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Quantification of Diclofenac Sodium in Enteric-coated Tablet Brands Using Three Standard Calibration Methods

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Overdoses of diclofenac sodium can be life threatening particularly to the elderly with cardio-vascular diseases. This drug is usually prescribed in high doses and must be consumed over a long period for effective treatment. Hence, quality control of the drug content in pharmaceutical products is essential to avoid overdosage. In this study, the diclofenac sodium content in three tablet brands were separated using RPHPLC-DAD and quantified using external standard calibration curve, internal standard calibration curve and standard addition calibration curve. The external standard method accurately quantified the diclofenac sodium in Voren® (error ≈ 4 %) and Remafen® (error ≈ 9 %) but not in Remethan® (error ≈ 27 %). The internal standard method was used to explore the possibility of volume error that may contribute to the discrepancies of accuracy in the sample brands. It was found that there was no improvement in the accuracy as no volume error was indicated, either in the sample preparation or in the volume injected to the RPHPLC-DAD. The discrepancies of accuracy for Remafen® and Remethan® due to matrix interferences were explored using the standard addition calibration curve. The accuracy of Remafen® (error ≈ 4 %) and Remethan® (error ≈ 2 %) was improved with this method. This suggests that the matrix interference in the two brands probably resulted from excipients used in the drug formulation. The excipient is unlikely to be present in Voren® considering that the diclofenac sodium content can be accurately estimated by merely using external standard method.

Keywords: External standard calibration curve; internal standard calibration curve; standard addition calibration curve; diclofenac sodium

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Diclofenac sodium belongs to non-steroidal anti-inflammatory drugs (NSAIDs) and is available in the form of tablets and capsules in local drug stores. This over-the-counter drug needs to be consumed in high doses in most treatment of osteoarthritis and rheumatoid arthritis. The dosage prescribed to patients is not only high (maximum daily dose for adults: 150 – 200 mg per day), but the drug must also be taken continuously over a long period of time to treat the pain effectively [1]. Consuming it in high doses may lead to overdose, if not managed properly, which will also result in several side effects such as stomach ulcer, heart attack, kidney damage, gastrointestinal bleeding, acute renal failure as well as coma [1]. Hence, a quality control of prescribed drugs is crucial to maintain the quality of the product and has recently been the number one issue in pharmaceutical industries [2]. A routine laboratory testing on the pharmaceutical products should be conducted not only to ensure the safety and efficacy of the products but also due to the widespread of counterfeit drugs in the local market. Counterfeit drugs can be a major public health concern as the consumers can be attracted to their low price [3,4] without considering the adverse effects of the drugs. The adverse effects of counterfeit drugs include increase in mortality and morbidity, and drug resistance that assists in the spread of infectious diseases [5,6].

Various methods have been applied to determine diclofenac sodium content in tablet samples [7–9]. However, chromatography has always been regarded as the standard method for analysis of drugs including diclofenac sodium. Thin layer chromatography (TLC) is the simplest and cheapest method compared to modern instrumental chromatographic methods such as gas chromatography (GC) and liquid chromatography (LC). Nevertheless, TLC can only be used for screening the presence of diclofenac sodium in pharmaceutical products but cannot be used to verify the analyte content as claimed by the manufacturer. TLC is also prone to error during screening analysis [10], where the hRf value used for confirming the presence of the analyte in the sample is not very reliable as it is sensitive to small changes (e.g., the hRf value can differ a lot in repetitive measurement), which is in contrast with the retention time unit used in GC and LC. The GC

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method can be easy as no optimal selection of mobile phase is required, which on the other hand, is crucial in LC. Nevertheless, LC is still the most used method for quantification of diclofenac sodium, probably due to no derivatization step required to increase the diclofenac sodium volatility compared to GC [11].

A calibration curve is essential in any analytical instrumentation method as the instrumentation did not directly measure the amount of diclofenac sodium per tablet. For example, the output data for LC and UV-VIS spectrophotometer are peak area of the analyte and the amount of light absorbed by the analyte (absorbance unit), respectively. The calibration curve is required to assess the relationship between the signal output of the analyte and its respective amount/ concentration that can be obtained by measuring a series of standard solutions of the analyte at various concentrations (e.g., mg/ L). There are three calibration curves that are commonly used in any analytical instrumentation, namely as external calibration curve, internal standard calibration curve and standard addition calibration curve. The selection of which calibration curve should be chosen highly depends on the sample matrices [12,13], the sample preparation procedure [14] and the condition of the instrumental for an analysis (e.g., manual injection versus auto-injection of sample solution in the LC). In this work, the diclofenac sodium content in three different brands of enteric-coated tablets commonly available in local pharmacies, was measured using reverse phase high performance liquid chromatography coupled with diode array detector (RPHPLC-DAD). To our knowledge, no studies have reported on the diclofenac sodium content in Voren®, Remafen® and Remethan® using any kind of analytical instrumental methods. Because different brands of the enteric-coated tablets may have different drug formulations, the sample matrices can affect the accuracy and precision of the calibration method used [15]. Thus, this work explores which calibration method is the most suitable method to determine the accuracy of diclofenac sodium content in each of the enteric-coated tablet brands.

EXPERIMENTAL

Chemicals and Reagents

Orthophosphoric acid, acetonitrile and methanol were obtained from Merck, Germany. All the solvents were LC reagent grade. Diclofenac sodium (95 % purity) is obtained from Sigma Aldrich, USA. The analytical grade of orthophosphoric acid (65 %) was diluted using ultra-pure water (18.2 MΩ) obtained from Smart2Pure Pro UV/UF 16 LPH (ThermoFisher Scientific, Germany). The solution pH was adjusted to the desired pH using hydrochloric acid and sodium carbonate solutions.

Preparation of Working Standard Solutions of Diclofenac Sodium

A stock solution of diclofenac sodium (1000 mg/ L) was prepared by dissolving the diclofenac sodium powder of 95 % purity in methanol. All working standard solutions for all three calibration methods were freshly prepared daily in methanol. The external standard calibration curve is constructed by diluting the stock solution at a concentration range of 5 – 100 mg/ L. To construct an internal standard calibration curve, the stock solution was diluted to final diclofenac sodium concentrations of 1, 5, 10, 15, 20, 25, 30 mg/ L with the presence of 10 mg/ L mefenamic acid in all the working standard solutions. For the standard addition calibration curve the stock solution was diluted at various concentrations and added into sample solutions to give the final concentrations between 0.5 and 5 mg/ L.

Preparation of Sample Solution

Diclofenac sodium enteric-coated tablets from three different brands (Voren®, Remethan®, and Remafen®) were purchased from a local pharmacy in Shah Alam, Selangor. For each brand, ten tablets were removed from their blister pack, weighed, and recorded. Then, the tablets were mixed homogeneously and ground finely by mortar and pestle. The sample powder obtained was dissolved in methanol and stirred for 30 min at 55 ^{0}C, followed by sonication for 30 min to aid the dissolution process. The sample solution was then filtered to remove any suspended particles that can damage the LC column. The filtered sample solution was used in the quantification of diclofenac sodium using external standard calibration method. For the internal standard method, the filtered sample solution was added with 10 mg/ L of mefenamic acid. While in the standard addition method, the filtered sample was added with different concentrations of standard diclofenac sodium solution prepared prior to the LC measurement. The filtered sample solution without any addition of the standard solution was also measured in the standard addition method.

LC Analysis

The RP-HPLC was carried out using Agilent Technology 1200 series (Waldbronn, Germany) equipped with diode array detector. The standard and sample solutions were injected in triplicates with injection volume of 20 µL using the autosampler system. The separation of diclofenac sodium was performed using Eclipse XDB-C18 column (4.6 x 150 mm, 5µm particle size) with mobile phase composition of 35 % orthophosphoric acid (pH 2) and 65 % acetonitrile at 2 mL/ min flowrate. All the solvents used in the LC analysis have been degassed using Branson Ultrasonic Cleaner

(Branson Ultrasonic Corporation, CT, USA). The diclofenac sodium detection was set at wavelength 210 nm. The chromatogram obtained was analyzed using Agilent Chemstation software.

Validation

All three calibration curves used in this study were generated using the least-square method. The calibration curves were validated by regression analysis. The best fit between the experimental data points and regression model line in the calibration curve can be interpreted by the coefficient of determination (R^2) and the standard deviation about regression (S_r) values. The reliability of each calibration method for estimating the amount of diclofenac sodium in the samples was assessed by the % recovery, % error and repeatability measurements. The % recovery and % error (Equation 1-2) was used to assess the accuracy. Meanwhile, the repeatability measurements indicate the precision of the calibration method assessed by the % relative standard deviation (RSD) value.

$$\% \text{ Recovery} = \frac{(A - B)}{C} \times 100\ \% \qquad \text{Equation 1}$$

A and B refer to the diclofenac concentration in a spike sample and an un-spike sample, respectively. C is the theoretical diclofenac standard concentration added into the spiked sample.

$$\% \text{ Error} = \left| \frac{M - N}{M} \right| \times 100\ \% \qquad \text{Equation 2}$$

M and N refer to theoretical (as claimed by the manufacturer) and average measured amount of diclofenac sodium per tablet, respectively.

RESULTS AND DISCUSSION

External Standard Method

Initially, quantification of diclofenac sodium in all sample brands were determined using external standard calibration method as this is the most common method used [16,17] and considered the simplest. Figure 1 shows the chromatogram of diclofenac sodium in standard and sample solutions obtained using external standard calibration method. Based on the standard solution, the diclofenac sodium in the samples was observed at ≈ 3.3 min. The retention time of diclofenac sodium observed in this work was also observed in previous studies [18,19]. The chromatograms of diclofenac sodium in all sample brands showed similar features as the chromatogram of diclofenac in the standard, with the unresolved methanol peaks observed at ≈ 1.2 and ≈ 1.4 min. The mean peak areas of diclofenac sodium measured at various concentrations of standard solutions were used to plot the external standard calibration curve (Figure 2). The diclofenac sodium concentrations in the samples were determined from the linear equation of the graph. The R^2 value (0.9999) indicates a very strong correlation between the mean peak area and diclofenac concentration, which suggests that the estimated diclofenac sodium concentration in the sample using the calibration curve is reliable.

The reliability of the external standard calibration curve in quantitative analysis can be deduced from Table 1 and Table 2. The accuracy of the external calibration method was assessed by the recovery test. The mean % recovery calculated for each sample in Table 1 was consistent with the accuracy of diclofenac sodium amount per tablet measured (Table 2). This trend is expected as the % recovery can reflect the accuracy of the analysis [20]. The external calibration method is the most suitable for Voren® sample as the estimated value is closest to the amount claimed by the manufacturer with the lowest % error (3.7 %). All sample brands showed a positive error and the acceptable recovery range for the positive error should not be more than 110 % [20]. Considering this factor, the external calibration method is acceptable for the Remafen® quantification but not for Remethan®. The positive error observed could be due to the interference of other chemical species present in Remethan® that appeared at the same retention time with diclofenac sodium and significantly interfered the signal of the diclofenac sodium which might not be present in other brands. The replicated measurements for all samples have % RSD less than 2 (Table 2) which showed that the HPLC method used in this study has very good repeatability. According to the Food and Drug Administration (FDA) guidelines [21], a method is considered very precise when the % RSD is less than 3.

External calibration method has been applied for quantification of diclofenac sodium in various product brands (Safediclo®, Qufen®, Diclorism®, Oxagesic®, Avenzor®, Vesalion®, Voltaren® and Dioxaflex®) [11,19,22–24]. The external calibration method in previously mentioned studies, has strong correlation between the diclofenac sodium peak area and its corresponding concentration ($R^2 > 0.999$), good accuracy with acceptable range of recoveries (97 – 102 %) and high precision (% RSD ≤ 3). In addition, the external calibration method can accurately estimate diclofenac sodium content measured using other instrumentation techniques such as UV-VIS and fluorescence spectroscopy [24–29]. Considering the good reputation and reliability of this calibration method, the result observed for Remethan® in this work is unexpected. To our knowledge, no previous studies have reported the diclofenac sodium content in Voren®, Remafen® and Remethan®. Thus, other common calibration methods were explored in this work to investigate whether the methods could improve the accuracy and precision for all sample brands and to identify the reasons why external calibration method was not suitable for Remethan®.

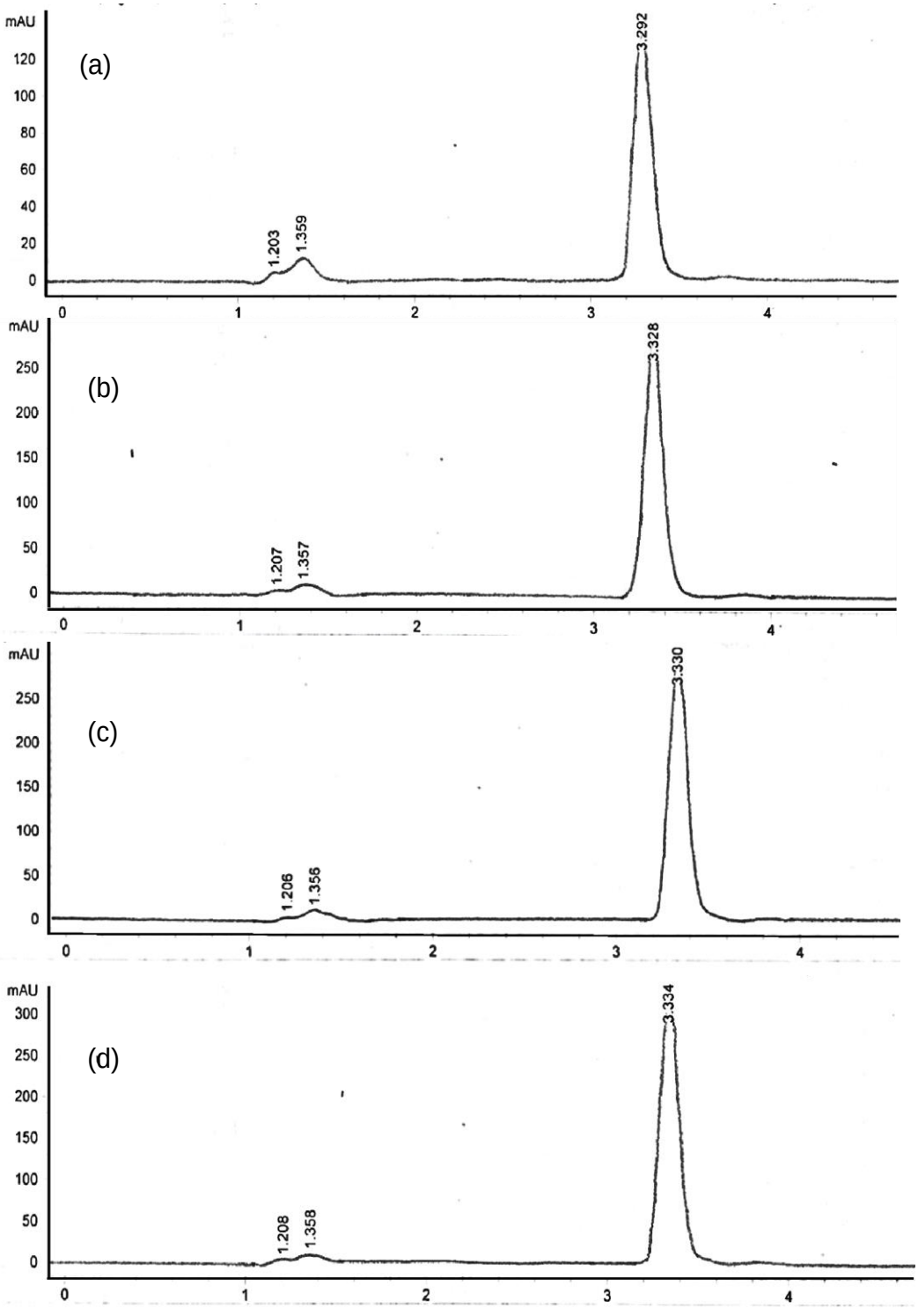

Figure 1. Chromatograms of diclofenac sodium in (a) standard solution (b) Voren® (c) Remafen® (d) Remethan®.

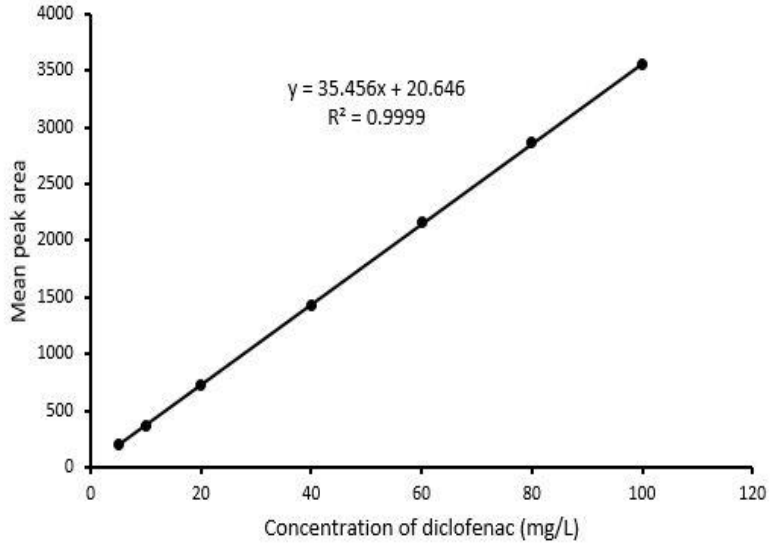

Figure 2. External standard calibration curve for diclofenac sodium.

Table 1. Recovery test for diclofenac sodium in tablets using external calibration method.

Brand name	Theoretical spiked concentration (mg/ L)	Mean spiked concentration measured (± SD) (mg/ L)	Mean Recovery (%)
Voren®	30	31.11 ± 0.08	103.69
Remafen®	30	32.76 ± 0.59	109.20
Remethan®	30	37.91 ± 0.41	126.37

Table 2. Diclofenac sodium content in samples estimates using external calibration method.

Brand name	Tablet expected content (mg)	Tablet measured content (± SD) (mg)	RSD, n=3 (%)	% Error
Voren®	50	51.84 ± 0.14	0.27	3.68
Remafen®	50	54.60 ± 0.99	1.80	9.20
Remethan®	50	63.52 ± 0.68	1.07	27.04

Internal Standard Method

Internal standard calibration method as previously reported was able to accurately quantify diclofenac sodium in various brands (Voltaren®, Clofen®, Voltaic®, Rapidus®, Rofenac® and Robinaxol®-D) [18,20,30]. Considering this, the calibration method was used in this work to explore whether the method can improve the accuracy and precision in Voren®, Remafen® and Remethan®. The internal standard method can compensate poor accuracy and precision that are due to volume errors resulted from either sample preparation or sample volume injected into the HPLC [31,32]. Hypothetically the internal standard will not improve the accuracy, as the sample preparation in this work is simple; did not involve any extraction, evaporation, and reconstitution that will attribute to subsequent volumetric losses. Nevertheless, the internal standard method is tested although the LC analysis was carried out using an autosampler injector, to double check whether the autosampler was working well. Mefenamic acid has been used as internal standard for quantification of diclofenac sodium [33] and indomethacin [34]. The mefenamic acid was chosen in this study due to several reasons: not found in the sample, has similar chemical structure with that of diclofenac sodium, and eluted with resolved peak latter than the target analyte. Figure 3 shows the chromatograms of diclofenac sodium and mefenamic acid in the standard solution and the three enteric-coated tablet brands obtained using the internal standard method. The diclofenac sodium (≈ 3.2 min) and mefenamic acid (≈ 4.4 min) peaks in the samples appeared at similar retention times with those of the standard solution. Wagih et al. [33] also observed a similar trend in their data with the diclofenac sodium peak eluting at 3.4 min and the mefenamic acid peak eluting at 4.8 min.

In the internal standard calibration method, the concentrations of diclofenac sodium in all samples were estimated by the linear equation (Figure 4) of the ratio of mean peak areas (diclofenac sodium (A_C) / mefenamic acid (A_{IS})) plotted as a function of the ratio of concentrations (diclofenac sodium (C_C) / mefenamic acid (C_{IS})). The R^2 value of 1 signifies perfect positive linear correlation between A_C/ A_{IS} and C_C/ C_{IS}. Application of internal standard method did improve the precision (% RSD less than 0.3) for all three tablet brands of replicated measurements. For Voren® sample, there is no significant improvement in the accuracy when using the internal standard method (Table 4), as the % error is within 3 - 4 % when compared to that of the external standard method. The accuracy for Remafen®, however, became worse but for Remethan® was improved when using the internal standard method. This trend suggests that the internal calibration method complicates and misleads the Remafen® and Remethan® quantifications. This can be supported by the inconsistent relationship between % recovery and accuracy of diclofenac sodium content observed in

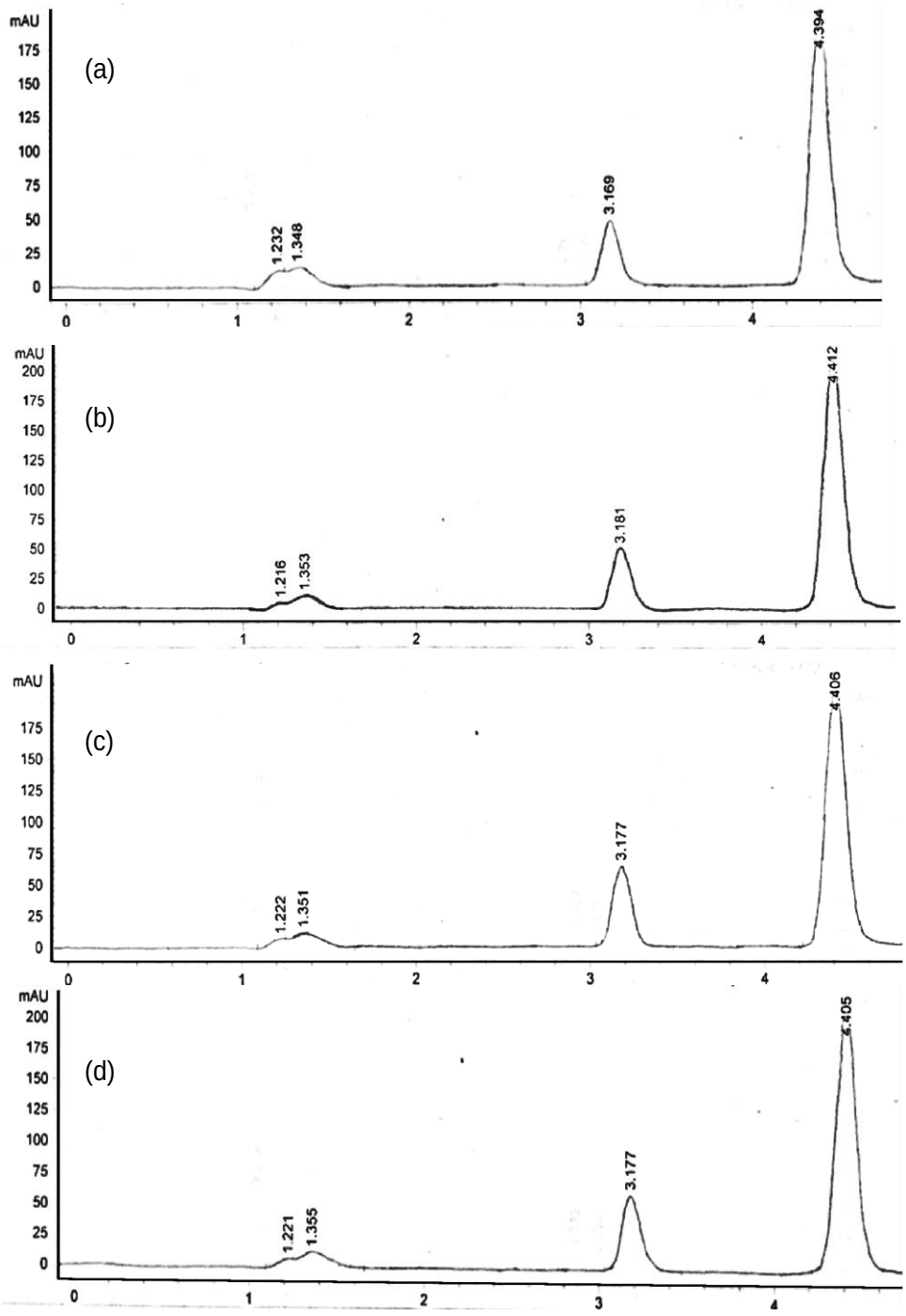

Figure 3. Chromatograms of diclofenac sodium in (a) standard solution (b) Voren® (c) Remafen® (d) Remethan® with presence of internal standard mefenamic acid.

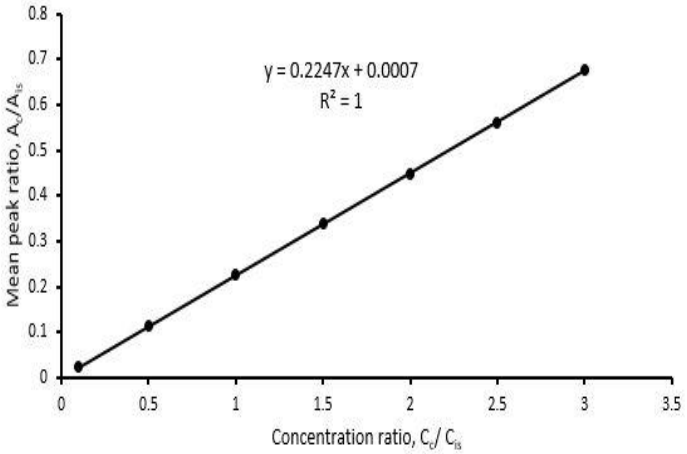

Figure 4. Internal standard calibration curve for diclofenac sodium.

Table 3. Recovery test for diclofenac sodium in tablets using internal standard method.

Brand Name	Theoretical spiked concentration (mg/L)	Mean spiked concentration measured (± SD) (mg/L)	Mean recovery (%)
Voren®	10	9.66 ± 0.02	96.62
Remafen®	10	12.20 ± 0.02	122.04
Remethan®	10	11.44 ± 0.08	114.44

Table 4. Diclofenac sodium content in samples estimates using internal calibration method.

Brand name	Tablet expected content (mg)	Tablet measured content (± SD) (mg)	RSD, n=3 (%)	% Error
Voren®	50	48.33 ± 0.11	0.24	3.34
Remafen®	50	57.22 ± 0.11	0.18	14.44
Remethan®	50	61.03 ± 0.05	0.08	22.06

both brands when using internal calibration method which was not seen when quantified using external calibration method. In addition, the internal calibration method makes the quantification of Remafen® worse (recovery of 122 %) as the acceptable range for the % recovery is within 90 and 110 % [20]. Based on the observation of the peak area of diclofenac sodium and the peak area of mefenamic acid in the replicated measurement of a particular sample, no volume error has occurred and the autosampler was working well in this work. Therefore, there is no requirement to use the internal calibration method as it may not add any benefits, instead may complicate the analysis, and increase the cost of purchasing the internal standard (mefenamic acid).

Standard Addition Method

The standard addition calibration method is not widely applied in quantification analysis as the other two calibration methods. This method is used to compensate the presence of sample matrix interferences [12,14] and has a better detection limit than the external calibration method [35]. The application of standard addition method in chromatography [14,36] is limited compared to other analytical instrumentation methods such as spectroscopy [37,38]. To our knowledge, no studies have been reported on the application of standard addition method in quantification of diclofenac sodium in enteric-coated tablets measured using chromatographic methods either GC or HPLC. The UV-VIS method is the only application of standard addition method that has been reported to determine the diclofenac sodium content [36]. In this work, standard addition method was not deemed necessary for the analysis of Voren® as the difference in accuracy and precision in the external standard and internal standard was comparable, suggesting that matrix interferences were not significant. Further investigation was carried out for Remafen® and Remethan® using the standard addition method due to poor accuracy observed in the external and internal calibration methods. This was done to explore whether the poor results were most likely caused by the sample matrix itself.

The diclofenac sodium in the three enteric-coated tablet brands used in this work may have different sample matrices resulted from different kinds of excipients added in the drug formulation [39–41]. Excipients are non-active substances, added in the enteric-coated tablets for many purposes such as for long-term stabilization and to maintain the tablet shape. Standard addition method can be used to compensate for errors in accuracy measurement of diclofenac amount per tablet and has been applied for estimation of drug content analyzed using LC [36,42]. In the standard addition calibration method, preparation of standard solutions must include the sample solution. To construct the calibration curve for standard addition method, chromatograms of Remafen® and Remethan® added with various concentrations of standard solutions were measured (Figure 5 and 6). This includes the chromatogram of the sample without any addition of the standard (Figure 5a and 6a). Both samples show similar features with methanol eluting at ≈ 0.8 – 0.9 min and the diclofenac sodium eluting at ≈ 2.3 min. The increment of peak area of diclofenac sodium is consistent with the increment of the analyte concentration added. Compared to the previous two calibration methods, interestingly the standard addition calibration method resulted in a faster analysis time (diclofenac sodium elution time is shorter than the one observed in the previous methods).

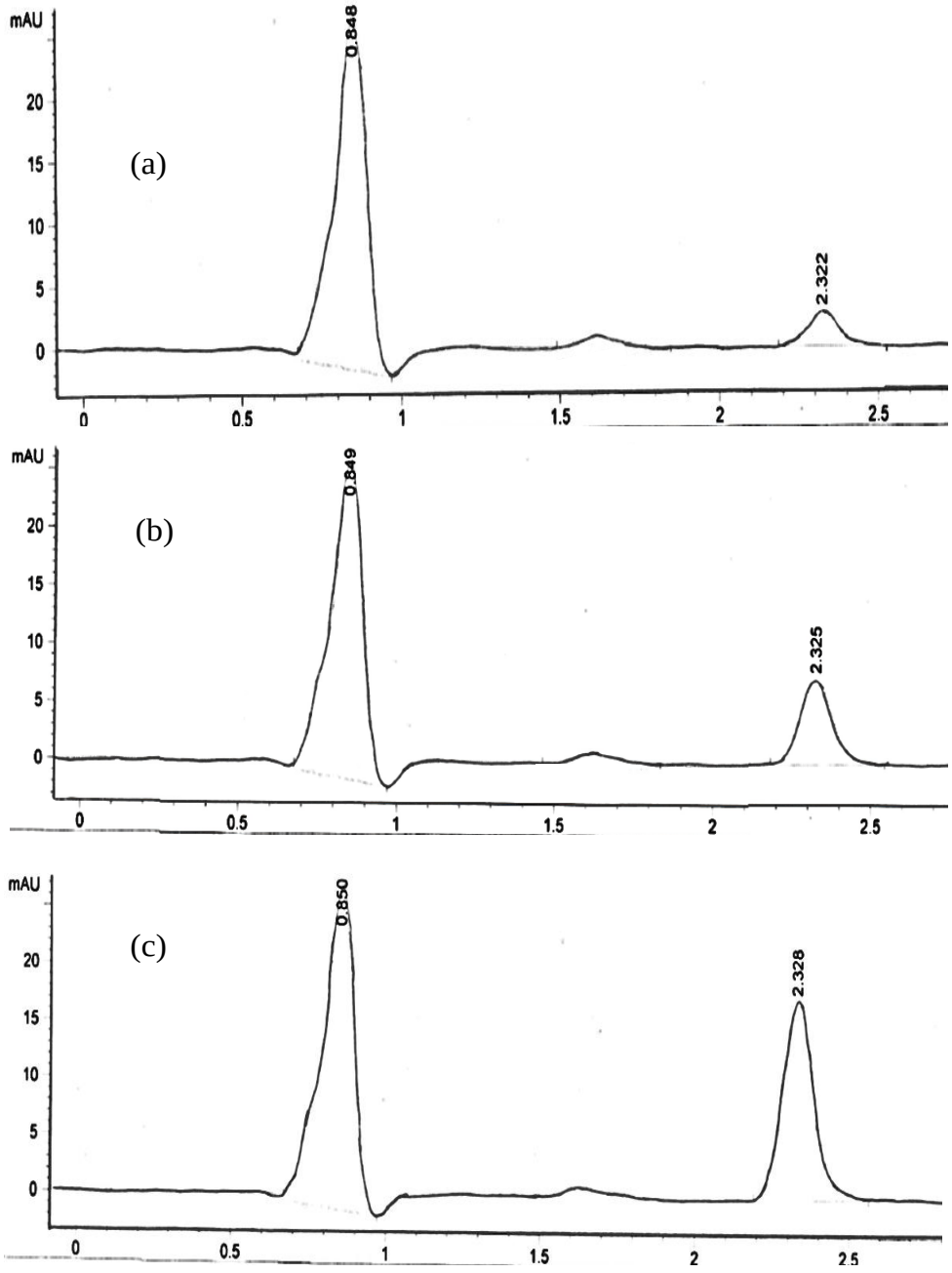

Figure 5. Chromatograms of diclofenac sodium prepared for standard addition method with (a) only Remafen® sample solution (b) the sample solution added with 1 mg/ L standard diclofenac and (c) the sample solution added with 3 mg/L standard diclofenac.

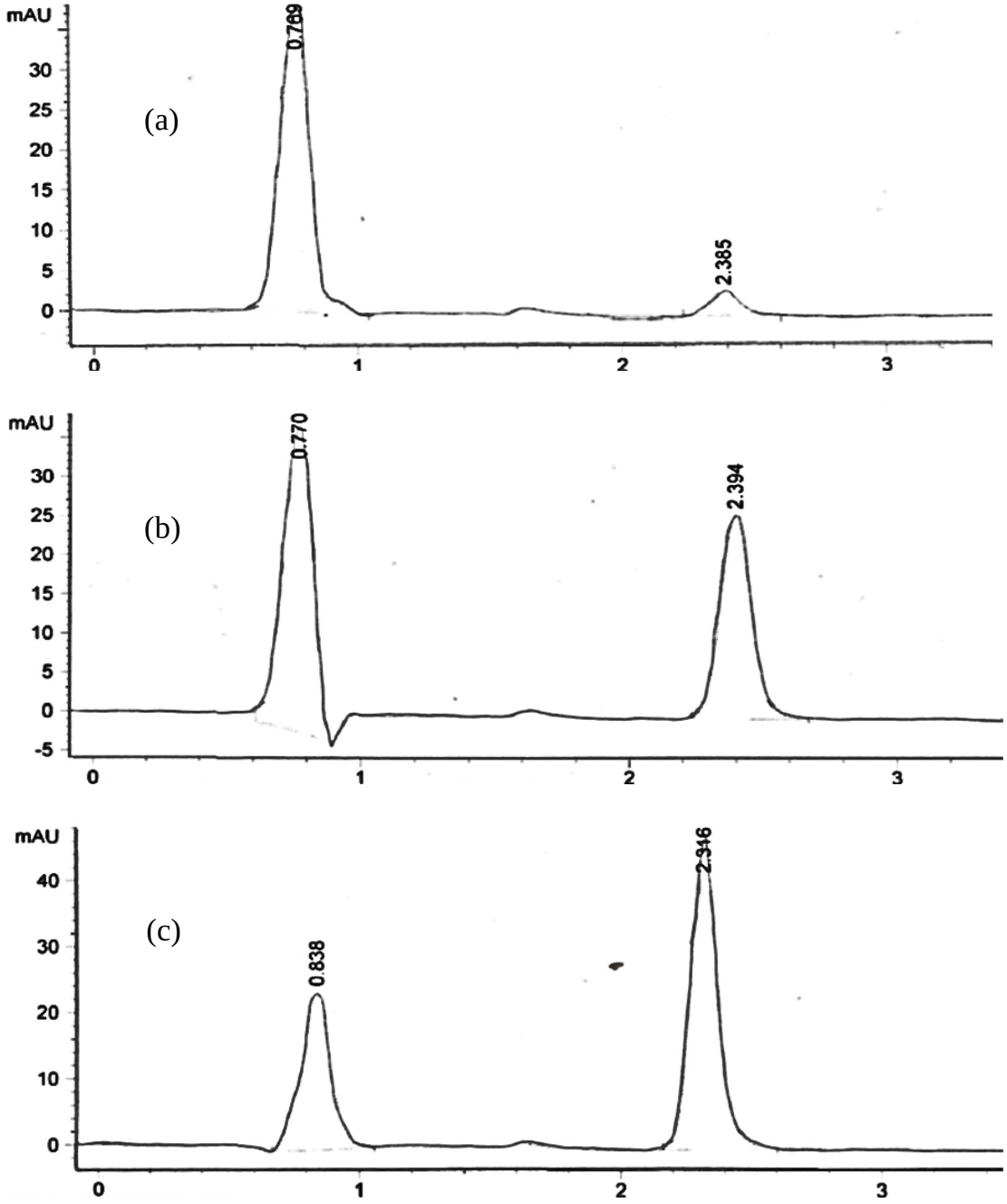

Figure 6. Chromatograms of diclofenac sodium prepared for standard addition method with (a) only Remethan® sample solution (b) the sample solution added with 3 mg/ L standard diclofenac and (c) the sample solution added with 5 mg/L standard diclofenac.

Figures 7 and 8 show the standard addition calibration curves for Remafen® and Remethan®, respectively. The amount of diclofenac per tablet in the samples (Table 5) was estimated from the linear equations with the R^2 values close to 1. The accuracy of diclofenac content in Remafen® and Remethan® was significantly improved when using the standard addition method. When compared to the external standard method, the error in Remafen® was improved from ≈ 9 % to ≈ 4 % and there was drastic improvement for Remethan® with an error decreasing from ≈ 27 % to ≈ 2 %. The added amount of standard in the x-axis (Figure 7 and 8) was plotted in volume instead of in concentration unit. When plotted in volume unit, the standard deviation of the analyte amount per tablet that obtained from the calibration curve can be obtained from the regression analysis using equations 3 and 4 [43,44]. The standard deviation calculated for both samples was less than 0.4. This indicates that any error associated with the regression model line that was used for estimating the analyte amount per tablet is insignificant. From this study we can deduce that there is a possibility of the presence of excipients in Remafen® and Remethan® samples that may interact with the diclofenac sodium and affect the polarity and partitioning degree of the diclofenac sodium between the mobile phase and the

stationary phase. When the standard addition is applied, the interference of the excipient is removed, and this affects the polarity of the diclofenac and thus changes the diclofenac sodium retention time (from ≈ 3.3 min to ≈ 2.3 min). This excipient seemed unlikely to be present in Voren® considering the diclofenac content can be estimated accurately (error ≈ 4 %) by merely using the external standard method. To the best of our knowledge, no studies have reported on the influence of excipients in the diclofenac sodium quantification from other diclofenac sodium brands. In addition, there are no accuracy studies on diclofenac sodium in Remafen® and Remethan® reported previously, thus this work contributes to a new knowledge on the quality control of diclofenac sodium products.

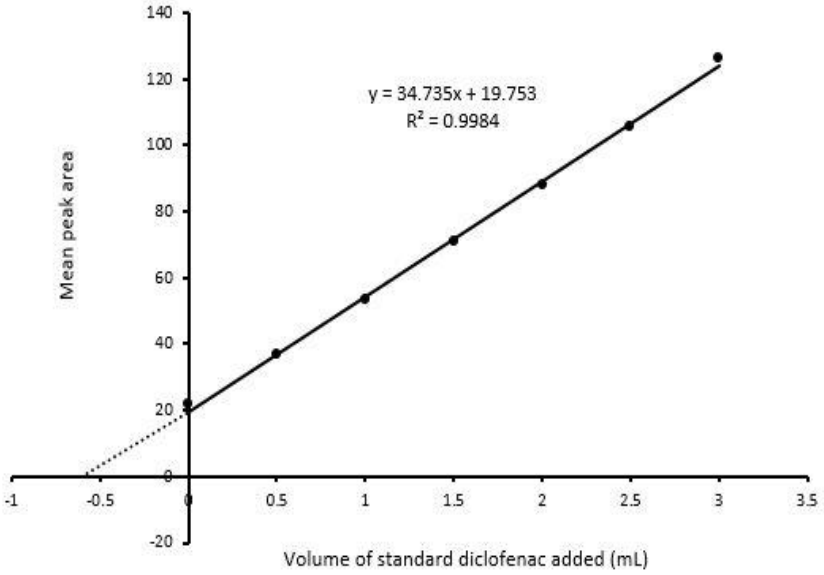

Figure 7. Standard addition calibration curve prepared for Remafen® measurement.

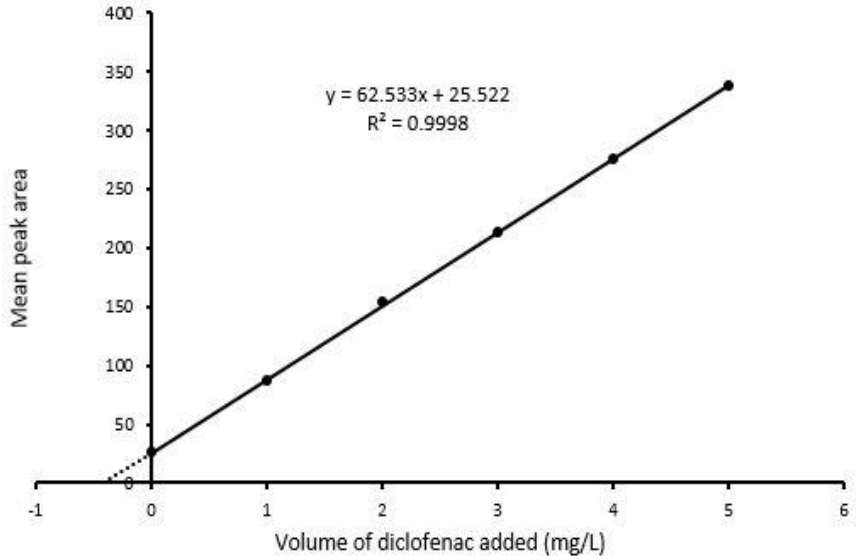

Figure 8. Standard addition calibration curve prepared for Remethan® measurement.

$$\text{std deviation in sample volume } (S_X) = \frac{S_r}{m}\sqrt{\frac{1}{N} + \frac{(y_c - y_{ave})^2}{m^2 S_{xx}}} \qquad \text{Equation 3}$$

S_r : standard deviation about regression
m : slope
N : number of data points in the calibration curve
y_c : peak area at the x-axis intercept

y_{ave} : average peak areas from the total areas of all datapoints in the calibration curve
S_{xx} : sum of the squares of the deviation from the mean for the individual values of x.

$$\text{std deviation in sample concentration } (S_c) = -\frac{C_x S_x}{b_v} \quad \text{Equation 4}$$

C_x : concentration of diclofenac in the sample
S_x : standard deviation in the sample volume
b_v : volume at the x-axis intercept

Table 5. Diclofenac sodium content in samples estimated using standard addition method.

Brand name	Tablet expected content (mg)	Tablet measured content (± SD) (mg)	% Error
Remafen®	50	48.05 ± 0.31	3.90
Remethan®	50	51.02 ± 0.11	2.04

CONCLUSION

The diclofenac sodium content in enteric-coated tablets analyzed by LC method can be quantified by three calibration methods: external, internal, and standard addition. In this study, simple external calibration method is sufficient for estimation of diclofenac sodium content in Voren® sample, with accurate measurement (error ≈ 4 %) and a very good repeatability (RSD of 0.27 %). The external method can be used for Remafen® (error ≈ 9 %) sample but not for Remethan® (error ≈ 27 %) although both brands have good repeatability. The internal standard method did not improve the accuracy of all enteric-coated tablet brands but improved the precision (% RSD of all samples less than 0.25 instead of 1.8 in the external standard method). The internal standard showed no significant volume error either in the sample preparation or in the sample injected to the RPHPLC-DAD. The accuracy of diclofenac sodium content in Remafen® and Remethan® improved when using standard addition method. When compared with the external standard method, the error reduced from ≈ 9 % to ≈ 4 % in Remafen® and from ≈ 27 % to ≈ 2 % in Remethan®. This suggests that the presence of matrix interference in Remafen® and Remethan® samples may affect the accuracy of diclofenac sodium content. The matrix interference may come from the excipient since each drug formulation can be differed by the non-active substances of the excipient added in the formulation. This excipient seemed unlikely to be present in Voren® considering that the diclofenac content can be estimated accurately (error of 3.6 % and % recovery within the acceptable range) by simply using the external standard method. In addition, the HPLC parameters used in this work were suitable for diclofenac sodium quantification in all brands (considering their low % errors (2 - 4 %) that are based on the calibration method suitable for each brand).

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