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Selective extraction of salbutamol from human plasma with the use of phenylboronic acid

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Abstract

An investigation was conducted on the usage of a single-step extraction procedure involving the retention of a phenylboronate–salbutamol complex on an end-capped C_{18} solid-phase sorbent to determine the level of salbutamol in human plasma samples. Propranolol, a β-blocker, was chosen as the internal standard for this assay. In this solid-phase clean-up method, 50 m*M* sodium carbonate buffer, pH 9.60, was used for conditioning the column as well as washing the endogenous interference. Under the optimal conditions, the recovery of salbutamol from spiked plasma samples was found to be high and reproducible with mean recoveries ($n=3$) of more than 90% after elution by using 50% 1 *M* trifluoroacetic acid in methanol. This sample clean-up step was effectively analyzed under reversed-phase high-performance liquid chromatography with fluorimetric detection. The method was successfully applied to the routine measurement of salbutamol in human plasma from the bioequivalence study on the different administration route of salbutamol. Quantification of salbutamol was convincingly reported with the correlation of coefficient of 0.9980 for the concentration range from 0 to 1000 ng ml^{-1}. An adequate precision was achieved with both between- and within-day precisions of less than 10% ($n=6$) for 100 and 1000 ng ml^{-1} and less than 15% ($n=6$) for 10 ng ml^{-1}.
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Keywords: Solid-phase extraction; Salbutamol; Propranolol; Phenylboronic acid

1. Introduction

The importance of determining salbutamol level in biological matrices for the investigation of banned substance in sport testing and growth promoter in animals is well recognized [1–4]. Determination of salbutamol in biological samples is usually done by high-performance liquid chromatography (HPLC) [5–9] and gas chromatography (GC) [1,10–13]. It requires an extensive sample purification step to remove potentially interfering compounds. This purification step has been performed mainly by using liquid–liquid or liquid–solid extraction procedures. Liquid–liquid extraction (LLE) [10,14–16] is labour intensive and time consuming and involves extraction of salbutamol into an organic phase and then back-extraction into an acidified aqueous phase. Solid-phase extraction (SPE) [10,14,17–19] utilizing weak or strong cation-exchange columns, octadecyl (C_{18}), octyl (C_8), cyano (CN), crosslinked polystyrene (ENV+) matrix or even internal surface boronate (ISBA) [20] and phenylboronate (ISPBA) [21] gels tends to use extensive column washing procedures and often requires two sequential extractions. In addition, optimal and reproducible recoveries with these procedures require careful adjustment of the pH of sample. Several on-line extraction

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methods have been reported for the purpose of simplification and automation. However, such methods require additional equipment such as column switching and automated sample processing systems, which are not readily available in clinical chemistry laboratories.

Soga and Inoue [20] studied the total automated methods for on-line sample pretreatment of catecholamines in urine and plasma using the synthesized ISBA gel as a pretreatment column in combination with an analytical column followed by electrochemical detection. Martin et al. [22] found that several β-blockers were retained on a solid-phase extraction cartridge containing phenylboronic acid. Ohta et al. [21] developed a method for the selective extraction of β-blockers from urine and plasma using an ISPBA precolumn combined with a reversed-phase analytical column followed by fluorescence detection. Boos et al. [30] introduced on-line sample processing using phenylboronic acid silica and a reversed-phase column and analyzed catecholamines and ribonucleosides in biological fluids. Grossi et al. described a rapid, one-step liquid–solid procedure for the extraction of catecholamines from urine employing diphenylboronic acid (DPBA) ethanolamine ester and a C_{18} SPE sorbent [23]. Urinary catecholamines are only weakly retained by the C_{18} matrix. However, DPBA–ethanolamine ester is dissociated into negatively charged diphenylboronate and ethanolamine [24]. Talwar et al. reported on the long-term performance of this method in the UK External Quality Assurance scheme for the measurement of urinary catecholamines. This method was found to be effective and reliable for the routine determination of free catecholamines in urine. The effectiveness of the extraction procedure was demonstrated by reversed-phase HPLC with electrochemical detection [25].

In this report, we conduct an investigation into the usage of the single-step extraction involving retention of phenylboronate complex on the end-capped C_{18} (EC-C_{18}) SPE sorbent to measure the salbutamol level in human plasma. This extraction method was validated on urinary salbutamol [26] and the reproducibility as well as reliability of this method was further explored on human plasma with minor modifications. Therefore, with this study, we hope to obtain a complete and precise pharmacokinetics profile of salbutamol in both human urine and plasma after two different administration routes namely oral and inhalation.

2. Experimental

2.1. Materials and reagents

Salbutamol sulfate and propranolol hydrochloride were purchased as pure drug standard from Sigma (St. Louis, MO, USA). Phenylboronic acid, trifluoroacetic acid (TFA) and triethylamine (TEA) were purchased from Fluka (Buchs, Switzerland). Orthophosphoric acid, perchloric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, sodium carbonate, sodium hydrogencarbonate, ammonium chloride, ethylenediaminetetracetic acid disodium salt and ammonium hydroxide purchased as 30% solution were obtained from R&M Chemicals (Essex, UK). The Isolute SPE cartridges (EC-C_{18}) were purchased from International Technologies, UK. All solvents used for liquid chromatography separation were HPLC grade and the solvents for sample preparation steps were analytical grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of the standard solutions

Stock standard solutions (1 mg ml^{-1}) of salbutamol sulfate and propranolol hydrochloride were prepared in both purified water and HPLC-grade methanol and were stored at 4 °C until use. The salbutamol stock standard solution was further diluted in purified water to obtain 100, 10 and 1 ng μl^{-1} working standard solutions for preparation of spiked salbutamol in blank human plasma. Propranolol stock standard solution was also diluted in purified water to obtain 1 ng μl^{-1} working standard solution as the internal standard (I.S.).

2.3. Preparation of spiked human plasma samples

Accurately measured aliquots of the diluted salbutamol standard solutions were each placed in silanized tapered tubes followed by the addition of 1-ml of blank plasma to give standard solutions

containing 0, 10, 20, 30, 50, 70, 100, 200, 500 and 1000 ng ml^{-1} of salbutamol.

2.4. Preparation of buffer and complexation reagent

Buffer stock solution pH 8.5 was prepared by weighing ethylenediaminetetracetic acid disodium salt (5.0 g) and ammonium chloride (106.98 g) into a 1-l volumetric flask. The chemicals were then dissolved in deionised water (950 ml). The pH of the buffer was adjusted to pH 8.5 (±0.04) with 30% ammonium hydroxide and made up to the mark with deionised water.

Buffer containing complexing reagent pH 8.5 was prepared by adding phenylboronic acid (1.0 g) to 500 ml buffer, pH 8.5 stock solution and stirring was carried out overnight. The pH of the reagent was adjusted to pH 8.5 (±0.04) with 30% ammonium hydroxide.

2.5. Sample pretreatment

Spiked plasma samples (1.00 ml) containing 0, 10, 20, 30, 50, 70, 100, 200, 500 and 1000 ng ml^{-1} salbutamol and clinical plasma samples (1.00 ml) from six healthy volunteers were added with 0.10 ml of I.S., 2.10 ml of 50 m*M* carbonate buffer, pH 9.60, and 1.00 ml of phenylboronic acid reagent. All samples were vortex-mixed for 1 min prior to loading on the SPE cartridges.

2.6. Solid-phase extraction procedure

The 1-ml Isolute SPE cartridges containing 100 mg of EC-C_{18} sorbent were positioned in the respective Luer fittings of the vacuum manifold. No vacuum pressure was applied throughout the extraction. The cartridges were preconditioned with 1 ml of methanol and 1 ml of 50 m*M* carbonate buffer, pH 9.60. The whole plasma sample mixture (4.2 ml) was loaded gradually to each cartridge and the cartridges were eventually washed with 1 ml of 50 m*M* carbonate buffer, pH 9.60. A vacuum of 100–200 mmHg (1 mmHg=133.322 Pa) was applied for 5 min to dry the cartridges. Elution of the analytes was performed with twice of 500 μl of 1.0 *M* trifluoroacetic acid–methanol (1:1, v/v). The eluates were transferred into 2-ml autosampler vials and then injected (100 μl) into the HPLC column using the autoinjector.

2.7. HPLC instrumentation and chromatographic conditions

The HPLC system for this assay consisted of a modular unit from Agilent Technologies (USA): a model HP 1100 series liquid chromatograph with autoinjector, a binary pump, solvent-delivery system and column oven. The separation was done on a NovaPak C_{18} analytical column, (150×46 mm I.D., 5 μm; Waters, USA) at ambient temperature. A mobile phase mixture of 50 m*M* phosphate buffer containing 1% TEA (pH 2.80)–MeOH (85:15, v/v) was delivered at flow-rate of 1 ml min^{-1}. A model HP 1100 fluorescence detector (Agilent Technologies) was operated at 230 nm for excitation wavelength and 320 nm for emission wavelength.

2.8. Extraction recovery

In order to determine the efficiency of the extraction procedure, an identical set of methanolic standard solutions was prepared with the concentrations equal to those in the spiked plasma. These methanolic standards were evaporated to dryness under a gentle stream of nitrogen at 37 °C. The dried standard was then reconstituted with 1 ml of eluting solvent and transferred into a 2-ml autosampler vial. The standard (100 μl) was injected into the HPLC column using the autoinjector. The peak area obtained was taken as an absolute recovery and compared with that obtained from spiked plasma.

2.9. Quantification and validation

In order to obtain standard calibration curves for analytical assay of human plasma, three stock solutions of salbutamol in distilled water were independently prepared and appropriate volumes added to 1.00-ml aliquots of drug-free plasma to give two replicate spiked standards 0, 10, 20, 30, 50, 70, 100, 200, 500 and 1000 ng ml^{-1}. The plasma standards were subjected to SPE and analyzed as described in Section 2.6.

The within-day variation of the assay at 10, 100 and 1000 ng ml^{-1} was assessed using aqueous standards by extracting and analyzing six replicates ($n=6$) of each independently prepared spiked plasma on the same day. Between-day variation was determined at 10, 100 and 1000 ng ml^{-1} by extracting and analyzing the spiked standard solutions on 6 subsequent days ($n=6$).

2.10. Sampling

Blood sampling was carried out in addition to urine during the bioequivalance study of different administration route of salbutamol [26]. Blood samples of about 10 ml volume were taken via an in-dwelling cannula placed in the forearm into heparinized tubes at 0 (predose), 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 16 h after dosing. The blood samples were centrifuged at 3500 rpm and the plasma samples were separated into plain tubes and then kept frozen at –20 °C until analysis.

3. Results

3.1. Evaluation of extraction method

The efficiency and robustness of this extraction procedure in terms of recovery and sample clean-up has been investigated for analytical variables such as extraction pH, sample loading, elution flow-rates, wash step, elution conditions, stability of the phenylboronate–salbutamol complex during the extraction procedure, the capacity of the C_{18} sorbent to adsorb the complex from urine, type of C_{18} sorbent used for extraction, effect of sample matrix and potential interferences by some structurally related compounds. Then, this one-step extraction procedure has been successfully applied to a urinary salbutamol study [26] and its application was further investigated after minor modifications to determine salbutamol level in human plasma samples in order to obtain a complete pharmacokinetics profile of bioequivalance study on different administration route of salbutamol in human.

Since Ohta et al. have also briefly studied salbutamol [21] and off-line complexation has been applied to the extraction of catecholamines from urine [25], we proposed to perform off-line complexation using phenylboronic acid instead of using the ISPBA that enables on-line isolation of the drug. Salbutamol forms cyclic boronate with phenylboronic acid under basic condition at its 1,3-diols group and not the hydroxyamino group. There is a high possibility that it is affected by adjacent bulky *tert*.-butyl groups (Fig. 1). The reaction involves the formation of ester complexes between phenylboronic acid and salbutamol and also propranolol under basic conditions by the addition of complexing buffer, pH 9.60. Propranolol has been selected as the internal standard for this assay because of its availability and more importantly isolation of propranolol by ISBA has been well established [20,21]. Unlike salbutamol, propranolol forms the cyclic boronate at its hydroxyamino group due to the absence of the 1,3-diols and bulky *tert*.-butyl groups. In the amounts used, it was found that the yield of phenylboronate complex for both salbutamol and propranolol were essentially quantitative [23]. In order to remove the extracted endogenous materials without washing the retained phenylboronate complexes, it was necessary to pass

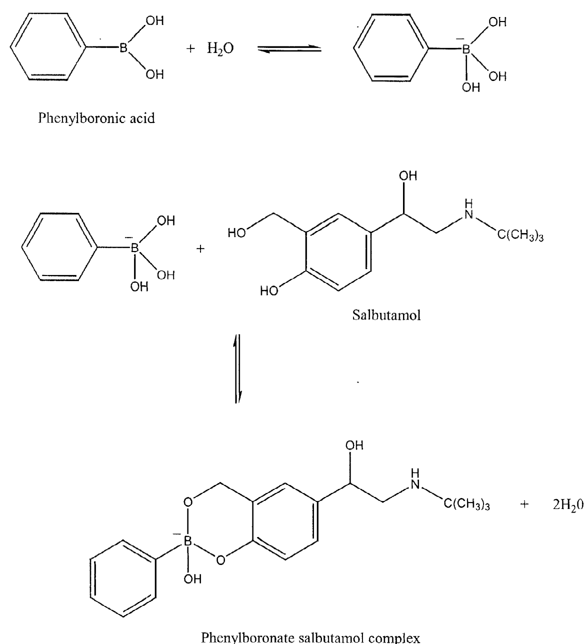

Fig. 1. Proposed mechanism of esterification between phenylboronic acid and salbutamol in aqueous solution under basic conditions.

the reaction mixture through a 1-ml SPE end-capped C_{18} cartridge that effectively removed the endogenous interference by washing the sorbent with the complexing buffer pH 9.60, resulting in cleaner chromatograms. Finally, the complexation process was reversed and the analytes were eluted with highest recovery from the SPE sorbent by using 1 *M* trifluoroacetic acid–methanol (50:50, v/v) after several types of elution solvents had been studied (Table 1). This SPE clean-up step was relatively rapid, simple, efficient and found to be more effective than back-extraction with 1 *M* HCl [14,15].

3.2. Chromatography

From the chromatograms of authentic and spiked samples as shown in Fig. 2, it can be seen that the peaks corresponding to salbutamol and propranolol eluted with retention times of 2.52±0.10 min and 11.20±0.10 min, respectively. Under the same HPLC conditions, the retention times were also in agreement with the results obtained from the similar spiked samples extracted using C_{18} SPE without phenylboronic acid complexation. Moreover, phenylboronic acid was also found to be eluted with retention time of 6.56±0.10 min. Thus, the peaks of salbutamol and propranolol were obviously free from interfering substances in their respective regions under the optimized HPLC conditions despite of the long run time of 14 min.

3.3. Quantification and validation

Linear regression coefficients of the daily calibration curves of spiked plasma samples are 0.9980 with the limit of detection (LOD) of 5 ng ml^{-1} ($S/N = 3$; RSD = 11.21%) and limit of quantification (LOQ) of 10 ng ml^{-1} ($S/N = 3$; RSD = 12.26%), although this can be lowered considerably by analyzing a larger volume of plasma or by injecting a larger proportion of the sample on-column. The equation describing the line is $y = 0.001x + 0.0231$.

The analytical procedure has shown good precision and accuracy at all three concentration levels. The within- and between-day relative standard deviations (RSDs) were determined by analyzing replicates of urine samples ($n = 6$) spiked with salbutamol at 10, 100 and 1000 ng ml^{-1} and the I.S. at 100 ng ml^{-1}. As shown in Table 2, the RSD values for both within- and between-days were between 10 and 15% for 10 ng ml^{-1}, indicating the assay was nearing its LOQ. On the other hand, the RSD was $<10\%$ for the other two concentrations for both within- and between-day precisions.

3.4. Assay application

The developed assay was successfully applied to clinical plasma samples from six healthy volunteers after two different administration routes of salbutamol. Fig. 3 shows the chromatograms of plasma samples at both 0 h and 3 h after oral salbutamol and Fig. 4 shows the chromatograms of plasma samples at both 0 h and 1.5 h after administration of salbutamol via inhalation, respectively. The retention times for salbutamol and the I.S. are 2.57±0.10 and 11.30±0.10 min, respectively. The pharmacokinetic profile of salbutamol in all volunteers shows the time of maximum plasma salbutamol concentration, T_{max} was 1.0–1.5 and 2–3 h after administration of salbutamol via inhalation and oral route, respectively. The plasma salbutamol concentrations at T_{max}, C_{max} were calculated as 200–230 ng ml^{-1} for plasma samples from volunteers administered with

Table 1
Mean recovery of salbutamol using different types of elution solvent

Elution solvent	Mean recovery (%) ($n = 3$)	
	1st elution (500 μl)	2nd elution (500 μl)
0.1 *M* Perchloric acid–methanol (50:50, v/v)	49.90±0.40	16.10±0.30
1 *M* Trifluoroacetic acid–methanol (50:50, v/v)	65.30±0.40	25.40±0.50
0.5 *M* Acetic acid–methanol (50:50, v/v)	44.40±0.50	20.80±0.50
Phosphate buffer, pH 2.8–methanol (50:50, v/v)	27.80±0.30	22.10±0.40
1 *M* Hydrochloride acid–methanol (50:50, v/v)	38.60±0.50	17.40±0.30

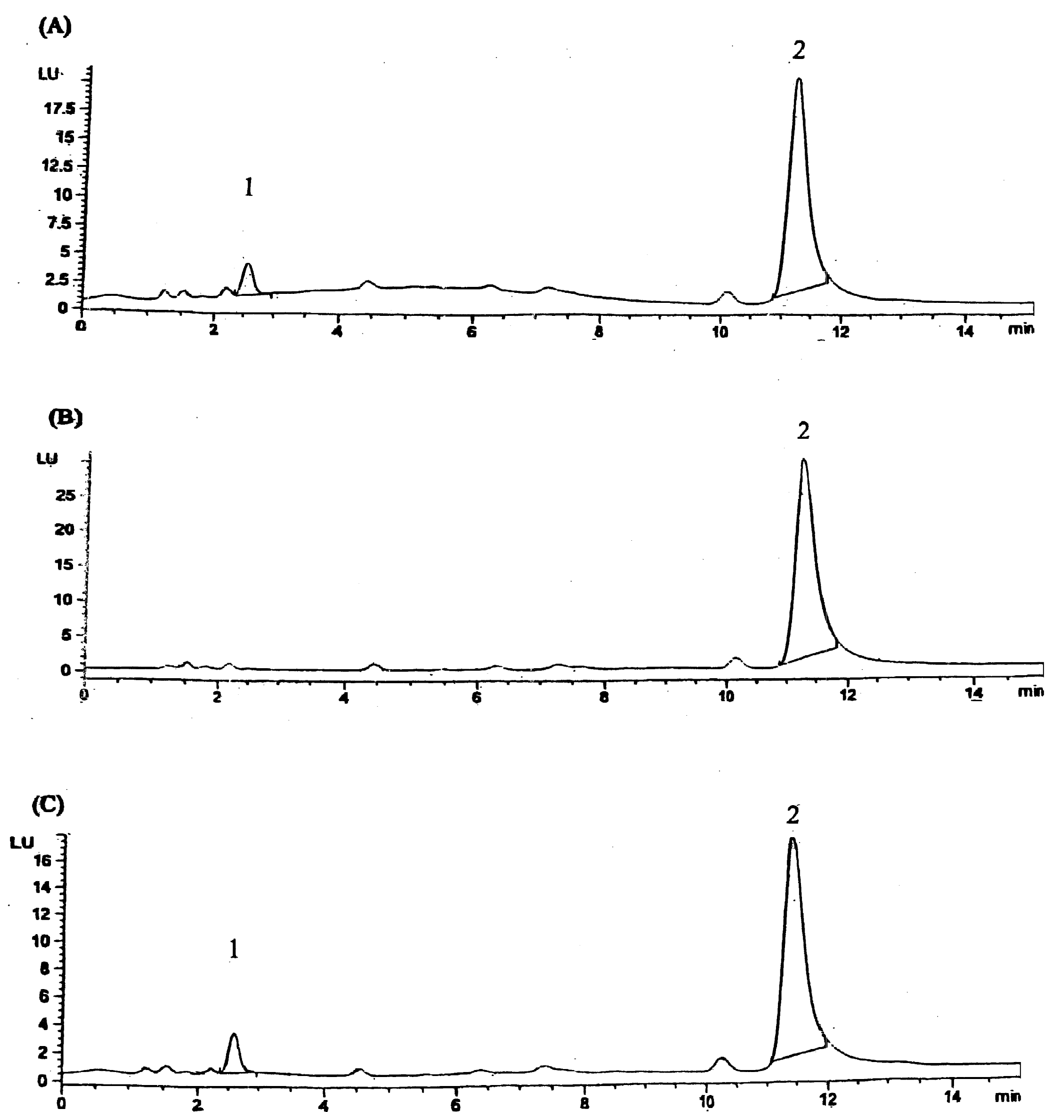

Fig. 2. Typical chromatograms of (A) salbutamol aqueous standard (1 ng μl^{-1}), (B) blank human plasma extract, (C) human plasma extract spiked with salbutamol at 1 ng μl^{-1}. Peaks: 1 = salbutamol; 2 = propranolol (I.S.).

Table 2
Within-day and between-day variation, accuracy and extraction recoveries of salbutamol in spiked plasma samples ($n=6$)

Concentration (ng ml^{-1})	RSD (%)		SD		Mean recovery (%)	
	Within-day ($n=6$)	Between-day ($n=6$)	Within-day ($n=6$)	Between-day ($n=6$)	Within-day ($n=6$)	Between-day ($n=6$)
10	14.30	11.94	0.0035	0.0031	85.90	86.20
100	8.38	6.11	0.0022	0.0154	87.70	90.10
1000	4.28	3.80	0.0856	0.0743	91.30	90.50

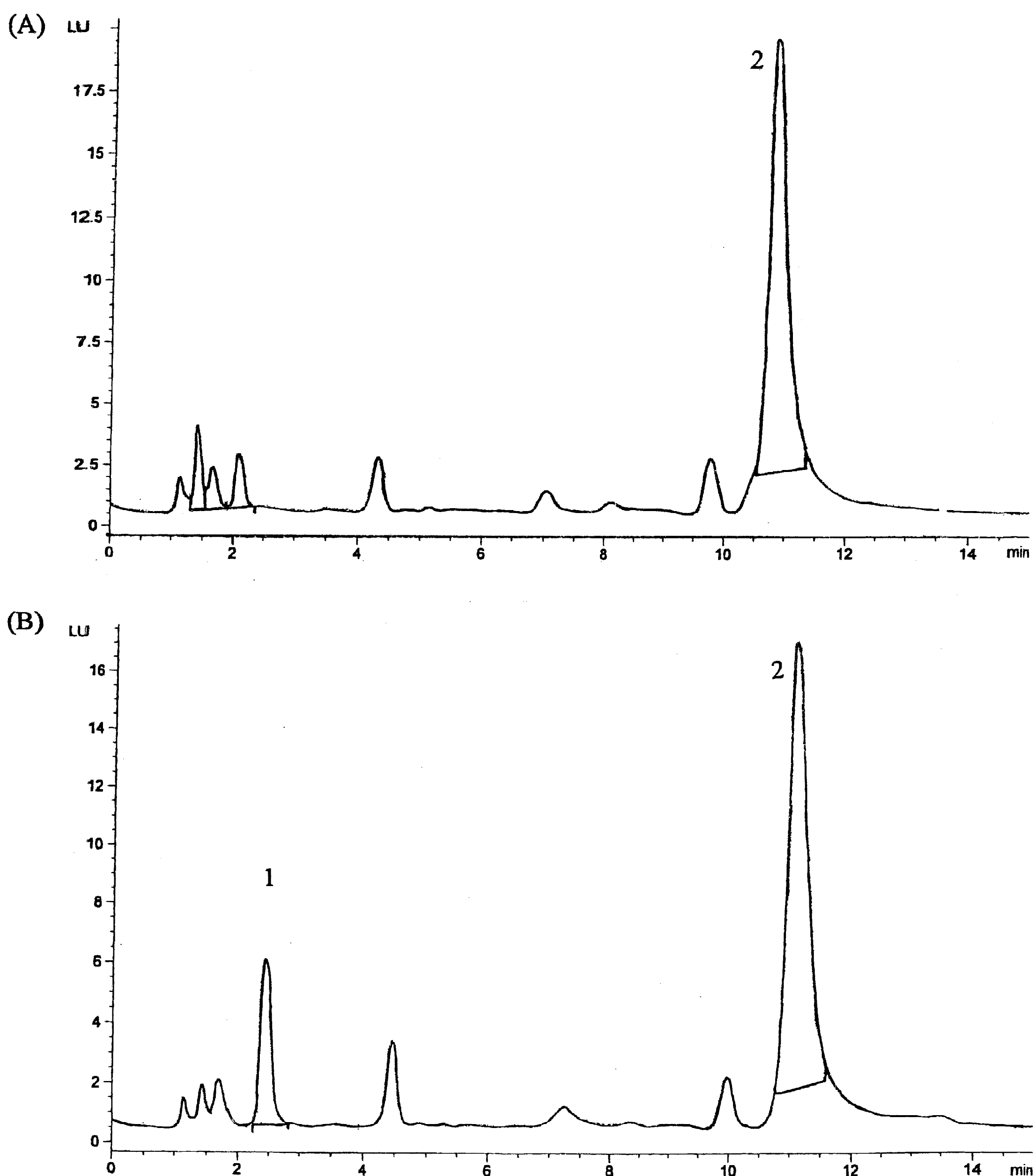

Fig. 3. Chromatograms of plasma extract from a healthy volunteer at (A) 0 h and (B) 3 h after 8 mg oral dose of salbutamol. Peaks: 1 = salbutamol; 2 = propranolol (I.S.).

8 mg oral salbutamol and 50–65 ng ml^{-1} for plasma samples from volunteers inhaling 2 mg salbutamol.

4. Discussion

Weith et al. first reported separation of nucleic acid components and carbohydrates by using boronate affinity chromatography in 1970 [27]. The specificity of boronate, therefore, has been further studied in the separation of a wider range of compounds with *cis*-diol as functional group that included catecholamines, nucleosides, nucleotides, nucleic acids, carbohydrates, glycoproteins and en-

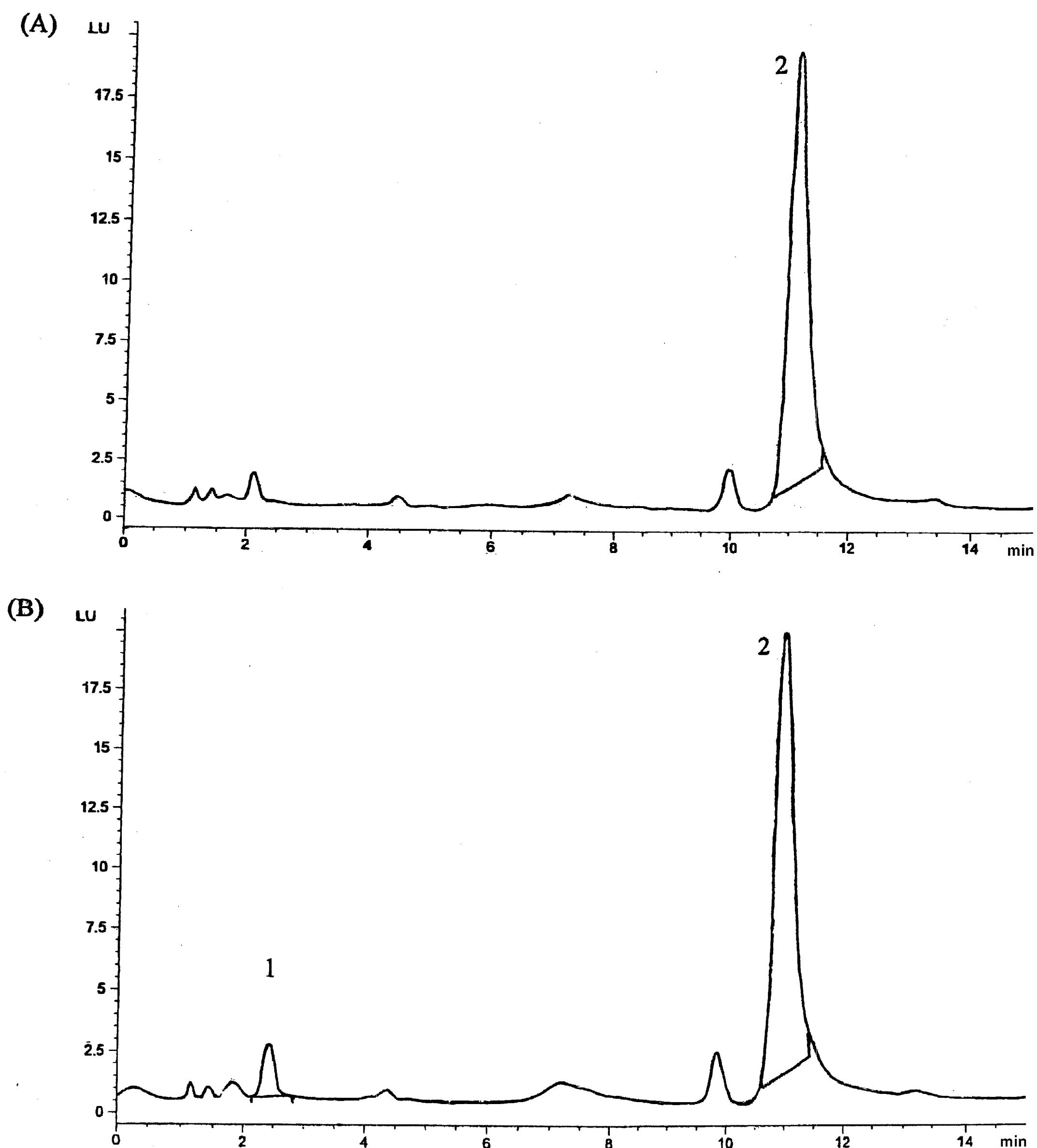

Fig. 4. Chromatograms of plasma extract from a healthy volunteer at (A) 0 h and (B) 1.5 h after 2 mg dose of salbutamol via inhalation. Peaks: 1 = salbutamol; 2 = propranolol (I.S.).

zymes [28]. Basically, these compounds undergo esterification between the boronate ligands and *cis*-diols. However, the major structural requirement is the two hydroxyl groups which should be on adjacent carbon atoms and in an approximately co-planar configuration, that is a 1,2-*cis*-diol. The boronate ester bonds formed are the strongest although interaction also occurs with 1,3-*cis*-diols and *cis*-inositols or triethanolamine [29]. Under basic conditions, boronate in aqueous solution, which has a trigonal co-planar geometry, is hydroxylated yielding a tetrahedral boronate anion that eventually form esters with *cis*-diols as illustrated in Fig. 1. The reaction can be reversed by hydrolysis of the cyclic diester

under acidic condition. Unfortunately, only a few dissociation constants for phenylboronic acid diesters have been reported because the boronate diester bond strength has not been well studied.

Although the basis interaction for boronate affinity chromatography is through boronate and *cis*-diol ester formation, secondary interactions such as hydrophobic, ionic, hydrogen bonding and charge transfer interaction can also play important roles in the process. Hydrophobic interactions occur because almost all boronate ligands used are aromatic boronate ligands. The phenyl ring can also contribute to the $\pi-\pi$ interaction. These interactions cause non-specific adsorption of analytes especially with proteins. Therefore, ionic strength should be low, usually about 50 m*M* in order to reduce the hydrophobic effect. The negative charge of the active tetrahedral boronate can cause ionic attraction or repulsion. The ionic strength should be high in order to reduce the effect, but still should be lower than 500 m*M* to avoid hydrophobic effects. Boronic acid has two hydroxyls (three in the active tetrahedral anion) and therefore contributes to the hydrogen bonding effect. Besides, the boron atom has an empty orbital in a trigonal uncharged boronate and it can serve as an electron receptor for charge transfer interaction. Unprotonated amines are good electron donors and when an amine donates a pair of electrons to boron, the boron atom becomes tetrahedral. This may explain why amines may serve to promote boronate and *cis*-diol esterification. However, if there is a hydroxyl group adjacent to the amine, this can block boronate/*cis*-diol esterification. For this reason, Tris and ethanolamines should be avoided in boronate chromatography binding buffers. Carboxyl groups can also serve as electron donors for charge transfer interaction. Carboxyls can form stable complexes with boronates together with α-hydroxyl groups as demonstrated by the esterification of lactic acid or salicylic acid with boronate in boronate chromatography.

We have previously examined the effect of various analytical variables on the efficiency of the one-step extraction procedure for the extraction of salbutamol from urine samples. The extraction efficiency in terms of recovery and effective sample clean-up was found to be influenced by analytical factors such as the pH of the sample, type of washing solvents, the concentration of acid used for elution and the type of C_{18} SPE sorbent used [26]. The phenyl boronate extraction procedure described has several advantages over conventional liquid phase and solid-phase extraction methods utilizing cation-exchange, cyano, C_{18}, C_8 or boronate sorbent. Firstly, the extraction method described is rapid—requiring only a single purification step to yield sample preparations that show low residual interference when analyzed by HPLC. Secondly, extraction of salbutamol as their phenylboronate complexes using end-capped C_{18} SPE sorbent shows uniform recoveries. Stability and recovery studies showed that once the complexing agent was added to samples, there was no significant loss of salbutamol at alkaline pH.

However, there are some limitations with the proposed extraction method. Since urinary salbutamol recoveries with this extraction procedure fall within the pH range of 9–10, it is necessary to check the pH of the sample prior to extraction. Also, like many other extraction methods previously described for salbutamol, the method suffers from interference by some structurally related drug metabolites e.g. metabolite of paracetamol and labetalol and potentially methyldopa.

The mixture containing the phenylboronate complex was introduced to solid-phase clean-up in order to remove endogenous interference in plasma by retaining the complex in the nonpolar end-capped C_{18} sorbent. A few conditions in SPE extraction steps were studied including the most suitable washing solvent and the most efficient eluting solvent to be used in order to achieve background-free chromatogram and highest recoveries of salbutamol, respectively. We have discovered that washing the sorbent with the same conditioning buffer, 50 m*M* carbonate buffer, pH 9.60, has managed to remove most of the endogenous interference without desorbing the complex. Hence it has provided a rather clean background chromatogram as compared to the other studied washing solvents [26]. Finally, the complex was dissociated and the analyte was eluted from the sorbent. The complexation process can be reversed under acidic condition that led towards the study of using different types of acids. Grossi et al. [23] described a one-step extraction of catecholamines from plasma employing diphenylboronic acid (DPBA) ethanolamine ester and monofunctional

(MF) C_{18} SPE sorbents. Perchloric acid (0.1 *M*)–methanol (9:1, v/v) was used to elute the analytes from the SPE sorbent. Trifluoroacetic acid was preferred to other type of acids because it is quite volatile and the level of protein contamination in plasma can be lowered to the minimum, besides obtaining the highest recovery of salbutamol with 50% of methanol. Although the highest recovery was achieved by using methanol, the chromatograms obtained were having higher background noise compared to the rest of the eluting solvent selection that led to difficulty in peak integration due to the interference caused by the co-eluting peaks. Therefore, a mixture of 1 *M* trifluoroacetic acid–methanol (1:1) was considered as much better desorbing solvent to obtain highest recovery for salbutamol in plasma samples compared to the rest of the possible eluting solvents.

5. Conclusion

In summary, the method described here for the extraction of salbutamol from human plasma and their subsequent measurement by HPLC with fluorimetric detection has shown acceptable precision and adequate sensitivity for use in bioequivalance study of different administration routes of salbutamol. With the obtained results from this study, a complete pharmacokinetics profile that based on both urinary [26] and plasma profiles could be generated to compare the different administration routes of salbutamol. It is an excellent alternative to other published methods to analyze drug residue in biological fluids with its main advantages of being simple, robust, efficient clean-up, effective chromatographic separation and relatively high sample extraction capacity.

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