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Determination of Salbutamol in Human Plasma and Urine by High-Performance Liquid Chromatography with a Coulometric Electrode Array System

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Abstract

A method is developed to determine salbutamol in human plasma and urine using high-performance liquid chromatography (HPLC) with a coulometric electrode array system, based on the electrochemical behavior of salbutamol at graphite electrode. The mobile phase component A is 30mM sodium dihydroxy phosphate–30mM triethylamine and is adjusted to pH 6.0 with 20% phosphate acid. The mobile phase component B is methanol. The optimized mobile phase composition was A and B in the proportion of 90:10 (v/v). Paracetamol is selected as the external standard. The human plasma and urine samples are pretreated using solid-phase extraction cartridges (Sep-Pak Silica), and the eluting solution is monitored by the coulometric electrode array system. The electrode potentials are set at 300, 400, 550, and 650 mV, respectively. Calibration curves show good linearity, and the recovery of salbutamol proves to be constant and unaffected by the concentration of the drug. This method, developed using HPLC–electrochemical detection, is reproducible and sensitive enough for the determination of salbutamol in human plasma and urine.

Introduction

Salbutamol is a selective β_2-adrenoceptor agonist in animal and man and has been suggested for the treatment of asthma (1). Recently, the growth promotion effects of β_2-agonist and their illegal use in animal feed have drawn substantial attention. When animals are treated with β_2-agonist, residues can accumulate in their body, which can result in human toxicity (2). Therefore, the use of β_2-agonists in meat-producing animals is now banned (3). Moreover, the use of salbutamol, as well as other agonists, has been banned by the International Olympic Committee because the misuse of β_2-agonists by athletes has been demonstrated (4).

Several analytical methods have been carried out by many laboratories for the detection of salbutamol in biological fluids, such as high-performance liquid chromatography (HPLC) with fluorescence detection (5–9), HPLC–mass spectrometry (MS) (10,11), and gas chromatography (GC)–MS (12,13). Plasma and urine were the samples mostly used for the analyses of salbutamol. Both liquid–liquid extraction (7,14) and solid-phase extraction (SPE) (5,6,8,9,12) can be used as the sample cleanup procedure.

The low plasma concentration of salbutamol following therapeutic doses required an assay with high sensitivity. Because salbutamol contains an electroactive phenolic hydroxyl group, electrochemical detection (ECD) is an ideal choice. The amperometric detection was employed in all HPLC–ECD methods (14–16) reported to determine salbutamol existing in biological matrices. The efficiency of an amperometric electrode is greatly affected by surface area contamination caused by electro-deposition and adsorption. The coulometric electrode used in this paper, however, due to its enlarged surface area, remains unaffected until more than 90% of the electrode is fouled. Thus, it need not be polished daily, as the amperometric electrode. The coulometric electrode array detection consisted of 16 electrochemical cells arranged in series, and the potential of each cell can be set independently. The porous-graphite-working, palladium-reference, and platinum-counter electrodes were used in each cell. A piece of chromatogram collected from the system contained a number of curves, which allowed for the identification of the compound of interest based on the retention time and its oxidation (reduction) characteristic on several traces. Thus, the coulometric detection system can offer superior sensitivity over other amperometric detectors commonly used with HPLC, and coeluting analytes can be resolved by differences in their electrochemical behavior. Therefore, coulometric electrode array detection was chosen to analyze the concentration of salbutamol.

This paper presented an accurate and reproducible HPLC assay for salbutamol in human plasma and urine using the coulometric electrode array detector. Samples were successively pretreated using SPE cartridges to remove endogenous

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interference. The influence of the pH of the mobile phase on the retention factor of salbutamol was considered, and the optimized potentials of electrodes were selected based on the current-potential curve.

Experimental

Chemicals and reagents

Salbutamol (Figure 1) and paracetamol (Figure 2) were obtained from Sigma (St. Louis, MO). Sodium dihydrogen phosphate, triethylamine, acetic acid, and phosphate acid were analytical grade from Sigma. Methanol was HPLC-purity reagent from Fisher Scientific (Fair Lawn, NJ). The water used in all experiments had a resistivity of 18.2MΩ·cm and was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Sep-Pak Silica columns (200 mg/3mL) were purchased from Waters. Stock standard solutions of salbutamol and paracetamol (external standard) in a concentration of 0.2 mg/mL were prepared in methanol and were stable at 4°C for several months. The spiking solutions of 1 µg/mL were made by diluting 0.5 mL stock solutions to 100 mL with the mobile phase.

Apparatus

The HPLC system used was an ESA chromatographic system (ESA, Chelmsford, MA) equipped with two 582 pumps, an organizer chamber, a PEEK pulse damper, a manual injector fitted with a 20-µL loop (Rheodyne 7725i, Rohnert Park, CA), and a 5600A sixteen channels CoulArray detector (ESA). In this method, analytes were detected on four channels attached in series after the HPLC column. The ESA software was used for data acquisition and processing. In addition, a Sartorius BS 110S electronic balance (Beijing, China) and WTW inolab pH level 1 pH meter (Weiheim, Germany) were used in the study.

Chromatographic conditions

Salbutamol was analyzed by reversed-phase HPLC using an ODS Hypersil (150- × 4-mm i.d., 5 µm, Hewlett-Packard, Palo Alto, CA) column. A guard column (Hypersil, 5 µm, Alltech, Deerfield, IL) was used to protect the analytical column. The mobile phase component A was 30mM sodium dihydroxy phosphate–30mM triethylamine and was adjusted to pH 6.0 with 20% phosphate acid. The mobile phase component B was methanol. A 90:10 (v/v) mixture of mobile phase component A and B was used in the method, and the flow rate was held constant at 1 mL/min. The mobile phase was always freshly prepared, filtered, and sonicated before use. The column effluent was monitored using the CoulArray electrochemical detector with porous graphite electrodes operated in the oxidative screen mode. The potentials of the electrodes were set at 300, 400, 550, and 650 mV. Typical retention times obtained with the final system were 4.3 and 5.1 min for salbutamol and paracetamol.

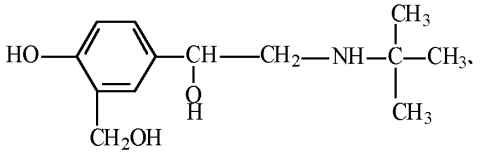

Figure 1. Chemical structure of salbutamol.

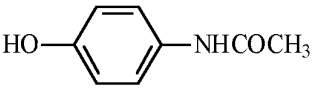

Figure 2. Chemical structure of paracetamol.

Solid–liquid extraction procedure

Plasma and urine samples were obtained by centrifugation at 3000 × *g* and stable at –20°C for up to one month. The Sep-Pak Silica column was pretreated with 1 mL 0.5% acetic acid–methanol solution, followed by 2 mL 1% acetic acid. One milliliter plasma or urine sample was applied to the cartridge at a flow of approximately 0.5–1.0 mL/min.The column was dried under high vacuum and washed with 2 mL 1% acetic acid. The column was dried again under high vacuum. Salbutamol was then eluted with 2 mL of 0.5% acetic acid–methanol solution. To the elute was added 20 or 100 ng working external standardfor plasma or urine sample and then vortex mixed and evaporated at 45°C under a continuous flow of nitrogen. The residue was redissolved in 1 or 5 mL of mobile phase for the plasma or urine sample, respectively, and 20 µL was injected in the HPLC–ECD system.

Method validation

The method was validated by determining the following operational characteristics: linearity range, selectivity, limit of detection (LOD), recovery, precision, and accuracy.

Blank plasma and urine samples were spiked with salbutamol over the ranges 0.84–84.0 and 4.2–420.0 ng/mL, respectively, to determine the linearity of the method. The calibration samples in each concentration were extracted in triplicate using the outlined method and then analyzed. The sum of the peak area of salbutamol and paracetamol at all sensitive channels was measured, respectively, and the peak area sum ratio of salbutamol over the external standard was plotted against concentration.

The selectivity of the method was studied by analyzing ten blank plasma and urine samples and checking any interference presented at the retention time of salbutamol and paracetamol at the same potential.

The LOD was estimated using a signal-to-noise ratio of 3:1. The extraction recovery of salbutamol was assessed at three concentration levels: 2, 20, and 80 ng/mL for blank plasma and 10, 100, and 400 ng/mL for blank urine. Similarly, two sets of aqueous standards were prepared with concentrations equal to those of spiked plasma and urine standards. To simulate the incurred samples, salbutamol was added to the blank plasma and urine, mixed, and the mixtures stored in the refrigerator for 24 h. The spiked plasma and urine samples were analyzed

with the method described. The aqueous standards were dried by rotary evaporator, and the residues were dissolved in 1- or 5-mL mobile phase. The spiked plasma and urine samples were run in triplicate, whereas the spiked water solutions were run in duplicate in three separate runs.

Precision and accuracy were assessed at performing replicate analyses of spiked samples. Three different concentration samples within the calibration range were prepared and analyzed with single-point calibration to determine intraday (six replicates per concentration) and interday (six replicates per concentration over 1–3 days) variability. The intra- and interday precisions were determined as the relative standard deviation (%RSD) and accuracy as the percentage relative error (%RE).

Results and Discussion

Optimization of the pH of the mobile phase

Initial experiments were carried out using a mobile phase containing 30mM sodium dihydrogen phosphate and methanol in different proportions, however, satisfactory chromatographic separation could not be achieved because of a poor peak shape for salbutamol. This was because the amido group in salbutamol molecule can unite with the sorbent of the column, which lead to the tailed peak. Triethylamine is an organic modifier added to the mobile phase in order to reduce the tailing effect. After that, the pH of the mobile phase was found to have an affect on the retention factor of salbutamol and paracetamol (Figure 3). From Figure 3, it is shown that the retention factor of salbutamol increased with the rise of pH, but the retention factor of paracetamol increased gradually before pH 6.0, and, after that, it decreased sharply. In order to achieve the appropriate retention time and desired resolution factor, the following experiments were carried out at pH 6.0.

Voltammetric behavior of salbutamol and paracetamol

Proper selection of applied electrode potentials was critical for accurate, interference-free measurement. A 1-µg/mL solution of salbutamol and paracetamol was prepared, respectively, by diluting 0.5 mL of stock standard solution (0.2 mg/mL) to 100 mL with mobile phase. Two electrodes were selected. The potential of electrode 1 (upstream electrode) was set at 50 mV, and the potential of electrode 2 (downstream electrode) at 800 mV, initially. Twenty microliters of the 1-µg/mL standard solution was injected, and the peak height of salbutamol at electrode 1 was recorded. The potential of electrode 1 was increased by 50 mV. The procedure was repeated until increases of potential did not result in significant changes in peak height. The relationship between the peak height (current) and set potential (voltage) is shown in Figure 4. From Figure 4, it can be seen that salbutamol can be oxidized at 400 mV and can reach a plateau at 650 mV, whereas no increase in peak height occurred when the potential was increased. Paracetamol can be oxidized at 150 mV and can reach a plateau at 400 mV. Based on the curve, the following potentials were selected for electrodes: 300, 400, 550, and 650 mV. Both salbutamol and paracetamol contain an electroactive phenolic hydroxyl group in their molecules; therefore, they can oxidize at positive potential. The higher potential value for salbutamol is probably a consequence of the more electrophilic CH_2OH substituent in this compound. No oxidation of salbutamol occurred at the first and second electrodes. Although greater response could be obtained with higher potential than 650 mV, the background noise also increased. Therefore, the potentials of 550 and 650 mV were selected to provide adequate sensitivity for salbutamol determination. Paracetamol could be oxidized completely at 300 and 400 mV, and the first two electrodes also could be used to remove the interference that may coelute with salbutamol and can be oxidized at low potential.

Another characteristic obtained when using the array of electrodes at potentials along the oxidative curve of each analyte is that the peak area response ratios between adjacent channels were descriptive of the voltammetric behavior of each analyte. Comparison of these ratios between the authentic standardand sample provided an estimate of the purity of each analyte peak in the sample. The peak area response ratios obtained for salbutamol in the standard were automatically calculated to be 1.06 for E4/E3. The ratios obtained from the plasma and urine sample area identified as salbutamol were

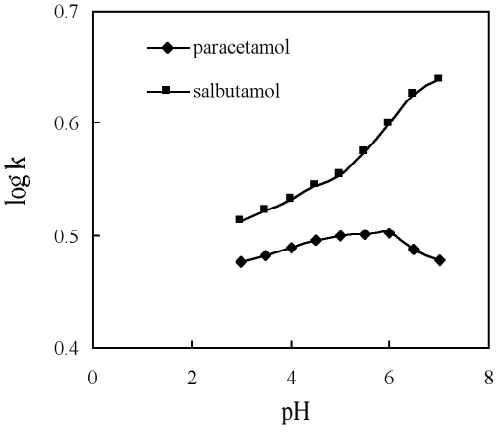

Figure 3. Log k-pH curve of salbutamol and paracetamol.

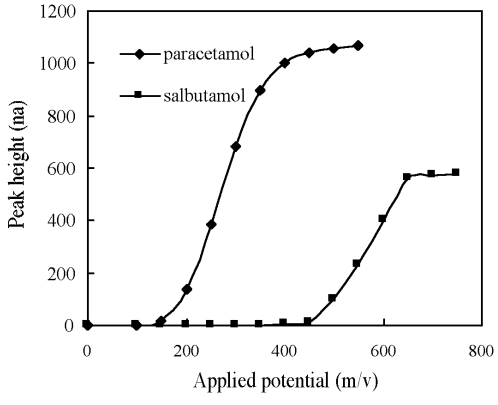

Figure 4. C-V curve for the oxidation of salbutamol and paracetamol.

Table I. Precision and Accuracy of the HPLC–ECD Analysis of Salbutamol from Spiked Human Plasma and Urine Samples (n = 6)

Sample	Theoretical concentration (ng/mL)	Concentration found (ng/mL)			Accuracy (%RE)
		Mean	SD	%RSD	
Plasma (intraday)	2	1.882	0.116	6.16	5.9
	20	19.64	0.85	4.33	1.8
	80	79.60	3.64	4.57	0.5
Plasma (interday)	2	1.746	0.129	7.39	12.7
	20	18.72	0.992	5.30	6.4
	80	75.84	3.84	5.06	5.2
Urine (intraday)	10	9.43	0.575	6.09	5.7
	100	99.0	4.56	4.60	1.0
	400	398.8	21.0	5.26	0.3
Urine (interday)	10	9.03	0.735	8.13	9.7
	100	94.8	4.83	5.09	5.2
	400	376.8	25.81	6.84	5.8

1.08 and 1.12 for E4/E3. The E4/E3 ratio in the standard divided by the E4/E3 ratio in the unknowns equaled 1.02 and 1.06. Because the sample ratio accuracy was within 20% of 1.06, the samples were deemed as the same compound as the standard.

Method validation

The concentrations of calibration standards for plasma were 0.84, 1.68, 3.36, 8.4, 16.8, 33.6, and 84.0 ng/mL, and the concentrations for urine were 4.2, 8.4, 16.8, 33.6, 84.0, 168.0, and 420.0 ng/mL. These standards were treated under the experimental conditions described and analyzed in triplicate. The calibration curve showed good linearity in the selected concentration range. The correlation coefficient was 99.86% and 99.91%

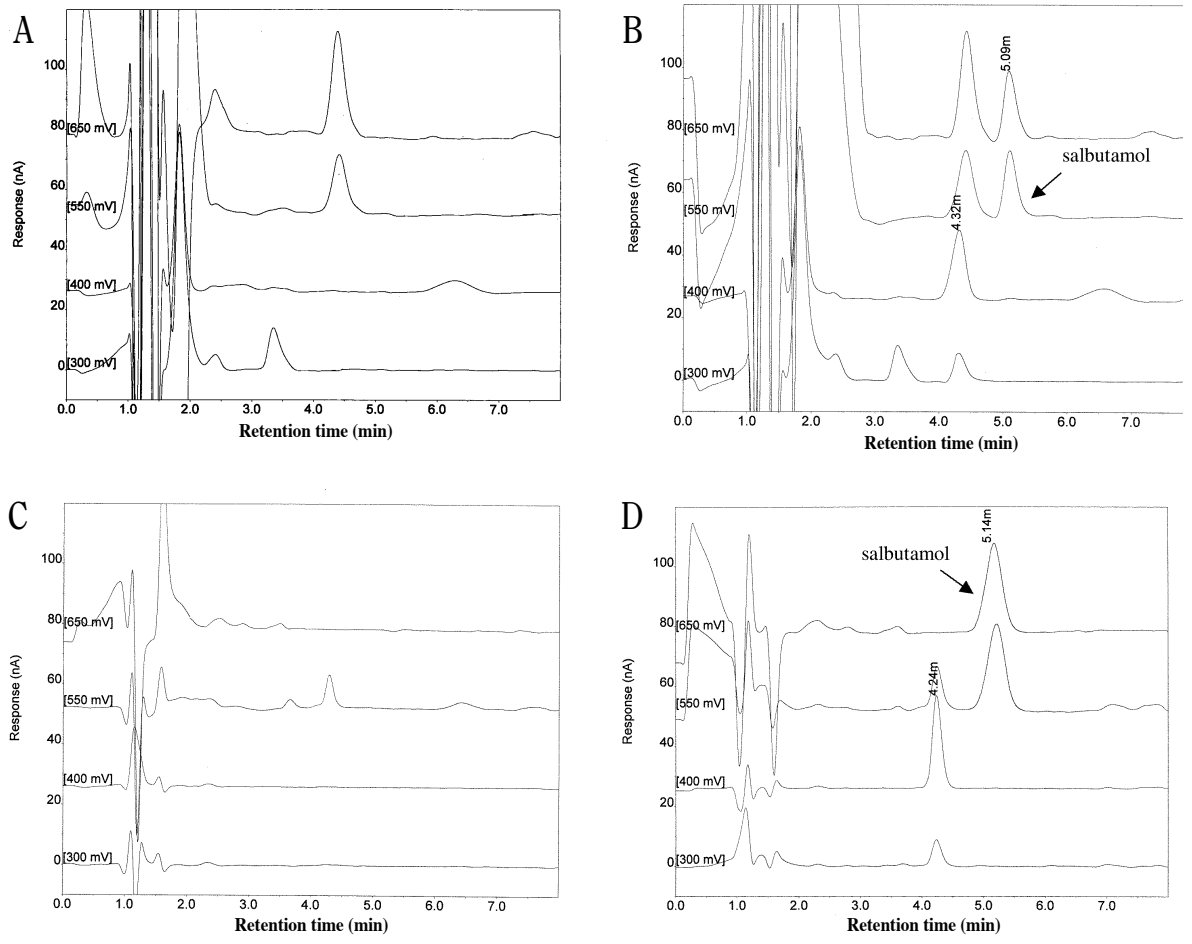

Figure 5. HPLC chromatograms from (A) blank plasma, (B) plasma collected from a patient administered with salbutamol, (C) blank urine, and (D) urine collected from a patient administered with salbutamol.

for the plasma and urine samples, respectively.

Ten blank plasma and urine samples were checked using the method developed, and no interference was found at the retention time of salbutamol and paracetamol at the same potential. Under the experimental conditions described, the minimum detectable concentration was 0.5 and 2.5 ng/mL for plasma and urine sample, respectively. The mean recovery of the extraction procedure was 77.32% and 81.25% for plasma and urine, respectively. Precision and accuracy results are shown in Table I, which demonstrate good precision and accuracy over the concentration range selected.

Sample analysis

Plasma and urine samples were collected from a single volunteer who was administered with salbutamol (8 mg) orally. The concentrations of the incurred samples were determined by the calibration curve. The chromatograms are shown in Figure 5. The samples tested positive for the presence of salbutamol, containing 12.6 and 23.2 ng/mL salbutamol in plasma and urine, respectively.

Electrode maintenance

The procedure devised showed a good reproducibility, however, before obtaining reproducible results, some care must be taken. For example, the sensitivity of the working electrodes gradually declined, and cleaning of these electrodes was necessary to restore them. Although the coulometric electrode remained unaffected by poisoning until more than 90% of the electrode surface was fouled, the electrodes were cleaned at the end of each working day in order to prolong the using time of electrodes and to get the reproducible results. The most rapid method for restoring the detector response was to keep all the electrodes at 900 mV for 1 or 2 min while the mobile phase continued to flow.

Conclusion

In summary, the method was precise and accurate for the determination of salbutamol in human plasma and urine. Electrochemical detection was chosen to enhance selectivity, as well as sensitivity, for salbutamol in biological matrix.

References

1. M.L. Powell, M. Weisberger, Y. Dowdy, and R.P. Gural. Comparative steady state bioavailability of conventional and controlled-release formulations of albuterol. *J. Biopharm. Drug Dispos.* **8:** 461–68 (1987).
2. L. Salleras, A. Dominguez, E. Mata, J.L. Taberner, I. Moro, and P. Salva. Epidemiologic study of an outbreak of clenbuterol poisoning in Catalonia, Spain. *Public Health Rep.* **110:** 338–42 (1995).
3. Council directive 96/22/EC. Concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists. *Off. J. Eur. Commun.* **L125:** 3 (1996).
4. International Olympic Committee. Prohibited classes of substances and prohibited methods. In *IOC Medical Code and Explanatory Document.* IOC, Lausannc, Switzerland, 1995.
5. D.W. Boulton and J.P. Fawcett. Determination of salbutamol enantiomers in human plasma and urine by chiral high-performance liquid chromatography. *J. Chromatogr. B* **672:** 103–109 (1995).
6. R.E. Bland, R.J.N. Tanner, W.H. Chern, J.R. Lang, and J.R. Powell. Determination of albuterol concentrations in human plasma using solid-phase extraction and high-performance liquid chromatography with fluorescence detection. *J. Pharm. Biomed. Anal.* **8:** 591–96 (1990).
7. D.J. Morgan, J.D. Paull, B.H. Richmond, E. Wilson-Evered, and S.P. Zicconc. Pharmacokinetics of intravenous and oral salbutamol and its sulphate conjugate. *Br. J. Clin. Pharm.* **22:** 587–93 (1986).
8. R. Berges, J. Segura, X. de la Torre, and R. Ventura. Analytical methodology for enantiomers of salbutamol in human urine for application in doping control. *J. Chromatogr. B* **723:** 173–84 (1999).
9. M. Hindle and H. Chrystyn. Determination of the relative bioavailability of salbutamol to the lung following inhalation. *Br. J. Clin. Pharmacol.* **34:** 311–15 (1992).
10. L. Malkki-Laine and A.P. Bruins. Structural characterization of the decomposition products of salbutamol by liquid chromatography-ionspray mass spectrometry. *J. Pharm. Biomed. Anal.* **12:** 543–50 (1994).
11. D.R. Doerge, M.I. Churchwell, C.L. Holder, L. Rowe, and S. Bajic. Determination and confirmation of β2-agonists in bovine retina using LC-APCI/MS. *Anal. Chem.* **68:** 1918–23 (1996).
12. X. Liu, Y.Z. Zhang, C.J. Zhang, and L. Ye. Determination of salbutamol in human urine. *Acta Pharm. Sin.* **29:** 454–58 (1994).
13. A. Solans, M. Carnicero, R. de la Torre, and J. Segura. Comprehensive screening procedure for detection of stimulants, narcotics, adrenergic drugs, and their metabolites in human urine. *J. Anal. Toxicol.* **19:** 104–14 (1995).
14. Y.K. Tan and S.J. Soldin. Determination of salbutamol in human serum by reversed-phase high performance liquid chromatography with amperometric detection. *J. Chromatogr.* **311:** 311–17 (1984).
15. T. Emm, L.J. Lesko, J. Leslie, and M.B. Perkal. Determination of albuterol in human serum by reversed-phase high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **427:** 188–94 (1988).
16. K.A. Sagar, C. Hua, M.T. Kelly, and M.R. Smyth. Analysis for salbutamol in human plasma by high-performance liquid chromatography with electrochemical detection using a micro-electrochemical flow cell. *Electroanalysis* **4:** 481–86 (1992).

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