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Determination of terbutaline in human plasma by coupled column chromatography

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커플드칼럼크로마토그래피에 의한 사람 혈장 중 테르부탈린의 정량

고미영 · 전상설 · 김경호★

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Abstract: A method was developed and fully validated for the determination of terbutaline, a β_2-receptor agonist, in human plasma. Plasma samples were prepared by solid-phase extraction with Sep-Pak silica, followed by high-performance liquid chromatography (HPLC). The terbutaline was pre-separated from the interfering components in plasma on a Luna C18 (2) column, and terbutaline and salbutamol as an internal standard were resolved and determined on a Luna Silica column. The two columns were connected by a switching valve equipped with silica pre-column. The pre-column was used to concentrate the terbutaline in the eluent from the C18 column before back-flushing onto the silica column with fluorescence detection at an excitation/emission wavelength of 276/306 nm. The method was shown to be specific by testing six different human plasma sources. Linearity was established for a concentration range of 0.4-20.0 ng/mL with a correlation coefficient of 0.9999. The lower limit of quantitation was 0.4 ng/mL with a precision of 10.1% as C.V.%.

요 약: 사람혈장 중 베타투 수용체 작용약 테르부탈린의 정량법을 개발하고 밸리데이션을 실시하였다. 혈장시료를 Sep-pak 실리카를 이용하여 고상추출한 후 HPLC로 측정하였다. C18 칼럼을 사용하여 혈장 중의 방해물질로부터 테르부탈린 분획을 분리하여 실리카칼럼으로 보내어 테르부탈린과 내부표준물질을 분리 정량하였다. 두 칼럼은 짧은 실리카 전칼럼이 달린 절환밸부로 연결하여 사용하였다. C18 칼럼의 이동상에 녹아있는 테르부탈린의 분획은 전칼럼에서 농축되어 실리카칼럼으로 보내지고 형광 여기파장 276 nm와 들뜸파장 306 nm로 측정하였다. 이 분석법은 여섯 명의 혈장에서 특이성이 있음을 확인하였다. 혈장 중 테르부탈린 0.4-20.0 ng/mL 농도범위에서 상관계수 0.9999로 양호한 직선성을 나타내었다. 정량한계농도는 0.4 ng/mL 농도로 정밀도가 10.1% 를 나타내었다.

Key words: terbutaline, coupled column chromatography, HPLC, solid phase extraction, plasma

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1. Introduction

Terbutaline, a sympathomimetic drug-selective β_2-receptor agonist, is used in the treatment of asthma and lung disease. It is known chemically as ±-α-[(*tert*-butylamino) methyl]-3,5-hydroxybenzyl alcohol (*Fig.* 1).

High-performance liquid chromatography (HPLC) methods have been used extensively to quantify terbutaline in its dosage forms. HPLC methods using ultraviolet (UV) detectors have been identified as the most acceptable for terbutaline.[1-2] In the USP and BP, the HPLC system consisted of a C18 column and a mobile phase made of a mixture of an ion pair solution, sodium hexanesulfonate in ammonium formate (pH 3.0), and methanol (77:23, v/v) with UV detection at 276 nm.[3-4]

There are two problems in the determination of terbutaline in plasma for pharmacokinetic study using chromatography. First, the concentration of terbutaline in plasma is too low. Second, injection of biological samples after liquid-liquid extraction or solid phase extraction results in interference by plasma matrix components.

Due to its low concentration, terbutaline in samples from pharmacokinetic studies has been analyzed by gas chromatography-mass spectrometry (GC-MS).[5-7] However, most investigators have used HPLC. An isocratic paired ion HPLC method with electrochemical detection was reported for the determination of terbutaline in plasma, in which the limit of quantitation was 1 ng/mL.[8] Several HPLC methods have been developed using MS[9-10] or fluorescence detection[11-12] to ensure adequate analytical sensitivity.

To overcome the interference by biological matrix components, coupled column chromatography has been used.[13-15] The principle of coupled column chromatography is that the selective fraction of the mobile phase is transferred from the outlet of one column to the inlet of another. The column switching technique combines an automated clean-up and pre-concentration step with a separation step. Automated column-switching LC systems have the advantage that the enrichment and clean-up of the sample is performed by on-line liquid-solid extraction via a pre-column for the removal of other matrix components. With this automated column-switching technique, the drug level in plasma is easily determined without loss of accuracy and sensitivity.

A column-switching HPLC method was reported to monitor terbutaline in dog plasma with UV detection. The system consisted of a C2 pre-column and a C18 analytical column connected in series via a switching valve. The limit of quantitation was 3.0 ng/mL and the approach was used for pharmacokinetic study.[16]

The objective of the present work was to develop a more sensitive method with a column-switching system and fluorescence detection for the analysis of terbutaline in human plasma. This method is sensitive with a limit of quantitation of 0.4 ng/mL, which can meet the requirements of pharmacokinetics studies. This method was applied to the pharmacokinetics of terbutaline after oral administration of terbutaline tablets.

2. Experimental

2.1. Chemicals and reagents

Terbutaline sulfate and the internal standard,

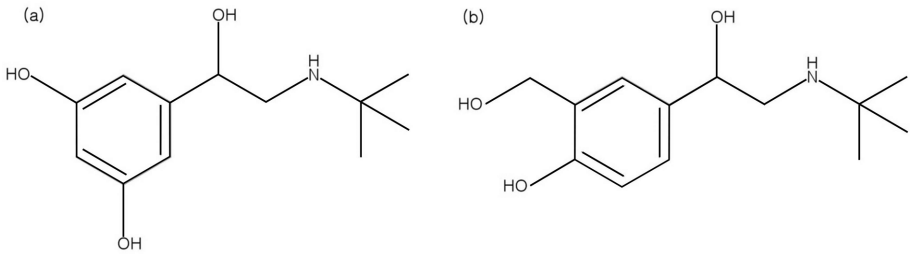
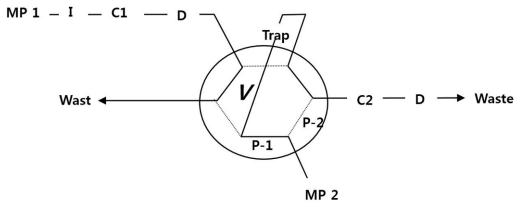


Fig. 1. Structures of (a) terbutaline and (b) salbutamol (I.S.).



Valve Position	Time(minute)	Function
P-1	0.00 – 9.50	Waste & Data acquisition from C18 column
P-2	9.51 – 12.00	Load terbutaline and salbutamol on silica trap column
P-1	12.01– 30.00	Elute terbutaline and salbutamol from silica column

Fig. 2. Schematic diagram of the column-switching apparatus in the HPLC system. P-1: valve position 1 (bold line), P-2: valve position 2 (dotted line), MP 1: mobile phase 1, MP 2: mobile phase 2, I: injector, C1: C18 column, C2: silica column, D1: UV detectors, D2: fluorescence detectors, Trap: silica guard column, V: switching valve.

salbutamol, were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade n-hexane, 1,2-dichloroethane, ethyl acetate, acetonitrile, and methanol were obtained from the Duksan Pure Chemicals Co. (Ansan, Kyeonggi, Korea). Deionized water was prepared in the laboratory. All other chemicals were of analytical reagent grade.

2.2. Equipment

The HPLC equipment (*Fig.* 2) consisted of the following components: two LC-9A pumps, two RF-10AXL fluorescence detectors with excitation/emission wavelengths of 276/306 nm, and a FCV-2AH six-port switching valve driven and time controlled by a SCL-6B controller (Shimadzu, Kyoto, Japan). The samples were applied using a Rheodyne model 7725i sample loop injector with an effective volume of 200 μL.

For the LC columns, a Phenomenex Luna C18 (2) column (250×4.6 mm I.D., 5 μm, Torrance, CA, USA) and a Phenomenex Luna Silica (2) column (250×4.6 mm I.D., 5 μm, SCAS, Osaka, Japan) were used as the C18 column and silica column, respectively. A Phenomenex silica guard column (30×4.6 mm I.D., 5 μm, Torrance, CA, USA) was chosen as the trap column. The acquisition of chromatograms and integration were obtained using a C-R4A integrator (Shimadzu, Kyoto, Japan). The vacuum manifold chamber and Sep-Pak Plus Silica, Sep-Pak C18, and OASIS HLB solid phase extraction cartridges were obtained from the Waters Corporation, USA.

2.3. Sample preparation

Ten microliters of internal standard solution (salbutamol, 0.25 μg/mL, in water) were spiked into 1 mL of plasma. The mixture was vortex-mixed for 10 s and applied to a Sep-Pak silica cartridge pre-conditioned with methanol (6 mL) and water (6 mL). The cartridge was washed with acetonitrile (2 mL). The analytes were eluted with 7 mL of methanol. The eluate was concentrated to dryness, and the residue was reconstituted in mobile phase 1 (500 μL) for an assay sample.

2.4. Chromatographic procedures

The column-switching system was able to introduce the objective compounds into the silica column and remove the large biological materials from the HPLC system. A schematic diagram of the column-switching system is shown in *Fig.* 2.

Two hundred microliters of plasma sample solution were injected into the C18 column, and terbutaline and the internal standard were separated from plasma interferences with a mobile phase consisting of 5 mM ammonium phosphate buffer (pH 4)-methanol (90:10, v/v) at a flow rate of 1.0 mL/min. After 9.5 min, the fraction containing the compounds of interest, terbutaline, and the internal standard was sent into the trap column for 2.5 min. The compounds were then eluted from the trap column and transferred to the silica column with the mobile phase consisting of 20 mM ammonium phosphate buffer (pH 4)-methanol (20:80, v/v) at a flow rate of 1.0 mL/min. Ratios of terbutaline to the internal standard versus concentrations of terbutaline were used for quantitative computations.

2.5. Validation studies

The method was validated according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance (FDA,

2001 and 2003). Spiked plasma samples were prepared by adding known amounts of terbutaline and the internal standard (salbutamol) to drug-free plasma at seven concentration levels (0.4, 0.5, 1.0, 2.0, 5.0, 10, and 20 ng/mL) and used for evaluation of linearity, accuracy, and precision. The precision and accuracy of the method were assessed through the analysis of spiked plasma samples (n = 5) at three concentrations (0.5, 2.0, and 10 ng/mL). Inter-day assays were evaluated via the analysis of samples for five different days.

2.6. Application of the method

The analytical method was applied to a pharmacokinetic study. The study was conducted on eight healthy male subjects administered two tablets of Beta-2® containing 2 mg of terbutaline sulfate from CG Pharm Ltd. (Republic of Korea) after they had been informed of the purpose protocol and risk of the study. All subjects gave written informed consent and the local ethics committee approved the protocol. The study was conducted strictly in accordance with the current Good Clinical Practices (GCP), International Conference on Harmonization (ICH), and FDA guidelines. The protocol for the study was approved by the Institutional Review Board (IRB) of the Research Institute of Pharmaceutical Science, Kangwon National University, which comprised a panel of medical practitioners. The subject were not allowed to consume any other medicine or alcohol for 2 weeks prior to or during the study. The health of the participants was judged via their clinical history, physical examination, and laboratory testing, that is, hemoglobin, hematocrit, white blood cell (WBC) and red blood cell (RBC) counts, platelets, differential counting of WBC BUN, creatinine, total protein, albumin, SGOT, SGPT, total bilirubin, cholesterol, glucose fasting, alkaline phosphatase-specific gravity, color, pH, sugar, albumin, bilirubin, ability to communicate efficiently with the researcher, and willingness to adhere to the protocol requirements. Plasma samples were obtained at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 15, and 24 h after intake of the drug and were stored at -70 ^{o}C until analysis.

3. Results and Discussion

3.1. Chromatography

The compositions of the mobile phase were investigated to determine the optimal chromatographic conditions. In the column switching technique, similar mobile phases have to be used for the two HPLC system because the solvent employed for the C18 column system could flow into the silica column system. Terbutaline and salbutamol (I.S.) were separated by HPLC on the silica column using 20 mM ammonium phosphate buffer (pH 4)-methanol (20:80, v/v) as a mobile phase. A similar mobile phase was investigated for HPLC on the C18 column. We selected 5 mM ammonium phosphate buffer (pH 4)-methanol (90:10, v/v) as the optimum mobile phase for C18 column. Under these conditions, terbutaline and the internal standard were eluted as a single peak, with a peak width of approximately 1.2 min; a total fraction containing them was transferred to the trap column via a six-port switching valve. The trapping capacity of the silica pre-column was examined by altering the transfer time. shows the relationship between peak areas of the analytes and trapping windows. The peak areas of terbutaline and salbutamol reached the highest levels at the transfer window of 1.5 min, and neither their peak areas nor the peak area ratio of terbutaline to the internal standard changed when a transfer time of 2.5 min was used. The peak areas decreased with a transfer time $\geq$ 3.0 min. These results indicate that the transfer of a peak containing terbutaline and internal standard was complete within 2.0 min and the trapping capacity was not less than 2.5 min. Thus, the 2.5 min window for the switch was selected and satisfactory results were obtained in validation studies.

The use of 20 mM ammonium phosphate buffer (pH 4)-methanol (20:80, v/v) as a mobile phase for silica column resulted in the baseline separation of terbutaline, the internal standard, and the interference peak. Under silica column conditions, the retention times of terbutaline and the internal standard were 24.5 and 27.0 min, respectively. *Fig.* 3 shows chromatograms obtained from the analysis of plasma samples

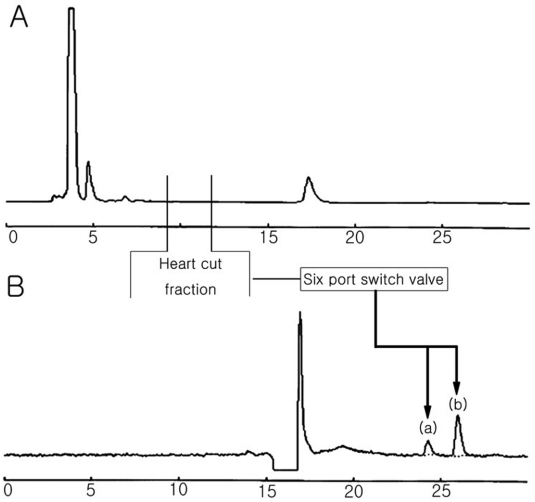


Fig. 3. Representative chromatograms of plasma samples at 1 h after oral administration of 4 mg terbutaline sulfate on the column switching system. (A) C18 column chromatogram, (B) silica column chromatogram after column switching. Peak a: terbutaline, peak b: internal standard (salbutamol). C18 column system: column: Phenomenex Luna C18(2) column (250 × 4.6 mm I.D., 5 μm), mobile phase: 5 mM ammonium phosphate buffer (pH 4)-methanol (90:10, v/v), detection: UV 276 nm, flow rate 1.0 mL/min. Silica column system: column: Phenomenex Luna Silica (2) column (250 × 4.6 mm I.D., 5 μm), mobile phase: 20 mM ammonium phosphate buffer (pH 4)-methanol (20:80, v/v), detection: fluorescence detector (excitation wavelength 276 nm, emission wavelength 306 nm, flow rate 1.0 mL/min).

at 1 h after oral administration of 4 mg of terbutaline sulfate. No interferences with terbutaline and the internal standard from blank plasma were detected.

3.2. Linearity and limit of detection

The calibration curves were obtained by analyzing spiked plasma samples. Calibration curves showed good linearity in the concentration range of 0.4~20 ng/mL in plasma. The equation of the calibration line obtained was as follows: $Y = 0.1834\ X + 0.0244$. The correlation coefficient was 0.9999 (*Fig.* 4). The limit of detection was 0.1 ng/mL and the limit of quantitation was 0.4 ng/mL, which was sensitive enough for the pharmacokinetic study of terbutaline.

3.3. Accuracy and precision

The accuracy and precision of the method were

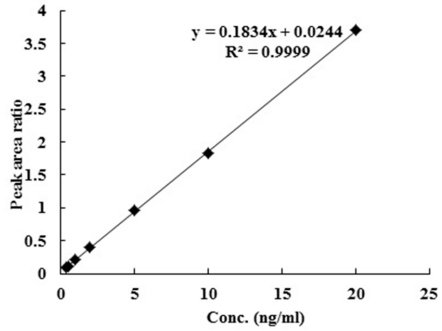


Fig. 4. Calibration curve of terbutaline.

Table 1. Precision and accuracy for determining terbutaline in human plasma (n = 5)

Conc. (ng/mL)	Precision (mean ± SD)		Accuracy (%)
	Intraday (n=5)	Interday (n=5)	
0.5	0.512±0.008	0.551±0.008	102.4±6.5
2.0	1.982±0.009	2.006±0.016	99.1±3.9
10.0	10.04±0.05	9.976±0.042	100.4±4.5

determined through a replicated analysis of blank human plasma spiked with terbutaline at three concentrations (0.5, 2.0, and 10 ng/mL). Five replicates of each concentration were analyzed on five separate days. The results obtained are shown in *Table* 1. The intra-day precision for each concentration was C.V. 2.4-7.8% and the inter-day precision was C.V. 5.2~6.3%. The accuracy determined for each concentration ranged from 99.1% to 102.4% (*Table* 1).

3.4. Recovery

The absolute recoveries were evaluated for terbutaline by comparing peak areas of extracted samples to the unextracted pure authentic standard solutions' peak areas at three concentrations (1.0, 7.5, and 15 ng/mL). The results ranged from 93.1% (C.V. 5.7%) to 99.9% (C.V. 9.9%).

3.5. Application

This analytical method was applied to analyze plasma samples obtained after the administration of a single dose of two 2 mg tablets of terbutaline sulfate to eight healthy male volunteers for pharmacokinetic study. Plasma sample were obtained from 0 to 24 h

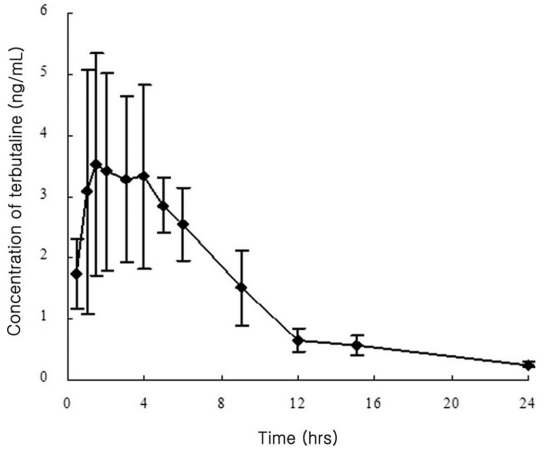


Fig. 5. Representative data showing the mean plasma concentration-time profile of eight healthy volunteers after the administration of an oral single dose of two 2 mg terbutaline sulfate tablets. The error bars represent± standard deviation (SD).

after intake of the drug and were stored at -70 °C until analysis. The mean plasma concentration-time profile of the eight volunteers is represented in *Fig.* 5.

The pharmacokinetics of terbutaline were demonstrated in the extent (AUC0-t and AUC0-inf) and rate (Cmax and Tmax) of absorption. The mean Cmax of terbutaline was 4.61±1.20 ng/mL. The mean Tmax for terbutaline was 3.13±1.87 h. The mean AUC0-t and AUC0-inf value of terbutaline was 28.16±5.75. The mean t1/2 value of terbutaline was 3.99±0.98.

4. Conclusions

One of the major problems encountered in the application of HPLC stationary phase to the analysis of biological samples is the co-elution of the target compound and interfering compounds from the matrix. This problem could be solved by the use of coupled column systems, in which the target compound is separated from interferences in the biological matrix on one stationary phase and then switched to another stationary phase.

A simple, specific, sensitive, stable, and relatively rapid HPLC method was successfully developed for the determination of terbutaline in human plasma. Both optimization and validation of the method were performed and the obtained results demonstrated that the method is suitable for pharmacokinetic studies of terbutaline.

A simple, specific, sensitive, stable and relatively rapid HPLC method has been successfully developed for determination of terbutaline in human plasma. Both the optimization and validation of the method were performed and the obtained results demonstrated that the method is suitable for pharmacokinetic studies of terbutaline.

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