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DETERMINATION OF SALBUTAMOL IN HUMAN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A simple and sensitive method for the quantitative determination of salbutamol in human serum using reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection is described. The method involves the combined use of Sep-Pak® cartridges and ion-pair extraction for sample clean-up, and subsequent separation of salbutamol and the internal standard from interfering compounds on a reversed-phase column. An amperometric detector incorporating a glassy carbon electrode was employed for detection. The inter-assay coefficients of variation at plasma concentrations of 2.0, 6.0 and 20.0 ng/ml were 7.3%, 7.2% and 8.5%, respectively (n = 20). The minimum detection limit was 400 pg/ml from a 0.5-ml sample of serum. The method can be readily utilised for clinical pharmacokinetic studies.

INTRODUCTION

Salbutamol, 2-*tert.*-butylamino-1-(4-hydroxymethyl)phenylethanol, is a β_2-adrenoreceptor agonist widely used in the treatment of asthma. Although its pharmacology is well documented [1, 2], pharmacokinetic information on salbutamol is limited largely due to the difficulty in measuring the drug at therapeutic concentrations. Thus far, reported assays for salbutamol have employed liquid scintillation spectrometry [3], gas chromatography—mass spectrometry [4, 5], high-performance liquid chromatography (HPLC) with a rotated disc amperometric detector [6] and more recently, HPLC with fluorescence detection [7, 8]. Some of these methods suffer from the disadvantages of a lack of sensitivity [3], the use of laborious extraction procedures and elaborate equipment [4, 5] and the apparent short lifespan of column and electrode [6]. We have exploited the sensitivity of amperometric detection

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which is capable of measuring catecholamines in the picogram range [9] to quantitatively determine the low therapeutic levels of the electrochemically active salbutamol.

In this report, a sensitive and simple HPLC method coupled with amperometric detection for the determination of salbutamol in human serum is described. This method includes initial sample clean-up with a Sep-Pak® cartridge followed by ion-pair extraction, and subsequent separation on a reversed-phase column with a phosphate buffer—methanol mixture. Selectivity was enhanced by the use of 1-heptanesulfonic acid, an ion-pair reagent in the mobile phase which thereby created a weak cation-exchange column. Pharmacokinetic studies have been carried out on human subjects receiving oral salbutamol using this HPLC—amperometric detection method.

EXPERIMENTAL

Chemicals and standards

Salbutamol sulphate and the internal standard fenoterol bromide were a generous gift from Glaxo Canada (Toronto, Canada) and Boehringer Ingelheim (Burlington, Canada), respectively. Di(2-ethylhexyl) phosphate (DEHP) was a synthetic-grade reagent obtained from Sigma (St. Louis, MO, U.S.A.). Glass-distilled ethyl acetate, Accusolv methanol and chloroform (without ethanol preservative) were purchased from BDH (Toronto, Canada), Anachemia (Mississauga, Canada) and Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), respectively. HPLC-grade sodium 1-heptanesulphonic acid used as ion-pair reagent was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Reagent A was a 70 m*M* sodium phosphate buffer ($NaH_2PO_4 \cdot H_2O$ from Fisher Scientific), pH 6.8, containing 1 m*M* chloride ions, and 2.0 m*M* sodium 1-heptanesulphonic acid. The buffer was filtered through a 0.45-μm membrane before use. Stock 10 mg/ml solutions of fenoterol and salbutamol were prepared in 0.1 *M* hydrochloric acid and stored at 4°C. All working solutions were prepared by dilution with distilled water.

Chromatographic conditions

A high-performance liquid chromatograph, Model 6000A solvent delivery system equipped with a Model U6K injector, both from Waters Assoc. and an Altex Ultrasphere 3-μm ODS column (7.5 cm × 4.6 mm, Beckman, Berkeley, CA, U.S.A.) were employed. The detector system comprised a BioAnalytical System Model LC-4 amperometric detector incorporating a TL-5 thin-layer glassy carbon working electrode and Ag/AgCl reference electrode (BioAnalytical Systems, West Lafayette, IN, U.S.A.) and connected to a Perkin-Elmer Model 024 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.). The detector was typically used at a sensitivity of 10 nA full scale and an applied potential of +0.80 V.

The mobile phase was a mixture of 25% (v/v) methanol in reagent A. Before use, it was degassed in an ultrasonic bath for about 2 h. The flow-rate was 0.5 ml/min and all separations were performed at room temperature.

Sample preparation

To 0.5 ml serum sample diluted with 1.0 ml distilled water 15 μl of internal standard solution (100 ng/ml) were added. This solution was slowly forced through a Sep-Pak cartridge which was previously washed with 10 ml of methanol and 10 ml of water. The cartridge was washed twice with 2 ml of water and the drug and internal standard were then eluted with 2 ml of methanol — the first two drops of eluate were discarded. The methanol was evaporated to dryness under a nitrogen stream at 40°C. Salbutamol and fenoterol in the dry residue were extracted as ion-pair with DEHP by vortexing vigorously for 1 min with 70 μl of reagent A and 300 μl of 0.05% (v/v) solution of DEHP in ethyl acetate. After centrifugation (5000 *g* for 30 sec) the organic phase was transferred to a second microtube (6 × 50 mm) containing 40 μl of reagent A and the vortexing step was repeated. The phases were again separated by centrifugation and the ethyl acetate phase transferred to a third microtube containing 70 μl of 10 m*M* hydrochloric acid into which salbutamol and fenoterol were back-extracted by vortexing for 1 min. Following centrifugation the organic layer was discarded and the acid was briefly washed with 150 μl of chloroform. A 40—60 μl volume of the aqueous phase was injected into the chromatograph after centrifugation.

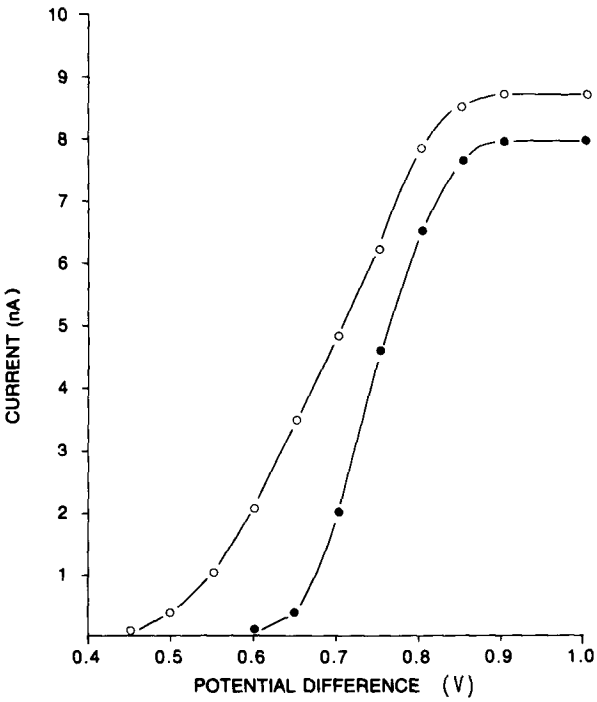

Fig. 1. Hydrodynamic voltammograms for salbutamol (•) and fenoterol (○) on injection of 4.0 ng of salbutamol and 2.0 ng of fenoterol employing the chromatographic conditions described in the text.

RESULTS AND DISCUSSION

Chromatography

Hydrodynamic voltammograms (i.e. profiles of current versus potential) for salbutamol and fenoterol were determined over the range of 0.40—1.00 V in the mobile phase described and are shown in Fig. 1. An applied potential of +0.60 V is required to initiate an electrochemical response for salbutamol whereas a response to fenoterol is initiated at a considerably lower potential. The applied potential was chosen as +0.80 V because sufficient sensitivity could be obtained at this voltage with minimal background noise.

Fig. 2 shows typical chromatograms of extracted blank plasma and plasma containing salbutamol at a concentration of 3.0 ng/ml and at a detector sensitivity of 10 nA full scale. Because the internal standard has almost twice the retention time to that of salbutamol, one chromatographic run requires about 14 min.

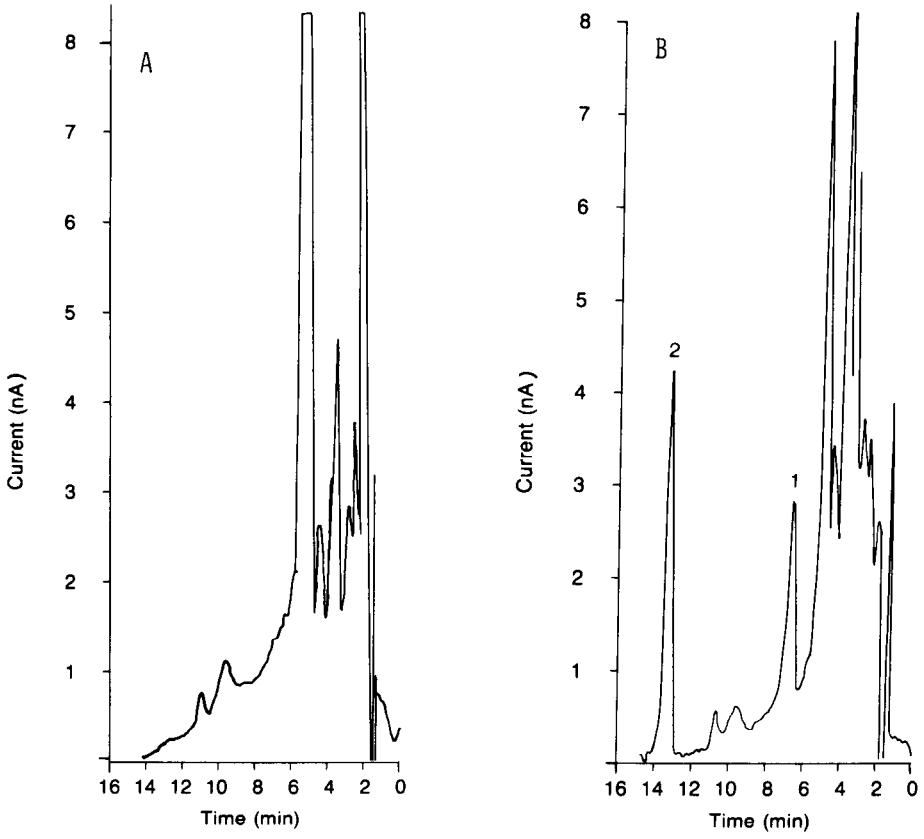

Fig. 2. Chromatograms obtained with (A) blank plasma and (B) plasma containing 3.0 ng/ml salbutamol. Peaks: 1 = salbutamol, 2 = fenoterol (internal standard).

Selectivity

The selectivity of the method was investigated at the retention times of salbutamol and fenoterol. No endogenous interference was found in

chromatograms of samples extracted from 25 pools of plasma. Also, replicate analyses of serum samples by this method containing therapeutic concentrations of theophylline, carbamazepine, phenytoin, phenobarbital, ethosuximide, primidone, valproic acid and gentamycin revealed no interfering peaks. Furthermore, injection of solutions of sympathomimetic agents such as epinephrine, phenylephrine, terbutaline, isoproterenol, metaproterenol, metaraminol, isoxsuprine, dobutamine, buphenine, phentolamine and oxymetazoline did not reveal any peaks with retention times similar to those of salbutamol or fenoterol. However, when heparin is used as an anticoagulant it was found to give an interfering peak with the same retention time as the internal standard. Heparinised plasma is therefore not recommended for the analysis described above.

Recovery

The percentage analytical recovery of salbutamol and fenoterol was measured by comparing the peak heights obtained from the injection of known quantities of the pure compounds with those obtained from the direct injection of extracted plasma samples spiked with three different concentrations of salbutamol. Using this procedure the percentage analytical recovery of salbutamol at various concentrations averaged 79% and that of fenoterol 73% (Table I).

TABLE I

RECOVERY DATA FOR SALBUTAMOL ASSAY (n = 12)

Plasma salbutamol concentration (ng/ml)	Percentage recovery (mean ± S.D.)	Coefficient of variation (%)
20.0	79 ± 4.8	6.1
6.0	79 ± 4.9	6.2
2.0	80 ± 4.3	5.4
Amount of internal standard fenoterol added		
1.5	73 ± 4.4	6.0

Precision and accuracy

The precision and accuracy of the method were assessed by the repeated analysis of a drug-free plasma pool to which salbutamol had been added to provide a series of concentrations ranging from 2.0 to 20.0 ng/ml. Twelve replicate samples at each of the three concentrations were used in the assessment of the within-day variability while between-day variability was assessed for twenty days over a one-month period employing samples which were stored frozen at −20° C; the results are presented in Table II.

Quantitation

The peaks on the chromatogram are identified by their retention time

relative to that of the internal standard. Quantitation was done by comparison of the peak height ratio of salbutamol to fenoterol in the unknown sample to those of control samples containing known quantities of salbutamol, extracted and chromatographed in exactly the same way. Concentration and peak height ratio were verified to be linearly related throughout the concentration range investigated, 1.0—20 ng/ml, yielding a correlation coefficient of 0.9946 and a linear regression equation of $Y = 0.266X - 0.036$.

TABLE II

WITHIN-DAY AND BETWEEN-DAY VARIABILITY OF SALBUTAMOL ASSAY

Salbutamol concentration (ng/ml)	Within-day precision (n = 12)		Between-day precision (n = 20)	
	Mean ± S.D. (ng/ml)	Coefficient of variation (%)	Mean ± S.D. (ng/ml)	Coefficient of variation (%)
2.0	1.97 ± 0.14	7.1	2.06 ± 0.17	8.3
5.0	5.04 ± 0.31	6.2		
6.0			5.84 ± 0.42	7.2
10.0	9.80 ± 0.66	6.7		
20.0			19.9 ± 1.47	7.4

Sensitivity and detection limit

With the above analysis set at a sensitivity of 10 nA full scale and a detector potential of +0.80 V versus Ag/AgCl reference electrode, the detection limit taken as a signal-to-baseline noise ratio of 2 was judged to be 400 pg/ml. This limit can be further lowered by either doubling the detector sensitivity or employing a larger serum volume.

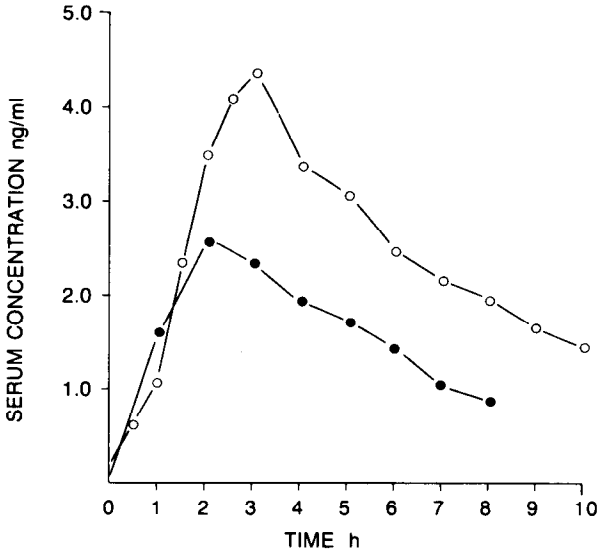

Fig. 3. Serum concentration—time curves following oral administration of 2 mg of salbutamol to two healthy adult volunteers with a body mass of 53 kg (○) and 62 kg (●).

Clinical studies

The HPLC—amperometric detection method described above has been applied to acquire clinical pharmacokinetic data. Fig. 3 shows two serum concentration—time curves in two healthy adults following an oral administration of 2 mg of salbutamol. The peak serum concentration occurred between 2 and 3 h post-dose and the elimination half-lives were between 4 and 5 h. The parameters can be expected to vary between individuals. As fenoterol, also an antiasthmatic agent, is used as the internal standard, the present method cannot be applied to serum from patients receiving simultaneous medication. The assay described for salbutamol can be readily adapted to investigate the fenoterol serum concentration in an asthmatic patient receiving the latter drug. However, we have found that it lacked adequate sensitivity to measure fenoterol concentration following a 2.5-mg oral dose, even with a detection limit of 100 pg/ml. Salbutamol pharmacokinetic studies including concentration—response and bioavailability assessment in healthy and/or asthmatic subjects are currently in progress.

CONCLUSION

We have successfully developed a simple and yet highly sensitive HPLC—amperometric detection assay for the determination of salbutamol in human serum. The method possesses good selectivity and reproducibility and superior sensitivity over all existing salbutamol assays to date [4—8]. The procedure generally requires only 0.5 ml of serum. Acquisition of salbutamol pharmacokinetic and pharmacodynamic information is now feasible.

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