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Development of a high-performance liquid chromatography method for warfarin detection in human plasma

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Aim: Most of the high-performance liquid chromatography (HPLC) methods that have been developed to measure warfarin levels use the preextraction spiked internal standard method. We developed a new liquid-liquid extraction (LLE) and reversed-phase HPLC method that uses a postextraction spiked internal standard for the determination of plasma warfarin.

Materials and methods: The effect of varying the 1) mobile phase pH, 2) type and composition of organic solvents, 3) type and concentration of buffer solutions, 4) column temperatures, 5) flow rates, 6) organic modifier, and 7) ultraviolet wavelengths were tested.

Results: Optimum separation was achieved by using 30:70 (v/v) acetonitrile and potassium dihydrogen orthophosphate (0.01 M) at pH 6.5 without the addition of an organic modifier at 300 nm. The column temperature was fixed at 30 °C and the flow rate at 1.0 mL/min. Phenylbutazone was the most suitable internal standard. For LLE, the optimum plasma pH was 4.5, using 2 volumes of 2.5 mL of diethyl ether. The average retention times of warfarin and phenylbutazone were 7 and 11 min, respectively.

Conclusion: The postextraction spiked internal standard method saved a significant amount of development time and gave an excellent recovery of nearly 90%. This method was successfully tested using spiked human plasma. On the whole, the developed method is simple, economical, and suitable for routine application.

Key words: Method development, HPLC, warfarin, postextraction

Introduction

Warfarin is one of the most commonly administered oral anticoagulants used in the prophylactic treatment of various thromboembolic disorders. Due to its wide interindividual variability and narrow therapeutic index, optimal determination of its dose is difficult to achieve and suboptimal dosings remain the norm (1). Because different ethnic groups may show different responses to warfarin (2), many researchers throughout the world have developed their own detection methods for warfarin pharmacokinetic studies for their local populations.

Some researchers have utilised gas chromatography-mass spectrometry (GC-MS) to quantitate warfarin levels in both biological samples and pharmaceutical products (3-5). However, these methods require prior derivatisation steps, which are tedious. The heating process for sample volatilisation may also contribute to some sample loss.

Other reports include the use of high-performance liquid chromatography (HPLC) analyses coupled with ultraviolet (UV) (6-8) and fluorescence (9) detectors. Another group of researchers achieved an exceptionally low limit of detection (0.3 ng/mL)

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by utilising a mass spectrometer detector (10) due to its high sensitivity. However, not all laboratories have mass spectrometry or fluorescence detectors. Therefore, HPLC coupled with a UV detector remains an easier and cheaper option that is popular among many researchers.

Chiral separation of the (S)- and (R)-warfarin enantiomers is useful for drug metabolism studies. Correlation between genetic variations of cytochrome *P450 2C9* gene (*CYP2C9*) and warfarin clearance can be elucidated by measuring the plasma level of (S)- and (R)-warfarin in individuals that have different *CYP2C9* genotypes (11). However, a chiral separation method can be expensive as special β-cyclodextrin or silica-based chiral columns are needed (12,13). Furthermore, it is not always necessary to run chiral separations if the HPLC result is to be correlated with a parameter such as the International Normalised Ratio (INR) that is influenced by both (S)- and (R)-warfarin (7,8).

The pretreatment of samples is another important step in methodology development as it also influences the sensitivity of the method. The most common pretreatment methods are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). SPE has an advantage over LLE in that it can easily be automated and consumes less organic solvents. However, LLE is preferred over SPE by some researchers because SPE may suffer from batch-to-batch manufacturing variations that influence reproducibility (14). Furthermore, SPE is a more expensive option due to the need for single-use columns.

In this research, our goal was to develop a new but simple HPLC method coupled with a UV detector and to find a new extraction method for plasma warfarin, which can be used routinely in the laboratory for the detection of warfarin plasma samples.

Materials and methods

Equipment

The HPLC machine consisted of the Waters 2695 HPLC system (Milford, MA, USA) integrated with a column oven, an autosampler, and a vacuum degasser. The detector was a Waters 2996 PDA detector (Milford, MA, USA). Empower Pro® 5.0 software (Waters, Milford, MA, USA) was used to control the equipment as well as integrate the peaks in the chromatograms. The analytical column was a Purospher® STAR (250 × 4.6 mm i.d., 5 µm) endcapped RP-18 column (Merck, Darmstadt, Germany), coupled with a guard cartridge (Purospher® STAR; 4 × 4 mm i.d., 5 µm) (Merck).

Chemicals and reagents

The warfarin standard was purchased from US Pharmacopeia (Rockville, MD, USA). The diethylstilbestrol, eserine, indomethacin, lignocaine, phenylbutazone, and sulphadiazine standards were purchased from Sigma Chemical Company (St Louis, MO, USA).

The acetonitrile (ACN), chloroform, dichloromethane, ethyl acetate, hexane, methanol (MeOH), and sulphuric acid (H_2SO_4) were from Merck, while the diethyl ether and formic acid were from Fisher Scientific (Leicestershire, UK).

The potassium dihydrogen orthophosphate (KH_2PO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), and sodium hydroxide (NaOH) were purchased from Ajax Chemicals (Auburn, NSW, Australia), and the potassium acetate (CH_3COOK) was from BDH Chemicals (Poole, England).

The ACN, MeOH, and diethyl ether were of HPLC grades while the other chemicals were of analytical grades. Deionised water was purified by using a Millipore Milli-Q® Integral 3/5/10/15 System (Molsheim, France).

Preparation of standards

Standard stock solutions (1000 µg/mL) of warfarin were prepared by desiccating the drug standard with phosphorous pentoxide (Merck) before diluting 10 mg of the desiccated drug standard with 10 mL of ACN in a conical flask. Working standard solutions were prepared fresh daily by further dilutions of the stock solution.

Other drug standards (namely diethylstilbestrol, eserine, indomethacin, lignocaine, phenylbutazone, and sulphadiazine in concentrations of 100 µg/mL) were investigated as possible internal standard candidates and were prepared by diluting 1 mg of each drug standard in 10 mL of ACN in a similar manner.

Preparation of buffer solutions

All buffer solutions were prepared fresh daily by diluting the buffer salts in 1 L of deionised water. The buffer's pH was adjusted to 6.5 by using a NaOH (5 M) solution. Before use, the organic solvents and buffer solutions were filtered through a polypropylene membrane filter (0.45 μm) (Pall Life Sciences, Ann Arbor, MI, USA) and degassed for 20 min by using an ultrasonic sonicator (Elma GmbH, Singen, Germany).

Preparation of samples

The plasma was allowed to thaw naturally on the bench at room temperature. It was then vortexed for a few seconds to ensure a uniform distribution. Plasma standards were prepared by spiking 100 μL of warfarin standard working solution (100 μg/mL) into 1 mL of the thawed plasma, followed by vortexing the solution for 5 s. The samples were prepared in duplicates.

Sample extraction

A simple 2-step LLE method was used to extract the spiked drugs from the plasma standards. The pH of the plasma standards was adjusted by using 1 N H_2SO_4. The plasma was then vortexed for 5 s before the addition of the organic solvent (2.5 mL). The mixture was centrifuged at 2000 rpm for 15 min before separating the organic layer into a v-tube. A fresh organic solvent (2.5 mL) was again added to the raffinate and the steps were repeated. The organic solvents were collected and dried under a gentle stream of nitrogen gas at 40 °C. The residue was reconstituted with 100 μL of the internal standard working solution (10 μg/mL) before being injected into the HPLC system (10 μL).

Optimisation steps

In the beginning, a 30:70 (v/v) combination of MeOH and KH_2PO (0.01 M) as the mobile phase at 0.75 mL/min was arbitrarily set for the initial parameters of the HPLC. The PDA detection wavelength was set at 190-800 nm. The warfarin standard solution (100 μg/mL) was injected (10 μL) into the HPLC system for each investigation, and the parameters were varied according to the steps below.

(a) Optimisation of the buffer solution pH

Five different pH levels of the KH_2PO_4 buffer solution (pH levels of 3.5, 4.5, 5.5, 6.5, and 7.5) were prepared in separate reservoir bottles and the solutions were individually filtered and degassed. With each change of mobile phase, the column was allowed to be reequilibrated with at least 20 column volumes of mobile phase before the experiment was repeated using different buffer pH levels each time.

(b) Optimisation of the type of organic solvents

To investigate the effect of using 2 different types of organic solvent, MeOH was replaced by ACN in the mobile phase at the same composition. The peak area of warfarin produced was then compared in the 2 different procedures.

(c) Optimisation of the mobile phase composition

Using the 2 different types of organic solvent as noted above, 2 compositions of organic solvent and buffer of the mobile phase were tested at ratios of 30:70 and 40:60.

(d) Optimisation of the type of buffers

Using the selected ratios of the mobile phase composition above, 3 different types of buffers, namely KH_2PO_4, NaH_2PO_4, and CH_3COOK buffer solutions (0.01 M), were further tested.

(e) Optimisation of the buffer concentration

The best buffer solution determined above was further tested by varying its concentration at 0.01, 0.05, and 0.1 M.

(f) Determination of the suitable internal standard candidates

Several potential internal standard candidates (such as diethylstilbestrol, eserine, indomethacin, lignocaine, phenylbutazone, and sulphadiazine) were tested (all at 100 μg/mL). They were chosen on the basis of their similar physicochemical properties to warfarin.

(g) Optimisation of the column temperature

To investigate the effect of temperature on warfarin's peak resolutions, column oven temperatures of 25 and 30 °C were tested.

(h) Optimisation of the mobile phase flow rate

For this purpose, 3 flow rates of 0.5, 0.75, and 1.0 mL/min were investigated and warfarin's peak area and retention times were then compared to determine the flow rate that yielded the largest peak area or the shortest retention time.

(i) Optimisation of the type of organic modifier

An organic modifier, acetic acid (1%), was also added to the mobile phase to determine whether it could reduce warfarin's peak tailing.

(j) Optimisation of the PDA detection wavelength

To increase the sensitivity and specificity of the detection of warfarin and its internal standard, photodiode array (PDA) detection at individual specific wavelengths (280, 290, 300, and 309.4 nm) was tested. A UV spectrum that covered the whole detection range of the PDA detector (between 190 and 800 nm) was also investigated. Since this experiment was also designed to test the detection sensitivity, lower concentrations of warfarin (500 ng/mL) and phenylbutazone (10 μg/mL) were injected into the HPLC system.

(k) Optimisation of the suitable plasma pH for LLE

In this experiment, diethyl ether was first arbitrarily selected as the extraction solvent. In LLE, the pH of the plasma needs to be varied to suppress the analyte's ionisation, thereby allowing its migration from the aqueous to the organic layer. In this experiment, the pH of the plasma samples was adjusted to pH 3.5, 4.5, 5.5, and 6.5 each time, in 4 sets of duplicate tubes. The mean percentage recovery (calculated by comparing the area ratio of extracted samples to the area ratio of standard samples) and the standard deviation of warfarin extracted from each tube was then calculated and compared.

(l) Optimisation of the type of organic solvent used for LLE

Using the most suitable plasma pH determined above, the optimisation of LLE was further carried out by varying the type of organic solvent. In this experiment, diethyl ether was replaced with 1) ethyl acetate, 2) dichloromethane, 3) chloroform, and 4) diethyl ether and *n*-hexane (2:1).

(m) System suitability tests

To ensure the quality of the chromatograms produced, a system suitability test was performed according to the parameters set by the FDA (15), where:

Resolution (R_s) = $(t_{R2} - t_{R1})/([W_{b2} + W_{b1}]/2)$,

Tailing factor (T_f) = $W_{0.05}/2f$,

Plate count (N) = $5.546(t_R/W_{1/2})^2$.

Here, t_{R1} = retention time of the first analyte, t_{R2} = retention time of second analyte, W_{b1} = base width of the first analyte, W_{b2} = base width of the second analyte, $W_{0.05}$ = peak width at 5% of the peak height, f = front width at the peak base, $W_{1/2}$ = peak width at ½ of the peak height, and t_M = void time.

Results and discussion

A new optimised method suitable for the routine analysis of plasma warfarin in human samples was successfully developed.

Theoretically, warfarin, which is an acidic compound, will be ionised when the pH of the buffer solution is increased above its pK_a value. This will lead to its early elution from a reversed-phase system (16). Another technique is to use a mobile phase pH that has been adjusted to 2 units above and below its pK_a values (the pK_a of warfarin is 5.0) (17). In this experiment, 5 buffer solutions with different pH levels were tested. However, when buffer solution of pH 3.5, 4.5, and 5.5 were used, warfarin was not eluted even after 30 min of total analytical time. It is possible that at these 3 pH levels, warfarin is not sufficiently ionised and therefore binds strongly to the stationary phase. Both pH 6.5 and 7.5 are suitable as they produced tall warfarin peaks within 30 min (Figure 1). However, even though buffer solution of pH 7.5 gave an earlier retention time, pH 6.5 was selected because it produced a larger peak area for warfarin.

ACN was favoured as the organic solvent because it produced a significantly earlier retention time and sharper peak (Figure 2). This may be attributed to its higher solvent strength (18). The low viscosity of ACN is an additional advantage over MeOH (18), which produces a lower system pressure (2384 psi) on the chromatographic system when compared to MeOH (3065 psi).

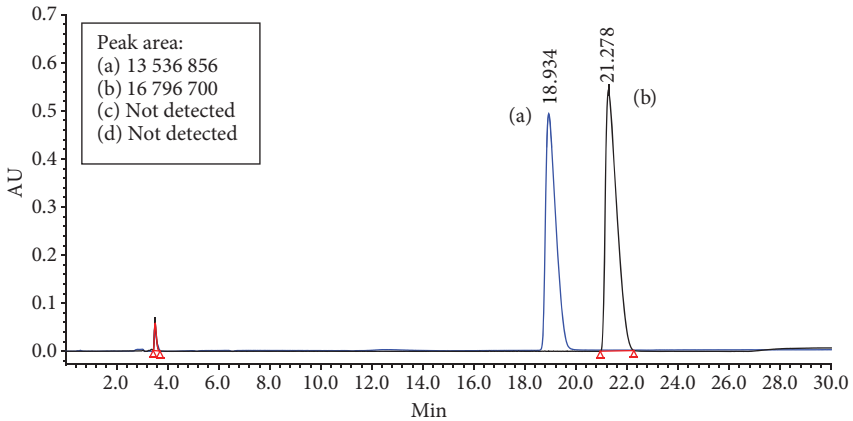

Figure 1. Superimposed warfarin peaks when the pH of the KH_2PO_4 buffer was adjusted to (a) 7.5 and (b) 6.5. Warfarin peaks were not detected when the pH was adjusted to 5.5 (c) and 6.5 (d).

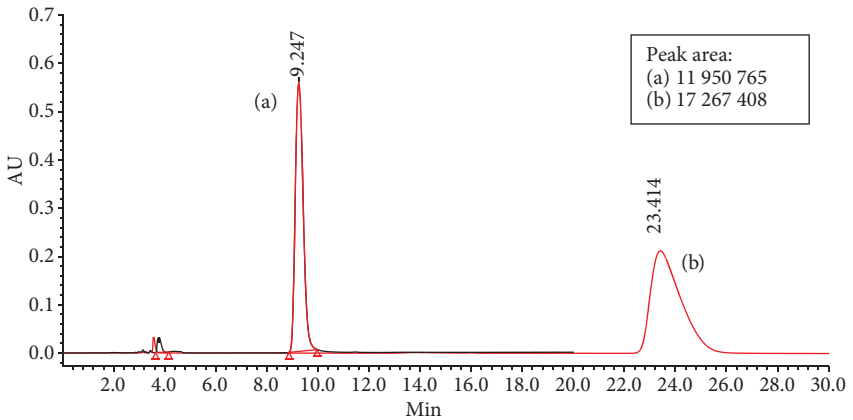

Figure 2. Superimposed warfarin peaks when (a) ACN and (b) MeOH were used as the mobile phase. MeOH gave an average system pressure of 3065 psi while ACN's was lower at 2384 psi.

A higher proportion of organic solvent tends to increase the mobile phase's solvent strength (19). Due to this fact, it was not surprising that the 40:60 composition of organic solvent and buffer gave a faster retention time. However, warfarin's peak area was similar to that of a 30:70 composition. Due to the fact that 40:60 is less economical since a higher proportion of organic solvent is involved, the composition of 30:70 was selected (Figure 3).

Using the right type of buffer solution is also a pivotal part of the development of methodology. From the 3 buffer solutions tested, the KH_2PO_4 buffer was selected because it gave the largest peak area (Figure 4). Besides this, the pH of the mobile phase previously selected (pH 6.5) fell within the buffering range of phosphate buffers (pH 1.8-3.5 and pH 5.8-8.0) (19). This is desirable because a mobile phase buffer with the correct buffering range tends to resist pH changes more efficiently and therefore may reduce analytical variations.

Theoretically, increasing the buffer concentration tends to allow polar samples to elute faster (18). However, this was not true for warfarin, perhaps due to the fact that it is a weakly polar compound. Too high a buffer concentration may also be harmful as it can form hard crystals abrasive to pump seals (20). In this experiment, even though a buffer salt concentration of 0.05 M gave the highest peak area for warfarin (Figure 5), a lower concentration of 0.01 M, which produced an earlier retention time, was selected as it is also economical and may preserve column life in the long run.

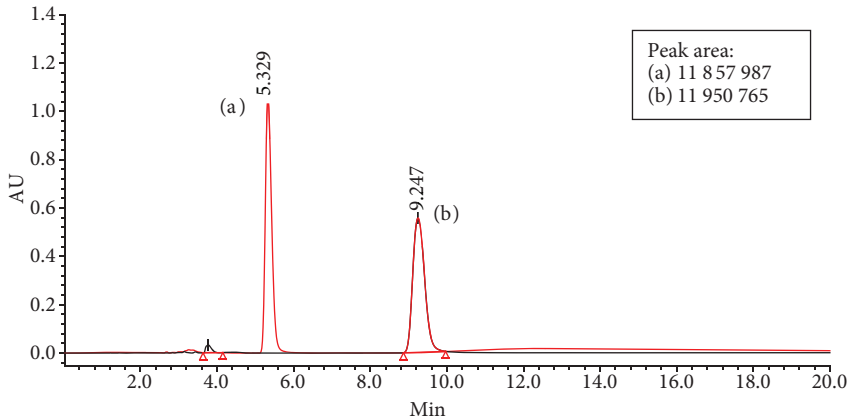

Figure 3. Superimposed warfarin peaks when the composition of the mobile phase was (a) 40:60 and (b) 30:70.

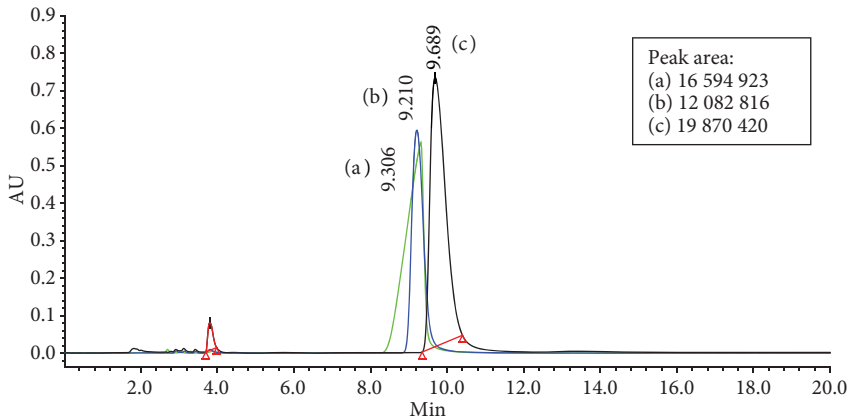

Figure 4. Superimposed warfarin peaks when (a) CH_3COOK, (b) NaH_2PO_4, and (c) KH_2PO_4 were used as the buffer solutions.

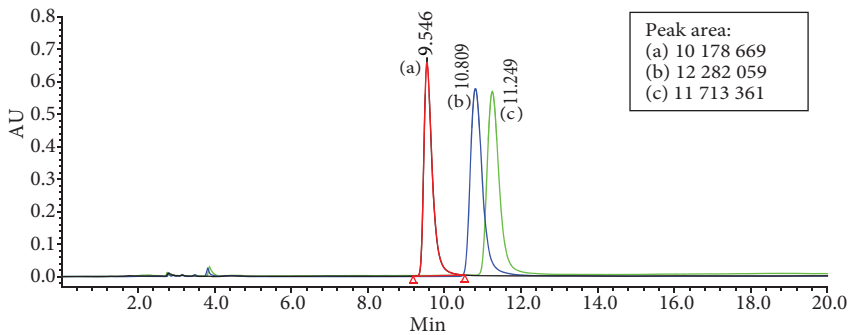

Figure 5. Superimposed warfarin peaks when the buffer concentration was adjusted to (a) 0.01 M, (b) 0.05 M, and (c) 0.1 M.

Lehrer (21) stated that a suitable internal standard should: 1) be completely resolved from all peaks in the sample, 2) be eluted near the analyte, 3) behave similarly to the analyte in pretreatment so that losses can be corrected, 4) have a peak area approximately equal to the standard in the concentration desired, 5) not normally be present in the sample, 6) be commercially available in a pure form, and 7) be easily added as a liquid. The search for an internal standard was carried out based on these principles. Previous reports

recommended the use of preextraction spiked internal standard methods (8,9,13). In our investigation, however, a high but false percentage recovery was obtained when using this method. We found the postextraction spiked internal standard method to have a similar analytical bias to the preextraction spiked internal standard, as also shown by Reagen et al. (22). Therefore, the simpler preextraction spiked internal standard method was selected.

During the determination of the suitable candidates for the internal standard, we found that the peaks of diethylstilbestrol and lignocaine did not appear when injected by using the developed HPLC condition (Figure 6). Sulphadiazine's peak coeluted with the solvent front while indomethacin showed a relatively long retention time of 26 min. Eserine's peak may coelute with warfarin as it appeared at 7.87 min, which is very close to warfarin's retention time. Therefore, phenylbutazone was selected as the internal standard of choice because its peak did not coelute with the solvent front or warfarin's peak. Furthermore, it showed an acceptable retention time and a symmetrical peak.

Higher column temperatures tend to lower mobile phase viscosities, which is desirable because a lower systemic pressure is produced (18). This will allow a lower linear velocity for the chromatographic system and produce a sharper peak (14). The heat will also provide some kinetic energy to the samples to propel them faster within the column, thereby decreasing the total analytical time (18). A column temperature of 30 °C was finally selected because it gave a faster retention time without compromising the peak area (Figure 7). A higher temperature than this was not tested in order to preserve column life since high temperatures may be detrimental to the column's packing when used over time.

Increased flow rates tend to shorten retention time, but at the same time may contribute to band broadening and a decrease in the column's efficiency (19). Even though both flow rates of 0.5 and 0.75 mL/min produced significantly larger peak areas than 1.0 mL/min did, they were not chosen because of the increase in retention time. In fact, the retention time at 0.5 mL/min was double that at 1.0 mL/min (Figure 8).

The function of an organic modifier is mainly to reduce peak tailing. In this experiment, acetic acid (1%) was added because, theoretically, an acetate organic modifier will decrease the band-broadening effect for acidic compounds such as warfarin (19). However, we found that it was not useful to add 1% acetic acid as it produced baseline noises while reducing warfarin's peak area to about one-third, and it even increased the retention time (Figure 9).

The use of a photodiode UV detector is advantageous as it allows for the viewing and selection of wavelengths in real time. This will greatly minimise the duration of method development. From our analyses, we found that a broad-spectrum range such as 190-800 nm tends to be suitable for the analysis of concentrated samples (such as warfarin at 100 µg/mL), but it produced larger baseline noises and was therefore unable to detect lower concentrations of warfarin (such as a concentration of 500 ng/mL) (Figure 10). In order to eliminate

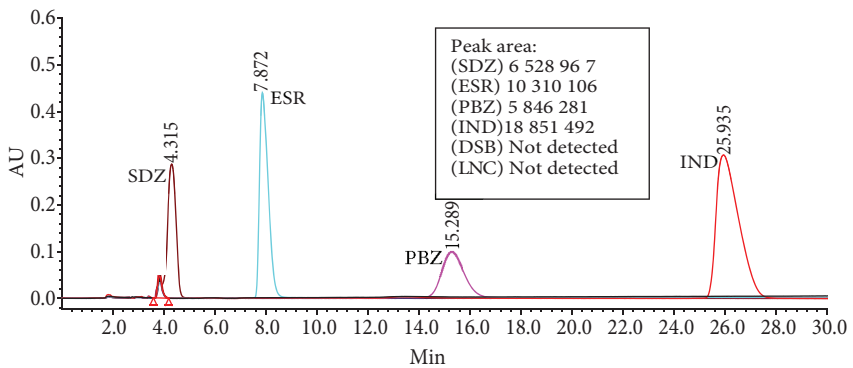

Figure 6. Superimposed chromatograms when sulphadiazine (SDZ), eserine (ESR), phenylbutazone (PBZ), and indomethacin (IND) were injected into the HPLC system. Diethylstilbestrol (DSB) and lignocaine (LNC) were not eluted when injected under similar chromatographic conditions.

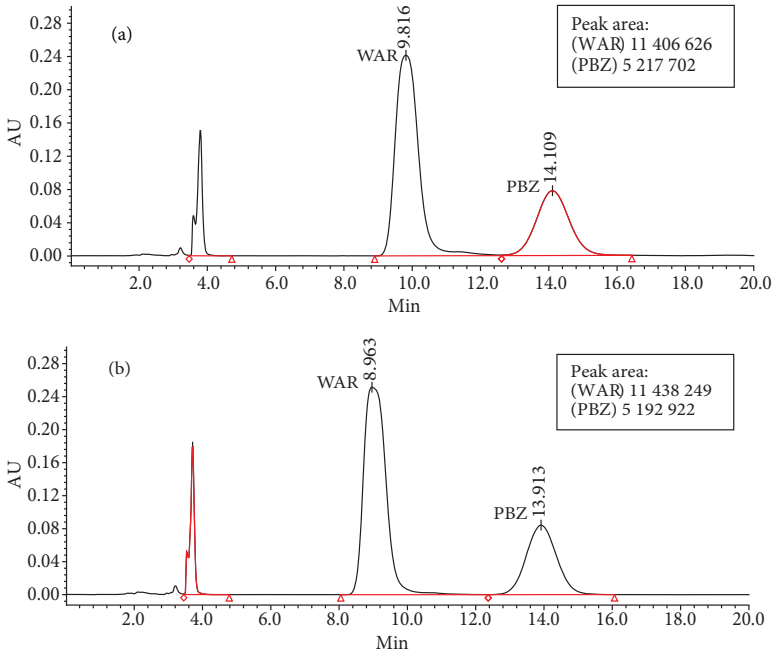

Figure 7. Chromatograms produced when the column temperature was maintained at a) 25 °C and b) 30 °C. The concentrations of both warfarin and phenylbutazone in this test were 100 µg/mL.

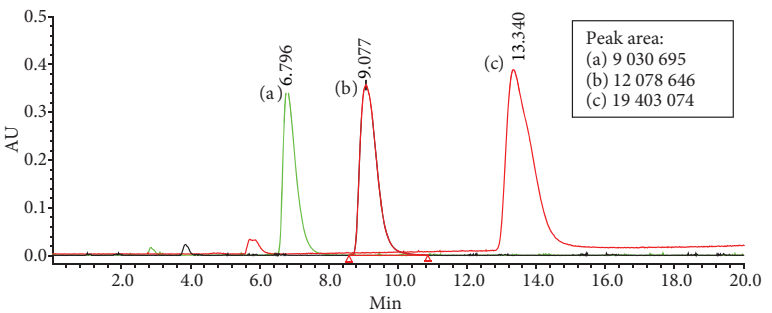

Figure 8. Superimposed warfarin peaks when the flow rates were set at (a) 1.0 mL/min, (b) 0.75 mL/min, and (c) 0.5 mL/min.

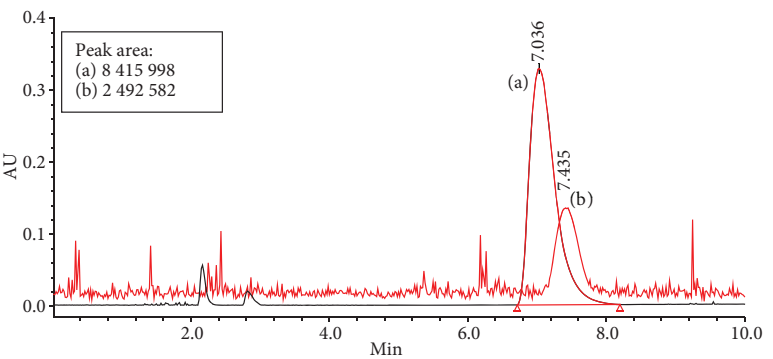

Figure 9. Superimposed warfarin peaks when using the mobile phase with (a) no acetic acid and (b) 1% acetic acid.

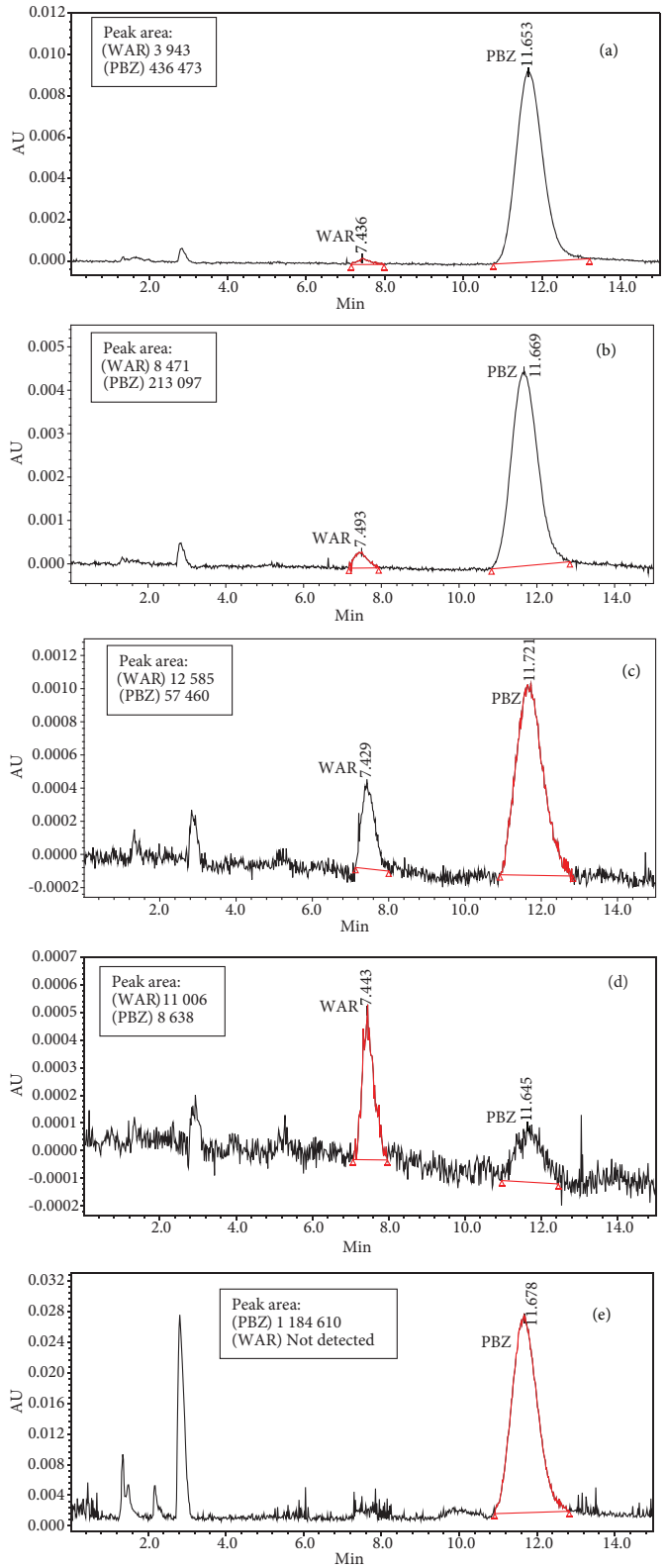

Figure 10. Chromatograms produced when the PDA detection spectrum range was set at a) 280 nm, b) 290 nm, c) 300 nm, d) 309.4 nm, and e) 190-800 nm. The concentrations of warfarin and phenylbutazone in this test were 500 ng/mL and 20 μg/mL, respectively.

baseline noise generated by endogenous compounds in plasma samples, it is preferable to do a single-point absorbance, which is more specific. Finally, 300 nm was determined to be the best UV wavelength for the detection of both warfarin and phenylbutazone.

Common pretreatment methods for warfarin are LLE and SPE. To ensure that the consistency of the results is not affected by day-to-day instrument variations, an internal standard is often employed (14). By far, the most popular internal standard method for warfarin analysis is the preextraction spiked internal standard, where the internal standard is added into the biological sample before the extraction process. In contrast, the postextraction internal standard method requires the addition of the internal standard compound after the extraction process is completed.

A preextraction method has the advantage of being able to compensate for sample loss during sample pretreatment (14). The postextraction method, while it may not incur the same advantages as the preextraction method, is much easier to carry out because the optimisation of the extraction process is only required for warfarin instead of both warfarin and the internal standard. Furthermore, the advantage of a preextraction method will only materialise if the chosen internal standard behaves similarly to the analyte in pretreatment (21), which means that the recovery of warfarin and the potential internal standard must be similar. However, in reality, the search for such an ideal internal standard can be difficult and time-consuming. Locatelli et al. (23) managed to obtain a similar recovery for racemic warfarin and their selected internal standard, albeit at lower percentage recoveries (below 80%).

In a new HPLC method development, the extraction step is also very important as it can affect the sensitivity of the overall analysis. Since pH 4.5 gave the largest percentage recovery for warfarin, it was selected as the pH of choice for the subsequent LLE method investigation (Figure 11).

With the pH of the plasma fixed at 4.5, the effects of using different organic solvents for warfarin extraction were further investigated. The combination of *n*-hexane and diethyl ether yielded the highest percentage recovery (89.96%) for warfarin (Figure 12). This may be due to the low polarity nature of

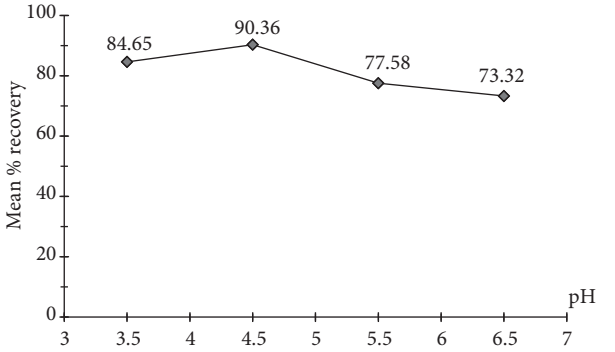

Figure 11. Mean percentage recoveries of warfarin at different plasma pH levels. The sample was extracted by using diethyl ether as the organic solvent.

n-hexane (polarity index = 0.1), which can attract the less polar warfarin compound from the aqueous layer more effectively than by more polar organic solvents such as ethyl acetate (polarity index = 4.4) (24). However, since *n*-hexane and diethyl ether have very different boiling points (69 °C and 35 °C, respectively) (24), they are not practical to use. Therefore, diethyl ether alone (which was the second-best alternative at 88.24%) was selected. Warfarin was extracted using 2 volumes of 2.5 mL of the organic solvent instead of using 1 large volume of 5 mL because a repeated small-volume extraction is said to yield a better recovery than a single large-volume extraction (25).

The final optimised chromatography method met the system suitability parameters requirement as outlined by the FDA (Table 1). The limit of detection was 20 ng/mL. The method was successfully tested by analysing plasma samples spiked with 100 µg/mL of warfarin and the internal standard (Figure 13).

To summarise, the HPLC detection of warfarin in plasma was accomplished at 300 nm with a 30:70 (v/v) ACN and KH_2PO_4 buffer solution (0.01 M) as the final mobile phase. The pH of the buffer solution was adjusted to 6.5 and the internal standard of choice was phenylbutazone, which was spiked postextraction. The column temperature was maintained at 30 °C and the flow rate was 1.0 mL/min. By using these optimised chromatographic conditions, the average retention times of warfarin and phenylbutazone were 7 and 11 min, respectively, with a total run time of 14 min following a 10-µL injection. The postextraction spiked internal standard method saves a significant amount of

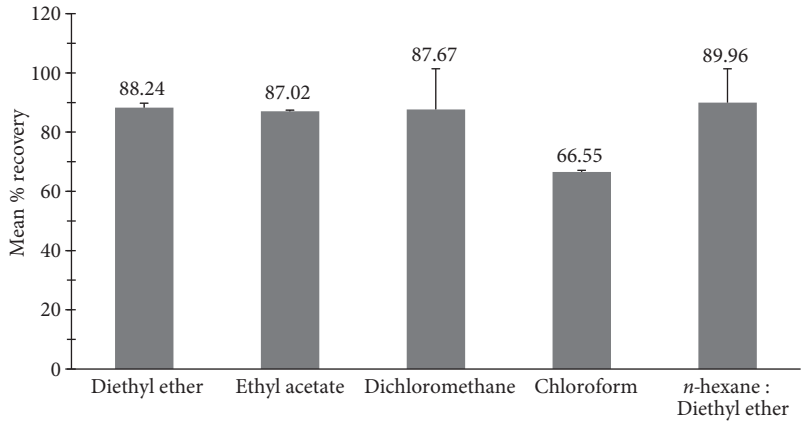

Figure 12. Mean percentage recoveries of warfarin and their standard deviations when the sample was extracted using different organic solvents with pH fixed at 4.5.

Table. System suitability parameters.

Parameters	Warfarin	FDA requirements
Resolution (R_s)	3.33	>2.0
Tailing factor (T_f)	1.86	≤2.0
Plate count (N)	2550	>2000

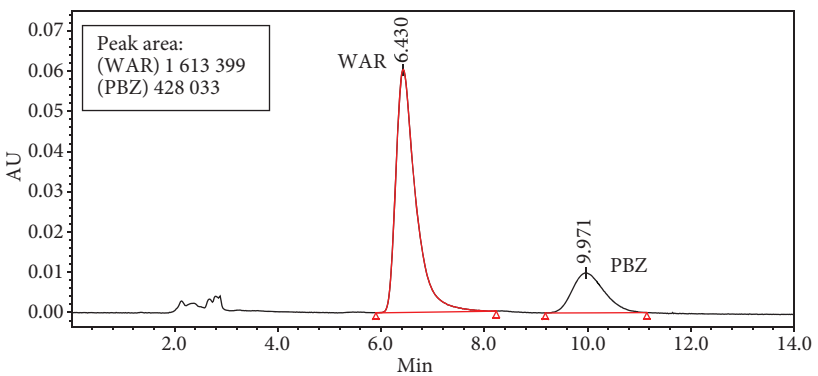

Figure 13. Representative chromatogram of plasma warfarin and phenylbutazone extracted by using the optimised LLE method with the optimised chromatographic condition. The concentrations of both warfarin and phenylbutazone in this test were 100 µg/mL.

time. Furthermore, this method gave a percentage recovery of nearly 90% and is cost-efficient. On the whole, the method is simple, economical, and allows both warfarin and phenylbutazone to achieve good resolutions, producing chromatograms that comply with the FDA's guideline.

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