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High-performance liquid chromatographic determination of phenylephrine in human serum with coulometric detection

Vusumuzi Vuma, Isadore Kanfer*

Biopharmaceutics Research Institute, School of Pharmaceutical Sciences, Rhodes University, Grahamstown 6140, South Africa

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Abstract

A high-performance liquid chromatographic method for the determination of phenylephrine (PE) in human serum using coulometric detection is described. PE and internal standard, orciprenaline, were extracted from serum by solid-phase extraction and separation achieved on a coupled column system consisting of two C_{18} cartridge columns (250×4.6 mm I.D. coupled to a shorter 50×4.6 mm I.D. column) using a mobile phase of methanol–50 m*M* phosphate buffer (pH 3.2; 10:90) at 36°C. Dual electrode coulometric detection was used in the "oxidative screen" mode. Calibration curves were linear over the range 0.3–4 ng/ml with a limit of quantification (LOQ) of 0.35 ng/ml. The method has a greater degree of sensitivity, precision and accuracy compared to previously published methods for PE and is suitable for use in pharmacokinetic and bioequivalence studies in humans.

Keywords: Phenylephrine

1. Introduction

Phenylephrine {1-*m*-hydroxy-[(methylamino)-methyl]benzyl alcohol (PE)} is a sympathomimetic amine that undergoes extensive first pass metabolism [1,2]. As a result, the bioavailability of PE is low and reported to be approximately 38% [2] or even lower [3]. In view of the above, low plasma concentrations of PE are found following oral administration, hence an extremely sensitive analytical method is necessary in order to determine the drug in biological fluids. A gas chromatographic method has been reported [4] but it involved tedious sample manipulation. In view of the reported lower limit of 12 ng/ml, it is, however unlikely that it would have the requisite sensitivity to quantitatively determine PE in biological fluids after oral administration of the drug. Gumbir and Mason [5] were unable to detect free PE in blood following the administration of a 20-mg oral dose using an HPLC method with a lower limit of quantification of 2 ng/ml. Various chromatographic methods have been described in the literature [6–12] but most of these [6–9] have been used for the analysis of PE in dosage forms and thus do not have the necessary sensitivity to determine the low concentrations of PE in biological fluids following oral administration. Other HPLC methods have also been reported for the determination of PE in biological fluids, two of which involved solid-phase extraction [10,11], the former utilising a lyophilization step [10]

*Corresponding author.

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whereas the latter method utilised a relatively large injection volume (200 μl), a low pH and no internal standard [11]. Recently, an HPLC method using column switching and fluorescence detection has been reported [12] but does not offer any improvement in sensitivity since "total" PE concentrations were measured following the hydrolysis of PE conjugates (mainly sulphate and glucuronide) which results in higher concentrations and thus does not require such a high degree of sensitivity. This approach to determine PE would not be suitable for use in bioequivalence assessments since only the free circulating drug is of interest in such comparative studies. Furthermore, the exact metabolic routes and proportions of PE metabolites formed remain unclear [13–15]. The proportions of metabolites are reported to be greatly dependent on the route of administration [2,14] with the major portion (>50%) of PE being deaminated to *m*-hydroxymandelic acid (MHMA) following intravenous administration.

PE has two pK_a values (8.9 and 10.1 for the –OH and –NH groups, respectively) and thus remains ionized over a broad pH range making efficient extraction from aqueous biological media (blood, serum, plasma, urine etc.) extremely difficult using the common approach of ion suppression coupled with extraction into an organic phase. Furthermore, the heating step during concentration of the extract has possible thermodegradation implications [10–13].

The present report describes the successful development of an HPLC method using electrochemical detection which has the required sensitivity, accuracy and precision to determine the concentrations of unconjugated PE in human serum following oral dosing and is eminently suitable for pharmacokinetic and bioequivalence studies.

2. Experimental

2.1. Materials

All chemicals were of analytical grade. The acetonitrile and methanol were distilled in glass (Burdick and Jackson, Muskegon, MI, USA) and the water was purified through a Milli-RO 15 water purification system (Millipore, Bedford, MA, USA).

Phenylephrine hydrochloride and orciprenaline hemisulphate (OC) were purchased from Sigma (St. Louis, MO, USA) and sodium hydrogen carbonate and sodium hydroxide were purchased from Holpro Analytics (Port Elizabeth, South Africa). The sodium salt of sulphonic acid was obtained from FSA Laboratory Services (Loughborough, UK) and the phosphoric acid from SaarChem (Krugersdorp, South Africa). Isolute PH solid-phase extraction columns (International Sorbent Technology, Mid Glamorgan, UK, Part No. 360-0010-A, Lot No. 4250206AB) were supplied by Anatech (Johannesburg, South Africa).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of an Isochrom pump (Spectra-Physics, San Jose, CA, USA), a WISP Model 710B autosampler (Waters Assoc., Milford, MA, USA), a column system comprising a Spherisorb 250×4.6 mm 5-μm ODS1 cartridge column (Phase Separations, Norwalk, CT, USA) coupled to a 50×4.6 mm 5-μm ODS1 cartridge column (Phase Separations). A Coulochem Model 5100A dual electrode electrochemical detector with a Model 5010 analytical cell and a Model 5020 guard cell (Environmental Sciences Assoc., Bedford, MA, USA) were used and the chromatograms were recorded on a Perkin Elmer Model 561 strip chart recorder (Hitachi, Tokyo, Japan).

A guard column with 2-μm replaceable frits (Upchurch Scientific, Oak Harbour, WA, USA) packed with 30–40 μm Perisorb RP 18 pellicullar packing (Upchurch Scientific) was employed and the packing was routinely changed after approximately 100 injections or as soon as the pressure of the system increased by 15–20% of the initial value (165 bar).

The mobile phase was prepared by mixing methanol and 50 m*M* sodium phosphate buffer (pH 3.2) containing 100 mg/l of heptane sulphonic acid (10:90, v/v). The sodium phosphate buffer was prepared by adding 1.6 ml phosphoric acid to 1 litre of HPLC-grade water and adjusted to pH 3.2 with sodium hydroxide pellets.

The mobile phase was deaerated and filtered through a 0.45-μm filter (TYPE HVLP, Millipore). An Erma ERC 1033 online degasser (Erma Optical Works, Tokyo, Japan) was used and separation was achieved at a flow-rate of 1 ml/min. The column system was maintained at a temperature of 36°C using an in-house built column heater. The guard cell was set at a potential of +1.0 V, and the analytical cell potentials at +0.6 V for the first electrode and +0.9 V for the second electrode at a gain of 300 and a chart speed of 2.5 mm/min using a recorder input of 10 mV full scale.

2.3. Standard solutions and calibration curves

Aqueous stock solutions of PE (200 ng/ml) and OC (20 ng/ml) were prepared in HPLC-grade water. A serum spike stock solution was prepared by adding an appropriate volume of the aqueous stock solution of PE and made up to volume with drug-free serum in a volumetric flask (20 ng/ml). Standard samples for calibration were consequently prepared from the serum stock solution (0.3–4 ng/ml) to afford the expected working range for this assay. Six calibration standards were prepared in this range and calibration curves for PE were constructed, after extraction of serum samples, by linear regression of plots of peak height ratios of PE/OC versus PE concentrations.

2.4. Extraction procedure

To 1 ml of serum were added 1 ml of sodium bicarbonate buffer (25 m*M*, pH 9.2), 200 μl of an aqueous OC solution (20 ng/ml) and the mixture vortexed for 30 s (Thermolyne Maxi Mix, Sybron, IA, USA). The entire sample was then loaded onto an Isolute PH (Phenyl) solid-phase extraction column (SPE) previously conditioned with 5 ml acetonitrile and 5 ml water and mounted on a Vac-Elut SPE extraction system (Analytichem, Harbour City, CA, USA). Samples were allowed to drain under gravity and then washed with 0.5 ml of sodium bicarbonate (25 m*M*, pH 9.2). Finally, the SPE column was washed with 2 ml water followed by 1 ml acetonitrile–water (10:90) and then dried under vacuum (10–15 cmHg, ca. $0.13 \cdot 10^5$–$0.2 \cdot 10^5$ Pa).

PE and internal standard were eluted with 2×0.5 ml aliquots of acetonitrile–50 m*M* sodium phosphate buffer pH 3.2 (70:30) and the eluate evaporated to dryness under vacuum in a Savant Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA). The sample was reconstituted in 200 μl of HPLC-grade water previously purged with helium and then transferred into a 1.5-ml disposable polypropylene tube (Eppendorf, Germany) and centrifuged at 16000 *g* in a centrifuge (Eppendorf). A 50-μl aliquot was injected onto the column using the autosampler.

2.5. Recovery, accuracy and precision

Intra-assay precision and accuracy were determined at three concentrations of PE (0.35, 0.71, 1.43 ng/ml) (n=6). Recovery was calculated by comparing peak height ratios of spiked serum samples containing internal standard with similarly extracted samples where the internal standard was only added in the final step when re-constituting the dried extract.

2.6. Application of method in a pharmacokinetic study in a human volunteer

To demonstrate the utility of the developed method for the study of PE pharmacokinetics, a pilot clinical study was carried out in an 80-kg human volunteer. A single dose of an aqueous solution of PE hydrochloride (20 mg) was administered orally with 250 ml of water and blood samples were collected at the following times; 0, 10, 20, 30, 40, 50, 60, 70, 90, 120, 180 and 240 min following the dose. The samples were centrifuged, the serum separated and stored at −15°C until the time of analysis. Samples were then processed as described above and a concentration versus time profile generated.

3. Results

3.1. Choice of internal standard

Previously reported HPLC methods for PE have not utilised an internal standard [11,12]. Although modern analytical technology has greatly improved the precision of volume measurement using auto-

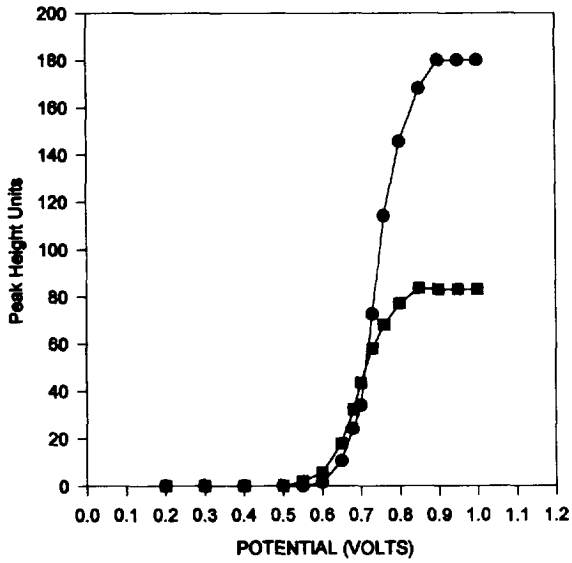

Fig. 1. Hydrodynamic voltammograms for PE (●) and OC (■).

samplers thereby enabling excellent precision without use of an internal standard, judicious selection of an appropriate internal standard offers distinct advantages [16]. A number of compounds including isoprenaline, dopamine and salbutamol were tested but rendered unsuitable because of instability (isoprenaline and dopamine) and late elution (salbutamol). Orciprenaline was chosen as internal standard because of similar physicochemical properties, greater stability and also its degree of electroactivity. Hydrodynamic voltammograms were generated for both PE and OC in order to determine optimal detector potential settings (Fig. 1).

3.2. Selectivity

Fig. 2 shows typical chromatograms obtained from extracts of blank human serum and serum spiked with PE (1 ng/ml) and internal standard. Under the conditions described above the peaks

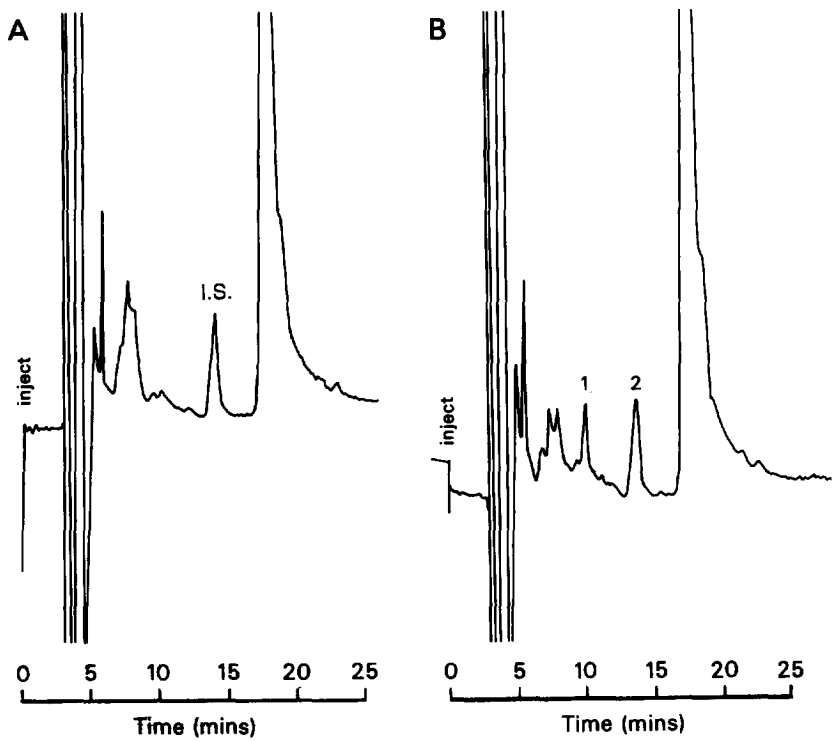

Fig. 2. HPLC chromatograms of (A) blank human serum spiked with internal standard and (B) human serum spiked with (1) phenylephrine (1 ng/ml), (2) orciprenaline (20 ng/ml).

representing PE and OC were symmetrical, well separated and free from interference from the solvent front. The retention times for PE and OC were approximately 10 and 14 min, respectively. A relatively large endogenous peak eluted at about 18 min but its presence did not interfere with either of the compounds of interest. Other drugs often administered concurrently with PE ($k'=2$) such as phenylpropanolamine and ephedrine (both of which are not detectable under the conditions of this assay using electrochemical detection), and similar sympathomimetics such as terbutaline ($k'=6.38$) and salbutamol ($k'=8.25$) did not interfere with this assay.

3.3. Linearity

Calibration lines using five different concentrations of PE in serum were linear over the concentration ranges studied. The linear regression equation was $y=0.059322+0.43748x$ with a correlation coefficient of 0.9980.

3.4. Precision and accuracy

As shown in Table 1, overall mean precision, as defined by the percent relative standard deviation (%R.S.D.) ranged from 3.4% to 9.3%. Analytical accuracy, expressed as the percent difference of the mean observed values compared with known concentrations varied from −1.2% to +4.4%.

3.5. Recovery

The recovery of PE from serum was 67.3–69.9% at concentrations ranging from 0.5 to 4 ng/ml (Table 2).

Table 2
Mean percentage recovery of PE from serum at varying concentrations ($n=6$)

Theoretical concentration (ng/ml)	Concentration found (ng/ml)	Recovery (%)	R.S.D. (%)
0.5	0.346	69.2	6.3
2	1.398	69.9	5.8
4	2.692	67.3	7.4

3.6. Stability

Freeze–thaw

In order to asses the stability of PE upon repeated cycles of freezing and thawing (i.e. sample processing on different days), two serum spikes of PE were prepared at two concentrations (2 ng/ml and 1 ng/ml) and assayed on the first day (day 0). Subsequently the samples were frozen and thawed over three cycles. The samples were stable over all three cycles at both concentrations (94–98% at 2 ng/ml, $n=3$; 98–112% at 1 ng/ml, $n=3$).

Stability of processed samples

The stability of PE during long analytical runs (e.g. 24 h or greater), was assessed by extracting samples as previously described. Following reconstitution a 50-μl aliquot was injected at the start of the analysis. The samples ($n=3$, 2 ng/ml) were retained in the autosampler for 24 h after which a 50-μl aliquot from each sample was re-injected. The concentration of PE in each sample was determined and mean data compared. In addition, extracted samples (n=3) were stored at ambient temperature (average temperature 22°C) and processed in the same manner (Table 3).

Based on these data (Table 3), extracted samples stored at room temperature and in the autosampler

Table 1
Precision and accuracy for the determination of PE in human serum

Prepared concentration (ng/ml)	Mean concentration found (ng/ml)	R.S.D. (%)	Percentage difference[a]
0.357	0.369	9.33	+3.36
0.714	0.705	4.40	−1.26
1.428	1.491	3.40	+4.41

$n=6$.
[a] Percentage difference=[(mean concentration found−prepared concentration)/prepared concentration]×100.

Table 3
Autosampler and bench-top stability of processed samples

Day	Concentration (ng/ml)	R.S.D. (%)
Autosampler		
0	1.98	3.2
1	2.01	5.6
Benchtop		
0	1.95	4.8
1	1.97	6.2

showed no significant degradation over a 24-h period.

Long-term stability of serum samples

As there is often a lag time between the time that serum samples are collected during a bioavailability/pharmacokinetics study and the time of bioanalysis, it is important to investigate the stability of the stored serum samples over long periods of time. Typically, samples may be stored up to 4–6 weeks prior to assay, although in some instances earlier assay may be desirable.

In this study, serum was spiked at 2 ng/ml and 1 ng/ml, divided into aliquots of about 10 ml and stored at −15°C in Kimax culture tubes protected from light. Samples of both serum spikes were removed on days 0, 1, 2, 7, 14 and 28 and assayed to assess long-term stability.

The samples were stable over the period of the experiment (28 days) with the stability ranging from 92–96% (1 ng/ml, $n=3$) and 96–102% (2 ng/ml, $n=3$).

3.7. Sensitivity and detection limit

Under the conditions of the assay the detection limit was 0.3 ng/ml and the lower limit of quantification (LOQ) was 0.35 ng/ml. At this concentration the response was approximately 3 times the baseline and %R.S.D. was within acceptable limits (9.3%).

3.8. Application of method

The applicability of the developed method was demonstrated by the determination of PE in serum samples from a single human volunteer who received an oral dose of 20 mg of PE. The method had sufficient sensitivity to quantitate PE levels after oral administration (Fig. 3).

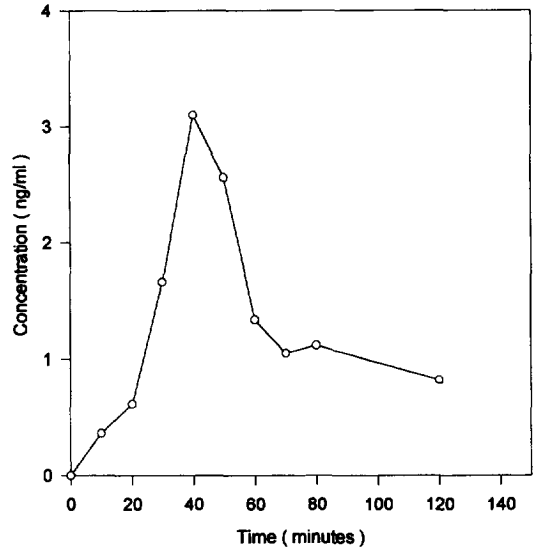

Fig. 3. Serum concentration versus time profile of PE following a 20-mg oral dose.

4. Discussion

In this study, electrochemical detection was chosen over fluorimetric detection due to the higher sensitivity and selectivity of the former. Coulometric detection was chosen over previously used amperometric detection [10] because of its efficiency and selectivity, in particular the provision to screen out interferences using a second electrode. In the coulometric mode it is possible to minimise interfering peaks by careful selection of the potential of detector 1 which functions as a screen thereby improving selectivity.

During the development of the method, there was a consistent problem of an unknown early eluting peak which did not return to the baseline prior to the elution of PE thereby rendering the measurement of PE difficult (Fig. 4). A similar problem has previously been reported with salbutamol using coulometric detection [17]. After extensive investigations to try and eliminate or reduce the peak, we found that by purging the water for reconstitution with helium, the

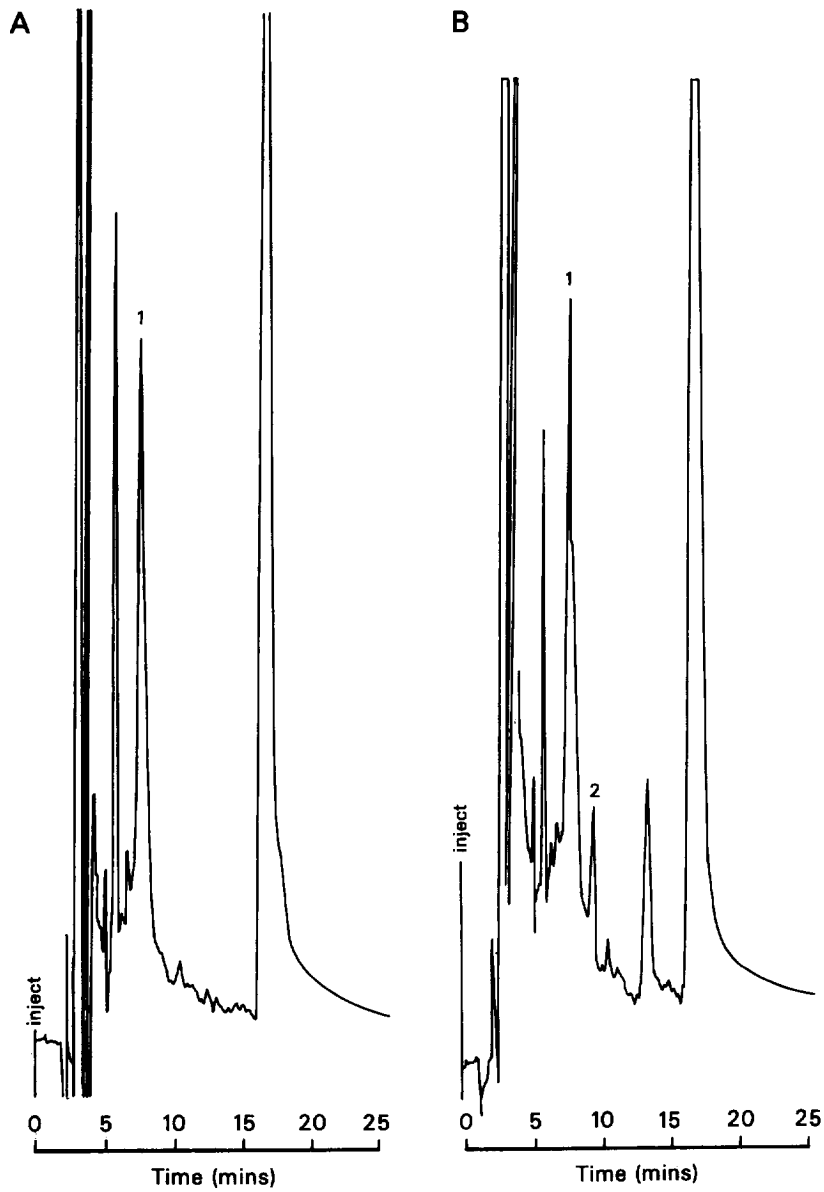

Fig. 4. HPLC chromatograms of (A) blank human serum extracts reconstituted with unpurged HPLC water showing an unknown early eluting peak (1) and (B) human serum extract spiked with PE (2), showing interference of (1) with measurement of PE (2).

size of the peak was significantly reduced such that the PE peak could be accurately measured (Fig. 2).

During the early stages of method development, a Spherisorb ODS1 250×4.6 mm I.D. cartridge column was used. Although this resulted in good sensitivity, there was a persistent problem of an interfering peak eluting at approximately the same time as PE. The coupling of a 50×4.6 mm Spherisorb ODS1 cartridge onto the 250×4.6 mm column, although somewhat increasing the backpressure of the system with a resultant slight decrease in sensitivity, resulted in a considerable

improvement in resolution thereby removing the problem of the interfering peak.

Whilst a high recovery of PE from serum samples was desirable, especially since the concentrations after oral dosing are extremely low, recoveries in the range of 60–70% were considered satisfactory and were similar to findings in other studies [9]. Recovery from extracts of aqueous solutions of PE were however, higher (80–85%) and the disparity between the serum and aqueous values may be due to the presence of substances in serum which interfere with the retention of PE on the solid-phase extraction columns. Although C_{18}, C_8 and phenyl extraction columns gave approximately the same recovery, phenyl columns were chosen because of their greater selectivity and cleaner extracts. The selectivity of the phenyl columns may be due to additional polar secondary interactions enhancing the retention of basic compounds during extraction [18].

Higher buffer content (e.g. buffer–acetonitrile, 50:50) of the eluent resulted in slightly higher recovery but it also significantly increased the time to dry the samples. Whilst concerns about the stability of PE during evaporation have previously been expressed [10,11], PE proved fairly stable under the conditions used in the study. The final step of centrifuging before injection greatly extended the lifespan of the guard column packing obviating the need for frequent changes and also extended column life.

5. Conclusions

An accurate, precise and reproducible analytical method has been developed for the analysis of PE in human serum. The method has a lower limit of quantitation (LOQ), greater precision and accuracy than previously published methods [10–12] and is suitable for bioavailability/bioequivalence studies and hence readily applicable to characterise the pharmacokinetics of PE in humans.

Acknowledgments

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