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# Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of phenylephrine in human plasma and its application to a pharmacokinetic study

P. Ptáček *, J. Klíma, J. Macek

*Pharmakl s.r.o., Seydlerova 2451, CZ-15800 Prague 13, Czech Republic*

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**Abstract**

A sensitive and reliable method was developed to quantitate phenylephrine in human plasma using liquid chromatography–electrospray tandem mass spectrometry. The assay was based on solid-phase extraction with $C_{18}$ cartridges and hydrophilic interaction chromatography performed on a pentafluorophenylpropylsilica column (50 mm × 4 mm, 3 μm particles), the mobile phase consisted of methanol–10 mM ammonium acetate (90:10, v/v). Quantification was through positive-ion mode and selected reaction monitoring at *m/z* 168.1 → 135.0 for phenylephrine and *m/z* 182.1 → 135.0 for internal standard etilefrin, respectively. The lower limit of quantitation was 51 pg/ml using 0.25 ml of plasma and linearity was observed from 51 to 5500 pg/ml. Within-day and between-day precision expressed by relative standard deviation was less than 12% and inaccuracy did not exceed 8% at all levels. The assay was applied to the analysis of samples from a pharmacokinetic study.


*Keywords:* Phenylephrine; Etilefrin; Mass spectrometry; HILIC; Liquid chromatography

## 1. Introduction

Phenylephrine (Fig. 1a) is a sympathomimetic vasoconstrictor that has been used as a nasal decongestant in various cold preparations for many years. The majority of its action is due to a direct stimulation of adrenoceptors and relatively little is due to an indirect effect via release of noradrenaline. At therapeutic doses, it does not cause significant stimulation of the central nervous system [1,2]. Following oral administration it is readily absorbed from gastrointestinal tract with approximately 40% systemic bioavailability. Peak plasma concentrations are achieved in 1–2 h and the mean plasma half-life is in the range of 2–3 h [3]. Reliable pharmacokinetic data of phenylephrine are scarce in the literature; a single $C_{max}$ value of 3 ng/ml following 20 mg dose is reported as an example in the analytical method description [4].

In spite of a wide use of the drug in medicinal products only a few published chromatographic methods are available for phenylephrine determination in human plasma. Solid phase extraction is mostly used for sample preparation and various detection modes are utilized including fluorimetric [5,6] and electrochemical [4,7] ones. All published methods suffer from an insufficient sensitivity for pharmacokinetic or bioequivalence studies, as the best limit of quantitation achieved is equal to 0.35 ng/ml [4]. Some of them evade this drawback by determining total phenylephrine after hydrolysis of the conjugates (mainly sulphate and glucuronide) but this approach is not supported by bioequivalence guidelines, which prefer the determination of a parent drug released from the dosage form. Usual phenylephrine single dose in combination pharmaceutical products ranges from 5 to 10 mg and pharmacokinetic studies of such products call for development of adequately sensitive analytical method.

The aim of this study was to develop a simple and sensitive LC–MS/MS method for determination of phenylephrine in plasma. The method was applied to a pilot pharmacokinetic study and the pharmacokinetic data will be used in future bioequivalence studies.

* Corresponding author. Tel.: +420 251 081 773; fax: +420 235 524 176.
*E-mail address:* ptacek@pharmakl.cz (P. Ptáček).

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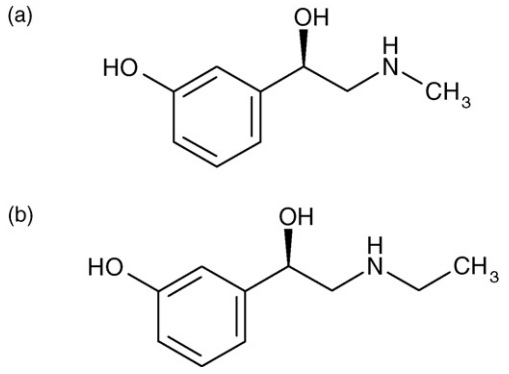


Fig. 1. Chemical structure of (a) phenylephrine and (b) etilefrin—internal standard.

## 2. Experimental

### 2.1. Chemicals

Methanol (for chromatography) was manufactured by Merck (Darmstadt, Germany). Ammonium acetate (puriss. p.a., ACS) was obtained from Fluka (Buchs, Switzerland), formic acid (puriss. p.a.) from Riedel de Haën (Seelze, Germany). Phenylephrine hydrochloride was manufactured by Iwaki Seiyaku (Tokyo, Japan), etilefrin hydrochloride (internal standard, Fig. 1b) was obtained from Boehringer Ingelheim as formulation Effortil (drops, concentration 7.5 mg/ml).

### 2.2. Apparatus and conditions

The HPLC/MS-MS system consisted of the P4000 pump, TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ion source, data station with Xcalibur software, Version 1.4 (all from Thermo Electron Corporation, San Jose, CA, USA). The Midas autosampler (Spark Holland BV, The Netherlands) was equipped with a 100 μl sample loop, methanol was used as a washing solution in the autosampler and the injection was performed in a partial-loop mode. The temperature of the column oven was 45 °C.

The separation was performed on a Discovery HS F5 column (3 μm, 50 mm × 4 mm, Supelco, Bellefonte, PA, USA) protected with a C18 4 mm × 3 mm precolumn (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol −10 mM ammonium acetate (90:10, v/v), the flow-rate was 0.6 ml/min. The column effluent was diverted to waste during first 4 min after injection, then it was switched to the ion source of the mass spectrometer.

The detection of the analytes was carried out using positive electrospray ionization technique and selected reaction monitoring mode to monitor the transitions (precursor → product) *m/z* 168.1 → 135.0 for phenylephrine and *m/z* 182.1 → 135.0 for etilefrin, respectively. The dwell time was 0.2 s for both analytes and scan width was set to 0.5 *m/z*. Ion spray voltage was set to 4500 V, temperature of the ion transfer capillary was 270 °C. Collision energy was 23 and 20 V for phenylephrine and etilefrine, respectively. The pressure of argon in the collision cell was 1.0 mTorr. The pressure of the sheath gas (sweep gas, auxiliary gas) was 40, 5 and 20 arbitrary units, respectively.

### 2.3. Standards

Stock solutions of phenylephrine were made by dissolving approximately 14–20 mg of phenylephrine hydrochloride in 25 ml of methanol. Separate solutions were prepared for the calibration curve samples and quality control ones. Further standard solutions were obtained by serial dilutions of stock solutions with methanol. The standard solutions were stored at −18 °C and were protected from light; they were stable at least 9 days under these conditions.

The calibration and quality control plasma samples were prepared by addition of standard solutions to drug-free plasma in volumes not exceeding 2% of the plasma volume.

The solution of the internal standard was obtained by serial dilution of etilefrin hydrochloride (drops, concentration 7.5 mg/ml) with water to a final concentration 1.876 pg/μl.

### 2.4. Preparation of the sample

The plasma samples were stored in the freezer at −18 °C and thawed at room temperature before processing of the sample. The preparation of the samples should be planned with respect to phenylephrine instability in plasma (see Section 3.4.3).

The solid-phase extraction (SPE) cartridges (Discovery DSC-18, 1 ml, 100 mg, Supelco) were washed with 1 ml of methanol followed by 1 ml of water. Two hundred and fifty microliters of plasma were pipetted to the polypropylene tube, 750 μl of the internal standard solution were added (the concentration of etilefrin hydrochloride in plasma was 5.628 ng/ml) and the tube was briefly shaken. This sample was applied to the SPE cartridge, which was subsequently washed with 1 ml of water and 1 ml of methanol–water (50:50, v/v). A clean tube was positioned below the SPE cartridge and the compounds were eluted with 1 ml of 3% formic acid in methanol–water (10:90, v/v). The eluate was transferred to an autosampler vial. Twenty microliters were injected into the chromatographic system.

### 2.5. Calibration curves

The calibration curve was constructed in the range 51.04–5531 pg/ml to encompass the expected concentrations in measured samples, the concentrations of individual calibration samples were 51.04, 121.0, 361.3, 776.1, 1940 and 5531 pg/ml. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$): the peak area ratio (analyte/internal standard) was plotted versus the analyte concentration. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

## 3. Results and discussion

### 3.1. Chromatography

Phenylephrine as a highly polar compound is poorly retained on standard reversed-phase columns. Resulting high water con-

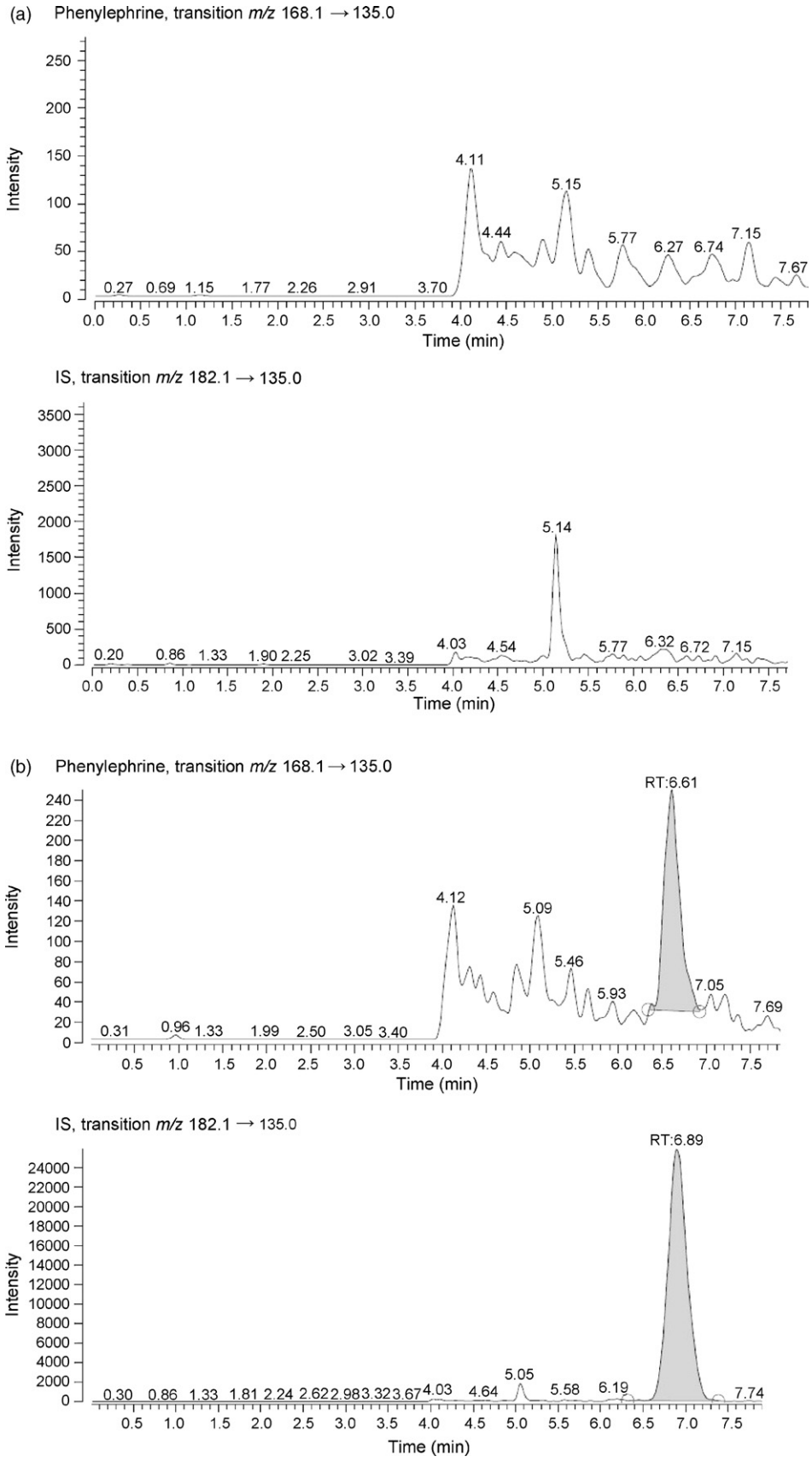


Fig. 2. Chromatograms of (a) drug-free human plasma, (b) spiked plasma at limit of quantitation (51 pg/ml) and (c) a plasma samples from a subject 2 h after administration of 10 mg of phenylephrine hydrochloride, the measured concentration was 395 pg/ml. The upper panel shows selected reaction monitoring of the transition *m/z* 168.1 → 135.0 (phenylephrine); the lower one shows the transition *m/z* 182.1 → 135.0 (etilefrin, internal standard).

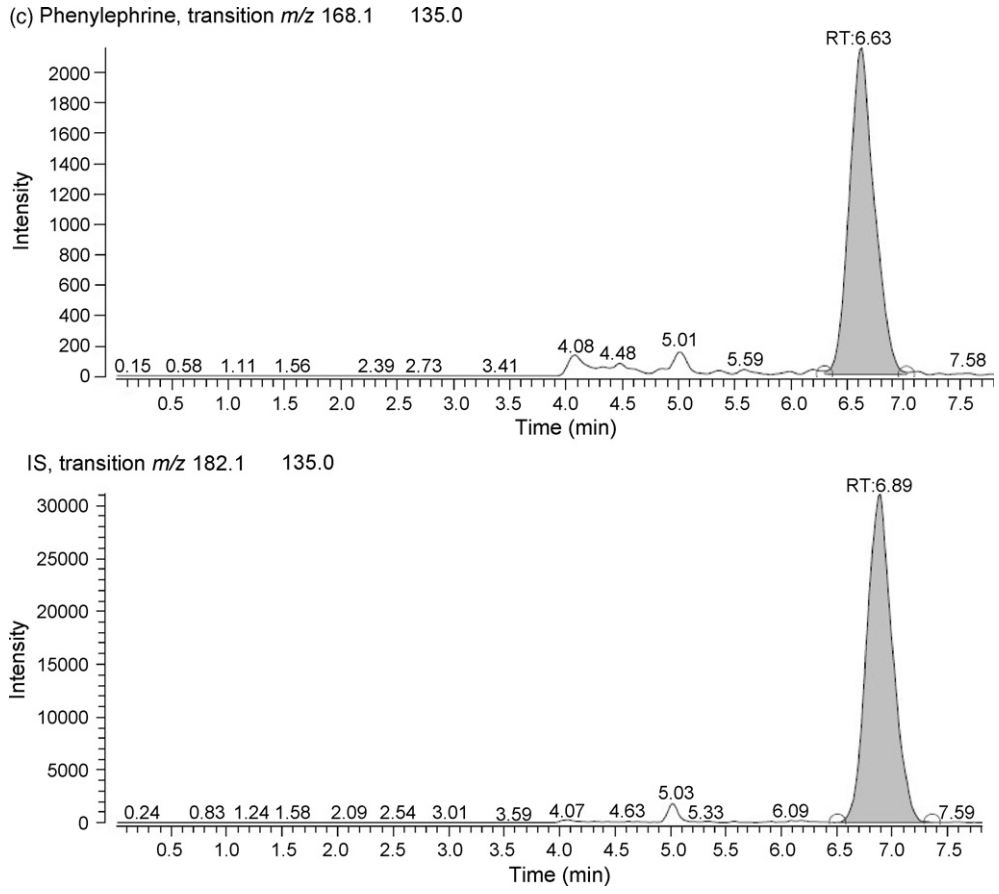


Fig. 2. (*Continued*).

tent in the mobile phase causes poor ionization efficiency in the mass spectrometric detection. Therefore, hydrophilic interaction chromatography (HILIC) was chosen to overcome this problem. Two columns were tried: silica column and a column with pentafluorophenylpropyl bonded phase (Discovery HS F5). The latter was finally chosen as it yielded well shaped peaks and generally more reproducible results.

The ion suppression with sample matrix was serious problem irrespective of used sample preparation. Chromatographic separation of coeluting interfering compounds was necessary; nevertheless the final run time is still acceptable: the retention time of phenylephrine and internal standard, etilefrin was ca. 6.5 and 6.7 min, respectively.

Typical chromatograms of drug-free plasma (a); spiked plasma at limit of quantitation 51 pg/ml (b) and plasma from a pharmacokinetic study containing 395 pg/ml phenylephrine (c) are shown in Fig. 2. The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks.

### 3.2. Sample preparation

Protein precipitation was initially tried as the most simple way of the sample preparation. However, severe problems with ion suppression were observed which could not be removed by a simple change of chromatographic conditions.

Liquid–liquid extraction was then employed as a more selective mode of sample preparation. Phenylephrine with p$K_a$ 8.8 (a secondary amine group) and 9.8 (phenyl group) is protonated practically at any pH value which makes its extraction from plasma difficult. Extraction efficiency was highest with *n*-butanol under high pH value as described in literature [5], addition of salts showed no gain in recovery. Another problem arose with loss of phenylephrine during evaporation at elevated temperatures which had to be prevented by addition of hydrochloric acid. The resulting 40–50% recovery would be enough due to the low detection limit but again strong ion suppression resulted in a low response.

Consequently, solid-phase extraction (SPE) was selected as a sample preparation technique. Extraction on Waters Oasis MCX SPE columns (combination of ion exchange and reverse phase mechanism) was tried at first, but final elution with methanolic ammonium hydroxide solution made the eluate inapplicable to direct injection on the column. Inserting of an evaporation step prolongs sample preparation and induces problems with analyte volatility. Finally, reversed-phase ($C_{18}$) SPE cartridges were selected, the sample preparation protocol requires no evaporation step.

### 3.3. Detection

The phenylephrine molecular ion fragments to several product ions with most intense ion transition *m/z* 168.1 → 150.1.

Table 1
Intra-assay precision and accuracy

| N | Concentration (pg/ml) | | | |
|---|---|---|---|---|
| | Added | Measured | Bias (%) | R.S.D. (%) |
| 6 | 96.06 | 96.35 | 0.3 | 11.5 |
| 6 | 509.0 | 499.2 | −2.0 | 3.5 |
| 6 | 4609 | 4280 | −7.7 | 1.5 |

Unfortunately high and noisy background signal was observed for the ion *m/z* 150.1 and any attempts to identify its origin were unsuccessful. Thus, monitoring of approximately five times less intensive transition *m/z* 168.1 → 135.1 was selected with better signal-to-noise ratio.

### 3.4. Method validation

#### 3.4.1. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y = bx + c$, where y represents phenylephrine/internal standard peak area ratio and $x$ represents concentration of phenylephrine in pg/ml. The mean equation (curve coefficients ± standard deviation) of the calibration curve ($N = 6$) obtained from six points was $y = 0.0001717$ ($\pm 0.0000076$)$x - 0.0002$ ($\pm 0.00011$) (correlation coefficient $r = 0.9983$).

The limit of quantitation was 51.04 pg/ml. The precision, characterized by the relative standard deviation, was 10.1% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was 6.0% at this concentration ($N = 6$).

#### 3.4.2. Intra-assay precision and accuracy

Intra-assay precision of the method is illustrated in Table 1. It was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The precision was at most 11.5% and the bias did not exceed 8% at all levels.

#### 3.4.3. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (three levels analysed twice, results averaged for statistical evaluation) on six separate runs. The samples were prepared in advance and stored at −18 °C. The respective data are given in Table 2. The precision was better than 12% and the inaccuracy did not exceed 6% at all levels.

Table 2
Inter-assay precision and accuracy

| N | Concentration (pg/ml) | | | |
|---|---|---|---|---|
| | Added | Measured | Bias (%) | R.S.D. (%) |
| 6 | 96.06 | 96.10 | 0.0 | 11.7 |
| 6 | 509.0 | 496.9 | −2.4 | 8.6 |
| 6 | 4609 | 4352 | −5.6 | 3.4 |

#### 3.4.4. Sample stability

Stability was generally concluded if the concentration change was less than 15% of the nominal concentration.

*3.4.4.1. Freeze and thaw stability.* Plasma samples with a low and high concentration of phenylephrine were prepared. The samples were stored at −18 °C and subjected for three thaw and freeze cycles. During each cycle triplicate 0.25 ml aliquots were processed, analyzed and the results averaged. The results are shown in Table 3. No significant substance loss during repeated thawing and freezing was observed.

*3.4.4.2. Processed sample stability.* Two sets of spiked samples with a low and a high concentration of phenylephrine were analyzed and left in the autosampler at ambient temperature. The samples were analyzed using a freshly prepared calibration samples 2 and 5 days later. The results are presented in Table 3. The processed samples are stable at room temperature for 5 days.

*3.4.4.3. Stability of plasma samples.* Phenylephrine decomposes in plasma at ambient temperature. Its stability was verified for 2 and 4 h at +20 and +4 °C, respectively, and all manipulation with thawed plasma samples should be kept within these limits. Stability of frozen plasma samples at −18 °C was demonstrated over 19 days period (Table 3), but almost 20% decomposition was observed after 7 weeks storage at −18 °C.

#### 3.4.5. Matrix-effects

In order to study matrix effects on the ratio of analyte/internal standard peak areas the following experiment was performed: six different plasma samples (five volunteers and pooled plasma for sample preparation) were spiked with phenylephrine (550 pg/ml) and internal standard (5750 pg/ml), processed and analyzed. The relative standard deviation of peak area ratios was 6% indicating no significant matrix effect on this parameter.

### 3.5. Application to biological samples

The proposed method was applied to the determination of phenylephrine in plasma samples from a pharmacokinetic study, which was approved by the local ethics committee. The plasma samples were collected following a single oral dose of 10 mg of phenylephrine hydrochloride (Beechams Flu Plus tablets, GlaxoSmithKline) administered to 12 healthy male volunteers: mean age of the group was 28 years (range 20–37), mean weight was 84 kg (range 71–103). Fig. 3 shows the mean plasma concentrations of phenylephrine; the error bars show standard deviations at individual time points.

The plasma levels reached their maximum 0.6 h (range 0.25–1 h) after the administration and thereafter the plasma level declined with an elimination half-time of 1.3 h (range 0.5–2.2 h). The maximum concentration ($C_{max}$) was 1.8 ng/ml (range 0.8–3.4 ng/ml) and the mean area under concentration–time curve (AUC) extrapolated to infinity was 1.8 μg h/l (range 1.5–2.4 μg h/l). The sampling interval during first hour after drug administration was 15 min. The shape

Table 3
Stability of phenylephrine

| | N | Concentration (pg/ml) | | | |
|---|---|---|---|---|---|
| | | 191.8 | | 4609 | |
| | | Measured | Bias (%) | Measured | Bias (%) |
| Freeze and thaw stability | | | | | |
| Cycle 1 | 3 | 192.7 | 0.4 | 4286 | −7.0 |
| Cycle 2 | 3 | 175.0 | −8.8 | 4306 | −6.6 |
| Cycle 3 | 3 | 186.1 | −3.0 | 4039 | −12.4 |

Stability of processed samples

| Sample | C (pg/ml) | N | C found (pg/ml) | R.S.D. (%) | Bias (%) |
|---|---|---|---|---|---|
| New | 96.06 | 6 | 96.35 | 11.5 | 0.3 |
| 2 days old | 96.06 | 6 | 93.58 | 8.5 | −2.6 |
| 5 days old | 96.06 | 6 | 81.95 | 8.2 | −14.7 |
| New | 4609 | 6 | 4280 | 1.5 | −7.2 |
| 2 days old | 4609 | 6 | 4154 | 2.6 | −9.9 |
| 5 days old | 4609 | 6 | 4371 | 2.9 | −5.2 |

Stability of plasma samples

| C (pg/ml) | Storage conditions | N | C found (pg/ml) | R.S.D. (%) | Bias (%) |
|---|---|---|---|---|---|
| 96.06 | 2 h/+20 °C | 5 | 90.97 | 12.8 | −5.3 |
| 4609 | 2 h/+20 °C | 5 | 4208 | 5.0 | −8.7 |
| 96.06 | 4 h/+4 °C | 5 | 85.55 | 11.6 | −10.9 |
| 4609 | 4 h/+4 °C | 5 | 4327 | 1.5 | −6.1 |
| 96.06 | 19 days/−18 °C | 6 | 94.38 | 10.8 | −7.9 |
| 4609 | 19 days/−18 °C | 6 | 4177 | 1.7 | −9.4 |

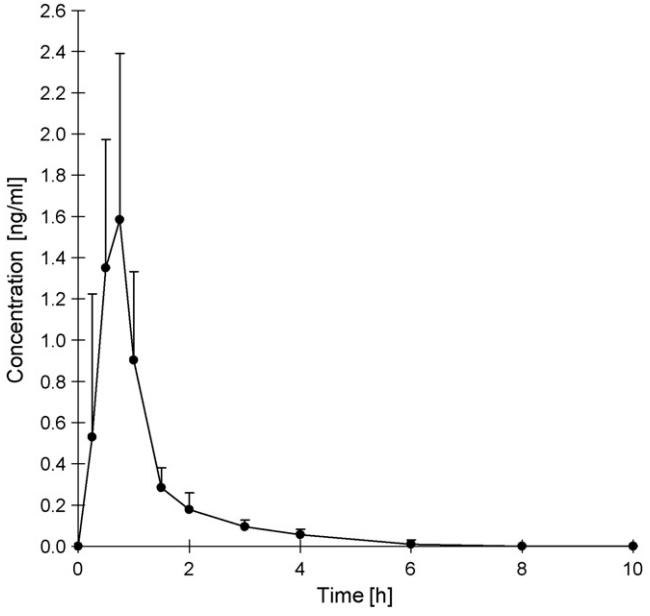


Fig. 3. Mean plasma concentrations (+S.D.) of phenylephrine after a single 10 mg oral dose of the drug administered to 12 healthy subjects.

of concentration–time curves around $t_{max}$ was very sharp and for more accurate estimation of $C_{max}$ more sampling points should be selected during the absorption phase, ideally 5 min intervals.

The mean AUC measured from 0 to the last non-zero sampling point was 94% of the value of AUC extrapolated from 0 to infinity. In all subjects this value was higher than 86% which indicates a suitability of the analytical method for pharmacokinetic studies.

## 4. Conclusions

The validated method allows determination of phenylephrine in the 50–5500 pg/ml range. About 150 samples can be prepared and analysed in one working day. The precision and accuracy of the method are well within the limits required for bioanalytical assays. The limit of quantification 51 pg/ml permits the use of the method for pharmacokinetic studies.

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