation of each of the curcuminoids peaks. While the effects of SLS and sodium phosphate buffer as dissolution media on the chromatographic separation of curcuminoids in dissolution samples were observed in this study, further investigation need to be conducted to understand on how the interaction of SLS and sodium phosphate with the curcuminoids occur, at which it was observed to reduced the peak separation.

**TLC Analysis of Dissolution Samples using The Optimized Mobile Phase.** The optimized mobile phase was tested on the TLC analysis of dissolution samples taken at different time intervals (5, 45, 90,120 min). Figure 4 shows the TLC bands of the dissolution samples taken at different time points showing well separated bands of curcumin from the adjacent bands after visualization with a UV light (366 nm).

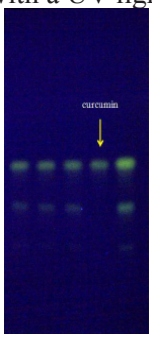


**Figure 4. Visual detection of TLC bands of dissolution samples taken at different time points showing separated curcumin band.**

The spots represent samples taken at 5 min (1), 45 min (2), 90 min (3), 120 min (5), and curcumin standard spiked in blank sample (4). The volume of sample application was 10 µL in all cases

**Specificity.** Specificity is defined as the ability of a method to measure the degree of interference with other components or sample matrix. Mobile phase optimization is necessary in part of achieving acceptable specificity. To evaluate specificity, values of Rf, $As_{0.05}$, and Rs were determined. Acceptable criteria for an analytical method specificity are $0.1 \leq Rf \leq 0.9$, $0.9 \leq As0.05 \leq 1.1$, and $Rs \geq 2.0$[30-32].

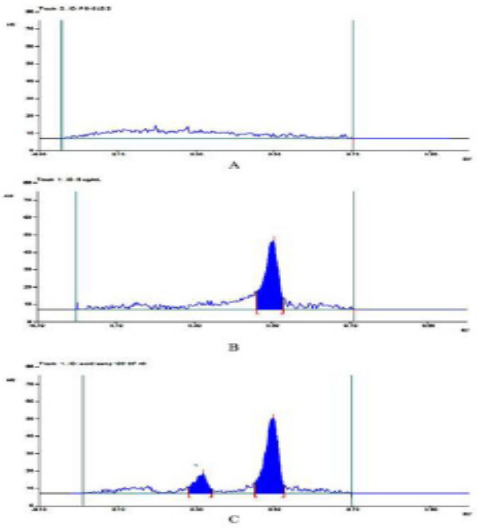


**Figure 5. Chromatogram of specificity test for dissolution samples.**

(A) blank sample;(B) curcumin standard spiked in blank sample consisted of dissolution medium (5 µg/mL); (C) dissolution sample taken after 120 min (40 times diluted prior to spotting on TLC plate). The volume of sample application was 10 µL in all cases.

The specificity of the developed TLC method is illustrated in Figure 5. As shown in Figure 5A, no peak was found in the blank sample containing dissolution medium. Spiking of the blank sample with curcumin standard solution to result a concentration of 5 µg/mL exhibited curcumin peak at Rf 0.50 (Figure 5B). To determine the potential influence of the adjacent peak,

the sample from the time sampling of 120 minutes was analyzed (Figure 5C). In Figure 5C, chromatogram shows that the curcumin peak is clearly separated from the adjacent peak. The Rf value of curcumin was found to be 0.50 (Figure 5C) which is in agreement with the Rf value of curcumin standard spiked in blank sample (Figure 5B). The value of $As_{0.05}$ was 0.87 and the Rs value appeared to be 2.63 showing an excellent peak separation of curcumin from the other curcuminoids.

Two peaks instead of three typical *C.longa* peaks appeared in the chromatogram presented in the Figure 5.There was reported in the literature that commercial available curcumin extract contains a mixture of curcuminoids with bis-demethoxycurcumin as the lowest content. The low curcuminoids concentration in the sample (40 times dilution) caused the appearance of only two peaks in the chromatogram in Figure 5C. The curcuminoid of the lowest concentration of *C.longa*, bis-demethoxycurcumin, appeared to be not detected by the developed TLC method. The 40 times dilution was conducted in this study, because in the determination of method specificity, the analysis of a sample at a low concentration of analyte which is still covered by the linear calibration curve is required[31,35].

The identity of curcumin in the dissolution sample was also confirmed by comparing the spectra of the sample and curcumin standard spiked in the blank sample. The spectra of the TLC bands of dissolution sample and curcumin standard are shown in Figure 6 demonstrates an overlay of the spectra of the two TLC bands at identical Rf, which thus confirms the identity of curcumin in the dissolution sample.

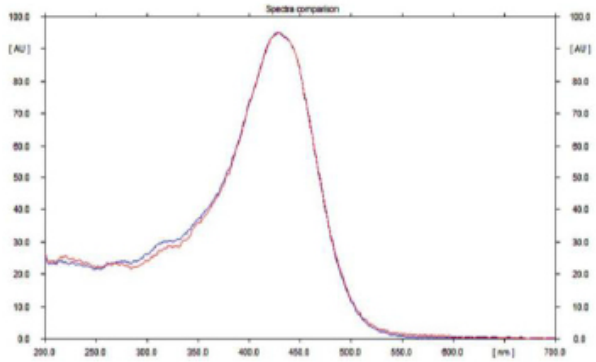

**Figure 6. TLC overlay spectra of curcumin from dissolution sample and standard.**

Regarding the found values of Rf, Rs and $As_{0.05}$ in the specificity evaluation, it can be concluded that the TLC method developed in this study for the quantification of curcumin concentration from dissolution samples of SD *C.longa* extract meets the requirements for acceptable specificity.

**Calibration Curve, Linearity and Range**.To construct a calibration curve, solutions of curcumin standard were prepared by spiking of the blank sample (dissolution medium) with curcumin stock solution (950 µg/mL in methanol) to achieve concentrations of 5, 10, 15, 20, 25, 30, and 35 µg/mL. The calibration samples were analyzed by the developed TLC method. Calibration curve was obtained by plotting peak area as a function of the concentration (Figure 7).


**Figure 7.Calibration curve of curcumin from 5-30 µg/mL.**

Regression parameters were calculated using the least-squares method. The regression of the concentration 5-35µg/mL resulted in correlation coefficient (r) of 0.98 which was lesser than 0.99, the acceptance correlation coefficient requested by the AOAC[36]. However, linearity was found at the concentration range of 5-30 µg/mL as indicated by correlation coefficient (r) of 0.9965, determination coefficient ($r^2$) was 0.9931, and the regression equation Y = 737.22x - 2779.4 (Table 1.). Moreover, the calibration curve of 5-30 µg/mL vs. peak area was considered suitable as tested by ANOVA ($P<0.05$).

**Table 1. Parameter for linearity for curcumin quantification (n=3).**

| Parameters | Data obtained |
|---|---|
| Linearity range (µg/mL) | 5-30 |
| Equation | 737.22x-2779.4 |
| Correlation coefficient (r) | 0.9965 |
| Determination coefficient ($r^2$) | 0.9931 |

**Precision**. Precision was evaluated at three different concentrations. Blank samples (dissolution medium) spiked with curcumin standard solution resulted in final curcumin concentrations of 10, 20 and 30 µg/mL. RSD or CV was evaluated for indication of precision. In general the value of RSD decreases with increasing analyte concentration[36], which means that the degree of precision is higher at higher

concentration of analyte. Therefore, according to the AOAC Guidelines for Standard Method Performance Requirements, acceptance criteria for RSD are concentration dependent, e.g.at categorized level of 100 ppm and 10 ppm of analyte, the RSD should not be more than 5.3 % and 7.3%, respectively[36]. The concentrations evaluated in the precision study i.e. 10, 20 and 30 µg/mL, which corresponds to 10, 20, and 30 ppm. Therefore, the RSD for the 20 and 30 µg/mL samples should be below 5.3% while the RSD for the 10 µg/mL can be below 7.3%. As shown in Table 2, the RSD for intraday analysis was less than 3.50% and for interday was less than 2.62%. Thus, RSDs obtained for the intraday and interday analysis of all investigated concentrations were below 5.3% which indicates that precision of the method fulfils the requirements.

**Accuracy**. Accuracy is a measure of closeness between the measured value and the true value. Table 2 shows the recovery values of the accuracy analysis for each concentration level (10, 20, 30 µg/mL). The intraday and interday accuracy analysis resulted in mean recovery of 94.00-102.60% and 97.54-105.00%, respectively. According to the AOAC, the requirement for the mean recovery at a concentration of 100 ppm is 90-107%, while at a lower concentration, a broader range is acceptable[36]. Thus, both the intraday and interday recovery found in this study meet the requirement of accuracy.

**Table 2. Intra and interday precision and accuracy of curcumin (n=3).**

| Nominal concentration (µg/mL) | Intraday | | | Interday | | |
|---|---|---|---|---|---|---|
| | Found (µg/mL) | Precision (%RSD) | Accuracy (%recovery) | Found (µg/mL) | Precision (%RSD) | Accuracy (%recovery) |
| 10 | 9.40 | 3.50 | 94.00 | 9.75 | 2.62 | 97.54 |
| 20 | 20.52 | 3.03 | 102.60 | 21.00 | 0.38 | 105.00 |
| 30 | 29.30 | 1.16 | 97.67 | 30.12 | 2.08 | 100.42 |

**LOD and LOQ**. Detection limit is necessary to be established especially during evaluation in early sampling. The LOD and LOQ value calculation based on standard deviation of response and slope was 1.3 µg/mL and 4.0 µg/mL, respectively.

**Application of Validated Method on The Estimation of Curcumin Dissolution.** The validated TLC method was applied for the determination of curcumin from dissolution samples without prior separation e.g. sample extraction procedures from the dissolution medium. The dissolution profiles of curcumin (from the source of *C.longa* extract), from PVP K30 based SD and PM are shown in Figure 8.

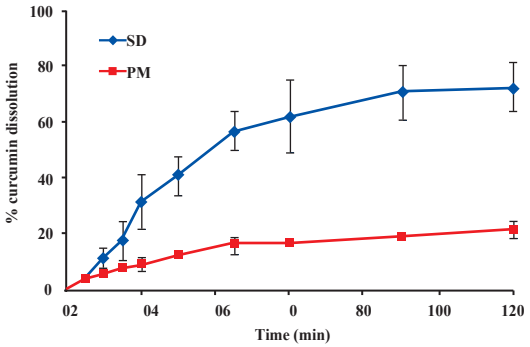


**Figure 8. Dissolution profile of SD C. longa extract and its corresponding PM formulation. Data is presented as mean and SD of n=3.**

As can be seen in Figure 8, curcumin dissolved faster from the SD formulation than from the PM formulation. Further assessment of dissolution performance was made by calculating the percentage of dissolution efficiency (DE). DE provides information for comparing dissolution behavior of different formulations. The DE values were calculated up to 120 minutes and it was found that the DE value was almost 4 times higher for the SD formulation (58.03 ± 8.49%) than for the PM formulation (15.21 ± 1.60%) ($p<0.05$).

The mechanisms of dissolution rate enhancement of solid dispersions have been discussed elsewhere. Increasing specific surface area by decreasing particle size of drugs and improved wetting as result of the intimate contact with hydrophilic carrier are the important keys in the dissolution enhancement of solid dispersions[37, 38]. When the intimate contact is accompanied with a molecular interaction between drug and carrier, the enhanced dissolution rate is further facilitated. It is well known that PVP interacts with many different lipophilic drugs on a molecular level. E.g. Srinarong et al. reported that the interaction between PVP K30 with a lipophilic drug (diazepam) contributed significantly to the high dissolution rate of drug. However, when they used other carriers where the interaction was absent, dissolution was slower[39]. Thus, although not investigated in this study, interaction of PVP K30 with curcumin on a molecular level in the SD might have occurred, which might have contributed to the enhanced dissolution rate.

## CONCLUSION

In conclusion, the TLC method developed in this study is applicable to determine curcumin concentrations from dissolution samples containing buffer and SLS with acceptable precision (RSD), accuracy (recovery) and linearity. Although not subjected to

sample extraction prior curcumin determination, the developed TLC method in this study results in a satisfactory separation of curcumin peak from curcuminoids peaks with an Rs value higher than 2.50. The developed TLC method provides precision less than 3.5% and accuracy of 94-105% recovery. Moreover this method is also highly sensitive having a low LOD (1.3 µg/mL) and LOQ (4.0 µg/mL).

## ACKNOWLEDGEMENT

The research was financially supported by Sanata Dharma Foundation and Doctoral Grant from Indonesian Directorate General of Higher Education (DIKTI) 2014. We expressed our gratitude to PT. Phytochemindo Reksa, Bogor, Indonesia for their kind gift of *Curcuma longa* extract and to PT. Zenith Pharmaceutical, Semarang, Indonesia for providing PVP K30.

## REFERENCES

1. Riswan S, Sangat-Roemantyo H. Jamu as traditional medicine in Java, Indonesia. South Pacific Study. 2002.23(1):1-10.
2. Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res.2003. 23(1A):363-98
3. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises.Mol Pharm. 2007.4(6):807-18.
4. Tonnesen H. Solubility, chemical, and photochemical stability of curcumin in surfactant solutions Studies of curcumin and curcuminoids, XXVIII Pharmazie. 2002.57(12):820-4.
5. Onoue S, Takahashi H, Kawabata Y, Seto Y, Hatanaka J, Timmermann B, et al. Formulation design and photochemical studies on nanocrystal solid dispersion of curcumin with improved oral bioavailability. J Pharm Sci. 2010.99(4): 1871-81.
6. Kanai M, Imaizumi A, Otsuka Y, Sasaki H, Hashiguchi M, Tsujiko K, *et al*. Dose escalation and pharmacokinetic study of nanoparticle curcumin, a potential anticancer agent with improved bioavailability, in healthy human volunteers. Cancer Chemother Pharmacol. 2012. 69(1):65-70.
7. Cui J, Yu B, Zhao Y, Zhu W, Li H, Lou H, et al. Enhancement of oral absorption of curcumin by self microemulsifying drug delivery systems. Int J Pharm. 2009.371(1-2):148-55.
8. Xu D-H, Wang S, Jin J, Mei X-T, Xu S-B Dissolution and absorption researches of curcumin in solid dispersions with the polymers PVP. AJPP. 2006. 6(4):343-9.
9. Wan S, Sun Y, Qi X, Tan F. Improved bioavailability of poorly water-soluble drug curcumin in cellulose acetate solid dispersion. AAPS PharmSciTech. 2012. 13(1):159-66.
10. Yadav VR, Suresh S, Devi K, Yadav S. Effect of cyclodextrin complexation of curcumin on its solubility and antiangiogenic and anti inflammatory activity in rat colitis model.AAPS PharmSciTech. 2009. 10(3):752-62.
11. Anand P, Thomas SG, Kunnumakkara AB, Sundaram C, Harikumar KB, Sung B, et al. Biological activities of curcumin and its analogues (congeners made by man and Mother Nature). Biochem Pharmacol. 2008.76(11):1590-611.
12. Mazzarino L, Bellettini IC, Minatti E, Lemos Senna E. Development and validation of a fluorimetric method to determine curcumin in lipid and polymeric nanocapsule suspensions. Braz J Pharm Sci. 2010.46(2):219-26.
13. Leung MHM, Colangelo H, W.Kee T. Encapsulation of curcumin in cationic micelles suppresses alkaline hydrolysis. Langmuir. 2008.24:5672-5.
14. Tonnesen HH. Solubility and stability of curcumin in solutions containing alginate and other viscosity modifying macromolecules. Studies of curcumin and curcuminoids. XXX. Pharmazie. 2006.61(8):696-700.
15. Zhang L, Cao F, Ding B, Li Q, Xi Y, Zhai G. Eudragit(R) S100 coated calcium pectinate microspheres of curcumin for colon targeting. J Microencapsul. 2011. 28(7):659-67.
16. Govindarajan VS, Stahl WH. Turmeric-chemistry, technology, and quality. CRC Crit Rev Food Sci Nutr. 1980-2015.12(3):199-301.
17. Jangle RD, Thorat BN. Reversed-phase high-performance liquid chromatography method for analysis of curcuminoids and curcuminoid-loaded liposome formulation. Indian J Pharm Sci. 2013.75(1):60-6.
18. Syed HK, Liew KB, Loh GOK, Peh KK. Stability indicating HPLC-UV method for detection of curcumin in Curcuma longa extract and emulsion formulation. Food Chem. 2015.170(0):321-6.
19. Wang YJ, Pan MH, Cheng AL, Lin LI, Ho YS, Hsieh CY, et al. Stability of curcumin in buffer solutions and characterization of its degradation products. J Pharm Biomed Anal. 1997.15(12):1867-76.
20. Collier JW, Shah RB, Bryant AR, Habib MJ, Khan MA, Faustino PJ. Development and application of a validated HPLC method for the analysis of dissolution samples of levothyroxine sodium drug products. J Pharm Biomed Anal. 2011.54(3):433-8.
21. Tonnesen H, Karlsen J. Studies on curcumin and curcuminoids VI. Kinetics of curcumin degradation in aqueous solution. Z Lebensm Unters Forsch.

1985.180(5):402-4.
22. Ansari MJ, Ahmad S, Kohli K, Ali J, Khar RK. Stability-indicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations. J Pharm Biomed Anal. 2005.39(1-2):132-8.
23. Pathania V, Gupta AP, Singh B. Improved HPTLC method for determination of curcuminoids from Curcuma longa. J Liq Chromatogr Relat Technol. 2006-2014.29(6):877-87.
24. Tonnesen H, Karlsen J. Studies on curcumin and curcuminoids VII. Chromatographic separation and quantitative analysis of curcumin and related compounds. Z Lebensm Unters Forsch. 1986.182(3):215-8.
25. Zhang JS, Guan J, Yang FQ, Liu HG, Cheng XJ, Li SP. Qualitative and quantitative analysis of four species of curcuma rhizomes using twice development thin layer chromatography. J Pharm Biomed Anal. 2008.48(3):1024-8.
26. Setyaningsih D, Murti YB, Martono S, Hinrichs WLJ, Hertiani T, Fudholi A. A novel reversed phase high performance liquid chromatography method to accurately determine low concentration of curcumin in rat plasma. International Journal of Pharmaceutical and Clinical Research. 2016.8(5Suppl):377-86.
27. Ermer J. Performance parameters, calculations and tests. In: Ermer J, Miller JHM, editors. Method validation in pharmaceutical analysis. Weinheim: Willey-VCH Verlag GmbH & Co. KGaA; 2005. p. 80-99.
28. Gustavo Gonzalez A, Angeles Herrador M. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. Trends in Analytical Chemistry. 2007.26(3):227-38.
29. ICH. ICH Harmonised tripartite guideline. Validation of analytical procedures: Text and methodology ed: Geneva; 2005.
30. Spangenberg B, Poole CF, Weins C. Quantitative thin layer chromatography: A practical survay. Heidelberg, Dordrecht, London, New York: Springer;2010.
31. Rashmin P, Mrunali P, Nitin D, Nidhi D, Bharat P. HPTLC method development and validation: strategy to minimize methodological failures. J Food Drug Anal. 2012.20(4):794-804.
32. Ferenzi-Fodor K, Vegh Z, Nagi-Turak A. Validation and quality assurance of planar chromatographic procedures in pharmaceutical analysis. J AOAC Int. 2001.84(4):1265-76.
33. Khan KA. The concept of dissolution efficiency. J Pharm Pharmacol. 1975.27(1):48-9.
34. Wall PE. Thin-layer chromatography a practical and modern approaches. Cambridge, UK: The Royal Society of Chemistry; 2005.
35. Ferenzi-Fodor K, Renger B, Vegh Z. The frustated reviewer-recurrent failures in manuscript describing validation of quantitative TLC/HPTLC procedures of analysis of pharmaceutical. JPC. 2010.23(3):173-9.
36. AOAC. Official Methods of Analysis of AOAC Interntional. In: George W. Latimer J, editor. Guidelines for Standard Method Performance Requirements. 19th ed. Gaithersburg, Maryland, USA: AOAC International; 2012. p. Appendix F, p9.
37. Leuner C, Dressman J. Improving drug solubility for oral delivery using solid dispersions. Eur J Pharm Biopharm. 2000.50(1):47-60.
38. Craig DQ. The mechanisms of drug release from solid dispersions in water-soluble polymers. Int J Pharm. 2002.231(2):131-44.
39. Srinarong P, Kouwen S, Visser MR, Hinrichs WL, Frijlink HW. Effect of drug-carrier interaction on the dissolution behavior of solid dispersion tablets. Pharm Dev Technol. 2009.15(5):460-8.