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Note

Determination of picogram levels of a novel α_2-adrenergic receptor antagonist in plasma using solid-phase extraction and capillary gas chromatography with nitrogen-selective detection

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(2*S*,12b*S*) - 1,3,4,5′,6,6′,7, 12b-Octahydro-1′, 3″ -dimethylspiro [2H-benzofuro(2,3-a)quinolizine-2,4′-(1′H)pyrimidin]-2′(3′H)-one (I, Fig. 1) is a potent competitive α_2-adrenergic receptor antagonist in vitro and in vivo [1] and has demonstrated the potential for use both as a hypoglycemic agent and as an antidepressent. In support of pharmacokinetic studies, it was desirable to have a reliable quantification of I at a level of 100 pg/ml to insure full mapping of plasma concentration–time courses. Therefore, a capillary gas chromatographic (GC) method with nitrogen-selective detection was developed which employs a solid-phase extraction scheme for the biological specimen. The sample extraction pro-

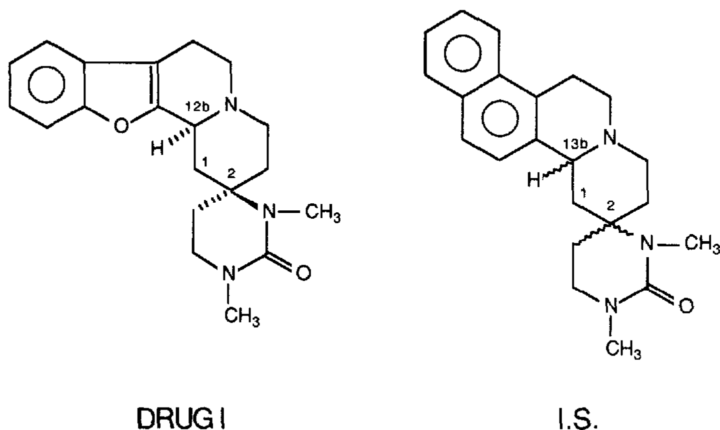

Fig. 1. Chemical structures of drug I and internal standard (I.S.).

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cedure yielded a mean drug recovery of 70% (see Table I) with sufficient sample clean-up to separate drug and internal standard from endogenous background.

EXPERIMENTAL

Materials and reagents

The following materials and reagents were used: drug-free human control plasma (Sera-Tec Biologicals, Harrisburg, PA, U.S.A.), Bond Elut C_2 1-ml solid-phase extraction columns with stainless-steel frit (Analytichem International, Harbor City, CA, U.S.A.), 4.5- and 10-ml polypropylene tubes with caps (Sarstedt, Princeton, NJ, U.S.A.), Vortexer (VWR Scientific, Bridgeport, NJ, U.S.A.), Beckman Microfuge II centrifuge (VWR Scientific), vacuum manifold SPE 21 (J.T. Baker, Phillipsburg, NJ, U.S.A.), Hamilton 701N syringe (Alltech Assoc., Deerfield, IL, U.S.A.), Hewlett-Packard Ultra 1 (25 m$\times$0.32 mm I.D.; 0.17 μm film thickness) (Hewlett-Packard, Avondale, PA, U.S.A.), methanol (HPLC grade) (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), toluene (nanograde) (Fisher Scientific), water (HPLC grade) (Burdick & Jackson Labs.), sodium bicarbonate (reagent grade) (Fisher Scientific), compound I and internal standard (Merck, Rahway, NJ, U.S.A.).

Instrumental

Analyses were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a nitrogen–phosphorus detector. The capillary jet inside the detector was standard bore with an end restrictor, therefore limiting the minimum distance between the capillary column end and the collector bead to 3–5 mm range. A 25 m$\times$0.32 mm I.D., 0.17 μm film thickness Hewlett-Packard Ultra 1 (100% dimethyl polysiloxane gum phase) was used. A splitless glass liner was installed in the injector and the splitter was turned off. The data collection and integration were performed on a Hewlett-Packard 3357 laboratory automation system. The analog-to-digital converter was sampling a rate of 8 Hz.

Instrumental conditions

The injection port and detector temperatures were set at 280 and 300°C, respectively. The oven temperature program was: (A) 60°C initial hold 1 min, (B) 30°C/min to 270°C, hold 10 min, (C) 30°C/min to 280°C, hold 5 min. Flow-rates of hydrogen, air, and nitrogen (make-up) gases were 3.0, 100, and 20 ml/min, respectively. The helium (carrier) gas had a column head pressure of 0.65 bar. The septum purge was off at time 0 and open at 0.5 min.

Assay procedure

Plasma (1.0 ml), internal standard (5 ng), and 0.3 ml of 0.2 *M* sodium bicarbonate were added to a 10-ml conical polypropylene centrifuge tube. The tube was capped and vortex-mixed for 30 s to insure mixing. A C_2 Bond Elut solid-phase extraction column was prepared by eluting 1 ml of methanol, 1 ml of water and 1 ml of 0.02 *M* sodium bicarbonate. The plasma sample was transferred to the C_2 extraction column and eluted with a vacuum gauge set between 12.5 and

25 cm of mercury. The sample tube was rinsed with 1.0 ml of 0.02 *M* sodium bicarbonate and transferred to the extraction column. The extraction column was washed with 1.0 ml of 0.02 *M* sodium bicarbonate and 2 ml of water and allowed to dry under vacuum. The analyte was then eluted off the column with 1 ml of methanol and collected in a 4.5-ml polypropylene tube. The elute was evaporated to dryness in a Savant Speed Vac concentrator. The sample was reconstituted in 20 μl of toluene, and 5 μl were injected onto the gas chromatograph.

Calculations

A standard curve of I in plasma was run daily with clinical specimens. The calibration curves for plasma were linear from 0.1 to 20 ng/ml. The equation for the resulting line was $y=0.2336x+4.189\cdot10^{-3}$ (with $r^2=0.99929$). The peak-area ratio of drug to internal standard from the unknown samples was employed to calculate their concentrations from the standard curve.

RESULTS AND DISCUSSION

The UV absorption spectrum of I indicated the molar absorption coefficient (ϵ) of 12 000 M^{-1} cm^{-1} at 247 nm (in water, hydrochloride salt), which was not high enough to develop a high-performance liquid chromatographic assay with UV detection, in the desired concentration range of 0.1–20 ng/ml. Also, no intrinsic fluorescence or electrochemical activity in the desirable and low oxidation potential range (below +1.0 V) were found for I. Therefore, the capillary GC method with nitrogen-selective detection has been developed for assaying I in biological fluids.

The within-day precision (repeatability) of the method and inter-day precision are presented in Table I. A typical human plasma concentration–time profile is presented in Table II. Typical chromatograms are presented in Fig. 2.

Compound I and the internal standard (I.S.) each contain two chiral centers

TABLE I

INTRA- AND INTER-DAY VARIABILITY AND RECOVERY OF DRUG I

Nominal concentration (ng/ml)	Mean concentration found (ng/ml)	Coefficient of variation (%)	Recovery (%)
Intra-day (n=5)			
0.10	0.10	5.3	71
0.25	0.24	9.8	66
0.50	0.52	8.0	68
1.00	1.08	9.8	72
5.00	4.89	7.4	74
10.00	9.92	4.0	70
20.00	20.07	3.0	69
Inter-day (n=48)			
0 5	0.47	8.3	
5.0	4.95	11.8	

TABLE II

PLASMA LEVEL–TIME PROFILE OF A SUBJECT RECEIVING A 2.0-mg DOSE OF DRUG I

Time (h)	Concentration (ng/ml)
−0.25	0
0.25	0.75
0.5	5.61
1.0	6.28
2.0	3.87
3.0	3.06
4.0	1.77
8.0	0.53
12.0	0.13
24.0	0

in their molecules (Fig. 1), creating the possibility of stereoisomerism. Compound I utilized in our studies was a single stereoisomer with the absolute configuration (2*S*,12b*S*) specified in Fig. 1. No indications of any in vivo conversion from I to its stereoisomers, separate from I under our GC conditions, were found. In the case of I.S., stereochemically pure analytical sample was not available and the racemic material (2*SR*,13b*SR*) utilized as I.S. contained about 5–10% of its racemic diastereomer (2*SR*,13b*RS*). This diastereomer of the I.S. was probably separated under our GC conditions to give a small peak with the retention time slightly shorter than the I.S. (Fig. 2B and C) on all chromatograms of the samples containing I.S. The presence of a small amount of the diastereomer in the I.S. sample had no effect on the assay methodology and the validity of the method since the same sample of the I.S. was utilized throughout the whole study. It demonstrates rather the high specificity of the GC method utilized and its potential for the separation of the diastereomers of the I.S. and/or I, if present in the samples analyzed.

The thermionic bead operating current was adjusted to give a baseline offset at 130 pA. Under these conditions, the thermionic bead had a useful lifetime of seven days, providing a minimum detection limit of 5 pg of neat drug at a signal-to-noise ratio greater than 5:1. In the presence of endogenous interferences, 100 pg of drug per ml of plasma could be quantified with absolute recovery of 70%.

A clean-up procedure which was more efficient in reducing the endogenous interferences relative to drug would, in principle, yield an even more sensitive method. However, as with other amines (tricylic antidepressants, etc.) the clean-up procedures can lead to losses of the drug to adsorptive phenomena [2]. Various liquid–liquid extractions were tried with relatively poor results. Either drug recovery was low or large endogenous interferences were encountered. By using a solid-phase extraction scheme, we were able to extract drug and minimize the endogenous background. During the solid-phase developmental work-up, it was found that 10–20% of the drug was adsorbing to the polyethylene frits of the

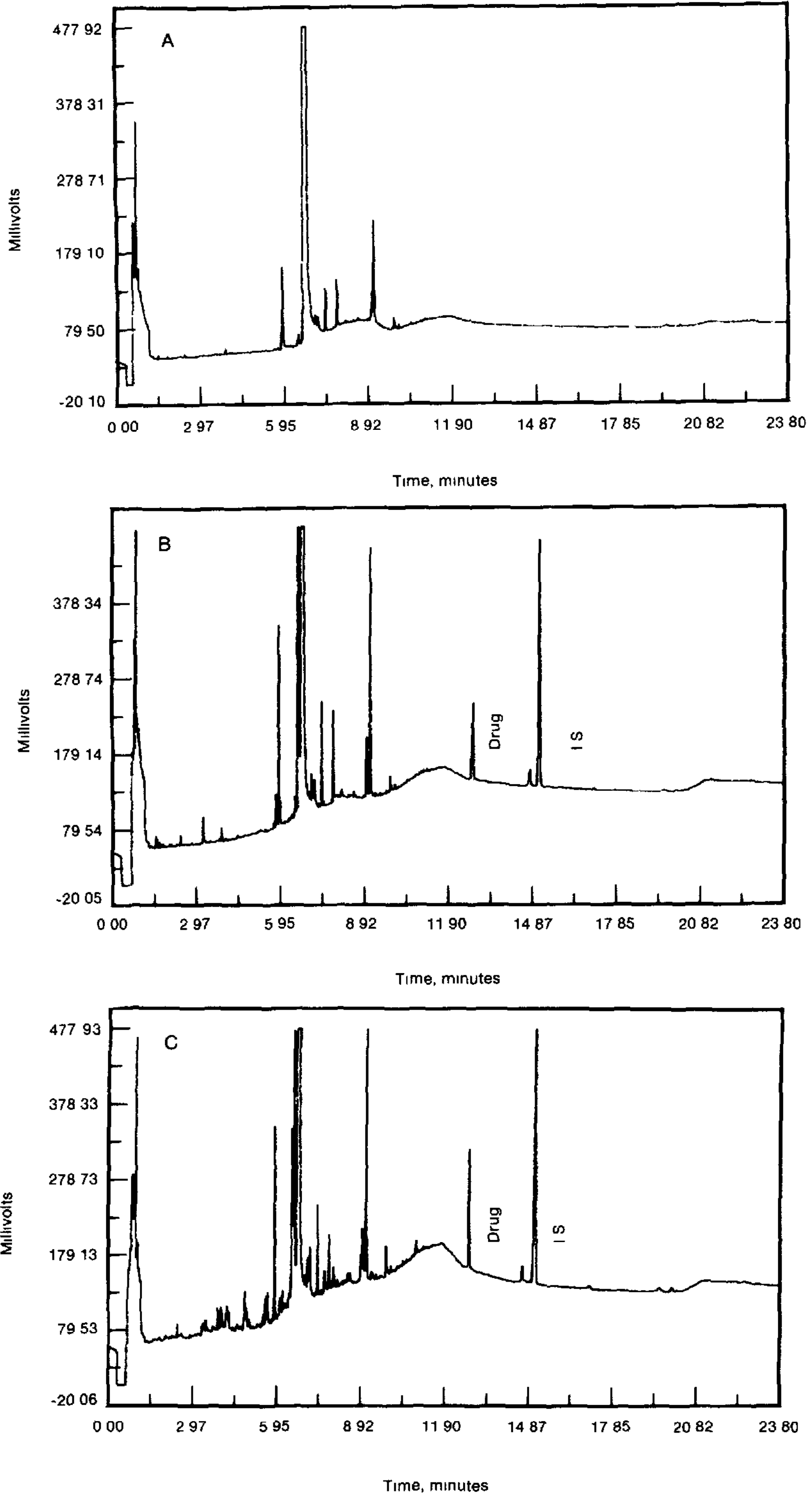

Fig. 2. Representative chromatograms of drug I and internal standard in plasma. (A) Blank plasma; (B) 0.1 ng/ml I and 5 ng/ml internal standard spiked into plasma; (C) clinical samples containing I (0.75 ng/ml calculated concentration) and 5 ng/ml I.S. spiked into subjects' plasma.

sample extraction column. This problem was easily eliminated by switching to stainless steel-fritted columns.

To maintain system integrity, the splitless liner must be changed on a daily basis to eliminate potential peak broadening (loss of separation efficiency) or drug loss due to active adsorptive sites (compounds) created during the injection process. To remove the residues, the column was back-flushed with varying polarity solvents on a weekly basis. Also, as needed, the inlet column end had to be shortened to maintain peak sharpness. In the future, a 1-m length of uncoated deactivated fused-silica tubing will be installed ahead of the column, acting as a retention gap [3,4]. Changing this deactivated fused silica 'precolumn' may prolong the useful life of the analytical column and retain peak integrity, while eliminating the time-consuming column back-flushes.

To operate the gas chromatograph consistently at low-level determination, much care must be taken to insure cleanliness of the capillary jet and the collector base housing. At the time of changing collector beads, the collector base was thoroughly cleaned with an alcohol-laden swab to eliminate any potential contamination build up. The cleaning procedure is important in eliminating noise spikes on the chromatogram. It is also important to have high-quality oxygen scrubbers on the carrier gas line to eliminate any oxygen-induced column stress [5–8].

This method has been employed for over 800 injections of plasma extract from human subjects over a four-month time period.

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