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Note

Normal-phase high-performance liquid chromatographic procedure for the determination of diprafenone in plasma

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Diprafenone, (±)-2'-[2-hydroxy-3-(*tert.*-pentylamino)propoxy]-3-phenylpropiophenone, is a class Ic anti-arrhythmic agent currently under development in several countries. The electrophysiological and anti-arrhythmic actions of diprafenone have been previously reported [1]. Suitable bioanalytical methods are needed in order to correlate drug plasma concentrations with pharmacologic response. A high-performance liquid chromatographic (HPLC) procedure for the quantitation of plasma diprafenone levels following a single oral dose is presented in this publication.

EXPERIMENTAL

Materials

Diprafenone, 5-hydroxydiprafenone, putative metabolites and the internal standard, CK-2624, 3'-[2-hydroxy-3-(*tert.*-pentylamino)propoxy]-3-(4-methylphenyl)propiophenone hydrochloride, were obtained from Helopharm-W. Petrik (Berlin, F.R.G.). [^{14}C]Diprafenone (2.0 MBq/mg, purity > 98%) was synthesized by New England Nuclear (Boston, MA, U.S.A.). Chloroform (preserved with amylene) and all other solvents were of HPLC grade. Sodium

dodecyl sulfate (SDS) (molecular biology reagent grade) was purchased from Sigma (St. Louis, MO, U.S.A.). Drug-free human plasma was obtained from New Jersey Blood Services (New Brunswick, NJ, U.S.A.). All other reagents were of ACS grade and were used as received.

Instrumentation

The HPLC system consisted of a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 400 pump, a WISP 712 automatic injector (Waters Assoc., Milford, MA, U.S.A.) and a Kratos 783 Spectroflow absorbance detector. The detector output was connected to a Hewlett Packard 3357 laboratory automation system via a Hewlett Packard Model 18652A A/D interface.

Chromatographic conditions

The mobile phase consisted of methanol–hexane–methylene chloride–distilled water–ammonium hydroxide (100:40:60:10:0.1, v/v). The mobile phase was filtered through a nylon filter (0.45 μm) prior to use. The flow-rate was 1.5 ml/min through a Nucleosil 50-5 (5-μm particles with a pore diameter of 50 Å) silica column (300 mm×4.0 mm I.D., ES Industries, Marlton, NJ, U.S.A.). The column was operated at ambient temperature (approximately 20°C). Column lifetime generally exceeded 1000 analyses. Injection volumes were 75 μl. Ultraviolet detection at 250 nm was used.

Preparation of plasma standards

Diprafenone plasma standards, ranging in concentration from 20 to 800 ng/ml, were prepared by diluting a 100 μg/ml aqueous solution of diprafenone with drug-free plasma. Individual 1-ml aliquots of standards were pipetted into screw-capped culture tubes and stored frozen until needed.

Radioactivity measurements

The radioactivity of recovery standards spiked with [^{14}C]diprafenone was determined by scintillation counting with a Packard Instruments (Dowers Grove, IL, U.S.A.) Model 2000 liquid scintillation counter.

Plasma extraction procedure

A 1-ml aliquot of plasma (sample or standard) was pipetted into a 100 mm×13 mm disposable glass culture tube. A 50-μl aliquot of internal standard solution (10 μg/ml in water) and 0.5 ml of an 0.5 M aqueous solution of SDS were added to the tube. The tube was vortex-mixed vigorously for 15 s. A 200-μl aliquot of a 5% aqueous solution of sodium carbonate was added and the tube vortex-mixed again. The entire contents of the tube were poured into a Clin Elut CE 1005 disposable extraction column (Analytichem, Harbor City, CA, U.S.A.) positioned on a Model UR1005 rack (Analytichem). The tube was washed with an additional 0.5 ml of distilled water, which was then added to

the column. The column was allowed to dry for 15 min. A 150 mm×25 mm disposable glass culture tube was positioned under the column. The column was washed consecutively with 15-ml volumes of diethyl ether–chloroform (3:1, v/v), diethyl ether and hexane–diethyl ether (1:1, v/v). The washes in the tube were placed in a water bath (40°C) and evaporated to dryness under a stream of nitrogen. The residue in the tube was reconstituted in 150 μl of methanol, rather than mobile phase, prior to injection into the HPLC system, as the analytes were found to be relatively insoluble in mobile phase. The injection of samples in methanol did not appear to adversely affect the chromatography.

Determination of extraction recovery

Radiolabeled plasma samples containing 40 and 400 ng/ml [^{14}C]diprafenone were extracted using the above procedure. Extraction recovery was calculated by comparing the radioactivity contained in the extracted sample to that contained in an equal volume of unextracted plasma.

RESULTS AND DISCUSSION

Diprafenone is a lipophilic compound with a pK_a of approximately 8.5. Equilibrium dialysis experiments have shown that most of diprafenone (>98.6%) is bound to the plasma proteins of humans. Initial experiments using liquid–liquid extraction with solvents such as benzene, methyl *tert.*-butyl ether, methylene chloride and chloroform to isolate diprafenone from the plasma matrix were unsuccessful because of low or irreproducible recoveries or the presence of interfering peaks in chromatograms of drug-free plasma. Use of a Clin Elut column and elution with a non-polar solvent, i.e. diethyl ether, yielded absolute recoveries of approximately 60%; however, the precision of the recovery across different human plasma pools, where each pool represents the plasma from a different anonymous human donor, was poor (Table I). Recovery of the drug from the Clin Elut column was improved with the addition of extra solvent washes, but the precision of the diprafenone recovery between plasmas remained poor. The high affinity of diprafenone to plasma proteins was believed to contribute to the poor precision of the extraction recovery between pools of plasma. Addition of an anionic surfactant, SDS, to plasma samples prior to extraction yielded an increase in extraction recovery as well as significantly improved precision across plasma pools (Table II). The surfactant is believed to relax the plasma proteins and free diprafenone from its plasma protein binding sites.

Figs. 1–3 show chromatograms of extracted drug-free plasma, a plasma sample spiked with diprafenone, 5-hydroxydiprafenone (a known metabolite) and internal standard, and a plasma sample (spiked with internal standard) taken from a subject 2 h after receiving a 200-mg dose of diprafenone. A comparison

TABLE I

DIPRAFENONE EXTRACTION RECOVERY ACROSS DIFFERENT HUMAN PLASMA POOLS USING A CLIN-ELUT COLUMN WITH DIETHYL ETHER ELUTION

Values in parentheses are coefficients of variation (%), $n = 3$.

Spiked concentration (ng/ml)	Recovery (%)			
	Plasma pool 1	Plasma pool 2	Plasma pool 3	Mean within concentration
40	74.2 (4.8)	74.1 (1.7)	59.6 (12.9)	69.3
80	79.5 (6.9)	66.2 (11.7)	50.1 (18.3)	65.3
200	66.7 (9.7)	55.5 (7.9)	54.7 (1.4)	59.0
400	68.0 (0.6)	61.4 (11.2)	55.1 (13.1)	61.5
Mean within plasma pool	72.1	64.3	54.9	

TABLE II

DIPRAFENONE EXTRACTION RECOVERY ACROSS DIFFERENT HUMAN PLASMA POOLS USING A CLIN-ELUT COLUMN AND THREE-SOLVENT ELUTION AFTER ADDITION OF SDS TO SAMPLE

Values in parentheses are coefficients of variation (%), $n = 3$.

Spiked concentration (ng/ml)	Recovery (%)			
	Plasma pool 1	Plasma pool 2	Plasma pool 3	Mean within concentration
40	88.7 (8.7)	83.3 (3.6)	86.2 (6.3)	86.1
80	84.8 (3.4)	87.9 (3.6)	82.8 (5.3)	85.2
200	84.5 (6.3)	79.1 (12.3)	80.1 (4.4)	81.2
400	82.1 (5.5)	85.5 (4.4)	80.9 (5.2)	82.8
Mean within plasma pool	85.0	84.0	82.5	

of Fig. 1 with Fig. 2 illustrates that the retention times of diprafenone and internal standard are free of matrix interferences.

The possibility of assay interference by several commonly prescribed drugs was tested by analyzing plasma samples spiked with each of the following compounds: doxepin, indoramin, flurazepam, diphenhydramine, chlorpheniramine, halazepam, propoxyphene, acetylsalicylic acid, caffeine, ephedrine, ibuprofen, phenobarbital, theophylline, xanthine and quinidine. None of the compounds tested were found to interfere with the internal standard or diprafenone peak.

Diprafenone is extensively metabolized to a number of compounds that are
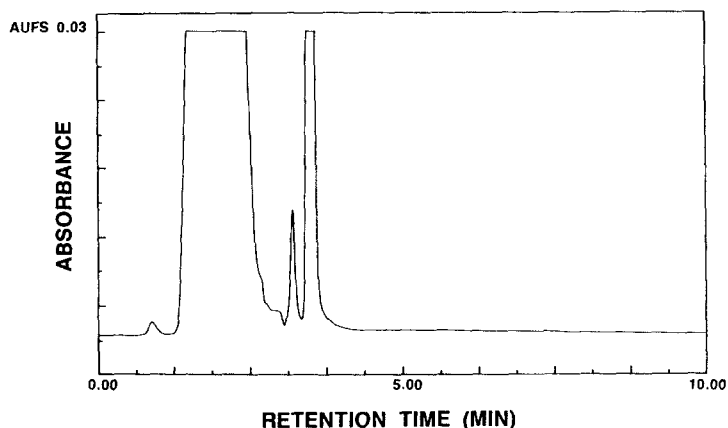
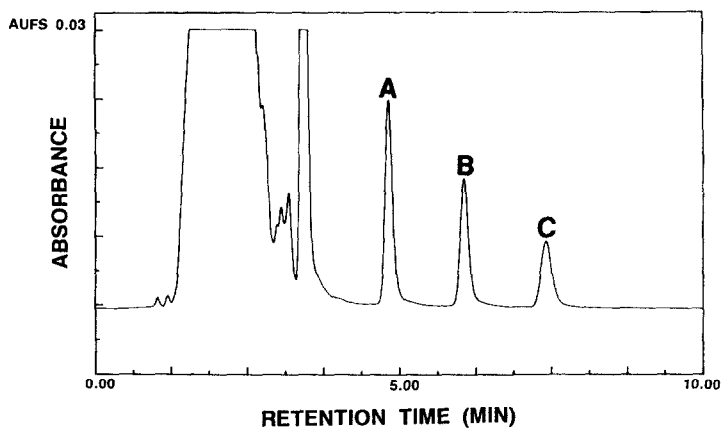Fig. 1. Chromatogram of extracted drug-free plasma.

Fig. 2. Chromatogram of plasma spiked with 500 ng/ml internal standard (A), 400 ng/ml diprafenone (B) and 400 ng/ml 5-hydroxydiprafenone (C).

of greater polarity than the parent compound, i.e. the metabolites will elute at retention times greater than that of diprafenone. Two putative metabolites are 4-methoxy-5-hydroxydiprafenone and n-desalkyldiprafenone. The chromatographic conditions specified in this method successfully resolved diprafenone, 5-hydroxydiprafenone and the two putative metabolites. N-Desalkyldiprafenone elutes at 7.9 min while 4-methoxy-5-hydroxydiprafenone elutes at 9.5 min. However, other unknown metabolites interfered with the quantitation of the metabolites in plasma samples taken from subjects receiving diprafenone. The major identified metabolite, 5-hydroxydiprafenone, can be quantitated with diprafenone if dual-wavelength UV detection at 250 and 340 nm is employed. Aromatic ring hydroxylation in the 5-position shifts the UV absorption max-
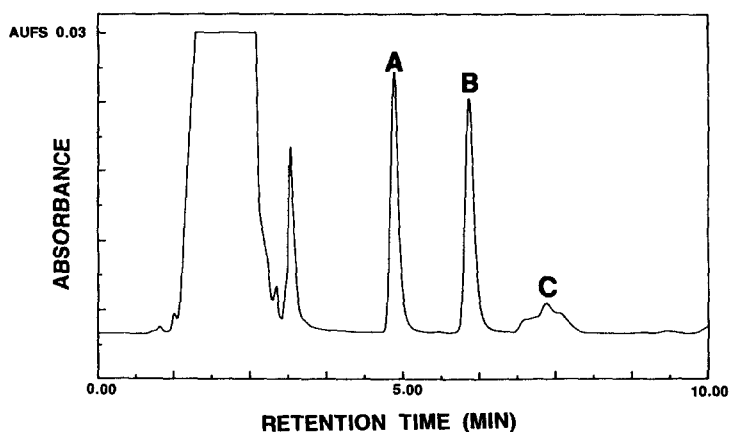
Fig. 3. Chromatogram of a plasma sample taken 2 h after a 200-mg oral dose of diprafenone. Peaks: A = internal standard (500 ng/ml); B = diprafenone (564 ng/ml); C = metabolites.

TABLE III

PRECISION AND ACCURACY OF THE METHOD

Spiked concentration (ng/ml)	Mean ($n=6$) analyzed concentration (ng/ml)	Coefficient of variation (%)	Mean error	Absolute relative error (%)
46.0	47.0	1.97	+1.0	2.2
91.9	89.8	1.33	−2.1	2.3
230	243	4.19	+13.0	5.7
460	451	1.29	−9.0	2.0

imum of diprafenone to 340 nm. The unknown interfering metabolites do not absorb at 340 nm as they probably lack substitution in the 5-position of the aromatic ring.

An additional consequence of the extensive metabolism of diprafenone was the need for an internal standard that eluted before diprafenone. Such an internal standard was required in order to eliminate the possibility of metabolite–internal standard interference.

Least-squares regression calibration curves, constructed by plotting diprafenone concentration versus the diprafenone-to-internal standard peak-height ratio were determined to be linear, based on an F-test [2], at plasma concentrations between 20 and 800 ng/ml diprafenone (8–320 ng diprafenone on-column). The limit of detection (signal-to-noise ratio = 3) of this assay was 5 ng diprafenone on-column, making it adequate for single- or multiple-dose pharmacokinetic studies in man.

The precision and accuracy of the method were determined from replicate

analyses ($n=6$) of four plasma pools spiked with diprafenone at concentrations within the linear range of the assay (Table III). The standards against which these samples were analyzed were prepared from the plasma of a separate pool. Method precision ranged from 1.2 to 2.0%. Accuracy, expressed as absolute relative error, ranged from 2.0 to 5.7%.

CONCLUSION

The normal-phase HPLC method described here has been found to be suitable for the analysis of plasma samples collected during single-dose bioavailability/pharmacokinetic studies. The addition of SDS to the samples effectively releases the highly bound drug from plasma proteins and eliminates the extraction recovery variability across different pools of plasma.

REFERENCES

1   S. Greenberg and A. Luisi, Drug Dev. Res., 17 (1989) 35.
2   M.G. Natrella, Experimental Statistics, U.S. Government Printing Office, Washington, DC, 1966.