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Note

High-performance liquid chromatography method for the determination of diphenhydramine in human plasma

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Diphenhydramine (2-diphenylmethoxy-N,N-dimethylethanamine) is an ethanolamine derivative with antihistaminic properties. It also has sedative, antiemetic and anticholinergic properties, and like the salt with 8-chlorotheophylline, under the name of dimenhydrinate, is used in the prevention and treatment of motion sickness. The drug is also available in the form of chewing gum, as the chewing process might have a calming effect on an individual with nausea.

Several methods have been described for the determination of diphenhydramine in biological fluids by gas chromatography (GC) [1–5]. At least two of these [2,3] are sensitive enough (limit of detection 1 ng/ml) to be used in a single-dose pharmacokinetic study.

Although many high-performance liquid chromatographic (HPLC) assays have been developed to measure diphenhydramine in various drug forms, methods published so far lack the sensitivity required in bioavailability projects, though the one by Skofitsch and Lembeck [6] shows good separation of diphenhydramine metabolites.

Diphenhydramine is metabolised to several products: monodesmethyldiphenhydramine and didesmethyldiphenhydramine, which are apparently intermediates in the formation of the major metabolite, diphenylmethoxyacetic

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acid [7]. The other major metabolite is the N-oxide of diphenhydramine [8]. However, not many investigators have isolated and measured those metabolites. Skofitsch and Lembeck [6] failed to detect any of them. More recently, Blyden et al. [9] have been able to quantitate a demethylated metabolite and provide pharmacokinetic data.

The goal of this study was to develop a simple and sensitive HPLC method to quantitate diphenhydramine in human plasma after a single dose of the drug.

EXPERIMENTAL

Materials

Diphenhydramine hydrochloride and imipramine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Desmethyldiphenhydramine hydrochloride and didesmethyldiphenhydramine were gifts from Parke Davis Pharmaceuticals, Research Division (Ann Arbor, MI, U.S.A.) and Chemofux (Wolfsberg, Austria), respectively. All other reagents and solvents were of HPLC grade and used without further purification.

Apparatus

The chromatographic system consisted of a Waters Model 590 programmable solvent delivery module, a Waters WISP 710B autosampler and a Model 481 UV detector, all from Waters (Milford, MA, U.S.A.). The column was 15 cm $\times$ 0.46 cm I.D. in-house packed with ODS-1 Spherisorb, 5 μm particle size, obtained from Phase Separations (Norfolk, CT, U.S.A.). Chromatograms were recorded by an SE-120 recorder (BBC Goerz Metrawatt). Data were collected and processed on a Hewlett Packard Model 3357 laboratory automation system.

Chromatographic conditions

The mobile phase was prepared by mixing 2200 ml of acetonitrile, 1400 ml of deionized water and 400 ml of 1 *M* sodium phosphate monobasic. After stirring, the mobile phase was filtered through a 0.45-μm Nylon filter. The flowrate was 1.8 ml/min, which resulted in a back-pressure of 170 bar. The detector wa set at 205 nm, 0.01 a.u.f.s. Retention times were 4.6 and 6.4 min for diphenhydramine and internal standard, respectively. The total run time was 10 min. The whole system was kept at room temperature (22 ± 3°C).

Standard and quality control (QC) sample preparation

The standard and QC samples were prepared by spiking human plasma with aqueous solutions of the drug. QC samples were prepared from separately weighed stock solutions. The volume of standard solution added to plasma was always less than 2% of the plasma volume to maintain integrity of the sample. The calibration curve ranged from 1.00 to 100 ng/ml. The standard and QC

samples were aliquoted (1.00 ml per tube) immediately after spiking and stored at −15 °C until needed.

Extraction procedure

To an aliquot (1 ml) of standard, QC or clinical sample, imipramine hydrochloride as internal standard (100 μl, 0.5 μg/ml) was added, as well as a saturated potassium carbonate solution (200 μl). After mixing, samples were extracted with 5 ml of hexane–isopropanol (98:2, v/v) by shaking on a reciprocating shaker at 230 rpm for 15 min and then centrifuged at 800 *g* for a further 10 min. The upper organic layer was transferred into a conical tube containing 100 μl of 0.5% (w/v) orthophosphoric acid. The tube was shaken for 15 min and centrifuged for 10 min at 800 *g* at 5 °C. The upper organic layer was aspirated off and the lower aqueous phase was transferred into conical microtubes. A 50-μl volume of the final extract was injected into the HPLC system.

RESULTS AND DISCUSSION

Precision and accuracy

A set of seven calibration standards, a zero, a blank and three QC samples, in duplicate, were analysed on each analytical day. The inter-assay and intra-assay precision and accuracy were assessed by the repeated analysis of QC samples containing different concentrations of diphenhydramine.

The results are shown in Table I. Linear response of the diphenhydramine to internal standard peak-height ratio was observed over the concentration range 1.00–100.0 ng/ml. A linear regression analysis using a least-squares fit

TABLE I

PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF DIPHENHYDRAMINE IN HUMAN PLASMA

Nominal concentration (ng/ml)	*n*	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Mean as percentage of nominal concentration
Inter-assay				
3.00	12	3.154 ± 0.1471	4.7	105 1
40.0	12	42.00 ± 1.275	3.0	105 0
90 0	12	94.41 ± 2.945	3.1	104.9
Intra-assay				
3.00	14	3.233 ± 0.1265	3.9	107 8
40.0	15	40 15 ± 0 723	1.8	100.4
90.0	15	87.15 ± 0.785	0.9	96 8

was performed with the reciprocal of the drug concentration as weight. The equation of the line was $y=mx+b$, where m was the slope of the calibration curve and b the intercept. The correlation coefficients, an indication of linearity, were equal to or better than 0.9984 ($n=6$).

Specifity

Plasma was collected from fifteen healthy donors and surveyed for interference at the retention times of diphenhydramine and the internal standard, imipramine. No interference peaks were seen in nine of them. A small interference peak representing less than 15% of the lowest standard value at the retention time of diphenhydramine was observed in five different drug-free samples of plasma. In one plasma this interfering peak represented 31% of the lowest standard value.

Recovery

Recovery was calculated by comparing extracted QC samples with unextracted standards, which represented 100% recovery, correcting for all the losses in volume due to sample transfer. The recovery of diphenhydramine was 90.1% [$n=6$; coefficient of variation (C.V.) 8.6%] at 3.00 ng/ml and 87.7% ($n=5$;

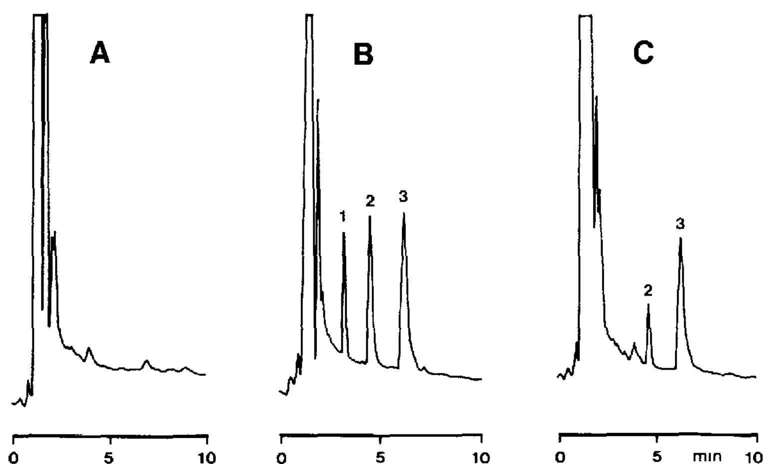

Fig. 1. Representative chromatograms of diphenhydramine analysis. (A) Drug-free plasma sample; (B) sample obtained from a volunteer 1.5 h after oral administration of 40 mg dimenhydrinate liquid; (C) standard of 12.5 ng/ml diphenhydramine in human plasma. Peak: 1= desmethyldiphenhydramine; 2=diphenhydramine; 3=internal standard (imipramine) Chromatographic conditions: wavelength 205 nm, 0.01 a.u.f.s., flow-rate, 1.8 ml/min; column, Spherisorb ODS-1 The small endogenous peak at retention time of 4.0 min appears only in some plasma samples and does not interfere with peaks of diphenhydramine (4.6 min) or desmethyldiphenhydramine (3.4 min)

C.V. = 2%) at 90.0 ng/ml. The recovery of imipramine was 92.9% (n=5; C.V. = 9.9%) at 50 ng per sample.

Chromatography

Fig. 1 shows a chromatogram of a drug-free plasma sample from a healthy volunteer (A), followed by a sample obtained from a healthy volunteer 1.5 h after oral administration of 40 mg of dimenhydrinate in liquid form (B). The final chromatogram represents standard 12.5 ng/ml.

There are several critical factors influencing the quality of chromatography. First, in order to achieve the necessary sensitivity, the detector is set at a lower range of the UV spectrum for the diphenhydramine shows only weak absorbance at 252 and 257 nm (ϵ=430). At a lower range of the UV spectrum, many substances, either endogenous or reagents, show a strong absorbancy. Hence, the glassware equipment and tube caps are washed with methanol to avoid incidental contamination. Second, the volume of injection has to be as low as possible. Should the injection volume be excessive (100–200 μl), the solvent front will overlap the peak of the drug.

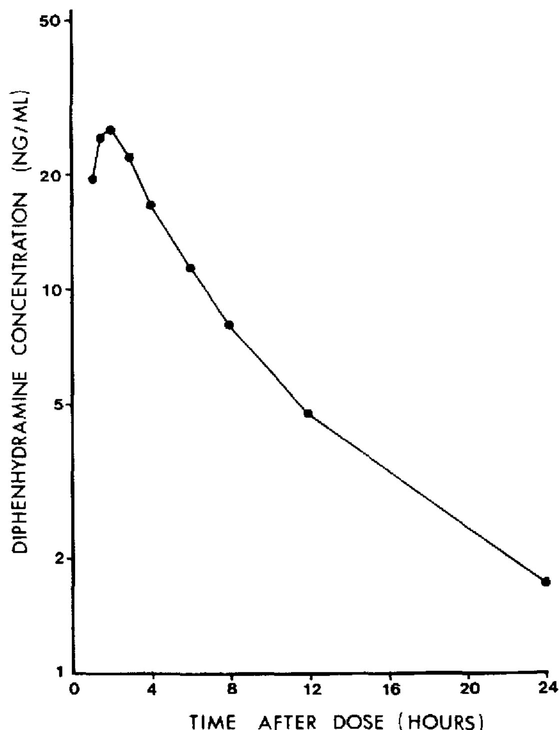

Fig. 2. Concentration–time profile of diphenhydramine in human plasma after a 40-mg oral dose of dimenhydrinate liquid.

Plasma concentration in humans

A healthy volunteer was administered 40 mg of dimenhydrinate liquid with 100 ml of water. Blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after administration. The plasma profile obtained in this way is shown in Fig. 2. The highest concentration in plasma was 25.3 ng/ml achieved 2 h after drug administration. The elimination half-life was 6.6 h. This value is within range of the half-lives reported by Gilsdorf et al. [10] (4.25–5.0 h), by Abernethy and Greenblatt [2] (4.1 h) and by Blyden et al. [9] (8.4 h).

Although measurement of tentative diphenhydramine metabolites was beyond scope of this study, it was interesting to observe the appearance of one more peak after drug administration, well separated from diphenhydramine, with a shorter retention time suggesting smaller molecular weight and/or greater polarity. The metabolite has been tentatively identified as monodesmethyldiphenhydramine by comparing the retention time (3.4 min) of the peak with that of the standard.

The other tentative metabolite, didesmethyldiphenhydramine, which, in the conditions described above, has a retention time of 2.1 min, was not observed.

The assay could easily be adapted for quantitation of the metabolite and the parent drug as well.

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