Journal of Chromatography A, 1216 (2009) 3526–3532

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantitative determination of fenoterol and fenoterol derivatives in rat plasma using on-line immunoextraction and liquid chromatography/mass spectrometry

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ARTICLE INFO

Article history:
Received 25 April 2008
Received in revised form 15 July 2008
Accepted 7 August 2008
Available online 19 August 2008

Keywords:
Fenoterol
Immunoextraction
Rat plasma
LC/MS

ABSTRACT

An on-line immunoextraction and liquid chromatography/mass spectrometry (LC/MS) method was developed and validated for the determination of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol in rat plasma. Sample preparation involved immunoextraction of analytes using an antibody raised against *R*,*R*′- and *R*,*S*′-aminofenoterol that was immobilized onto chromatographic support. LC was performed on a Waters hydrophilic interaction chromatography (HILIC) column (150 mm × 2.1 mm), using an isocratic mobile phase of methanol:ammonium acetate (10 mM, pH 6.8) (90:10, v/v) at a flow rate of 0.2 ml/min. The MS was operated in the single ion monitoring mode (*m*/*z* 304.2 for *R*,*R*′-fenoterol, *m*/*z* 318.1 for *R*,*R*′-methoxyfenoterol, and *m*/*z* 339.2 for *R*,*S*′-naphthylfenoterol). Optimization of analytes desorption process from the immunoextraction column was performed by factorial analysis and the sample calibration curves were made with spiked rat plasma samples containing 0.5–100 ng/ml of drugs. The cross-selectivity studies of the antibody were determined and the results suggested high selectivities toward *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol. The accuracy of assay was more than 96% while intra- and inter-day precision of assay were less than 12.4%. Stability studies (2 h benchtop, freeze/thaw, and autosampler stability) were conducted and the analytes were stable through out studies. The validated method was used to determine the plasma concentration–time profiles of drugs after oral administration to rats of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol.

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1. Introduction

Fenoterol, 5-(1-hydroxy-2{[2-4(hydroxyphenyl)-1-methylethyl]-amino}ethyl)-1,3-benzenediol is a selective β_2-adrenergic receptor (β_2-AR) agonist that has been clinically used for the treatment of asthma [1] and evaluated for use as a tocolytic agent in premature labor. Fenoterol has two asymmetric centers, Fig. 1, and the marketed drug is a racemic mixture of *R*,*R*′-fenoterol and *S*,*S*′-fenoterol.

Recent cellular membrane affinity chromatography studies and cardiomyocytes binding and contraction assays have demonstrated that the β_2-AR agonist activity resides primarily with *R*,*R*′-fenoterol while *S*,*S*′-fenoterol is essentially inactive at this receptor [2]. These observations lead to the conclusion that *R*,*R*′-fenoterol may be a useful agent in the treatment of congestive heart failure, and this agent is currently undergoing initial clinical trials.

The basic structural nucleus of fenoterol comprises a benzene ring substituted with an ethylamino group, Fig. 1. Like other selective β_2-AR agonists, the compound contains a bulky *N*-substituent moiety (*p*-hydroxyphenylisopropyl) that leads to an increase in the β_2-AR potency and decreased activity at α-adrenergic receptors [1]. The effect of the structure of the *N*-alkyl substituent on β_2-AR binding affinity and cardiomyocyte contractility had not been extensively studied and such a study was recently undertaken [3]. The data from this study indicated that *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol, Fig. 1, had the same activities as *R*,*R*′-fenoterol at the β_2-AR [3]. These results suggested that *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol could also be used in the treatment of congestive heart failure [4].

As a part of the drug development process, comparative pharmacokinetic and bioavailablity studies were designed following iv and oral administration of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol. As key component of these studies was the development of an analytical method to quantify these compounds in biological samples.

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0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2008.08.046

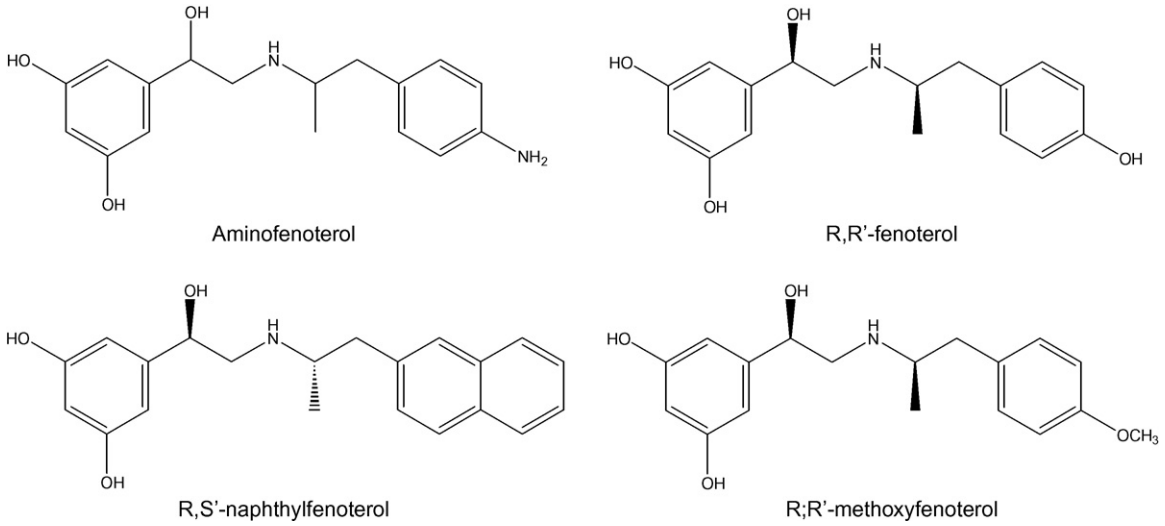

Fig. 1. Structures of *R,R′*-fenoterol, *R,R′*-methoxyfenoterol, *R,S′*-naphthylfenoterol and aminofenoterol used in this study.

A number of methods have been reported for the determination of fenoterol as well as other structurally similar β_2-AR agonists have been reported. These assays included absorptive stripping voltammetry [5] and enzyme immunoassay[6]. Rominger et al. reported a highly sensitive (0.02 ng/ml) radioimmunological assay for fenoterol in clinical samples during the drug development phase but this assay is no longer available, since the production of radiolabel has been abandoned [7].

There have been a number of chromatographic assays of fenoterol in biological fluids including derivatization with *N*-(chloroformyl)carbazole followed by HPLC separation and fluorescence detection, which was able to quantitated the compound at sub-nanogram per milliliters concentrations [8,9]. Liquid chromatography with tandem mass spectrometry (LC/MS/MS) has also been reported for the determination of fenoterol and other β_2-agonists in biological fluids with detection levels of less than 5 ng/ml in human urine [10] and human plasma [11,12]. Capillary electrochromatography with mass spectrometry using on-line isotachophoretic sample focusing of fenoterol [13] as well as capillary zone electrophoresis [14] were also reported and gas chromatography with mass spectrometric detection has been used to analyze fenoterol in postmortem blood [15].

Initial studies in this laboratory used solid phase extraction and LC/MS analysis as the basis of the bioanalytical system. This approach was validated and applied to the analyses of samples from the i.v. fenoterol arm of the study [16] but could not be used with samples obtained from the oral studies of the three test compounds. The key issue was the extraction of the target analytes from the biological matrix. In order to overcome this problem an online immunoextraction was developed and validated. This manuscript reports the results of this study.

2. Materials and methods

2.1. Materials

The rabbit anti-fenoterol polyclonal antibodies were prepared by Pacific Immunology Corp. (Ramona, CA, USA) using *R,R′*-and *R,S′*-aminofenoterol (Fig. 1) tagged KLH-carrier protein. *R,R′*-Fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol were synthesized and purified as previously described [3]. Ritodrine, ractopamine, metaproterenol, salbutamol, isoxsuprine and terbutaline were from Sigma (St. Louis, MO, USA). Sodium periodate, sodium cyanoborohydride and sodium borohydride were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was from Fisher (Pittsburgh, PA, USA). The Nucleosil Si-1000 silica (7 μm particle diameter, 1000 Å pore size) was purchased from Macherey–Nagel (Bethlehem, PA, USA). Phosphate-buffered saline (PBS) (pH 7.4) was from Invitrogen (Carlsbad, CA, USA). The rabbit immunoglobulin G (IgG) was from Sigma and reagents for the bicinchoninic acid (BCA) protein assay were obtained from Pierce (Rockford, IL, USA). All other chemicals were of the highest purity available. All other reagents were ACS grade or better. All aqueous solutions were prepared using water from a Milli-Q water system (Millipore, Billerica, MA, USA) and filtered using Osmonics 0.22 μm nylon filters purchased from Fisher.

2.2. Apparatus

A schematic diagram of the chromatographic system used in this study is depicted in Fig. 2. System 1 (SV1) consisted of two Shimadzu (Columbia, MD, USA) LC-10AD isocratic pumps, Rheodyne (Rohnert park, CA, USA) Lab Pro six-port 2 way switching valve, Agilent (Wilmington, DE, USA) 1100 series isocratic pump and auto sampler, a Shimadzu CTO-10AS column oven, the anti-fenoterol antibody column (30 mm × 4.6 mm I.D.). System 2 (SV2) consisted of an Agilent 1100 binary pump, an Alltech (Deerfield, IL, USA) Prevail C_{18} trap column (7.5 mm × 4.6 mm I.D.), a Waters (Milford, MA, USA) HILIC column (150 mm × 2.1 mm I.D.) and an Agilent 1100 mass-selective detector. Chromatographic data were collected and processed using Chemstation software version 3.01 from Agilent.

2.3. Immobilized antibody column

Nucleosil Si-1000 silica was converted into a diol-bonded form according to a previous procedure [17–19]. The anti-fenoterol antibodies were first purified from rabbit serum using Montage antibody purification kits from Waters and further purified by fenoterol immobilized affinity column. Anti-fenoterol antibodies were immobilized onto the diol-bonded support using the Schiff base method [17–19]. The immobilization reaction was allowed to proceed for 3 days at 4 °C. The silica was washed with PBS buffer three times and treated with three portions of 10 mg sodium borohydride to convert the excess aldehyde

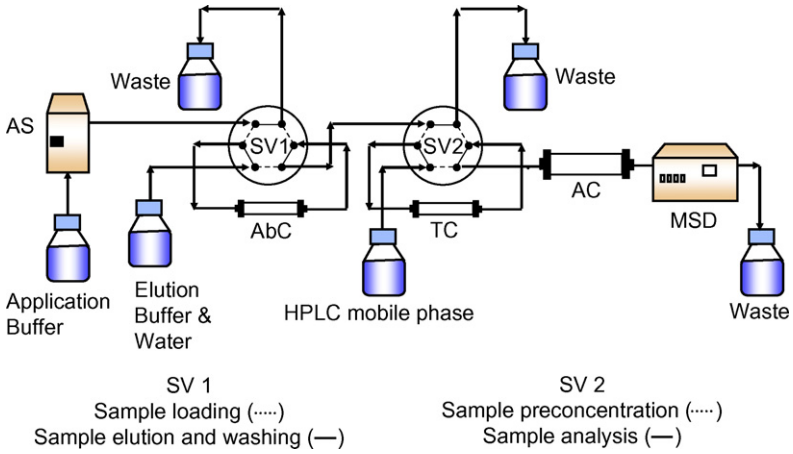

Fig. 2. Schematic diagram of on-line immunoextraction LC/MS system used in this study. MSD = mass-selective detector.

groups on the support into alcohols. The support was washed several times with PBS and stored in this buffer at 4 °C until use.

A control support was also prepared by performing the Schiff base method [17–19] on the diol-bonded silica, but with no antibodies being added during the immobilization step. This control material was washed and stored in the same manner as the immobilized antibody support.

A 0.5-ml portion of either the silica containing the immobilized antibodies or the control support was washed several times with deionized water (1.5 ml each) using Eppendorf centrifuge (Model 5415) and dried under vacuum at room temperature. The dried samples were weighed and analyzed in triplicate using a BCA protein assay kit in which rabbit IgG was the standard and the control silica acted as the blank. BCA protein assay was performed using a Model 680 microplate reader from Bio-Rad (Hercules, CA, USA). The absorbance of sample solution was determined at 560 nm by using the microreader plate. The anti-fenoterol antibody silica and control support were downward slurry packed at 3000 psi (183 bar) into 30 mm × 4.6 mm I.D. stainless steel columns using PBS as the packing solution. Empty columns used to prepare the antibody columns were purchased from Alltech and columns were downward slurry packed using an Alltech Slurry Packer.

2.4. On-line extraction of fenoterol with anti-fenoterol antibody column

Stock solutions (1.0 mg/ml) of *R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol were prepared in ethanol and further diluted with PBS to produce concentrations of 10–6000 ng/ml of working solutions. A 50-μl volume of working solution was spiked into 950 μl of blank rat plasma from Innovative Research (Southfield, MI, USA) to prepare 0.5–300 ng/ml standard rat plasma solutions. These standard rat plasma solutions were then diluted and vortex mixed with PBS (1:1, v/v, dilution) and filtered through a 0.22-μm regenerated cellulose acetate filter from Millipore using an Eppendorf microcentrifuge at 15,000 × *g* for 10 min. A 100-μl aliquot of the filtrate was injected at flow rate of 0.2 ml/min into the chromatographic system with the switching valve 1 (SV1) set at dashed line position (Fig. 2) using PBS as an application buffer. In this configuration, the sample was applied and target analytes were extracted to antibody column (AbC) and non-retained solutes were directed to waste. At SV1 solid line position, an elution buffer [Gly-HCl (0.2 M, pH 2.5):methanol, 90:10, v/v] was applied to antibody column at 1.0 ml/min and desorbed the extracted sample. The eluted sample was then directed to a trapping column (TC, Alltech Prevail C_{18}) where the sample was retained. The effects of application and elution flow rates on the extraction of samples from the antibody column were investigated. The temperature of antibody column was set to 37 °C using Shimadzu CTO-10AS column oven.

The extraction recovery study was performed by spiking a known amount (50 μl, 200 ng/ml) of test compounds (*R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol as well as structurally similar compounds in Fig. 3) to rat plasma (950 μl) to give a final concentration of 10 ng/ml. A 100-μl aliquot of PBS diluted (1:1, v/v) and filtered (0.22 μm regenerated cellulose acetate membrane) plasma sample is applied to the antibody column and recovery was determined by comparing MS responses (peak area) from this to neat standard samples without the antibody column.

2.5. HPLC separation

The TC was washed with water for 5 min to remove high concentrations of inorganic salts in elution buffer, the switching valve 2 (SV2) was rotated to position 2 (Fig. 2) and the samples retained in the TC were eluted onto an analytical column (AC, Waters HILIC) at 0.2 ml/min with HPLC mobile phase compositions of methanol:ammonium acetate (10 mM, pH 6.8) (90:10, v/v) for *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol and methanol:ammonium acetate (10 mM, pH 6.8) (90:10, v/v) for *R,R′*-fenoterol. The analytical separations were carried out at room temperature and the analytes were detected by MS. All mobile phases for the chromatographic studies were degassed at least 15 min prior to use. The MS system was operated in the single ion monitoring (SIM) mode (*m/z* 304.2 for *R,R′*-fenoterol, *m/z* 318.1 for *R,R′*-methoxyfenoterol, and *m/z* 339.2 for *R,S′*-naphthylfenoterol). The N_2 drying gas flow rate and temperature of MSD were 10 l/min and 350 °C, respectively. Other MS parameters: nebulizer pressure and capillary voltage were 20 psi (1.2 bar) and 3000 V, respectively.

The quantifications of *R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol were achieved by comparing to the response of a standard curve for corresponding compounds. The data was analyzed using GraphPad Prism 4 software and linear regression lines were obtained for *R,R′*-fenoterol with an $r^2 = 0.994$, *R,R′*-methoxyfenoterol with an $r^2 = 0.994$, and for *R,S′*-naphthylfenoterol with an $r^2 = 0.9998$. Standard rat plasma solutions containing the anlaytes were prepared daily.

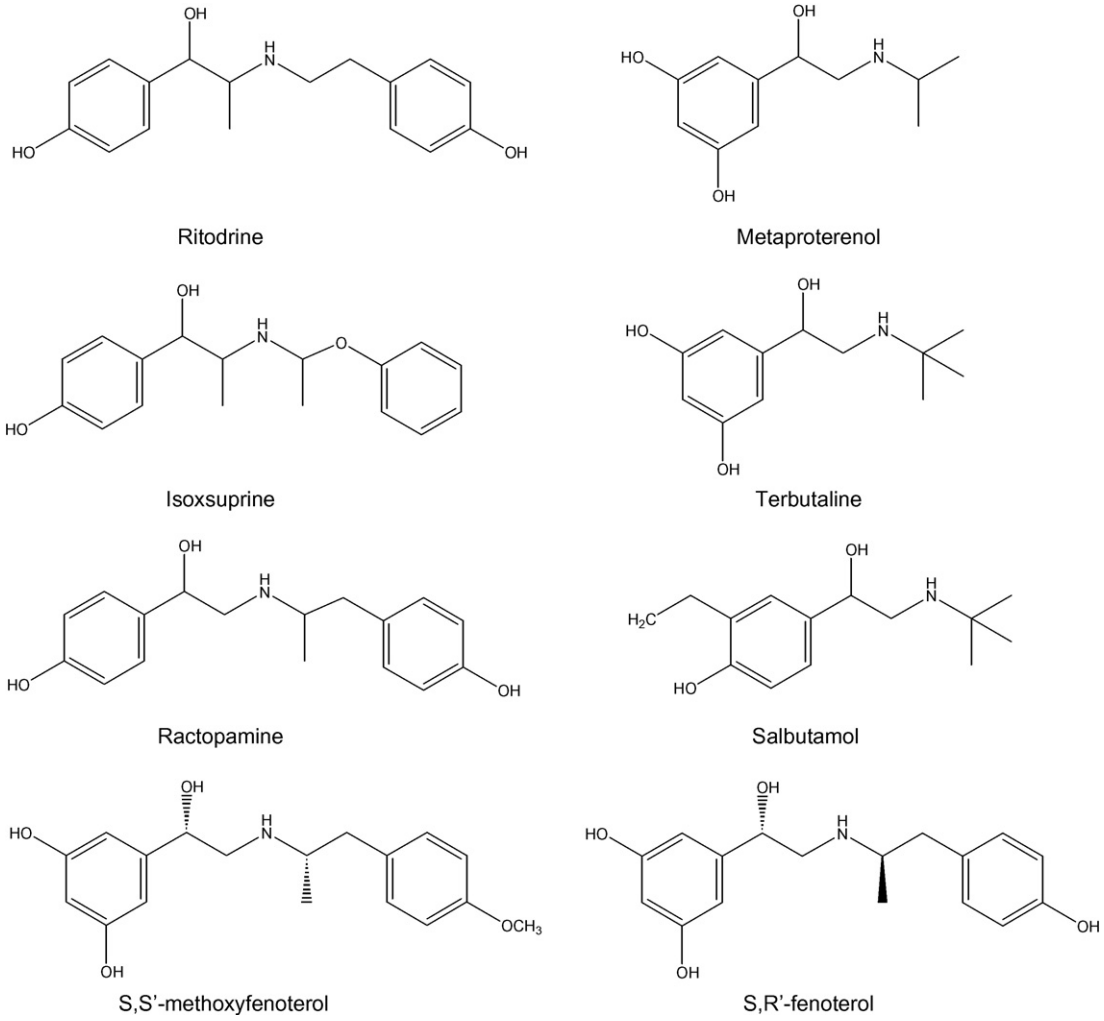

Fig. 3. Structures of fenoterol analogues used for cross-selectivity study.

2.6. Animal protocols

General procedures for animal care and housing were conducted in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Standards incorporated in 9CFR part 3, 1991. Each drug (*R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol) was given to a male Sprague–Dawley rat at 25 mg/kg. About 150 μl of blood was drawn at 0 (predose), 5, 15, 30, 60, 120, 240 and 300 min. Total six rats were used for each drug in this study. Animal care and blood collection were carried out by SRI International (Menlopark, CA, USA).

3. Results and discussion

3.1. General characteristics of the anti-fenoterol antibody column

The binding properties of the anti-fenoterol antibody column used in this study are listed in Table 1. The amount of immobilized antibody was determined by a protein assay to be approximately 92 pmol antibody/column. This is consistent with previous values reported for antibody on the same type of silica and under similar immobilization conditions [20–22].

The total binding capacity of antibody column was determined by performing frontal analysis of *R*,*R*′-fenoterol, which involves measuring the amount of analyte needed to saturate the antibody column (i.e., apparent binding capacities, $m_{L,app}$) at several different analyte concentrations ([A]). The plot of $1/m_{L,app}$ versus 1/[A] were made and the ratio of slope to intercept is used to estimate a binding capacity ($m_{L,app}$) of a given column, as described previously for other compounds [17,18]. The plot obtained for $1/m_{L,app}$ versus 1/[A] gave linear relationships with correlation coefficients of 0.995 ($n = 5$) suggesting one class of binding sites. This was expected since anti-fenoterol antibodies were purified with an immobilized *R*,*R*′- and *R*,*S*′-aminofenoterol affinity column.

The binding capacity of antibody column estimated from this plot was determined to 40 (±1) pmol *R*,*R*′-fenoterol/g silica, which was less than the amount of immobilized antibody determined from BCA protein assay. This was expected since the immobiliza-

Table 1
General properties of the anti-fenoterol antibody extraction support

General property	Corresponding value[a]
Amount of immobilized antibody[b]	92 (±3) pmol antibody/column
Binding capacity antibody column	40 (±1) pmol fenoterol/column
Specific activity of antibody column	0.43 (±0.02) mol fenoterol/mol antibody
Association constant for fenoterol	$3.9\ (\pm 0.5) \times 10^7\ M^{-1}$ for anti-fenoterol antibody

[a] All numbers in parenthesis represent ±1 SD.
[b] Determined using a molecular mass of 150,000 g/mol for rabbit IgG.

Table 2
Results of recovery studies performed on the immunoextraction/HPLC system

Compounds	Recoveries[a] (%)
R,*R*′-Fenoterol	98.4 (±8.4)
R,*R*′-Methoxyfenoterol	97.6 (±9.1)
R,*S*′-Naphthylfenoterol	98.9 (±7.5)
S,*R*′-Fenoterol	2.6 (±2.3)
S,*S*′-Methoxyfenoterol	4.2 (±3.0)
Metaproterenol	8.2 (±3.2)
Salbutamol	≤1%
Terbutaline	7.4 (±3.9)
Ritodrine	≤1%
Ractopamine	≤1%
Isoxsuprine	≤1%

[a] All values in parenthese represent ±SD ($n = 3$).

tion of antibody process was purely in random orientation mode and fenoterol binding site which might be completely or partially blocked (i.e., steric hindrance) during immobilization process. The specific activity (i.e., available antibodies for capturing *R*,*R*′-fenoterol) was determined from the binding capacity obtained from frontal analysis and the known antibody content of given column (BCA protein assay). It was estimated that 43% of the immobilized antibodies were able to capture fenoterol. This result was consistent for the three anti-fenoterol antibody columns that were prepared during this study with typical fenoterol binding capacities of 8–12 ng per column. Furthermore, the ratio of slope to intercept obtained from this plot was used to estimate the association equilibrium constant (K_a) for the binding of fenoterol to the immobilized antibodies. It was found to 3.9 (±0.5) × 10^7 M^{-1}. This large association constant suggested a relatively strong retention of fenoterol with antibody column, a feature that is critical for optimum extraction.

3.2. Cross-selectivities of the anti-fenoterol antibody column

The cross-selectivity or specificity of antibody against the target molecule and structurally related compounds is an important parameter in immunoextraction study because it is often in the case that that the antibody developed for the recognition of a small molecule were also able to recognize structurally similar compounds with different affinities [20,22]. Therefore in addition to *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol, the cross-selectivity property of the immobilized anti-fenoterol antibody to structurally similar β_2-AR agonists was investigated using a series of β_2-AR agonists, Fig. 3. The recoveries of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol were close to 100% in Table 2, which were expected since these compounds all shares common chemical groups except at the end (i.e., phenol, anisole and naphthyl groups). Among the examined test compounds, metaproterenol and terbutaline demonstrated more than 5% recoveries for anti-fenoterol antibody column in Table 2. Although they have the same chemical groups (hydroxyethylamino benzenediol group) compared to fenoterol, the overall length of these compounds are shorter than fenoterol, resulted in partial interaction to antibody binding sites All other structurally similar β_2-AR agonists showed minimal recoveries indicating that the antibody column is highly selective for *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol.

One interesting item observed in this study was chiral recognition of the antibody column to other stereoisomers of fenoterol. The *S*,*S*′- and *S*,*R*′-stereoisomers of fenoterol were not significantly retained indicating that an *R* configuration is necessary for recognition. This is partially due to the fact that the anti-fenoterol antibody was developed using *R*,*R*′- and *R*,*S*′-aminofenoterol conjugated to a KLH-carrier protein.

3.3. Optimization of desorption and separation

Several key parameters in optimization of on-line extraction for fenoterol to antibody column were investigated using 2^3 factorial analysis using design of experiment (DoE) approach [23,24]. The factors under the study were: sample application buffer flow rate, sample elution buffer flow rate and the effect of organic modifier. These factors were selected since they significantly affect absorption and desorption kinetics of antigen to antibody interaction. These factors were varied at 2 levels (min and max) and optimum conditions were determined from the Design Expert Software from Stat Ease (Minneapolis, MN, USA).

The DoE approach gave unexpected results. For all studied compounds, the extraction efficiency decreased as the elution buffer flow rate decreased even if the total elution volumes remained constant. The changes of application flow rate (0.1–0.5 ml/min) had no significant effect on the extraction efficiency of studied compounds. The amount of organic modifier (methanol) on the elution buffer was also an important factor since the interaction of antibody to small molecules are much harder to disrupt than macromolecules. As the amount of methanol increased (0–50%), the time required to achieve maximum extraction from antibody column decreased. The extraction efficiency, however, decreased as the amount of methanol increased over 10% since more than this amount negatively helps to elute fenoterol and its derivatives from the trapping column (i.e., faster extraction from antibody column but weak retention in the trapping column). The final optimum extraction

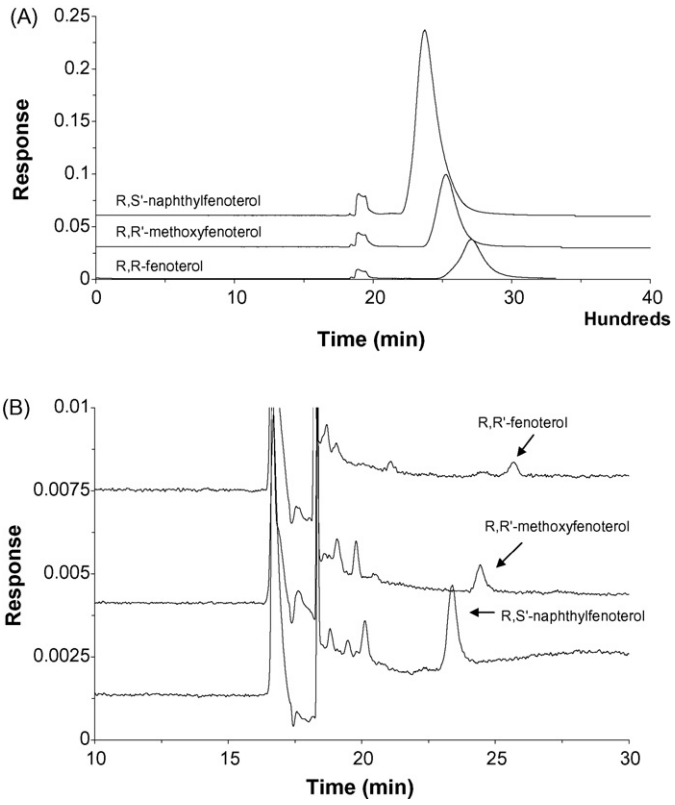

Fig. 4. (A) General chromatograms of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol after on-line extraction with subsequent LC/MS analysis. (B) Typical chromatograms of plasma samples of oral administration of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol to rats.

Table 3
Precision and accuracy results of *R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol on the immunoextraction/HPLC system

Compounds	Norminal concentration (ng/ml)	Precision		Accuracy	
		Intra-day RSD (%)	Inter-day RSD (%)	Concentration calculated	Average (%)
Fenoterol	1 (LQC)	9.4	11.3	0.98	98
	25 (MQC)	9.1	10.6	25.0	100
	100 (UQC)	7.2	8.4	96.4	96.4
Methoxyfenoterol	1 (LQC)	11.4	12.8	0.96	96
	25 (MQC)	9.5	10.1	25.1	100
	100 (UQC)	8.0	8.9	98.1	98.1
Naphthylfenoterol	1 (LQC)	10.8	12.4	0.99	99
	25 (MQC)	9.5	11.4	25.2	101
	100 (UQC)	7.4	10.8	98.9	98.9

conditions were 0.5 ml/min (3 min) for application buffer flow rate and 1.0 ml/min (10 min) for elution buffer (10% methanol).

3.4. System validation

An example of chromatogram obtained with the on-line immunoextraction-HPLC system the optimum extraction and HPLC separation conditions is shown in Fig. 4A. The total analysis time including application, elution and regeneration of the antibody column as well as wash of the trap column and separation of the sample took less than 30 min. The analysis of next sample, however, technically took less time since application, elution and washing steps of the next sample can be simultaneously performed with the separation of previously injected sample.

The calibration curves for *R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol obtained with this system showing linear response from 0.5 to 100 ng/ml ($n = 8$) for all three compounds with r^2 value of 0.997 (*R,R′*-fenoterol), 0.994 (*R,R′*-methoxyfenoterol) and 0.991 (*R,S′*-naphthylfenoterol). The limits of detection (LOD) for *R,R′*-fenoterol, *R,R′*-methoxyfenoterol and *R,S′*-naphthylfenoterol were determined at signal to noise ratio at 3, and they were 0.1, 0.2 and 0.2 ng/ml, respectively.

The upper calibration limit is determined by the capacity of the antibody column, which corresponds to the total number of accessible binding sites. As the amount of injected fenoterol increased over the number of accessible binding sites, a plateau was observed from 100 to 500 ng/ml range. The lower limit can be further improved by addition of bigger sample loop. In this study, a 100-μl sample loop was used because it can be maximally coupled to the Agilent autosampler used.

The lower limit of detection can, technically, be improved by the same amount if the sample loop size increased 10-fold. A sample application size of 45 ml to immunoextraction column coupled to HPLC system for analyzing herbicide residues from ground water has been reported [21].

The results of accuracy and precision for the immunoextraction/HPLC system were shown in Table 3. The accuracy and intra-day precision were examined by making 15 sequential injections of five groups, three quality control samples (LQC, MQC and UQC) of a spiked plasma sample. All three compounds showed more than 95% accuracy for all three concentrations. The intra-day precision of the system gave precisions of less than 12% for all three compounds at three level quality control samples. The inter-day precisions were evaluated over a 7-day period using three quality control samples prepared in the same manner as intra-day assay. These gave precision values of less than 13% for all three compounds.

Stability studies (2 h benchtop, freeze/thaw, and autosampler stability) of fenoterol and its derivatives were conducted to ensure stability of the drugs in the rat plasma. The results of sample stabilities studies conducted in this work are shown in Table 4. The long-term stability of the immunoextraction/HPLC system was also evaluated by comparing *R,R′*-fenoterol extraction recoveries. Less than 20% of antibody activities were lost over 3 months with more than 300 injections indicating this immunoextraction/HPLC system can be used for long-term studies.

3.5. Application

The validated immunoextraction/HPLC method developed in this work was used to determine the plasma concentration–time

Table 4
Results of sample stability studies on the immunoextraction/HPLC system

	R,R′-Fenoterol			*R,R′*-Methoxyfenoterol			*R,S′*-Naphthylfenoterol		
Norminal concentration (ng/ml)	Measured concentration (ng/ml)	RSD (%)	Accuracy (%)	Measured concentration (ng/ml)	RSD (%)	Accuracy (%)	Measured concentration (ng/ml)	RSD (%)	Accuracy (%)
2 h Benchtop									
1	1.1	8.9	110	1.2	6.2	102.4	1.1	5.9	100.9
25	24.8	11.3	99.2	23.9	11.8	92.1	25.6	3.3	102
100	103	10.0	103	98	4.3	106.6	97	6.9	97
Freeze and thaw									
1	1.1	9.2	109	1.1	2.4	109.6	1.0	10.2	100.8
25	25.1	9.8	100	26.1	11.8	92.1	26.2	8.9	104.8
100	99.8	11.6	99.8	109	4.3	106.6	102	6.9	102
Post-preparative									
1	1.1	6.9	110	1.2	4.1	106.7	1.0	4.6	100
25	25.6	7.6	102	24.6	7.7	105.3	25.2	4.9	101
100	108	8.5	108	105	1.7	106.5	98.4	8.1	98.4

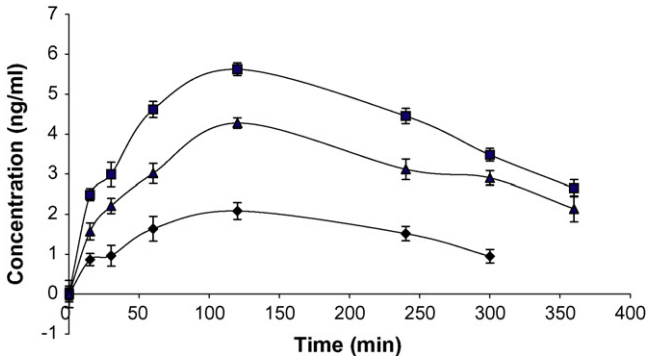

Fig. 5. The plasma concentration–time curve of *R*,*R*′-fenoterol (♦), *R*,*R*′-methoxyfenoterol (■) and *R*,*S*′-naphthylfenoterol (▲).

curve after oral administration of drugs to rats. Fig. 4B shows typical chromatograms of plasma samples obtained after the oral administration of *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol or *R*,*S*′-naphthylfenoterol to rats. The plasma concentration–time curves from a single rat for each compound are presented in Fig. 5. In these animals the maximum response was observed at 100 min and subsequently decreased thereafter. The results indicate that the immunoextraction/HPLC method developed in this work can be used to determine pharmacokinetic parameters (such as half life, clearance or bioavailability) of drugs administered in animals.

4. Conclusions

The data from the study demonstrated that affinity purified rabbit anti-fenoterol antibodies were successfully immobilized onto chromatographic support and packed into HPLC column. The specificity of immunoextraction column was studied using cross-selectivity studies using structurally related compounds and the immunoextraction column showed high selectivities toward *R*,*R*′-fenoterol, *R*,*R*′-methoxyfenoterol and *R*,*S*′-naphthylfenoterol. Furthermore, the recoveries of *R*,*R*′- and *R*,*S*′-stereoisomers of fenoterol and related compounds compared to *S*,*S*′- and *S*,*R*′-stereoisomers indicated that the developed immunoextraction column exhibited a high degree of stereoselectivity. Optimization of immunoextraction procedures were performed by using the design of experiment (DoE) approach and showed that the flow rate and amount of origanic modifiers in eluction buffer significantly affect the overall analysis time. The immunoextraction/HPLC system developed was also validated according to the US Food and Drug Administration (FDA) guidelines. The calibration curves were made using spiked rat plasma samples in the concentration ranges of 0.5–100 ng/ml. The LODs for these compounds were found to be 0.1–0.2 ng/ml. Accuracy and precision as well as sample stability studies performed on the developed immunoextraction/HPLC system were acceptable. This study illustrated that the immunoextraction/HPLC system developed here have several advantages over traditional extraction approaches such as solid phase extraction or liquid–liquid extraction. These include ease of automation, speed, selective enhancement of target analytes, and minimal matrix effects on mass spectrometry as well as improved accuracy and precision. The approach described here was successfully used to determine the plasma concentration–time profiles of the test compound after oral administration to rats.

Acknowledgment

This research was supported by the Intramural Research Program of the NIH, National Institute on Aging.

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