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SENSITIVE QUANTIFICATION OF PSEUDOEPHEDRINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An assay for the selective quantification of pseudoephedrine in human plasma and urine was developed using high-performance liquid chromatography with UV detection at 205 nm. Analyte and internal standard were extracted from alkaline plasma or urine into a mixture of *n*-hexane and diethyl ether, and the organic phase was back-extracted into dilute acid. The chromatographic system comprises microparticulate cyanopropyl-silica as stationary phase and a ternary solvent mixture with ion-pair reagents as mobile phase. Using 0.25 ml plasma, the lower limit of quantification was 25 ng/ml with excellent linearity up to 1000 ng/ml. In urine, the calibration ranged from 2.5 to 100 μg/ml. The selectivity of the method was demonstrated for several pharmaceuticals with similar structures. The validated method was applied to a pharmacokinetic study with a single oral dose of 100 mg of pseudoephedrine in two galenic formulations. Precision and accuracy data of the assay and calculated pharmacokinetic parameters are presented.

INTRODUCTION

Pseudoephedrine (1*S*, 2*S*-*threo*-2-methylamino-1-phenyl-1-propanol) is widely used either singly or in combination with other substances in the clinical treatment of the common cold, sinusitis, hay fever, bronchitis, rhinitis and other upper respiratory allergies. Pseudoephedrine is an indirect sympathomimetic with both peripheral and central stimulating effect and broncholytic and antiallergic properties. The usual daily doses of 30–240 mg of pseudoephedrine hydrochloride result in plasma levels in the nanogram range. High-performance liquid chromatography (HPLC) has been used to determine the content and stability in galenic preparations [1,2], and gas chromatography (GC) has been used to quantify the enantiomeric purity [3] after derivatization to diastereomers.

For quantification in plasma or urine, one method using GC with electron-

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capture detection has been published [4], as well as one stereoselective radioimmunoassay [5]. We are the first to publish a selective and sensitive method for the quantification of pseudoephedrine in human plasma and urine using HPLC with UV detection, which was successfully applied to samples from several pharmacokinetic studies. The selectivity to all possible coadministered synthetic and natural drugs is examined and demonstrated.

The method comprises extraction of alkalinized plasma with purified diethyl ether–hexane, and back-extraction into dilute acid. As the plasma will usually be re-used for analysis of other drugs, only 250 μl of substrate were taken for extraction of pseudoephedrine. Urine samples were worked up with bonded-phase columns for routine analysis. An alternative procedure with direct injection and pre-column switching for clean-up is described.

EXPERIMENTAL

Materials

Pseudoephedrine hydrochloride was supplied by Aldrich (Steinheim, F.R.G.). Acebutolol (internal standard) was supplied by Sigma (Munich, F.R.G.). Diethyl ether (E. Merck, Darmstadt, F.R.G.) was cleaned up by filtration through activated basic alumina, *n*-hexane (E. Merck) was cleaned up by distillation over sodium suspension. All chromatographic solvents were LiChrosolv grade (E. Merck), and all other chemicals were analytical-reagent grade.

The extraction columns for urine analysis were Baker-10 SPE 3-ml columns filled with octadecylsilica (J.T. Baker, Gross-Gerau, F.R.G.).

Apparatus

A Merck-Hitachi 655A liquid chromatographic pump (E. Merck, Munich, F.R.G.) and a Waters 710B WISP autosampler (Waters-Millipore, Eschborn, F.R.G.) were used. A LiChrosorb 100 CN 250×4.0 mm I.D. column (particle size 5 μm; E. Merck) was connected to a Kratos SF 757 variable-wavelength detector (Kratos, Karlsruhe, F.R.G.) operated at 205 nm and 0.02 a.u.f.s. The mobile phase was a degassed mixture of 20 g of methanol, 800 g of water, 160 g of acetonitrile, 10 g of potassium dihydrogenphosphate, 1.0 g of sodium pentanesulphonate and 1.0 g of sodium heptanesulphonate pumped at a constant flow-rate of 1.5 ml/min. All chromatography was carried out at ambient temperature.

For analysis of urine samples, the conditions were the same with the exception that the separation column was shorter (125×4.0 mm I.D.).

Internal standard

A stock solution of 10 μg/ml acebutolol (free base) was made weekly in water and stored at 4°C.

Spiked calibration samples and quality controls

For preparation of calibration samples, plasma from healthy volunteers was pooled and spiked with 1.00 mg/ml pseudoephedrine in water to give a concentration of 1000 ng/ml pseudoephedrine in plasma. Samples at concentrations of

500, 250, 100, 50.0 and 25.0 ng/ml were prepared by appropriate dilution with additional blank plasma. The samples were divided into 0.250-ml portions, frozen and stored with the subject samples of the studies. Quality controls (QCs) at concentrations of 25, 100 and 750 ng/ml were prepared daily and blinded to the study analyst.

For urine samples, the same was done in 1.00-ml portions at calibration concentrations of 2.50, 5.00, 10.0, 25.0, 50.0 and 100 μg/ml and QC concentrations of 2.50, 10.0 and 100 μg/ml.

Extraction of plasma samples

To a 10-ml screw-capped glass test-tube, 0.25 ml of plasma, 100 μl of internal standard solution, 0.25 ml of a solution of 10 g of sodium hydroxide and 40 g of sodium carbonate in 1000 ml of water and 6 ml of a 1:1 (v/v) mixture of *n*-hexane and diethyl ether were added. The sample was treated for 30 min using the overhead-shaker and centrifuged at 2000 *g* for 10 min. The organic phase was transferred to another conical glass tube containing 150 μl of 0.05 *M* sulphuric acid. The tube was treated for 10 min in the overhead-shaker and centrifuged for 10 min at 2000 *g*. The organic phase was aspirated, and the aqueous phase was transferred to the microinserts of the sampler. An aliquot of 100 μl was injected automatically.

The samples were extracted in sequences. One sequence consisted of 30–40 study samples, control blank, calibration samples and quality controls.

Extraction of urine samples (off-line procedure)

The extraction columns were conditioned with 2 column volumes of methanol and 2 column volumes of water. To 1.0 ml of urine, 75 μl of internal standard solution (1 mg/ml) and 1 ml of 0.1 *M* sodium hydroxide were added, and the mixture was transferred to the extraction tube. The column was washed twice with 20% aqueous methanol and dried. The analytes were eluted with two 500-μl aliquots of methanol and mixed with 1.5 ml of 0.05 *M* sulphuric acid. An aliquot of 150 μl was injected automatically.

Extraction of urine samples (on-line procedure)

A column-switching unit (Latek, Heidelberg, F.R.G.) was inserted between the sampler and the separation column. An additional pump was connected to the switching unit to deliver the mobile phase. In position "load" the sampler injected 100 μl of diluted urine (50 μl of urine mixed with 50 μl of internal standard solution). The pump connected to the sampler delivered phosphate buffer (0.1 *M*, pH 8) at a flow-rate of 0.5 ml/min. Thhe injected sample was purged on a short precolumn (50×4 mm I.D.) filled with preparative cyanopropylsilica (LiChroprep CN, 40 μm, E. Merck). After 180 s, the precolumn was switched to the analytical column till the end of the run. The reswitching and reconditioning of the precolumn took 6 min, and the total analysis time was 14 min.

Recovery from plasma

For the determination of the recovery, three replicate samples at 25.0, 100 and 1000 ng/ml for pseudoephedrine were run through the procedure with exactly

controlled volumes, as described for the extraction of samples. The peak areas obtained for the extracted samples were compared with those of fresh standards of the analyte in the mobile phase, with respect to the volumes handled during extraction.

Quantification

The integrator determined the peak areas of analyte and internal standard. After the run, it calculated the peak-area ratio of the analyte to the internal standard. This result was fed to a computer (Commodore CBM 8032, Commodore, Frankfurt, F.R.G.). The results of the calibration samples were used to calculate the calibration curve with linear regression after $1/x$ concentration weighting. This was done by LAB-CAL software. The calibration curve was characterized by regression coefficient, slope and intercept. Using the calibration curve, the concentrations of the samples and the QCs were calculated. The calibration was valid from the lower limit of quantification (LLQ) of 25.0 ng/ml to the upper limit of quantification (ULQ) of 1000 ng/ml. Blanks were not included in the calibration.

For urine samples the same was done from an LLQ of 2.50 μg/ml to a ULQ of 100 μg/ml.

Method validation

The procedure was evaluated in terms of sensitivity, linearity of response, accuracy, precision, recovery and specificity. For this purpose five calibration curves in five different sequences were measured. The sensitivity of the method was evaluated by analysing plasma samples at the presumed LLQ. The linearity of response was checked by means of the regression coefficients. The accuracy of the method was assessed by statistical evaluation of the mean value of five spikes of the same nominal concentration. The specificity was tested by injection of structurally related compounds, as well as common drugs, and evaluation of their retention times.

Pharmacokinetic study

In a pharmacokinetic study eight healthy volunteers (four male, four female, aged between 18 and 52 years) were dosed in a randomized two-fold cross-over with two different formulations of pseudoephedrine hydrochloride. Dosage A was 25 ml of syrup containing pseudoephedrine in combination with codeine phosphate, diphenhydramine, caffeine and natural plant extracts. Dosage B was two capsules with the same drug amount. The dosed amount was 100 mg of pseudoephedrine free base. The volunteers fasted overnight and received the drug together with 200 ml of water in the morning. Breakfast followed 2 h after drug administration. Blood samples (10 ml) were obtained by venipuncture and collected in heparinized tubes (Vacutainers, Becton and Dickinson, Kassel, F.R.G.) at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18 and 24 h following each dose. The blood samples were centrifuged immediately and the plasma was removed and stored at $-20\,^{\circ}$C until analysis.

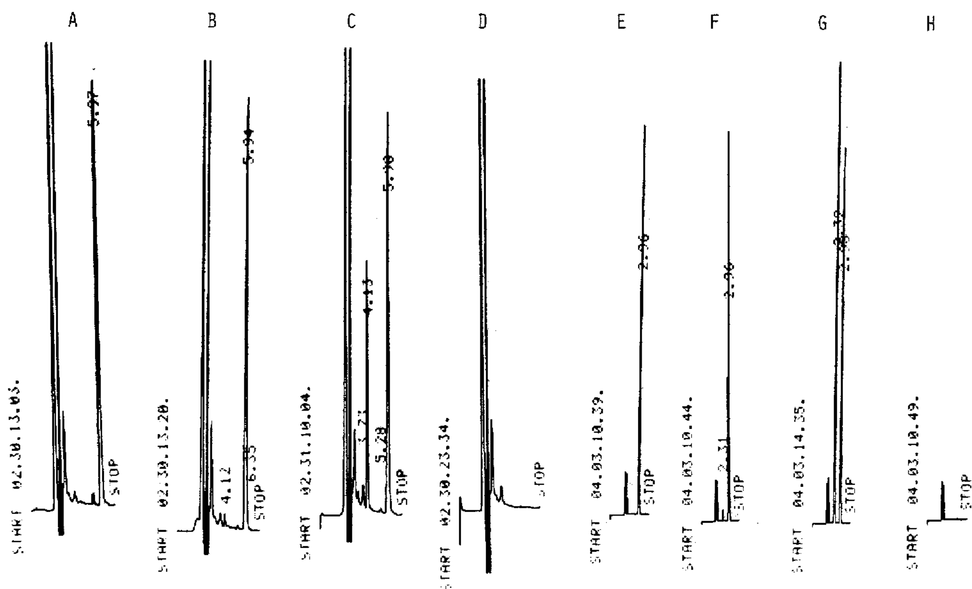

Fig. 1. Chromatograms of extracts from 0.25 ml of plasma. (A) Pre-dose sample; (B) plasma sample 24 h post-dose with 26 ng/ml pseudoephedrine; (C) plasma sample 2 h post-dose with 340 ng/ml pseudoephedrine (the peak at 4.13 is pseudoephedrine, that at 5.98 is internal standard); (D) blank plasma without internal standard; (E) urine blank sample; (F) lowest urine calibration concentration (2.5 μg/ml); (G) subject's urine with 95 μg/ml pseudoephedrine (the peak at 2.32 is pseudoephedrine, that at 2.98 is internal standard); (H) blank urine without internal standard.

RESULTS AND DISCUSSION

Analytical results

Pseudoephedrine and internal standard gave sharp, symmetrical, well resolved peaks with retention times of 4.1 and 5.9 min, respectively. The mixture of pentane- and heptanesulphonate separated pseudoephedrine from an endogeneous plasma interference peak eluted before, which was not the case for each alone. Fig. 1A shows the chromatogram of a pre-dose plasma extraction. Fig. 1B shows the chromatogram of a real sample with an analyte concentration near the LLQ, where the signal-to-noise ratio is >40. Fig. 1C shows the chromatogram of a subject's maximal concentration (C_{max}) sample with a calculated concentration of 340 ng/ml. In the urine system with a short separation column the analytes eluted at 2.3 and 3.0 min, respectively. Fig. 1E shows a urine blank sample. Fig. 1F shows a chromatogram of the lowest calibration concentration (2.5 μg/ml) and Fig. 1G shows a real sample with a concentration of 95 μg/ml. Table I shows the results of the plasma validation for accuracy and precision of the calibration and QC samples, as well as slopes, intercepts and regression coefficients of the calculated calibration curves. The calibration curves of the validation were linear from 25.0 to 1000 ng/ml, with a mean slope value of 0.008455, a mean intercept of 0.00015 and a mean r^2 of 0.9996. Table II shows the results for the calibration and QC samples of the clinical study. The calibration curves of the study were

TABLE I

STATISTICAL EVALUATION OF THE ANALYTICAL PARAMETERS DURING VALIDATION OF PSEUDOEPHEDRINE IN PLASMA ($n=5$)

Added (ng/ml)	Calculated (mean ± S.D.) (ng/ml)	Accuracy (%)	Precision (%)
25.0	24.2 ± 2.11	−3.2	8.7
1000	1004 ± 13.5	+0.4	1.3
QC 25.0	26.7 ± 2.33	+6.8	9.3
QC 100	104 ± 4.89	+4.1	4.7
QC 750	739 ± 11.8	−1.5	1.6

Mean r^2: 0.9996 ± 0.0003
Mean slope: 0.008455 ± 0.000402
Mean intercept: 0.00015 ± 0.00011
Equation: $y = 0.00015 + 0.008455\,x$

linear from 25.0 to 1000 ng/ml, with a mean slope value of 0.00836, a mean intercept of 0.00011 and a mean r^2 of 0.9995. Table III gives the results of the recovery study: at all the investigated concentrations the recovery from plasma is higher than 95%.

Table IV shows the results of the urine validation for accuracy and precision of the calibration and QC samples, as well as slopes, intercepts and regression coefficients of the calculated calibration curves. The calibration curves of the validation were linear from 2.50 to 100 μg/ml with a mean slope of 0.1266, a mean intercept of −0.08735 and a mean r^2 of 0.9999. Table V shows the results for the calibration and QC samples of the clinical study. The calibration curves of the study were linear from 2.50 to 100 μg/ml with a mean slope value of 0.1271, a mean intercept of −0.0803 and a mean r^2 of 0.9999. Table VI gives the results of

TABLE II

STATISTICAL EVALUATION OF THE ANALYTICAL PARAMETERS DURING STUDY OF PSEUDOEPHEDRINE IN PLASMA ($n=16$)

Added (ng/ml)	Calculated (mean ± S.D.) (ng/ml)	Accuracy (%)	Precision (%)
25.0	25.0 ± 1.69	−0.0	6.8
1000	1002 ± 10.8	−0.2	1.1
QC 25.0	25.1 ± 2.14	+0.4	8.5
QC 100	98.1 ± 5.33	−1.9	5.2
QC 750	756 ± 14.2	+0.8	1.9

Mean r^2: 0.9995 ± 0.0006
Mean slope: 0.008360 ± 0.000391
Mean intercept: 0.00011 ± 0.00019
Equation: $y = 0.00011 + 0.008360\,x$

TABLE III

RECOVERY OF PSEUDOEPHEDRINE FROM PLASMA

Three determinations at each concentration.

Pseudoephedrine added (ng/ml)	Pseudoephedrine recovered (ng/ml)	Recovery (mean ± S.D.) (%)
25.0	24.0	96.0 ± 3.8
100	99.1	99.1 ± 2.6
1000	988	98.8 ± 3.1

TABLE IV

STATISTICAL EVALUATION OF THE ANALYTICAL PARAMETERS DURING VALIDATION OF PSEUDOEPHEDRINE IN URINE ($n=5$)

Added (μg/ml)	Calculated (mean ± S.D.) (μg/ml)	Accuracy (%)	Precision (%)
2.50	2.50 ± 0.11	−0.0	4.4
100	100 ± 1.01	−0.0	1.0
QC 2.50	2.44 ± 0.18	−2.4	7.4
QC 10.0	10.3 ± 0.35	+3.0	3.4
QC 75.0	74.4 ± 1.22	−0.8	1.6

Mean r^2: 0.9999 ± 0.00003
Mean slope: 0.1266 ± 0.0012
Mean intercept: −0.0873 ± 0.0103
Equation: $y = -0.0873 + 0.1266\,x$

TABLE V

STATISTICAL EVALUATION OF ANALYTICAL PARAMETERS DURING STUDY OF PSEUDOEPHEDRINE IN URINE ($n=8$)

Added (μg/ml)	Calculated (mean ± S.D.) (μg/ml)	Accuracy (%)	Precision (%)
2.50	2.49 ± 0.17	−0.4	6.8
100	100 ± 0.58	−0.1	0.6
QC 2.50	2.51 ± 0.16	+0.4	6.4
QC 10.0	10.1 ± 0.13	+1.4	1.3
QC 75.0	75.5 ± 0.44	+0.6	0.6

Mean r^2: 0.9999 ± 0.00005
Mean slope: 0.1271 ± 0.0011
Mean intercept: −0.0803 ± 0.0092
Equation: $y = -0.0803 + 0.1271\,x$

TABLE VI

RECOVERY OF PSEUDOEPHEDRINE FROM URINE

Three determinations at each concentration.

Pseudoephedrine added (μg/ml)	Pseudoephedrine recovered (μg/ml)	Recovery (mean ± S.D.) (%)
2.50	2.44	98.4 ± 0.7
10.0	9.93	99.3 ± 1.3
100	100.5	100.5 ± 0.6

TABLE VII

RELATIVE RETENTIONS OF SELECTED DRUGS TO PSEUDOEPHEDRINE

Drug	Relative retention ratio to pseudoephedrine at 4.1
Ephedrine	1.1
Norpseudoephedrine	0.8
Doxylamine	2.7
Brompheniramine	2.8
Pheniramine	2.3
Diphenhydramine	2.5
Clemastin	2.9
Codeine	4.4
Clobutinol	1.6
Isoaminil	2.1
Pentoxyverin	4.2
Oxeladin	4.1
Bromhexin	> 5
Ambroxol	3.6
Butamirat	4.0
Caffeine	0.3
Theophylline	0.3
Amphetamine	1.4
Camphor	> 5
Menthol	Not detected
Eucalyptus oil	> 5, several peaks
Terpineol	No peaks detected

the recovery study: at all the investigated concentrations the recovery from urine is higher than 95%.

The results were obtained with off-line workup of urine samples. An on-line workup was rejected for following reasons: (i) the analysis time for on-line workup was unacceptable, as only 60–80 samples could be measured per day; (ii) the preconcentration column changed its capacity continuously, and after ca. twenty cycles the analyte began to bleed from the column during the washing phase, which implied permanent maintenance steps; (iii) the peak shapes of the analytes showed significant tailing, and unpredictable variations in retention times occurred frequently.

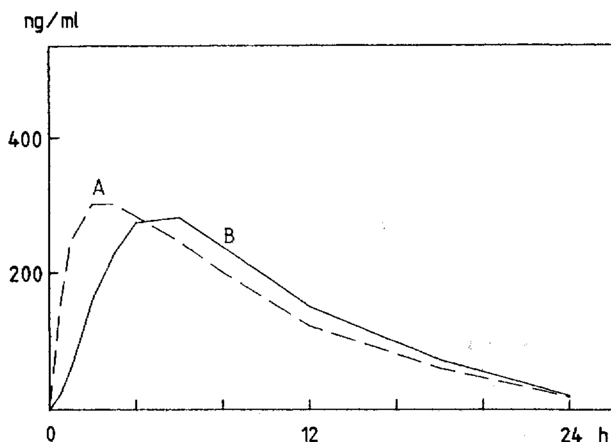

Fig. 2. Plasma concentration versus time profile. Values are the mean over eight subjects for two formulations: (A) 100 mg of pseudoephedrine in syrup; (B) 2×50 mg of pseudoephedrine in capsules.

The selectivity was tested by taking therapeutic concentrations of other broncholyte, antihistaminic and sympathomimetic drugs through the method. None of the cited drugs interfered either with the internal standard or the analyte. Table VII lists the drugs examined and their retention times.

Pharmacokinetic results

Fig. 2 shows the mean plasma concentration versus time profile for all eight subjects and two dosages. The collected pharmacokinetic parameters are listed in Table VIII.

In the absorption phase, the two formulations differ significantly in their plasma level versus time profile. Formulation A (syrup) has a mean maximal concentration (C_{max}) of 299 ng/ml after a mean maximal time (T_{max}) of 2.49 h. Formulation B (capsules) has a C_{max} of 275 ng/ml with a T_{max} of 5.35 h. After T_{max}, the mean plasma concentration curve of formulation A decreases significantly faster than the curve of formulation B. The terminal half-life times, determined with logarithmic/linear regression, differ only slightly: 5.7 h for formulation A and 6.0 h for formulation B. The area under the curve (AUC) was calculated by the trapezoidal rule. The ratio AUC formulation B/AUC formulation A was 98.42. In the ratio of the geometric means, formulation B has 95.5% of the area of formulation A. The Westlake confidence interval of 88–114% is well within the limits of 80–125%. The excreted amount in urine as unchanged analyte is 74% for treatment A and 72% for treatment B, i.e. very similar.

CONCLUSION

The method presented here for the quantitation of pseudoephedrine in human plasma and urine is sensitive, selective, precise and accurate enough to monitor

TABLE VIII

PHARMACOKINETIC PARAMETERS OF TWO PSEUDOEPHEDRINE FORMULATIONS

Formulation A: 100 mg of pseudoephedrine in syrup; formulation B: 2×50 mg of pseudoephedrine in capsules.

Parameter*	Treatment A		Treatment B	
	Mean	S.D.	Mean	S.D.
AUC	3034	625	2986	793
C_{max}	299	50.6	275	48.4
T_{max}	2.49	1.16	5.35	1.39
$t_{1/2}$	6.03	1.10	5.72	1.27
Excreted analyte after 24 h (%)	73.97	19.8	72.04	14.08

*AUC = area under the curve (h ng/ml); C_{max} = mean maximal concentration (ng/ml); T_{max} = mean time of maximal concentration (h); $t_{1/2}$ = terminal elimination half-life (h).

the pharmacokinetic profile of the analyte after normal single doses. Application of the method in a clinical study with eight healthy subjects gave useful results for several pharmacokinetic parameters of two different drug formulations.

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