Journal of Chromatography, 490 (1989) 206–212
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4653

Note

Determination of low concentrations of dibenzylamine in human plasma and urine by gas chromatography with a nitrogen–phosphorus detector

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(First received October 12th, 1988; revised manuscript received December 8th, 1988)

Numerous secondary effects that occur after the administration of drugs have been related to the vehicles used in elaborating the formulation [1]. Government Drug Regulatory Agencies have largely prevented the introduction of new toxic excipients. However, the use of new substances or the use of previously approved excipients (but not sufficiently studied) continue to cause tragedies such as the E-Ferol incident [2]. Most problems associated with excipients could be prevented by an appropriate knowledge of their toxicity and disposition characteristics in the organism following administration.

Dibenzylamine is a compound closely related to N,N-dibenzyl-β-chloroethylamine (dibenamine), a known adrenergic blocking agent used in the preparation of insoluble derivatives of certain antibiotics to obtain a sustained pharmacological action [3]. Owing to the introduction of this substance into the structure of these antibiotics, which behaves as an independent chemical entity after release from the complex in the organism, it is essential to have an analytical technique that will determine the levels of this substance in different body fluids, thus yielding information on its disposition properties and hence the safety characteristics of the formulation.

Currently, the techniques that have been developed for the determination of dibenzylamine levels are oriented towards biotransformation studies in homogenates of rabbit liver [4]. In these studies, a flame ionization detector is used, although with this it is not possible to detect the small amounts of diben-

0378-4347/89/$03.50

zylamine present in the organism after its joint administration with cephazoline. Accordingly, in this paper we describe a gas chromatographic (GC) technique which, with the aid of specific nitrogen–phosphorus detection (NPD), permitted the determination of benzylamine in plasma and urine after administration of the cephazoline–dibenzylamine complex (Daren®) at a dose of 1250 mg to healthy volunteers.

EXPERIMENTAL

Chemicals and reagents

Analytical-reagent grade sodium hydroxide, hexane and dibenzylamine were purchased from Merck (Darmstadt, F.R.G.), diphenylamine from Sigma (St. Louis, MO, U.S.A.) and Daren from Antibióticos (Madrid, Spain). All other chemicals were of analytical-reagent grade.

Stock solutions of dibenzylamine and diphenylamine (1 mg/l) in methanol were prepared weekly and stored at 5 °C, protected from light.

Apparatus and chromatographic conditions

A Varian (Palo Alto, CA, U.S.A.) Model 3300 gas chromatograph equipped with a nitrogen–phosphorus detector and a glass column (1.2 m × 4 mm I.D.) packed with 3% QF-1 on Gas-Chrom Q (100–120 mesh) was used in conjunction with a Varian 4270 computing integrator. The instrument was operated isothermally with the detector, injector port and oven temperatures at 250, 210 and 150 °C, respectively. The carrier gas was highly purified nitrogen at a flow-rate of 20 ml/min. The hydrogen and air flow-rates were 3.9 and 175 ml/min, respectively.

Analytical procedure

Volumes of 1 ml of plasma or urine diluted 1:25 in phosphate buffer (pH 7.4) were pipetted into a 15-ml borosilicate glass culture tube, then 100 μl (1 μg/ml) of a methanolic solution of diphenylamine (internal standard), 1 ml of 5 *M* sodium hydroxide solution and 7 ml of hexane were added, mixed well and shaken on a rotary shaker for 15 min at 20 rpm. After centrifuging at 300 *g* for 10 min, 6 ml of the organic phase were transferred into conical glass tubes and evaporated to dryness at 37 °C under a flow of nitrogen. The residue was dissolved in 50 μl of hexane and a 10-μl aliquot was injected into the GC system.

Calibration

Five to nine calibration standards, covering the appropriate concentration range of the unknown samples, were prepared by diluting a given volume of a suitable stock solution with methanol. For the preparation of spiked plasma samples, 100 μl of the appropriate spiked solution were pipetted into a 15-ml conical glass tube and evaporated to dryness under a gentle stream of nitrogen

at 37°C. Following this, 1 ml of plasma was added and extracted as described above.

A calibration graph was obtained by least-squares linear regression of the peak-height ratio of dibenzylamine to the internal standard versus the dibenzylamine concentration. The ratios of the measured peak heights of the compound and the internal standard in the unknown samples were interpolated on this calibration graph to obtain the dibenzylamine concentrations.

Plasma and urine samples

Venous blood samples were collected in heparinized tubes from normal subjects at various times before and after intramuscular administration of a dose of 1250 mg of Daren. After centrifugation at 850 *g* for 15 min, the plasma was separated and stored at −40°C until analysis. Urine samples were collected in polyethylene bottles over 72 h after administration and stored as described for plasma.

RESULTS AND DISCUSSION

Chromatography, linearity and sensitivity of the method

The chromatograms of two plasma and urine samples obtained from a volunteer before and after the administration of 1250 mg of Daren are shown in Fig. 1.

The chromatograms of plasma and urine blanks are free from peaks that interfere with dibenzylamine and the internal standard. These results indicate the specificity of the assay with respect to other endogenous components. The retention times were 2.8 and 3.6 min for diphenylamine and dibenzylamine, respectively.

Concentration and peak heights were linearly related throughout the concentration ranges investigated, i.e., 0–300 ng/ml for plasma and 0–500 ng/ml for urine. The regression equations obtained from data on the eight standards were $y=0.008x-0.043$ ($r=0.998$) for plasma and $y=0.013x-0.005$ ($r=0.999$) for urine. The limit of quantification, calculated as a signal twice the height of the noise level, was approximately 8 ng/ml for plasma and 5 ng/ml for urine.

Precision and accuracy

Within-day. The coefficients of variation for five plasma samples each in the 12.5–300 ng/ml range were between 0.82 and 9.09%. The overall inaccuracy of the plasma assay was calculated to be 3.15%.

The coefficients of variation for the urine assay ranged between 6.77% at 50 ng/ml and 0.55% at 500 ng/ml. The overall inaccuracy of the urine assay was calculated to be 3.58%.

Between-day. The coefficients of variation of the mean values for plasma observed over five days at concentrations of 12.5, 50, 100 and 300 ng/ml were 6.62, 3.43, 1.93 and 3.74%, respectively. For these concentrations, the devia-

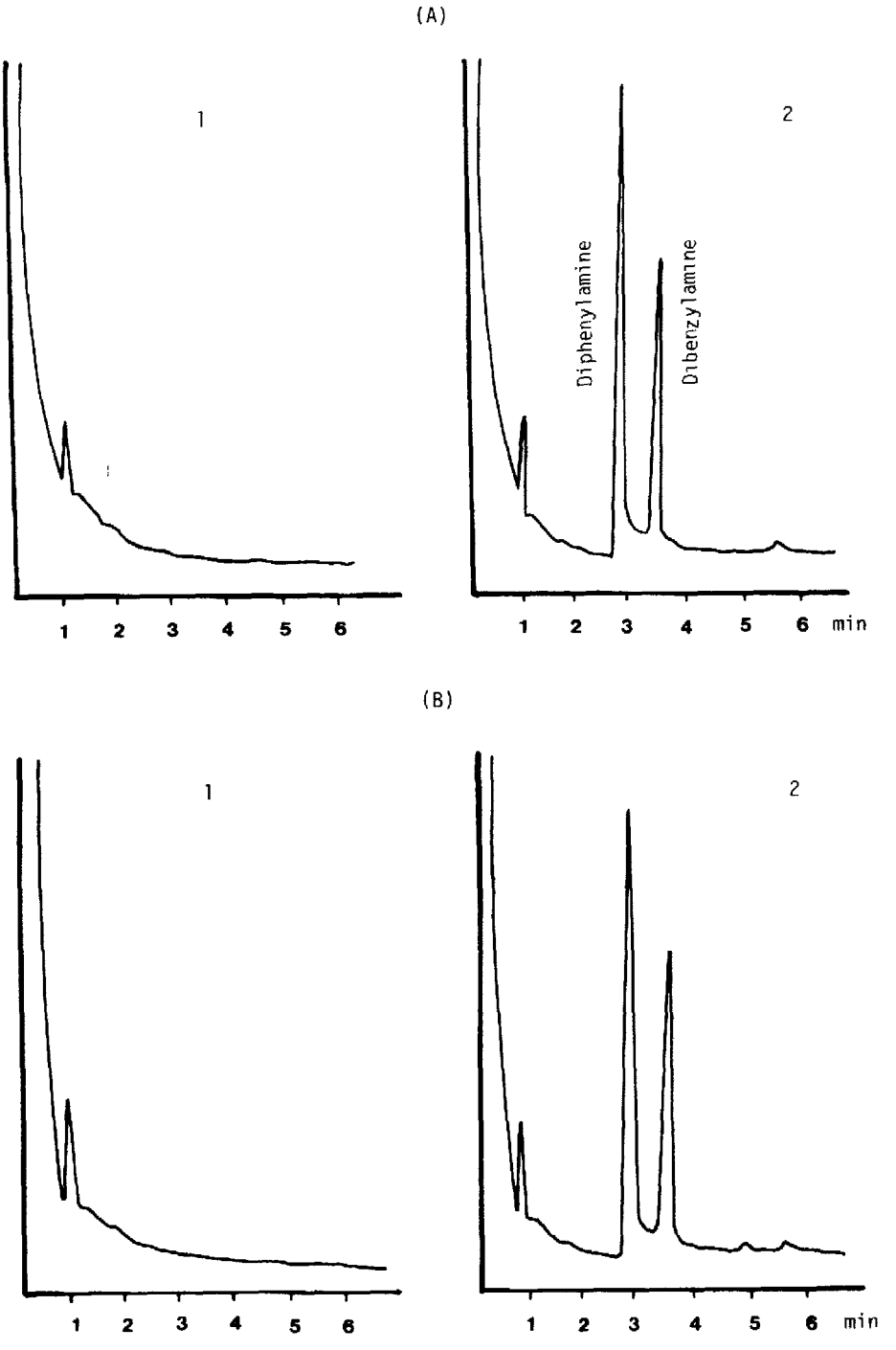

Fig. 1. Gas chromatograms of two (A) plasma and (B) urine samples obtained from a volunteer (1) before and (2) after taking the cephazoline-dibenzylamine complex (internal standard, diphenylamine).

tions from the amounts added were +2.56, +6.84, −4.69 and −5.87%, respectively.

The coefficients of variation of the mean values for urine observed over five days at concentrations of 20, 200 and 500 ng/ml were 5.43, 1.57 and 1.01%, respectively. The deviations from the amounts added were +6.4, +1.8 and −0.92%, respectively.

Applicability of the method

The method was used to measure the plasma and urine concentrations of dibenzylamine in three healthy volunteers receiving an intramuscular dose of 1250 mg of Daren. Fig. 2 shows the plasma levels of dibenzylamine. The pharmacokinetic parameters were calculated by non-linear regression using PCNONLIN [5] and are given in Table I.

The absorption rate constants were in the range 0.14–0.46 h^{-1}. The peak plasma concentration of 141.6 ± 28.7 ng/ml was achieved at 5.23 ± 1.5 h after administration. The mean terminal half-life of dibenzylamine was 2.95 h. According to these findings and using the normal dosage interval of 24 h, the

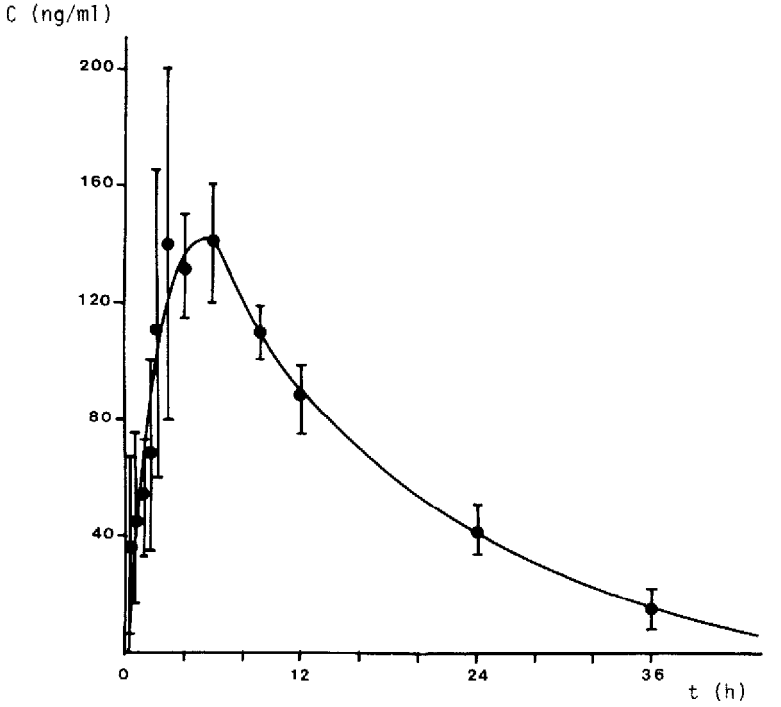

Fig 2. Plasma levels of dibenzylamine obtained in three volunteers after intramuscular administration of 1250 mg of the cephazoline–dibenzylamine complex.

TABLE I

PHARMACOKINETIC PARAMETERS OF DIBENZYLAMINE IN HEALTHY VOLUNTEERS AFTER INTRAMUSCULAR ADMINISTRATION OF DAREN

Volunteer	K_a (h^{-1})	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/ml)	AUC[a] (ng h/ml)	Excreted (%)
1	0.46	4.57	3.60	174.5	1983.1	7.14
2	0.38	9.74	5.40	129.4	2674.2	9.44
3	0.14	4.69	6.70	121.0	2232.1	3.60
$\bar{X}$	0.33	6.33	5.23	141.6	2296.5	6.73
σ_{n-1}	0.17	2.95	1.56	28.8	350.0	2.94

[a]Area under the curve.

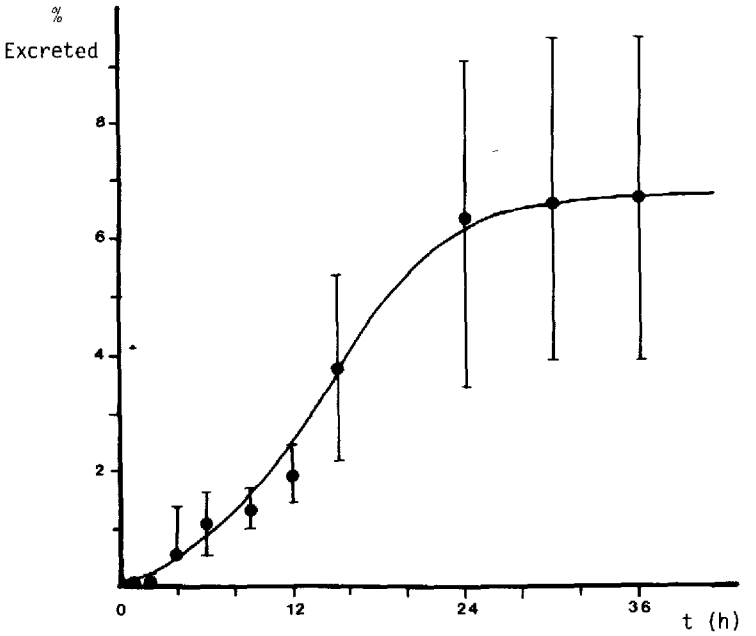

Fig. 3. Cumulative mean urinary excretion curve of dibenzylamine in three healthy volunteers after intramuscular administration of 1250 mg of the cephazoline–dibenzylamine complex.

degree of accumulation of dibenzylamine between the first dose and the steady state would be 1.07.

Fig. 3 shows the mean percentage cumulative urinary excretion curve of dibenzylamine. Only 6.7% of the dose administered was collected unaltered in urine 36 h after administration. Such results are in agreement with those of Beckett et al. [4], who showed that most of the dibenzylamine is transformed

from fortified rabbit hepatic homogenates, and suggest a need to study the biotransformation processes of the drug in humans.

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