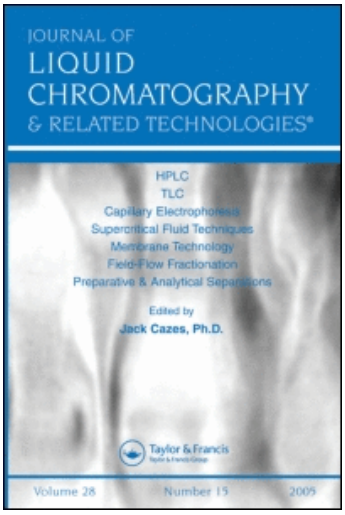

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Hydrophilic Interaction Chromatographic Determination of Epirubicin in Human Plasma using Solid Phase Extraction for Sample Clean-Up

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Abstract: An analytical method based on hydrophilic interaction chromatography (HILIC) using diode array detection was developed and validated for the determination of epirubicin in human plasma. Epirubicin and epidaunorubicin (internal standard) were collected from the plasma by solid-phase extraction (SPE) using Oasis HLB cartridges. The chromatographic separation was on a silica analytical column (250 × 4.6 mm I.D., 5 μm) with a mobile phase consisting of acetonitrile/40 mM ammonium formate buffer solution (pH 2.9) (90/10, v/v). The detection wavelength was 254 nm. Linearity of the method was in the concentration range of 0.050-2.5 μg/mL ($r^2 = 0.9991$). The limit of quantification (LOQ) and limit of detection (LOD) were 0.050 and 0.030 μg/mL using 0.2 mL plasma sample, respectively. Recoveries of the analyte ranged from 89.4% to 101.2% with intra- and inter-day precision (RSD); less than 7.0% were obtained at the concentrations above the LOQ. The proposed method is simple, accurate, and its mobile phase is compatible for mass spectrometry.

Keywords: Hydrophilic interaction chromatography, Epirubicin, Solid phase extraction, Method validation, Human plasma

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INTRODUCTION

Epirubicin (4′-epidoxorubicin) is an anthracycline antibiotic, which has been widely used in the treatment of neoplastic diseases not only due to its good therapeutic efficacy but also due to its low cardiotoxicity.[1] Up to now, several analytical methods for the determination of epirubicin and its metabolites in human plasma have been developed and published, including capillary electrophoresis (CE) with laser induced fluorescence detection[2] and high performance liquid chromatography (HPLC) with electrochemical,[3] fluorometric,[4–6] or mass spectrometric (MS) detection.[7–10] Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been proven to be the method of choice because of its sensitivity, selectivity, and speed of analysis. However, the speed for LC-MS/MS method development appears to have become the bottleneck, mostly owing to a lack of sufficient LC-MS/MS instruments. Methods published in literature to analyze epirubicin in biological fluids are based on reversed phase liquid chromatography (RPLC). So far, no hydrophilic interaction chromatography (HILIC) methods were reported in the literature for the quantification of epirubicin in biological fluids.

HILIC termed by Alpert[11] in 1990 shows separation similar to normal phase liquid chromatography (NPLC), but it is possible to use low aqueous–high organic volatile mobile phases, which are compatible for MS. Especially, it is very useful and effective for some polar compounds.[12] The separation mechanism of HILIC is opposite to that of RPLC.[11] In recent years, HILIC has been frequently applied in the determination of basic drugs and their metabolites with several advantages superior to RPLC.[12,13]

In our previous work, we have investigated the chromatographic behavior of epirubicin and its analogues on high purity silica in HILIC mode.[14] Also, we developed a novel method to determine epirubicin in human plasma by ultra performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS).[9] The aim of this study was to illustrate a practical example of the application of HILIC for the determination of epirubicin in biological samples, in which a high purity silica column and aqueous-organic mobile phase were employed. Solid-phase extraction (SPE) using Oasis HLB cartridge was applied to extract the analytes in human plasma prior to HILIC analysis. In order to obtain high and reproducible recoveries of the analyte, particular effort has been made in the SPE method development. The purpose of this paper is not to undermine the dominant position of reversed-phase separations, but to provide an alternative for anthracycline drugs.

EXPERIMENTAL

Chemicals and Reagents

Epirubicin (EPI) and epidaunorubicin (EPR), an internal standard (IS) for EPI as pure standards were kindly supplied by Hisun Pharmaceutical Inc.

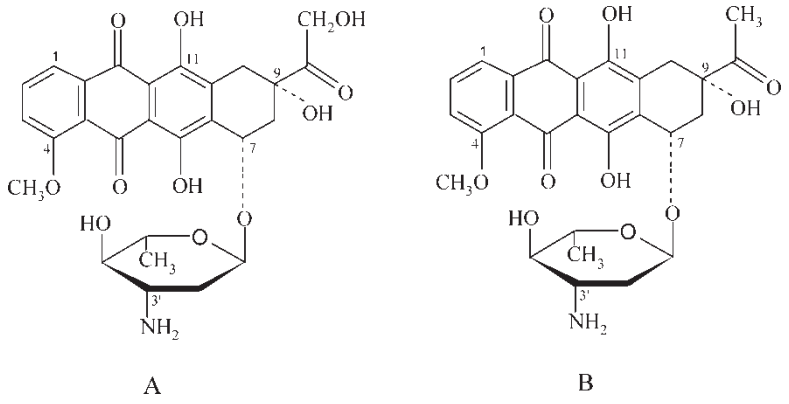

Figure 1. Chemical structures of (A) EPI and (B) EPR (IS).

(Zhejiang, China) and stored at +4°C in the dark (Figure 1). Acetonitrile (ACN) was of HPLC grade and all other solvents and reagents were of analytical grade. Deionized and distilled water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Oasis HLB 1 cc/30 mg extraction cartridges (Part No. WAT094225) were provided by Waters (Milford, MA, USA). Control blank human plasma was supplied by healthy donors.

Apparatus and HILIC Conditions

The TSP chromatograph (Thermo Separation Products, San Jose, CA, USA) consisted of a SpectraSYSTEM P4000 pump, a SpectraSYSTEM AS3000 autosampler with a fixed loop injection valve, and a Spectra FOCUS diode array detector (DAD). Chromatographic system control, data acquisition, and chromatographic analysis were exerted with TSP PC1000 Chromatography Manager software (3.0 version). The chromatographic separation was performed on a Kromasil KR100-5SIL column, 250 × 4.6 mm I.D., 5 μm (Eka Chemicals AB, Bohus, Sweden) with a mixture of ACN/40 mM ammonium formate buffer (pH 2.9) (90:10, v/v) as mobile phase, delivered at a flow rate of 1.0 mL/min. All separations were operated at ambient temperature. The injection volume was 20 μL. Solutes were detected at UV 254 nm.

Blood Sample Collection and Preparation

For the validation of the method, blood samples from healthy donors were collected in heparinized tubes and plasma was obtained by centrifugation at 3000 rpm (Jouan MR 18 22, France) at 4°C for 15 min. Pooled drug free plasma samples were aliquotted (0.2 mL) into polypropylene tubes, frozen

at −20°C, and then used in the preparation of standards and quality control (QC) samples.

Preparation of Calibration Standards and Quality Control Samples

Primary stock solutions of EPI and EPR (IS) (1 mg/mL) were prepared in methanol/water (1:1, v/v), respectively. The stock solutions were stored in polypropylene tubes with screw caps and kept in a refrigerator at 4°C. Working standard solutions of EPI were prepared by diluting the primary solution with formate buffer (20 mM, pH 2.9) to reach the concentrations of 0.40, 0.80, 1.6, 3.2, 6.4, 8.0, 16, and 20 μg/mL. In the same way, the working solution for IS (32 μg/mL) was prepared from the IS stock solution.

Drug free human plasma samples, 0.2 mL, were spiked with 25 μL of IS solution and 25 μL of the appropriate working solutions of EPI to produce the following calibration levels: 0, 0.050, 0.10, 0.20, 0.40, 0.80, 1.0, 2.0, 2.5 μg/mL. The quality control (QC) samples were prepared with the concentrations of 0.10 (low), 0.80 (medium), and 2.5 (high) μg/mL.

Sample Pretreatment Procedure

Sample pretreatment involved a solid phase extraction (SPE) procedure. Each of the above-prepared calibration standards and QC samples was briefly vortex mixed and applied to an Oasis HLB cartridge, and forced to pass through at 1 mL/min by applying reduced pressure. The cartridge has been previously activated and conditioned with 1 mL methanol followed by 1 mL deionized water. After loading, the cartridge was washed sequentially with 1 mL of 5% methanol (the first wash) and 1 mL of 40% (v/v) methanol containing 2% ammonium hydroxide (the second wash), and dried by passing a stream of air for 5 min. The analytes were eluted from the cartridge with 0.5 mL of 0.5% formic acid methanol solution in 1.5 mL Waters certified vials. The eluates were evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of 90% ACN in ammonium formate buffer (40 mM, pH 2.9) and vortexed for 20 s. A 20 μL aliquot was injected into the HILIC system.

Validation of the Method

Specificity

Specificity was assessed by the examination of peak interference from an endogenous substance. Blank human plasma samples collected under controlled conditions were carried through the extraction procedure and

chromatographed using the HILIC method with DAD detection. The result was compared with those obtained from EPI and the IS.

Calibration Curve

The calibration curve was constructed from eight concentrations of EPI ranging from 0.050–2.5 μg/mL. The peak area ratio of EPI to the IS versus the respective standard concentrations was used for plotting the graph and the linearity evaluated by a weighted $(1/x^2)$ least squares regression analysis.

Precision and Accuracy

The QC samples at three different concentrations (0.10, 0.80, and 2.5 μg/mL) were analyzed to evaluate the precision and accuracy of the method. The accuracy was expressed in terms of %recovery, which was determined by comparing the peak areas of the analyte extracted from the spiked samples with those of unextracted neat solutions at the corresponding concentrations. The precision of the method was obtained by analyzing three replicates of validation samples at 0.10, 0.80, and 2.5 μg/mL on 3 days, respectively. The precision was determined by calculating the relative standard deviation (RSD) for the repeated measurements.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The lowest standard concentration on the calibration curve should be accepted as the limit of quantification if the following conditions are met: the analyte response at the LOQ should be at least five times the response compared to the blank response. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

Stability

Analyte stability was tested by analyzing validation samples at 2.5 μg/mL stored at $-20^{\circ}C$ in the dark on every 7 days, for a month.

RESULTS AND DISCUSSION

Extraction and Recovery

SPE was employed to eliminate the destructive matrix compounds while extracting the analytes of interest. In this study, Oasis HLB cartridges have been used to extract the analytes from plasma matrix, because these cartridges are water wettable and easy to use, and the sorbents retain analytes even when the cartridges run dry. More importantly, the Oasis HLB cartridges have

unmatched advantages to capture polar basic compounds and metabolites over silica based sorbents.[15] Absolute recoveries of EPI were measured three times at two concentrations of calibration standards (0.10 and 2.5 μg/mL EPI, added with 4.0 μg/mL EPR, respectively) during all steps of SPE. The SPE method with two washes was employed for the extraction of the analytes. It was found that, of utmost importance for achieving high recoveries of the analytes and removing the interferences, was the choice of methanol concentrations in the second wash. Methanol concentrations ranging from 20% to 50% were investigated in the present study. As a result, a good compromise was reached at 40% containing 2% ammonium hydroxide, and the clean extract can be obtained with high recoveries. In addition, the following solutions were tested as the elution solvent, including methanol, 0.5% formic acid in methanol, 0.5% formic acid in ACN, and 90% ACN in formate buffer (40 mM, pH 2.9). The results showed that a large elution volume (>1 mL) of methanol or 0.5% formic acid in ACN was required to obtain acceptable recoveries of the interested analytes. With only 0.5 mL of 0.5% formic acid in methanol or 90% ACN in formate buffer (40 mM, pH 2.9) as the elution solvent, excellent recoveries with good reproducibility were reflected. However, 0.5 mL of 0.5% formic acid in methanol was chosen over 90% ACN in formate buffer because of the quite shorter evaporation time obtained for the former. After the overall extraction process (including two washes, evaporation, and reconstitution), the absolute recoveries were 85.6% for 4.0 μg/mL EPR, 83.5% and 88.1% for 0.10 μg/mL, and 2.5 μg/mL EPI, respectively.

HILIC Analysis

Because the analytes are charged compounds, the use of a buffered mobile phase is crucial to achieve acceptable repeatability. Figure 2 displays the chromatogram of a blank plasma sample taken from the healthy donors, and Figure 3 depicts the chromatogram of a standard EPI and IS. Figure 4 exhibits the representative chromatogram of blank plasma spiked with standard EPI and IS. Both EPI and IS were separated with excellent separation and peak shapes, and no interfering peaks were observed in the blank sample and in all samples examined. Moreover, the high purity silica column demonstrated excellent stability as shown by very stable retention time (t_R) and unchanged peak shape for both EPI and IS. The flow rate used for the formal validation was 1 mL/min. At least three hundred extracted samples could be injected onto the same column without loss of performance. In addition, the contaminated column can be effectively cleaned and regenerated by 60% ACN in 40 mM formate buffer (pH 2.9). The advantage of the current method is the use of a volatile mobile phase, which facilitates the mass spectrometry analysis of EPI in biological matrices. Also, because of higher organic content of the mobile phase in HILIC than in RPLC, a lower pressure drop is usually observed on the silica column.

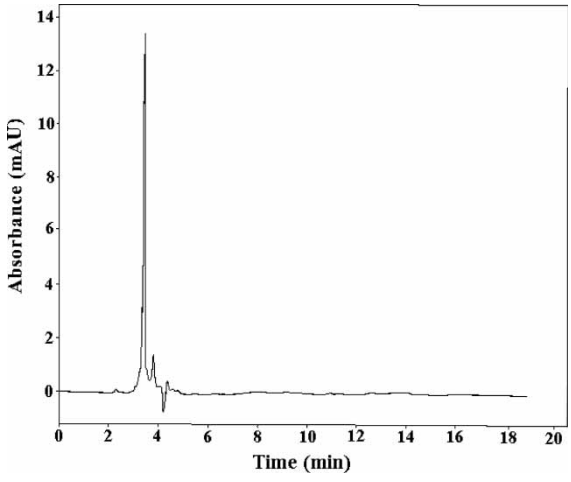

Figure 2. The chromatogram of blank plasma from the healthy donor. Conditions are the same as described in the text.

Validation of the Method

Table 1 shows the validation data for the analyte. The extraction recoveries ranged from 89.4 to 101.2%, while the precision values ranged from 3.4 to 7.0% over the three concentrations (i.e., 0.10, 0.80, and 2.5 μg/mL)

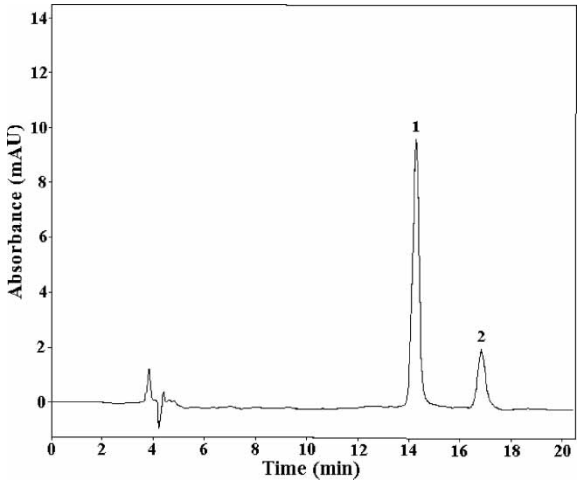

Figure 3. Typical HILIC chromatogram of standard EPI (2.5 μg/mL) and IS (4.0 μg/mL). Conditions are the same as described in the text.

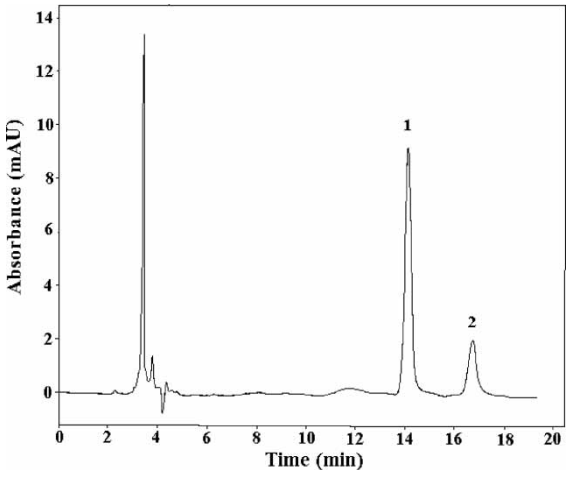

Figure 4. HILIC chromatogram of human plasma spiked with standard EPI (2.5 μg/mL) and IS (4.0 μg/mL). Conditions are the same as described in the text.

evaluated during 3 days. The regions of the analyte and IS peaks were free from interference. The stability tests showed that validation samples stored in a freezer at −20°C remained stable at least for 1 month. These results indicate that the proposed method is consistent and reliable. As demonstrated here, EPR worked well for tracking the analyte during the extraction and compensated for potential recovery inconsistency.

A calibration curve was constructed for EPI by plotting the peak area ratios (analyte/IS) against the concentration. A weighted ($1/x^2$) linear regression line was applied. Linear responses were obtained for EPI over the range 0.050–2.5 μg/mL with a regression coefficient of 0.9991, and intercepted the y-axis close to the origin. The estimated limit of detection (LOD) at a signal-to-noise ratio of 3 was about 0.030 μg/mL for EPI. The limit of quantification (LOQ) was 0.050 μg/mL.

Table 1. Validation data of the method (n = 3)

Concentration added (μg/mL)	Extraction recovery (%)	Intra-day precision RSD (%)	Inter-day		
			Determined μg (mL)	RSD (%)	Relative mean error (%)
0.1	101.2	5.1	0.106	7.0	6.0
0.8	89.4	3.4	0.772	4.9	−3.5
2.5	98.3	4.2	2.465	5.5	−1.4

CONCLUSION

A reliable and rugged HILIC method for the determination of EPI in plasma has been developed and validated. SPE was employed to extract the analyte and IS from the plasma with high recoveries. A silica column and a low aqueous/high organic mobile phase were used, which would lead to favorable MS conditions. This validated method could be useful not only for the quantitative analysis of other anthracyclines, but also for the development of HILIC coupled with mass spectrometric detection of EPI.

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