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High-performance liquid chromatographic determination of phenylephrine in human serum using column switching with fluorescence detection

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Abstract

A method for the determination of total phenylephrine (free plus conjugated) in human serum was developed using column-switching high-performance liquid chromatography (HPLC) with fluorescence detection. After serum was deproteinized with acetonitrile, the conjugated phenylephrine was hydrolyzed with diluted hydrochloric acid. The solution was evaporated to dryness. The reconstituted residue was analyzed with HPLC using a heart-cut technique. Good recoveries of the analytes from spiked human serum samples were obtained with small coefficients of variation. A good linear response was obtained for the concentration range 5–500 ng/ml. The lower limit of quantitation for phenylephrine in human serum was 5 ng/ml. The method was applied to the determination of phenylephrine in human serum after oral administration of phenylephrine hydrochloride.

1. Introduction

Phenylephrine (PL) hydrochloride {(−)-*m*-hydroxy-α-[(methylamino)methyl]benzyl alcohol hydrochloride}, a well-known sympathomimetic amine, has been in commercial use for many years. It has vasoconstrictive activity and has been employed as a nasal decongestant. Radioactive tracer studies revealed a very low serum concentration (lower than 1 ng/ml) of free (unchanged) PL in human serum after oral administration [1,2]. High-performance liquid chromatographic (HPLC) [3,4] and gas chromatographic [5] methods have been reported for the determination of free PL in serum. However, these conventional methods lack sensitivity for the determination of PL after oral administration considering the low concentration found in the literature [1,2]. Bruce and Pitts [6] have reported that PL is excreted to urine almost entirely (ca. 80% of dose) in a conjugated form, mainly as the sulfate, and not in its free form. Therefore, PL was presumed to exist in serum mostly in its conjugated form, although this has to our knowledge not yet been demonstrated. It is also essential to perform pharmacokinetic studies for the development of a new pharmaceutical formulation, e.g. a sustained-release dosage form. For this purpose, determination of the conjugated form instead of the free drug will suffice, when the drug exists in serum mostly in the conjugated form and the free existing drug has a negligibly low concentration. The aim of this study was to develop an HPLC method for the determination

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of total PL (conjugated plus free) in human serum and to demonstrate the applicability of this method to serum samples obtained after oral administration of PL hydrochloride.

2. Experimental

2.1. Reagents and materials

PL hydrochloride was of biochemical reagent grade (Wako, Osaka, Japan). Acetonitrile and methanol were of HPLC grade (Wako). Sodium 1-butanesulfonate (SBS) was of ion-pair reagent grade (Tokyo Kasei, Tokyo, Japan). All other reagents were of reagent grade and used without further purification.

2.2. Instrumentation and conditions

The column-switching HPLC system consisted of two LC-6A pumps, an SIL-6A autosampler with cooling system, an SCL-6A controller, a CTO-6A column oven, an SPD-6A UV detector, an FCV-2AH six-port switching valve (all from Shimadzu, Kyoto, Japan), a U-228 dual-pen recorder (Nippon Denshi Kagaku, Kyoto, Japan), an 821-FPS fluorescence spectrometer (Nippon Bunko, Tokyo, Japan) and a Maxima 820 integrator (Nippon Millipore, Tokyo, Japan). Both columns (C1 and C2) used were Inertsil ODS-2 (5 μm particle size, 150 × 4.6 mm I.D.; GL Science, Tokyo, Japan). An Inertsil ODS-2 column (5 μm particle size, 10 × 4.0 mm I.D.; GL Science) was connected in front of C1 as a precolumn. The mobile phase for C1 (MP 1) was 20 m*M* potassium dihydrogenphosphate–methanol (50:1, v/v) containing 5 m*M* SBS. The mobile phase for C2 (MP 2) was 20 m*M* potassium dihydrogenphosphate–methanol (50:1, v/v). The column temperature and the flow-rate were 40°C and 1 ml/min, respectively. The sample cooling system was set at 4°C. The column eluate was monitored with fluorescence detection at 270 nm excitation and 305 nm emission.

2.3. Analytical system and procedure

A schematic diagram of the HPLC system with the column-switching valve is shown in Fig. 1. The column-switching procedure was almost the same as that reported previously [7–12]. Briefly, the retention time of PL on the C1 column with UV detection (260 nm) was checked each day prior to analysis to determine the time program for column switching. The injected sample was first separated on C1 and the eluate fraction containing the analyte was transferred to C2 by valve operation. The introduced eluate was further separated on C2 followed by fluorescence detection.

2.4. Serum sample pretreatment

A human serum sample (200 μl) was deproteinized with 2 ml of acetonitrile. After centrifugation at 2000 *g* for 5 min, 50 μl of 1 *M* HCl was added to the decanted supernatant. The solution was incubated at 70°C for 1 h and then

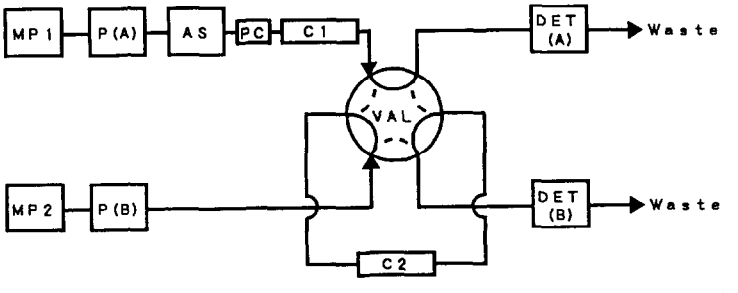

Fig. 1. Schematic diagram of the HPLC system. P(A), P(B) = pumps A and B; AS = autosampler; VAL = six-port valve; PC = precolumn; C1, C2 = columns 1 and 2; DET(A), DET(B) = UV detector and fluorescence spectrometer; MP1, MP2 = mobile phases 1 and 2.

evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was dissolved in 200 μl of MP 1 and a 100-μl aliquot of the solution was injected onto the HPLC system.

2.5. Hydrolysis of urine

The hydrolysis of human urine after oral administration of PL hydrochloride (5 mg) was carried out according to the method described by Bruce and Pitts [6]: to a sample of 100 μl urine 200 μl of 6 *M* HCl was added and the mixture was heated in a boiling water bath for 30 min. The solution was neutralized with 300 μl of 4 *M* NaOH and 400 μl of saturated $NaHCO_3$ solution. The solution was made up to 10 ml with MP1 and analyzed with the HPLC system described above.

Urine samples (100 μl) were hydrolyzed according to a method similar to that for serum. The evaporated residue was dissolved in 10 ml of MP1, which was analyzed with the same HPLC system.

2.6. Stability of PL during evaporation

A 50-μl aliquot of methanolic PL standard solution (400 ng/ml) and 50 μl of 1 *M*, 2 *M* HCl or water were added to 2 ml of acetonitrile. The solution was evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was dissolved in 200 μl of MP1 and a 100-μl portion of the solution was injected onto the HPLC system. As a control sample, diluted standard solution was prepared.

2.7. Calibration graph and quantitation

Drug-free human serum samples spiked with known amounts of PL hydrochloride were analyzed according to the procedure described above. The peak heights of PL were plotted against the serum concentrations to give the calibration graph. A weighted (1/concentration) linear regression was used to determine slope, intercept and correlation coefficient. The percentage recoveries of PL in human serum were calculated from the peak-height ratio of spiked standard samples relative to directly injected standard solutions. Intra- and inter-day accuracy and precision of the assay procedure were assessed in the concentration range 5–500 ng/ml in serum. The concentrations in samples were calculated using calibration graphs obtained with spiked standard.

3. Results and discussion

3.1. Pretreatment of serum and stabilization of PL during evaporation

PL, being a very hydrophilic compound, is difficult to extract with an organic solvent from aqueous media. Thus, serum samples were deproteinized with acetonitrile. Following deproteinization, evaporation and reconstitution were necessary to concentrate the analytes. However, PL was found to be unstable during evaporation under a stream of nitrogen gas at room temperature, as has also been reported previously [3,5]. Dombrowski et al. [5] prevented loss of PL during the evaporation process by acidifying the aqueous solution. Based on this result [5], the effect of addition of HCl to the PL-containing acetonitrile solution on the PL stability during the evaporation was investigated. As shown in Table 1, PL was completely stabilized by the addition of 1 *M* HCl during the

Table 1
Residual content of PL after evaporation under a stream of nitrogen gas at room temperature

Sample No.	Residual content[a] (%)		
	H_2O[b]	1 *M* HCl[b]	2 *M* HCl[b]
1	97.5	100.4	100.1
2	98.2	100.5	100.6
3	94.0	101.0	100.8
Mean	96.6	100.6	100.5
S.D.	2.3	0.3	0.4

[a] The peak height of diluted standard solution was taken as 100%.
[b] Each solution (50 μl) was added to the authentic sample dissolved in acetonitrile (2 ml).

evaporation under a stream of nitrogen gas at room temperature.

3.2. Hydrolysis of conjugated PL

As PL was predicted to be present mostly in a conjugated form in the supernatant of deproteinized serum, hydrolysis to its corresponding free base was necessary prior to HPLC analysis. On the other hand, as described above, addition of HCl to the supernatant was essential during the evaporation process. Conjugated PL would be hydrolyzed in heated acid solution since the conjugate was presumed to be principally sulfate [6]. Although a higher temperature probably would have been advantageous, hydrolysis was carried out at 70°C considering the boiling point of acetonitrile. The effect of incubation time on the hydrolysis of conjugated PL was investigated using a urine sample obtained after oral administration of PL hydrochloride (5 mg). The PL concentration in the urine sample reached a plateau after a 30-min incubation period (Fig. 2). To confirm whether the hydrolysis was completed or not, the present method was compared with that of Bruce and Pitts [6], in which strong acid and high temperature were employed. Almost identical PL concentrations in the urine samples were obtained by these two methods (Table 2), indicating that hydrolysis by the present method was complete. Based on the studies above, the conditions employed for hydrolysis of serum samples were as described in Experimental.

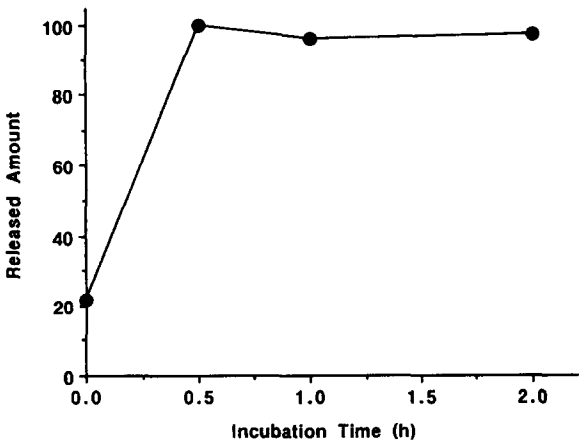

Fig. 2. Effect of incubation time on hydrolysis of conjugated PL at 70°C. Released amount of PL at 0.5 h was taken as 100.

Table 2
Comparison of PL concentrations obtained by both methods in the same urine sample

Sample No.	Concentration (ng/ml) in urine sample[a]	
	Method by Bruce and Pitts [6]	Present method
1	8896.0	9184.5
2	8909.0	9201.8
3	8447.1	8979.8
Mean	8750.9	9122.0
S.D.	263.2	123.5

[a] Urine sample was obtained from a human after oral administration of PL hydrochloride.

3.3. Chromatography

Fig. 3 shows typical chromatograms of a drug-free human serum and a standard solution equivalent to 5 ng/ml in serum obtained using only C2 without column switching, and monitored with fluorescence detection. To acquire a high sensitivity, PL should be eluted as rapidly as possible. However, because large interfering peaks were observed at the retention time of PL, the conventional HPLC analysis could not offer the required high sensitivity. As PL has a secondary amino group, precolumn fluorescence derivatization with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole was tried. Although several conditions were tested for the derivatization, the results were unsatisfactory (data not shown). Recently, Yamashita and co-workers [7–12] have reported an HPLC method for basic compounds in biological fluids using column switching combined with ion-pair chromatography. We applied this technique for the determination of PL, and it was shown to be effective for our purpose. Fig. 4 shows typical chromatograms of human serum

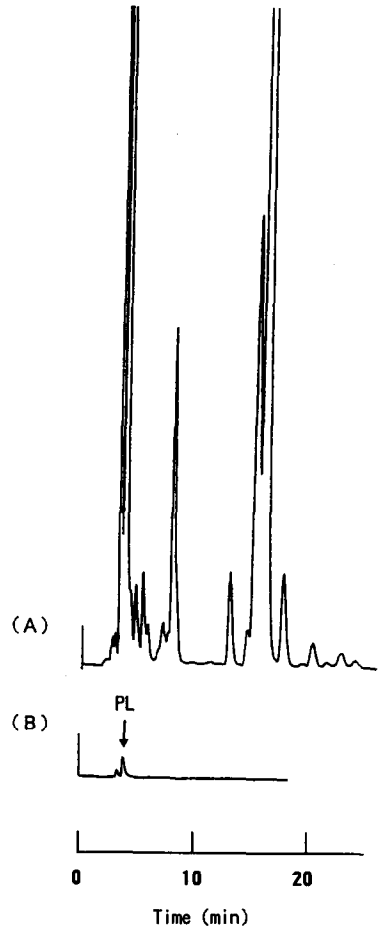

Fig. 3. Chromatograms of (A) a drug-free human serum and (B) a standard solution of PL equivalent to 5 ng/ml obtained with only column C2.

samples after column switching. No interference was observed at the retention time of PL.

3.4. Linearity, recovery and accuracy

The calibration graph was obtained by analyzing drug-free human serum samples spiked with PL in the concentration range 5–500 ng/ml. Good linearity was observed between the peak heights (y) and the corresponding serum concentrations (x). The equation of the regression line was $y = 78.2x - 34.3$ (correlation coefficient, $r = 0.99994$). The recoveries of PL from spiked serum samples were 88.0–95.3%. The accuracy

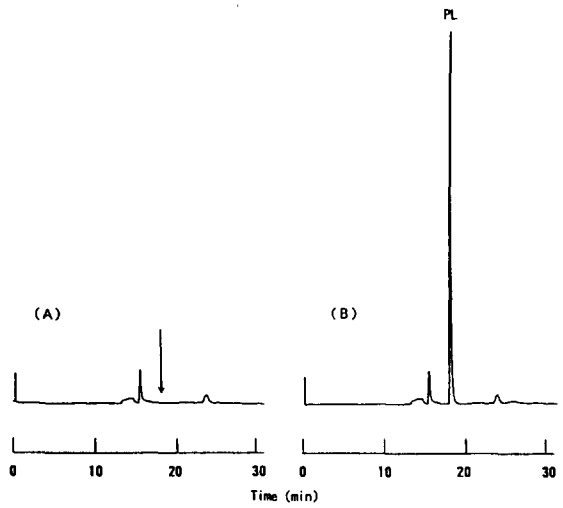

Fig. 4. Chromatograms of (A) a drug-free human serum and (B) human serum spiked with PL (100 ng/ml).

and reproducibility for PL in human serum are also shown in Table 3. In this method, the quantitation limit for PL was 5 ng/ml in serum, although the peak corresponding to 1 ng/ml was detectable at a signal-to-noise ratio of 3 (data not shown). Moreover, by using a larger amount of serum samples (e.g. 1 ml), a higher sensitivity would be obtained, because no interference was observed at the retention time of PL (Fig. 4).

3.5. Application of the method

The present method was applied to the determination of PL in human serum after its oral administration. The mean serum concentration–time profile of PL after oral administration of a single dose of 3.3 mg of PL hydrochloride is shown in Fig. 5. The mean serum concentration showed a peak of 67.4 ng/ml one hour after administration. Hengstmann and Goronzy [1] reported serum levels of free PL after oral administration of [^{3}H]PL, as determined by the radioactivity of PL, after isolation from serum by column chromatography. The concentrations of free PL were lower than 1 ng/ml. Our preliminary studies using a direct injection method also showed that free PL was not detectable (lower than 5 ng/ml) in human serum after oral ad-

Table 3
Accuracy and reproducibility of the determination of PL added to a drug-free human serum

Added concentration (ng/ml)	Concentration found (ng/ml)		
	Day 1	Day 2	Day 3
5.00	5.29 (6.81)	5.19 (4.05)	5.20 (3.46)
50.00	47.62 (3.28)	48.79 (0.70)	48.56 (1.67)
100.00	97.24 (2.16)	97.91 (0.86)	97.27 (1.43)
250.00	250.42 (0.70)	250.19 (0.34)	250.47 (1.19)
500.00	502.87 (1.82)	502.49 (0.48)	502.92 (0.76)

Values in parentheses are coefficients of variation (%) ($n = 5$).

ministration of a dose of 5 mg of PL hydrochloride (data not shown). On the other hand, Bruce and Pitts [6] described that conjugation with sulfuric acid was the major metabolic step after oral ingestion of PL. Judging from these results, the majority of the PL concentrations determined by the present method seemed to be derived from the conjugated form of PL. To our knowledge, this is the first report which shows the existence of conjugated PL in serum after oral administration of PL to man.

The analytical method was also applicable to the determination of PL in dog plasma. The chromatograms were free from interference at the retention time of PL (data not shown) and the validation data for dog plasma were also satisfactory [equation: $y = 80.2x - 17.1$, $r = 0.99995$, recovery: 85.9–99.3%, coefficient of variation at 100 ng/ml ($n = 4$): 4.1%].

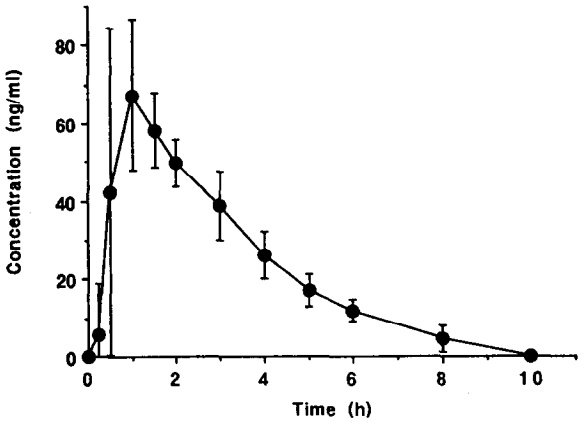

Fig. 5. Serum levels of PL in human volunteers after oral administration of a single dose of 3.3 mg of PL hydrochloride. Each point and bar represent the mean ($n = 6$) and the standard deviation, respectively.

In conclusion, a specific and sensitive method for the determination of PL in human serum was established by column-switching HPLC with fluorescence detection. This method was suitable for determination of the total PL concentration after oral administration of PL hydrochloride, and might be applicable even to animal plasma samples.

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