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DETERMINATION OF SALBUTAMOL IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A rapid, accurate and sensitive method for the determination of salbutamol in plasma and urine is described. Salbutamol is extracted using solid-phase techniques and converted to an indoaniline dye by reaction with dimethyl-*p*-phenylenediamine. The indoaniline is separated using high-performance thin-layer chromatography and quantified by absorption microdensitometry at 650 nm. The method is sensitive down to 20 ng/ml in urine and to 1 ng/ml in plasma and provides data in good agreement with that obtained by gas chromatography—mass spectrometry. The method can be used for analysis of pharmacokinetic studies.

INTRODUCTION

Salbutamol, 1-{[(1,1-dimethylethyl)amino]methyl}-4-hydroxy-1,3-benzenedimethanol (I), is a selective β_2-adrenoceptor agonist bronchodilator widely used for the relief of bronchospasm in asthma and bronchitis [1, 2]. The effective oral dose is 4 mg, providing peak concentrations in plasma, as determined by gas chromatography—mass spectrometry (GC—MS) of 5—15 ng/ml [3, 4]. Investigation of the pharmacokinetics of salbutamol requires measurement of concentrations in plasma below the therapeutic level and this has been achieved using GC—MS [4]. This method, however, although sensitive, precise and accurate, is labour-intensive and requires expensive instrumentation. There is

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therefore a need for an assay of similar performance that can be used more widely. Several methods based on high-performance liquid chromatography (HPLC) with either fluorescence [5, 6] or amperometric [7, 8] detection have been published but on evaluation none of these has proved satisfactory in our hands for analysing the large numbers of samples generated by pharmacokinetic studies in man.

In this paper we report the development and performance of a rapid, accurate and sensitive method for the determination of salbutamol in urine and plasma. The method depends on solid-phase extraction of salbutamol, conversion to an indoaniline dye which is separated using high-performance thin-layer chromatography (HPTLC) and quantified by absorption microdensitometry at 650 nm. The method is accurate and sensitive down to 20 ng/ml in urine and to 1 ng/ml in plasma, and has been used for both compliance and pharmacokinetic studies.

EXPERIMENTAL

Chemicals, standards and materials

Salbutamol base was supplied by the Pharmaceutical Sciences Division of Glaxo Group Research. Methanol, hexane and acetonitrile, all HPLC grade, were from Rathburn Chemicals (Walkerburn, U.K.); chloroform and ethyl acetate (Aristar®), sodium bicarbonate and potassium ferricyanide (AnalaR®) and N,N-dimethyl-*p*-phenylenediamine (DMPD) (II), sulphate salt, were from BDH (Poole, U.K.); 4-(2-dimethylaminoethyl)phenol sulphate (DMAEPS, analytical-reagent grade) was from Aldrich (Gillingham, U.K.).

Merck glass-backed silica gel 60 HPTLC plates (10 × 20 cm) without fluorescent indicator were from BDH, Sep-Pak® C_{18} cartridges were purchased from Millipore (Harrow, U.K.) and PREP-I® Type W XAD-2 cartridges from Dupont (Stevenage, U.K.). Microcap® volumetric capillary tubes (2 μl, Drummond) were obtained from Scientific Supplies (London, U.K.)

Sample preparation

Urine. A 1-ml sample of 0.5 *M* Tris buffer, pH 8.4, and a 1-ml sample of urine were pipetted into a PREP-I Type W cartridge and then placed in a PREP-I automated sample processor (Dupont) and run on programme 15 up to the end of step 12 [9]. Distilled water (2 ml per cartridge) and methanol (2 ml per cartridge) were used as washing and eluting solvents. After step 12 the vials containing the methanol eluates were transferred to a Savant Speed Vac® concentrator (Uniscience, London, U.K.) and the solvent was evaporated for 75 min at 45°C.

Plasma. Sep-Pak C_{18} cartridges were washed sequentially with 5 ml methanol and 5 ml distilled water, taking care not to allow the cartridges to dry. A plasma sample (2 ml) was applied to the cartridge, the eluate discarded and the cartridge allowed to dry. The cartridge was then washed sequentially with water (2 ml) and acetonitrile (2 ml), allowing the cartridge to dry. Salbutamol was eluted from the cartridge with 1.5 ml methanol and the solvent removed at 45°C on a Savant Speed Vac concentrator.

Derivatisation

Urine residue. Distilled water, 1.0 ml, was added to the residue followed by 0.30 ml aqueous sodium bicarbonate (5%, w/v), 0.30 ml aqueous DMPD (0.1%, w/v) and 0.30 ml aqueous potassium ferricyanide (8%, w/v). After vortex-mixing for 5 s the mixture was left in the dark at room temperature for 20 min. Chloroform, 0.20 ml, was added, and after vortex-mixing for 20 s the aqueous layer was removed by aspiration. Samples (2 μl) of the chloroform layer were applied to the HPTLC plate in duplicate using the data pairing technique [10].

Plasma residue. Distilled water, 0.20 ml, was added to each residue followed by 0.06 ml aqueous sodium bicarbonate (5%, w/v) and 0.06 ml aqueous DMPD (0.1%, w/v). The mixture was then vortex-mixed and 0.06 ml aqueous DMAEPS (0.2%, w/v) and 0.06 ml aqueous potassium ferricyanide (8%, w/v) were added. The solution of DMAEPS was prepared by dissolving the solid in one tenth the final volume of 5% sodium bicarbonate solution and diluting to volume. After vortex-mixing the reaction mixture was allowed to stand in the dark for 5 min at room temperature and then extracted for 20 s with 0.10 ml chloroform followed by centrifugal separation (Eppendorf 5412 centrifuge, 1.5 min). The aqueous layer was removed by aspiration and 6 μl of the chloroform extract were applied to the HPTLC plate, using the data pairing technique. Spots could be applied manually using 2-μl microcaps or automatically using a Camag TLC I automatic sample applicator (Camag, Muttenz, Switzerland).

Chromatography

Samples were applied 0.5 cm from the edge of the HPTLC plate. Development was carried out, immediately after spotting, in a Camag horizontal development chamber (Camag).

Two solvent systems were used: system A (urine analysis): chloroform—ethyl acetate (60:40, v/v); system B (plasma or urine analysis): ethyl acetate—chloroform—methanol (60:40:1, v/v).

Plates were developed for 3.5 cm beyond the origin. The derivative (III) had an R_F of 0.3 to 0.35 in system A and 0.3 in system B. Chromatograms could be preserved by dipping the plate in a solution of liquid paraffin in hexane (20%, v/v).

Detection and quantitation

Plates were scanned from origin to solvent front using a Zeiss KM3 densitometer with micro-optics using the reflectance/20% transmission mode, a monochromator wavelength of 650 nm and a scan speed of 20 mm/min. This densitometer is no longer available commercially and in later experiments we have used the Camag TLC II scanner (Camag) with equal success. The signal output was recorded on a Spectra Physics SP 4100 or on a Trivector Trilab 2000 chromatography data system. The concentration of salbutamol in applied samples was calculated by comparison of peak heights with those of calibration standards.

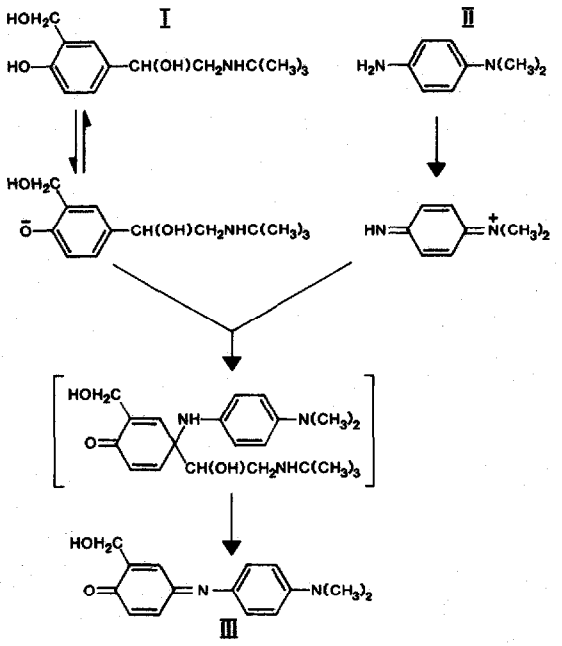

Fig. 1. Proposed scheme for the reaction of salbutamol (I) with dimethyl-*p*-phenylenediamine (II) to produce 4-{[4-(dimethylamino)phenyl]imino}2-(hydroxymethyl)-2,5-cyclohexadien-2-one (III).

RESULTS AND DISCUSSION

Formation and identity of the indoaniline

The derivatisation is based on the reaction of phenols with DMPD under oxidising conditions to form an indoaniline [11, 12]. The proposed reaction sequence is shown in Fig. 1. Proton NMR of the product of this reaction gave a spectrum consistent with a 63:37 mixture of the *Z* and *E* geometric isomers of 4-[4-(dimethylamino)phenyl]imino}2-(hydroxymethyl)-2,5-cyclohexadien-1-one (III). The isomers were not resolved in the solvent systems used. In ethanol the product had an ultraviolet—visible spectrum with maxima at 280 nm (*E* 15 750) and at 598 nm (*E* 19 500). On the HPTLC plate absorption maxima were observed at 250 and 650 nm.

Formation of this indoaniline is not specific to salbutamol. Any metabolite of salbutamol containing the saligenin nucleus would be converted to the same indoaniline. However, the principal metabolite of salbutamol in man is the sulphate [13] and this ester would not be expected to react with DMPD.

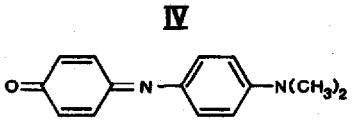

Fig. 2. Indoaniline, 4-{[4-(dimethylamino)phenyl]imino}2,5-cyclohexadien-1-one (IV), formed from the reaction of dimethyl-*p*-phenylenediamine with 4-(2-dimethylaminoethyl)-phenol.

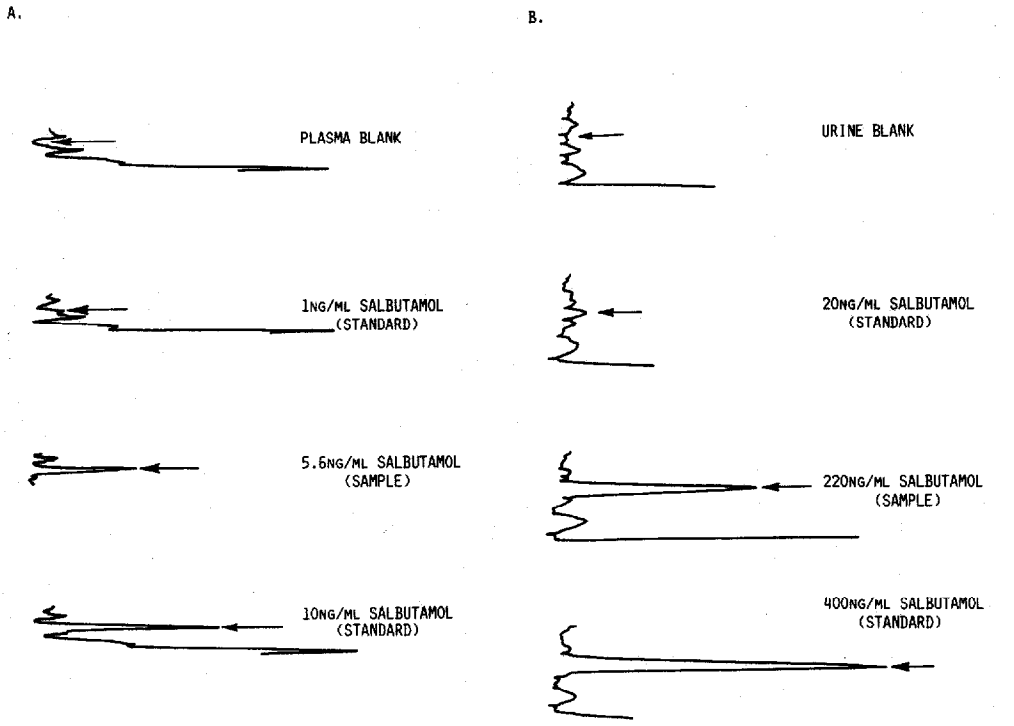

Fig. 3. Chromatogram traces obtained from analysis of salbutamol calibration standards and from dosed volunteers. (A) Plasma samples; (B) Urine samples.

In chromatograms derived from extracts of plasma but not from urine extracts, red streaks or spots that interfered with the assay were observed. These were considered to be due to the presence of excess DMPD when plasma samples were being assayed and were eliminated by the routine addition of 4-(2-dimethylaminoethyl)phenol to the reaction mixture. This reagent reacts with DMPD to form an indoaniline (IV) (Fig. 2) which is separated on the plate from that derived from salbutamol (III).

Quantitation

Standard solutions of salbutamol in urine and plasma were analysed, peak heights were recorded and calibration lines constructed using least-squares regression. Salbutamol concentrations in quality control samples (spiked samples and from dosed subjects) and in unknown samples were quantified by comparison with these standard lines. Fig. 3 shows typical chromatograms obtained from both urine and plasma.

Precision and accuracy

Assay precision and accuracy were determined by assay of spiked samples and by comparison of concentrations determined by HPTLC and those obtained by GC—MS for the same samples. In both matrices intra-assay precision was good (Table I). When results obtained by HPTLC were compared with those obtained by GC—MS agreement between the two methods was excellent for both matrices (Tables II and III).

TABLE I

INTRA-ASSAY PRECISION OF THE HPTLC ASSAY FOR SALBUTAMOL IN URINE AND PLASMA

Matrix	Nominal concentration (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Urine	33.3	8	33.6 ± 2.0	6.0
	333	8	353.8 ± 6.2	1.7
Plasma	1.0	5	0.9 ± 0.10	11.1
	10	5	8.8 ± 0.23	2.6

TABLE II

COMPARISON OF GC—MS AND HPTLC IN THE ASSAY OF SALBUTAMOL IN SPIKED HUMAN URINE (SPIKED SAMPLES)

Nominal concentration (ng/ml)	Concentration found (ng/ml)	
	HPTLC*	GC—MS**
30	31.9	26.5
60	57.3	59.5
80	72.4	82.8
150	157.9	147.2
300	324.8	318.2
500	513.8	526.9
700	695.6	659.9

*Single assays.
**Mean of duplicate assays.

TABLE III

COMPARISON OF GC—MS AND HPTLC IN THE ASSAY OF SALBUTAMOL IN HUMAN PLASMA

Quality control pools from dosed subjects. n = 6.

Pool	Concentration found (mean ± S.D.) (ng/ml)		Coefficient of variation* (%)	
	HPTLC	GC—MS	HPTLC	GC—MS
A	7.36 ± 0.171	7.82 ± 0.12	2.32	1.53
B	7.02 ± 0.092	7.71 ± 0.08	1.32	0.98
C	1.16 ± 0.078	1.21 ± 0.025	6.74	2.05

*The coefficient of variation represents the inter-assay variation.

TABLE IV

COMPARISON OF GC—MS AND HPTLC ASSAYS IN URINE FROM VOLUNTEERS GIVEN INTRAMUSCULAR SALBUTAMOL

Volunteer No.	Dose (μg)	Sample time	Concentration (ng/ml)	
			HPTLC	GC—MS
1022	500	0—2	250	262
		2—4	503	519
		4—6	626	642
		6—8	357	353
		8—12	151	153
		12—24	98	79
1211	250	0—2	122	117
		2—4	204	195
		4—6	269	255
		6—8	269	250
		8—12	126	130
		12—24	22	29

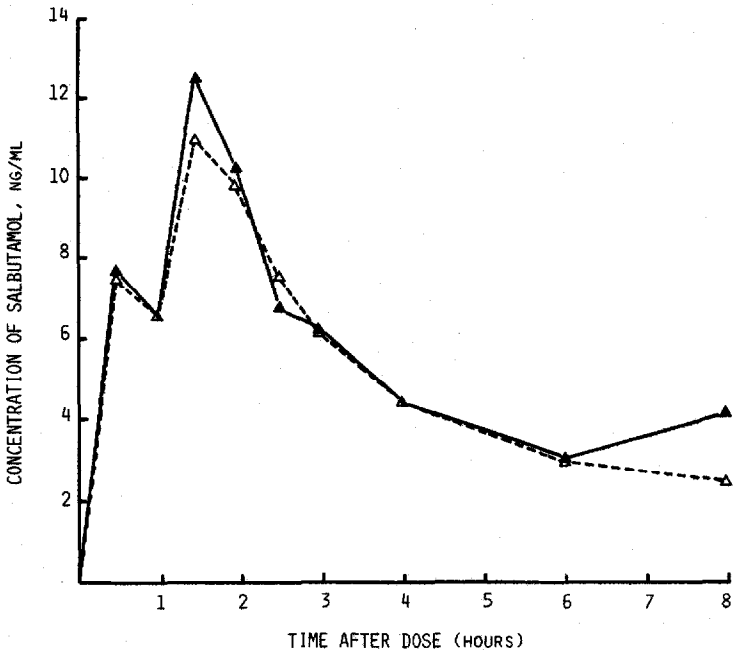

Fig. 4. Concentration of salbutamol in plasma from a volunteer given an oral dose of 4 mg. Comparison of HPTLC (▲) and GC—MS (△) data.

Analysis of samples from pharmacokinetic studies

The main metabolite of salbutamol in man is the phenolic sulphate [13] and in both plasma and urine this is present in greater amounts than the parent compound. However, the good agreement between GC—MS and HPTLC

achieved with pooled plasma samples (Table III) indicates that the HPTLC method is specific for unmetabolised salbutamol. This was confirmed by the analysis of salbutamol in urine from volunteer subjects given either 250 or 500 μg of salbutamol intramuscularly (Table IV) and in plasma from a volunteer subject given an oral dose of 4 mg salbutamol (Fig. 4). In both studies there was excellent agreement between values obtained by HPTLC and GC—MS with the exception of a single outlier at 8 h in the plasma curve. There was insufficient plasma for a repeat assay of this sample.

CONCLUSIONS

This assay method for salbutamol in plasma and urine using conversion to an indoaniline dye and separation with HPTLC is rapid, sensitive and precise. It gives values in good agreement with the established GC—MS method allowing analysis of samples from pharmacokinetic studies and has the advantages of simplicity, lower cost, and improved throughput.

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