

*Full Length Research Paper*

# Simultaneous spectrophotometric determination of phenacetin and paracetamol in human plasma for the quantitative assessment of liver function reserve

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**A simple, economical and reliable spectrophotometric method was developed for the simultaneous determination of phenacetin and paracetamol in human plasma. We purified the plasma samples by solid phase extraction procedure. The analytes reacted with chromogenic sodium 1,2-naphthoquinone-4-sulfonate and cetyltrimethyl ammonium bromide, and the products conformed to Beer's law over the range 2 to 24 µg/ml for phenacetin and 1 to 16 µg/ml for paracetamol. The method was validated according to the Food and Drug Administration (FDA) guideline. There was no statistical difference between the proposed method and the modified high performance liquid chromatography (HPLC) method with regard to accuracy and precision. The proposed method is suitable for the quantitative assessment of liver function reserve in patients with liver cirrhosis.**

**Key words:** Spectrophotometric method, phenacetin, paracetamol, liver function reserve.

## INTRODUCTION

The assessment of liver function reserve is critically important for the prediction of the safety of partial hepatectomy and the efficacy of a therapeutic intervention in patients with liver cirrhosis. Phenacetin test is a useful method for the quantitative assessment of liver function reserve (Schneider, 2004; Qu et al., 2007; Liu et al., 2012). The test is to measure the ratio of plasma concentration of the metabolite-paracetamol (PAR) to the probe drug-phenacetin (PHN) at a time after oral administration of phenacetin (Xiong et al., 2010). Hence, it is essential to develop a simple, economical and reliable method for simultaneous determination of PAR and PHN in plasma.

A few methods have been reported for simultaneous

determination of PHN and PAR in plasma including high performance liquid chromatography (HPLC) (Gotelli et al., 1977; Uges et al., 1981; Mineshita et al., 1989; Bartoli et al., 1996; Cui et al., 2002), liquid chromatography-mass spectrometry (LC-MS/MS) (Xiong et al., 2010; Whiterock et al., 2012) or gas chromatography-mass spectrometry (GC-MS) (Murray et al., 1991). It is too complicated and costly of each of these methods to be applied in routine clinical work.

Although some simple spectrophotometric methods have been reported for determination of PHN or PAR in pharmaceutical preparation (Michael et al., 1989; Domagalina et al., 1997; Nagaraja et al., 1998; Yousefinejad and Hemmateenejad, 2012), there is not

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any spectrophotometric method reported for simultaneous determination of PHN and PAR in complicated samples such as plasma. In this paper, a simple and economical spectrophotometric method was developed for simultaneous determination of PHN and PAR in plasma and validated according to the United States Food and Drug Administration (US FDA's) Guidance for Industry Bioanalytical Method Validation.

The PHN and PAR in the plasma samples were purified by solid phase extraction (SPE) and reacted with sodium 1,2-naphthoquinone-4-sulphonate (NQS) and cetyltrimethyl ammonium bromide (CTAB) to increase the absorptivity. The concentrations of PHN and PAR were determined by the simultaneous equation method. Analytical accuracy and precision of the proposed method were compared statistically with a modified HPLC method considering its high selectivity and sensitivity. The proposed method was successfully applied in the phenacetin test to quantitatively assess liver function reserve of eight healthy subjects and twenty-two cirrhotic patients. The proposed method is suitable for the phenacetin test to be used in routine clinical work.

## MATERIALS AND METHODS

### Chemicals and reagents

Pure phenacetin, paracetamol and acetanilide were purchased from Sigma-Aldrich (Prague, Czech Republic). Sodium 1,2-naphthoquinone-4-sulphonate and cetyltrimethyl ammonium bromide were purchased from Aladdin (China). All other reagents were of analytical grade.

### Apparatus

A SHIMADZU model OV-265 double beam UV-Vis spectrophotometer with a fix slit width of 1 nm coupled 1 cm quartz cell was used for spectral measurements. The chromatographic system consisted of an Agilent 1200 series LC system equipped with an G1322A solvent degasser, G1311A pump, G1314B detector, G1329A autosampler, Agilent ChemStation B.04.02 and a reversed phase 5  $\mu$ m Xterra ODS column (250  $\times$  4.6 mm, i.d.). All pH measurements were made with a SARTORIUS model PB-10 digital pH meter. Empty SPE tubes and frits for Solid-phase extraction (SHENZHENBIOCAMMA BIOTECH CO., LTD), and AMBERCHROM™ CG161M macroporous adsorbent (Rohm and Haas) were used.

### Solution preparation

#### Preparation of calibration standards

A 10 mg/ml stock solution of PAR was prepared by dissolving accurately weighted pure PAR in ethanol. It was serially diluted to give working solutions at the concentrations over 0.1 to 1.6 mg/ml, after that the calibration standards of PAR at the concentrations of 1, 2, 4, 8, 12, 16  $\mu$ g/ml were prepared by spiking 2 ml of blank human plasma with 20  $\mu$ l of working solutions. Calibration standards of PHN at the concentrations of 2, 4, 8, 12, 16, 24  $\mu$ g/ml were prepared using the same method.

#### Preparation of quality control (QC) samples

The QC samples were prepared by spiking blank human plasma at concentrations of 2, 10, 24  $\mu$ g/ml for PHN and 1, 10, 16  $\mu$ g/ml for PAR, which represented low, medium, and high concentration QC samples, respectively. The QC samples were prepared independently of the calibration standards.

#### Preparation of reaction reagent solutions

A 0.1% (M/V) aqueous solution of NQS was freshly prepared and protected from sunlight. A 1% (M/V) aqueous solution of CTAB, 40% (M/V) sodium hydroxide solution (NaOH) and 0.4 % (M/V) sodium hydroxide solution (NaOH) were used.

#### Sample preparation and determination for spectrophotometric method

An aliquot of 5 ml of calibration standard, QC sample or unknown plasma sample was loaded onto the CG161 cartridge that was preconditioned with 2 ml of 95% ethanol and 9 ml of water in order. It was then washed with 6 ml of deionized water. The elution was made with 2 ml of 65% ethanol. The elution collected was mixed with 0.8 ml of 4 M HCl, and refluxed for 1 h. The solution was cooled to room temperature and transferred to a 5 ml calibrated flask, and 1.16 ml of 40% NaOH, 0.25 ml of 0.4% NaOH and 200  $\mu$ l of 0.1% NQS were added sequentially. The mixture was vortexed, left to stand for ten minutes, and 200  $\mu$ l of 1% CTAB was then added. The mixture was filled to the mark with deionized water. After mixing the solution thoroughly, an aliquot of this analyte was taken and the absorbance was measured at 500 and 565 nm against the reagent blank according to the standard procedure. Absorbance value (A) was substituted into the corresponding equation to get the plasma concentrations (C).

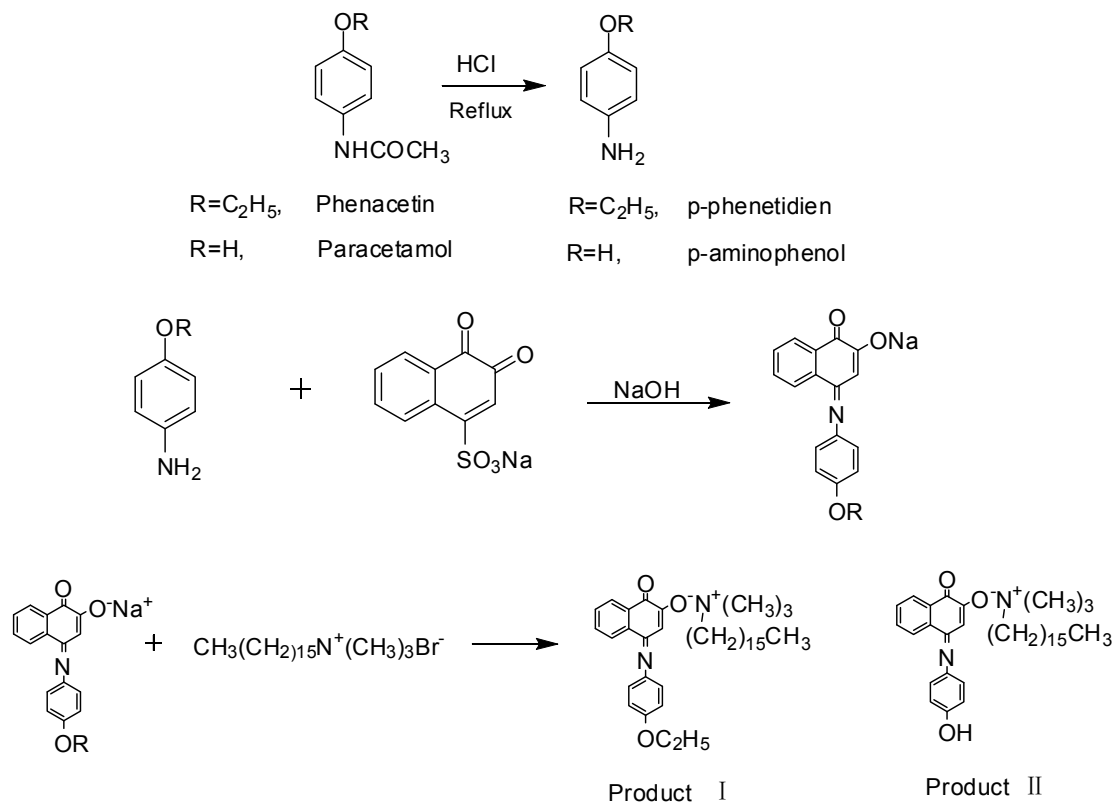
### Chromatographic conditions

The chromatographic conditions of the modified HPLC method were as follows: Plasma sample of PHN and PAR was mixed with acetanilide as an internal standard and purified by the SPE procedure shown earlier; an aliquot of 20  $\mu$ l of the elution was injected into the HPLC system and analysed at 245 nm; the mobile phase was water and methanol (63.5:36.5, v/v), which run at a flow rate of 1 ml/min at 37°C.

## RESULTS AND DISCUSSION

### Development of a solid-phase extraction procedure (SPE)

The previous purification methods reported for the determination of PAR and PHN in plasma included methanol deproteinization (Uges et al., 1981) and more complex liquid-liquid extraction (LLE) using acetoacetate (George et al., 1977). Both have obvious disadvantages. Too much organic solvent is added into the plasma in protein precipitation. Besides that, LLE has a difficulty in the stable reproducibility of the extraction due to the



**Figure 1.** The reaction mechanism.

incomplete phase separation. A solid-phase extraction (SPE) was developed for the sample preparation of PHN and PAR in plasma. Different kinds of nonpolar sorbents were tested in this research including polystyrene (PS), octyl (C8) and octadecyl (C18). The best purification and satisfied recovery values for PHN and PAR obtained were using polystyrene column (AMBERCHROM™ CG161M macroreticular adsorbent). The absolute extraction recoveries for low, medium and high concentration QC samples were consistent at 98% using 0.165 g CG161M with 65% ethanol as elution at room temperature. The absolute extraction recoveries of PAR and PHN from the SPE procedure were much higher than the average 80% of the LLE reported in literature (George et al., 1977), and no organic solvent was involved in the SPE procedure.

### Reaction mechanism

Because the analysing sensitivity of spectrophotometric method is generally lower than HPLC, LC-MS/MS and GC-MS, NQS and CTAB have been added as the chemical derivative chromogenic reagents to increase the analysing sensitivity of the proposed method. PHN reacted with NQS and CTAB to form product I that had higher absorptivity than PHN itself, and PAR was handled

in the same way to form product II. The stoichiometric ratio of reagent and corresponding product in this reaction was 1:1. The reaction mechanism is shown in Figure 1.

### Influence of pH

The effects of reaction pH on the absorbance of products were studied, and pH 8 to 9 was selected as the optimal experimental conditions. When pH was above nine, the absorbance of the solution could hardly stabilize but increased slowly. A previous paper (Nagaraja et al., 1998) reported that, PAR solution was stable with NaOH, but PHN solution was not. Na<sub>2</sub>CO<sub>3</sub> was used instead of NaOH in their paper. However, we found that the stability of absorbance was only influenced by the pH of the solution, not influenced by the chosen of pH regulator. In this study, we chose NaOH as the pH regulator in the reaction.

### Influences of temperature

Keeping pH at nine, the influence of temperature on the reaction was studied. We found that the absorbance of solution was maximal at room temperature and decreased

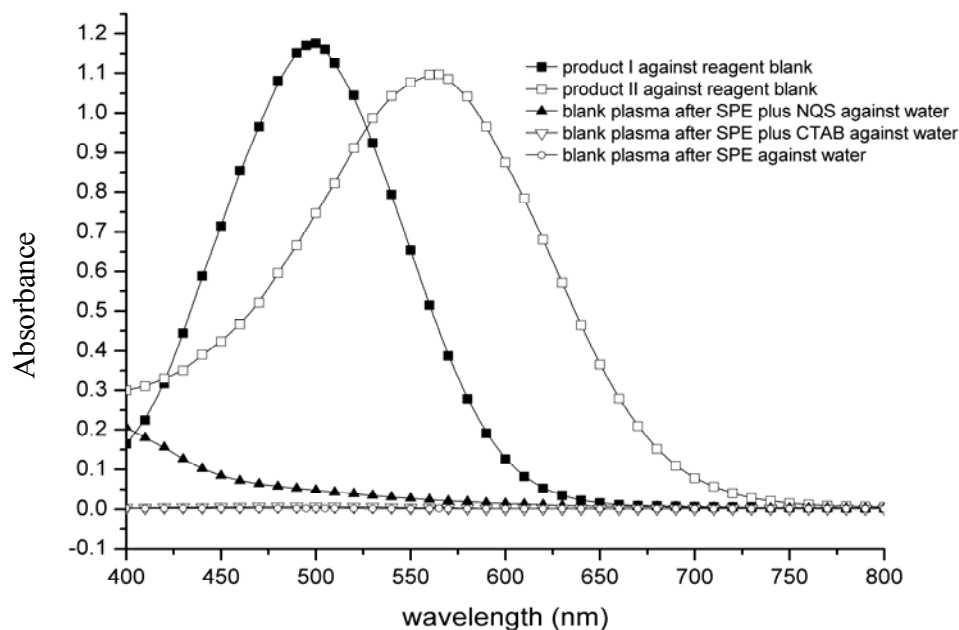


Figure 2. Absorption spectra of I, II, NQS, CTAB, and blank plasma.

decreased with the increased temperature. The absorbance of the solution fell to zero in water bath at 80°C for 10 min. Therefore, we chose room temperature as the optimum reaction condition in order to make the method reproducible and simple.

### Influence of reaction time

Keeping other conditions unchanged, the absorbance of the product was measured at different reaction time. The results showed that both p-aminophenol and p-phenetidine reacted quickly with NQS and CTAB. The absorbance increased to the top within 5 min and kept stable in 24 h. Therefore, no less than 10 min was necessary for the reaction to complete considering the robustness.

### Spectral characteristics

The spectra of the product I and product II were determined separately. Overlain spectra (Figure 2) suggested that the product I and product II showed maximum absorbance at 500 and 565 nm, respectively. The solutions were found to be sufficiently stable throughout the experiment.

### The establishment of simultaneous regression equations

Both the product I formed from PHN and product II formed

formed from PAR interfered with the absorption of each other. However, Figure 2 showed different absorption maxima. Therefore, modified Beer's law (Chaudhari et al., 2006) (law of additivity) was used for their estimation in mixture (Dave et al., 2007; Garg et al., 2008).

$$A_{500} = 0.0325C_{PHN} + 0.0282C_{PAR} \quad (1)$$

$$A_{565} = 0.0126C_{PHN} + 0.0594C_{PAR} \quad (2)$$

Where,  $C_{PHN}$  and  $C_{PAR}$  were the concentrations of PHN and PAR in the plasma,  $A_{500}$  and  $A_{565}$  were absorbance values of the sample solution at 500 nm and 565 nm, respectively.

Values of 0.0325 and 0.0126 were the mean apparent absorptivity of PHN at 500 nm and 565 nm, respectively. Values of 0.0282 and 0.0594 were the mean apparent absorptivity of PAR at 500 nm and 565 nm, respectively. The mean apparent absorptivity of PHN and PAR were determined by measuring the absorbance of different concentrations of calibration standards at the selected wavelengths (Table 1).

By applying the Cramer's rule to Eqn. 1 and 2, the  $C_{PHN}$  and  $C_{PAR}$  can be obtained as follows:

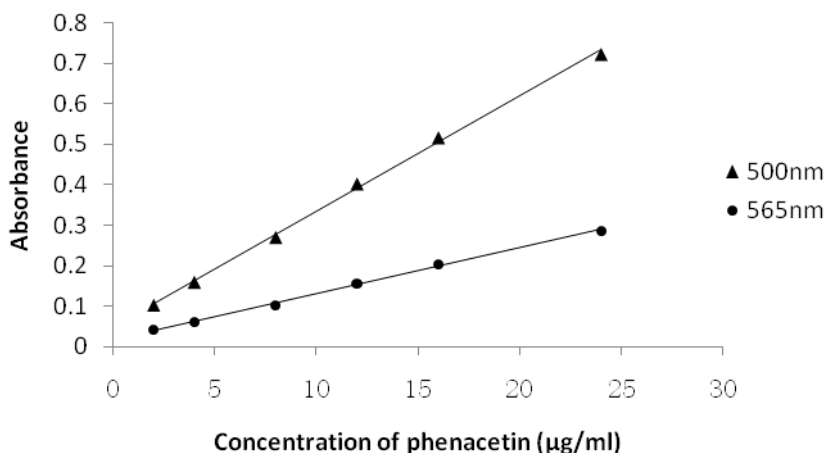
$$C_{PHN} = \frac{0.0594A_{500} - 0.0282A_{565}}{0.00157} \quad (3)$$

$$C_{PAR} = \frac{0.0325A_{565} - 0.0126A_{500}}{0.00157} \quad (4)$$

So, the concentrations of PHN and PAR in unknown samples can be calculated by Eqn. 3 and 4, respectively.

**Table 1.** Absorptivity values of PHN and PAR at 500 nm and 565 nm (mean ± SD, n = 6).

Absorptivity at 500nm		Absorptivity at 565nm	
PHN	PAR	PHN	PAR
0.0325 ± 0.0013	0.0282 ± 0.0011	0.0126 ± 0.0005	0.0594 ± 0.0026



**Figure 3.** Calibration curve of PHN at 500 and 565 nm.

**Method validation**

The method was validated according to the US FDA’s Guidance for Industry Bioanalytical Method Validation (2000). The calibration curves were constructed for both PHN and PAR at 500 and 565 nm using the calibration standards (Figures 3 and 4). Beer’s law hold well over the range 2 to 24 µg/ml for PHN and 1 to 16 µg/ml for PAR and the calibration curves covered the entire range of clinical expected concentrations (Qu et al., 2007). The precision of the proposed method was determined at low, medium and high concentrations by analysing six QC samples over 1 day in random order (Intraday precision or within-run precision), and analysing each control once on each of the six different days (Interday precision or intra-batch precision). The sensitivity of the method was measured by determination of the lower limit of detection (LLOD) and lower limit of the quantification (LLOQ). LLOD and LLOQ of PHN and PAR at 500 and 565 nm were calculated, respectively, according to the Eqn. 5 and 6, where  $\sigma$  is the standard deviation of reagent blank and  $s$  is the slope of the calibration curve.

$$LLOD = \frac{3.3\sigma}{s} \tag{5}$$

$$LLOQ = \frac{10\sigma}{s} \tag{6}$$

The results are satisfactory and presented in Table 2. The absolute extraction recoveries were the absorbance

values obtained from three concentrations of PHN and PAR added to and extracted from QC plasma samples, compared with the absorbance values obtained for the true concentrations of the pure authentic unextracted standards prepared with 65% ethanol instead of blank human plasma. The absolute extraction recoveries were consistent at 98% of the low, medium and high concentrations indicated the good extraction efficiency of the SPE procedure. Method’s accuracy was evaluated by means of recovery studies of QC samples at three concentration levels (n = five for each level). The results are satisfactory: mean recovery values are always higher than 95%; the deviations of the mean (%Bias) from the true recovery values are within 4% that is much less than the 15% requested in the FDA guideline (Table 3).

**Statistical comparison of the results of the proposed method and the HPLC method**

Spiked QC samples at concentrations of 2, 12, 24 µg/ml for PHN and 1, 8, 16 µg/ml for PAR were measured by both the proposed method and the HPLC method. The results obtained were presented in Table 4 and compared statistically by the statistical package for social sciences (SPSS) 15.1. Student’s *t*-test and variance ratio *F*-test were examined for accuracy and precision at 95% confidence level, respectively. The calculated *t*-values and *F*-values at all the three concentrations did not exceed

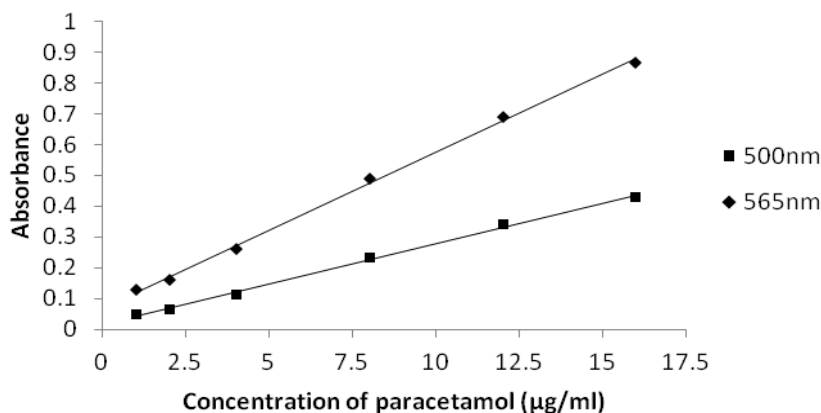


Figure 4. Calibration curve of PAR at 500 and 565 nm.

Table 2. Calibration data of PAR and PHN.

Parameter	PHN		PAR	
	500	565	500	565
Analytical wavelength (nm)	500	565	500	565
Molar absorptivity (l/mol/cm)	$5.10 \times 10^3$	$2.02 \times 10^3$	$3.97 \times 10^3$	$7.65 \times 10^3$
Linearity range (µg/ml)	2.0–24.0	2.0–24.0	1.0–16.0	1.0–16.0
Regression equation	$0.0285x+0.0491$	$0.0113x+0.0178$	$0.0263x+0.0169$	$0.0506x+0.0703$
Slope	0.0285	0.0113	0.0263	0.0506
S.E. of slope	0.0005	0.0002	0.0007	0.0011
Intercept	0.0491	0.0178	0.0169	0.0703
S.E. of intercept	0.0056	0.0022	0.0027	0.0053
Correlation coefficient ( $R^2$ )	0.9984	0.9976	0.9971	0.9981
LLOD (µg/ml)	0.583	0.620	0.312	0.291
LLOQ (µg/ml)	1.766	1.879	0.947	0.882
<b>Intraday precision (%RSD)</b>				
Low concentration	5.2		4.3	
Medium concentration	2.4		2.8	
High concentration	1.2		1.4	
<b>Interday precision (%RSD)</b>				
Low concentration	6.7		5.7	
Medium concentration	2.7		2.4	
High concentration	1.3		1.4	

exceed the theoretical values ( $t = 2.776$ ,  $F = 19.00$ ;  $n = 3$ ) indicating that there was no significant difference between the proposed method and the HPLC method with regard to accuracy and precision. The chromatograms of the pure analyte and QC sample are shown in Figure 5.

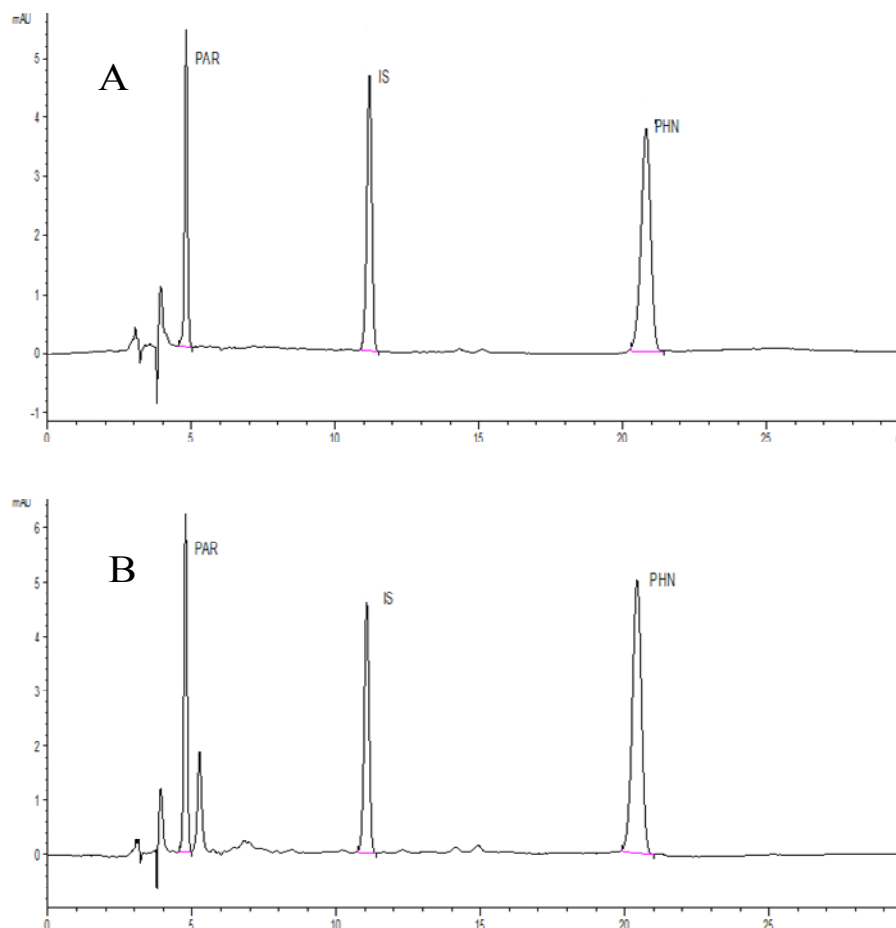
#### Analytical application in phenacetin test

Eight healthy subjects and twenty-two cirrhotic patients participated in phenacetin test to assess the liver function reserve from November, 2011 to February, 2012. All of

the participants were required to fast overnight. Phenacetin 1 g was ingested with 200 ml water in the morning. The blood sample was drawn from antecubital vein at 2 h after the oral administration of phenacetin. The contents of phenacetin and paracetamol in plasma were determined by both the proposed method and the HPLC method. The results are shown in Table 5. Significant differences in the ratio of plasma concentration of PAR to PHN were observed between the controls and the cirrhotic patients using two-sample t-test at 95% confidence level. The ratio of plasma concentration of PAR to PHN calculated with the proposed method decreased 60.8% in the cirrhotic patients (0.381) compared

**Table 3.** Accuracy of the proposed method calculated at low, medium and high concentration of the spiked QC plasmas (mean  $\pm$  SD, n=5).

Sample NO.	Added ( $\mu\text{g/ml}$ )		Found ( $\mu\text{g/ml}$ )		Recovery (%)		%Bias		%RSD	
	PHN	PAR	PHN	PAR	PHN	PAR	PHN	PAR	PHN	PAR
1	2	16	1.94 $\pm$ 0.12	16.28 $\pm$ 0.22	97.0	101.8	-3.0	1.8	6.18	1.35
2	10	10	10.12 $\pm$ 0.23	9.81 $\pm$ 0.16	101.2	98.1	1.2	-1.9	2.27	1.63
3	24	1	24.43 $\pm$ 0.36	1.04 $\pm$ 0.07	101.8	104.0	1.8	4.0	1.47	6.73

**Figure 5.** Representative chromatograms obtained from (A) pure PHN, PAR and I.S. (B) QC sample.

compared with the controls (0.972). There was no statistical difference of the ratio between the proposed method and the HPLC method (60.8 vs. 58.8%) using paired t-test at 95% confidence level. The decreased ratio indicated that the liver function reserve in cirrhotic patients had been much lower. The spectrophotometric method was successfully applied in the phenacetin test of cirrhotic patients and healthy subjects.

### Conclusion

A simple and reliable spectrophotometric method for the

simultaneous determination of phenacetin and paracetamol for the quantitative assessment of liver function reserve has been developed and validated. A major improvement of the proposed method is that it can be used for analysis in human plasma compared with the spectrophotometric methods reported before. In contrast with the HPLC, LC-MS and GC-MS methods, the proposed method has many advantages: the extraction recovery is higher; it avoids using organic reagent; the test fee per sample is lower. What is more, spectrophotometers are more popular than HPLC, LC-MS or GC-MS equipped in hospitals, and spectrophotometric method

**Table 4.** Comparison of QC samples determined by the proposed method and the HPLC method.

Sample	Concentrations analysed (mean $\pm$ SD, n=3)		Statistic		
	Proposed method	HPLC method	$F_{\text{calculated}}$	$t_{\text{calculated}}$	
Low	PHN	2.04 $\pm$ 0.11	2.02 $\pm$ 0.06	3.36	0.276
	PAR	0.96 $\pm$ 0.06	0.98 $\pm$ 0.02	9.00	0.548
Medium	PHN	12.12 $\pm$ 0.26	12.06 $\pm$ 0.13	4.00	0.358
	PAR	8.03 $\pm$ 0.17	8.01 $\pm$ 0.11	2.39	0.171
High	PHN	23.73 $\pm$ 0.32	24.03 $\pm$ 0.20	2.56	1.377
	PAR	16.28 $\pm$ 0.21	16.12 $\pm$ 0.14	1.25	1.098

**Table 5.** Determination of paracetamol and phenacetin in phenacetin test compared with the HPLC method (mean  $\pm$  SD).

Sample	PHN ( $\mu\text{g/ml}$ )	PAR ( $\mu\text{g/ml}$ )	Ratio	PHN ( $\mu\text{g/ml}$ , HPLC)	PAR ( $\mu\text{g/ml}$ , HPLC)	Ratio (HPLC)
Controls	4.68 $\pm$ 2.69	4.55 $\pm$ 2.64	0.972 $\Delta$	4.74 $\pm$ 2.72	4.52 $\pm$ 2.23	0.954
Cirrhotic patients	7.06 $\pm$ 3.03	2.69 $\pm$ 1.88	0.381* $\Delta$	7.03 $\pm$ 2.63	2.76 $\pm$ 1.69	0.393*

\* $P < 0.05$  vs. controls;  $\Delta P > 0.05$  vs. HPLC method.

may save more time to analyse batches of samples. Hence, the proposed method is more practical and suitable for the phenacetin test to be used in routine clinical work.

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