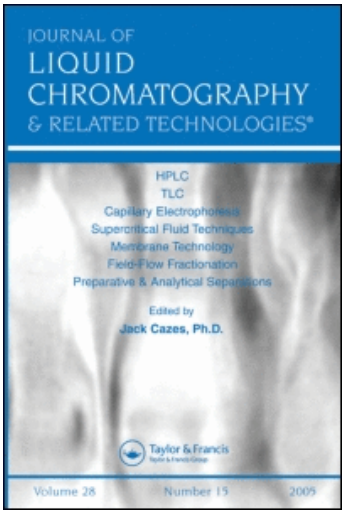

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High Performance Liquid Chromatographic Analysis of Some Antiarrhythmic Drugs in Human Serum Using Cyanopropyl Derivatized Silica Phase

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME ANTIARRHYTHMIC DRUGS IN HUMAN SERUM USING CYANOPROPYL DERIVATIZED SILICA PHASE

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ABSTRACT

A high performance liquid chromatographic determination of antiarrhythmic agents: propafenone (PPF) with its principal active metabolite 5-hydroxypropafenone (5-OHPPF), amiodarone (AD) with an active metabolite desethylamiodarone (DEAD), mexiletine (MEX) and diltiazem (DILT) in human serum is presented. The compounds are separated on Supelcosil LC-CN column at ambient temperature under isocratic conditions. For PPF, 5-OHPPF, MEX and DILT analysis the mobile phase: CH_3CN : H_2O : 0.5 M KH_2PO_4 (360:620:20 v/v) is pumped at a flow rate of 1.8 mL/min and the ultraviolet detection is performed at 210 nm. AD and DEAD are eluted with stronger mobile phase: CH_3OH : CH_3CN : H_2O : 0.5 M KH_2PO_4 (480:136:360:24 v/v) at a flow rate of 1.5 mL/min and detected at 240 nm. Sample preparation is based on simple liquid-liquid

extraction from acidified serum for AD and DEAD and on liquid-liquid extraction from alkalized serum followed by back extraction for PPF, 5-OHPPF, MEX and DILT analysis. Sensitivity of the assay was set at 5 ng/mL for PPF and MEX and at 10 ng/mL for 5-OHPPF, DILT, AD and DEAD. Coefficient of variation for intra- and interassay precision was below 10% for all the tested compounds in all the tested concentrations. The method is convenient for therapeutic monitoring of those four antiarrhythmics. Described assay may be easily adapted for the determination of verapamil, gallopamil, quinidine, flecainide and a few beta-blockers.

INTRODUCTION

Propafenone (PPF), amiodarone (AD) and mexiletine (MEX) are well-known antiarrhythmic agents, diltiazem (DILT) is a calcium channel blocker used in ischemia, hypertension and also in supraventricular arrhythmias.[1-4] Narrow therapeutic range with large, especially evident for PPF, intersubject concentration variability (in part associated with CYP2D6 mediated polymorphic metabolism), severe side effects as well as often occurring interactions render monitoring serum concentration very helpful in the therapy with these drugs.[4-8] Recently, in some patients the PPF-MEX combination was found effective for suppressing of arrhythmias.[9-11] For some antiarrhythmics i.e. flecainide, quinidine, lidocaine, procainamide, N-acetylprocainamide immunological assay kits are commercially available for therapeutic drug monitoring. However, our drugs of interest are quantified by chromatography and HPLC is often used. Many methods have been described for the determination of PPF, AD, MEX and DILT alone or with several of their metabolites.[12-28] The assay established in our laboratory has been done for serum monitoring of antiarrhythmics saving both cost and labour. Presented assay puts us also in the way of determining the main active PPF metabolite, 5-hydroxypropafenone (5-OHPPF) which is also the marker of individual metabolic activity co-segregated with CYP2D6 function.[5,29]

MATERIALS

The pure substances of PPF hydrochloride, 5-OHPPF hydrochloride, N-depropylPPF fumarate and an internal standard LU41616 {2'-[2-hydroxy-3-(3"-hydroxy-3"-methylbuthylamino)-propoxy]-3-phenylpropiophenone hydrochloride} were obtained from Knoll (Ludwigshafen, Germany). MEX

hydrochloride, its p-hydroxy (KOE 2127), hydroxymethyl (KOE 2259) and their corresponding alcohol metabolites (KOE 2618 and KOE 2619) were from Boehringer Ingelheim (Ingelheim, Germany). DILT hydrochloride with its deacetyl metabolite as well as AD, DEAD and L8040 hydrochlorides were supplied by Sanofi-Winthrop (Gentilly, France).

Stock solutions of PPF, 5-OHPPF, MEX, DILT, LU41616, AD, DEAD and L8040 (1 mg/mL) were obtained by dissolving appropriate amounts of their hydrochlorides in methanol (except DILT - in 0.01 M HCl) and were stable for at least six months when stored at 4°C in the dark.

HPLC grade acetonitrile and diisopropyl ether were from Merck (Darmstadt, Germany), methanol, hexane and KH_2PO_4 were from JT Baker (Deventer, Netherlands) and water was obtained from BDH (Poole, England). All other chemicals were of analytical grade.

METHODS

An HPLC isocratic system (LKB, Bromma, Sweden) consisted of a pump (model 2150), injector with 50 μL loop (model 7125, Rheodyne, Cotati, CA, USA), variable wavelength UV monitor (model 2141) and integrator (model 2221). The separation of compounds was made on Supelcosil LC-CN (150x4.6 mm, 5 μm) column protected with Supelguard LC-CN (20x4.6 mm, 5μm) precolumn (Supelco, Bellefonte, PA, USA) at ambient temperature. The weaker mobile phase: CH_3CN : H_2O : 0.5 M KH_2PO_4 (360:620:20 v/v) for PPF, 5-OHPPF, MEX and DILT quantification was pumped at a flow rate of 1.8 mL/min. Detection was accomplished at 210 nm. The stronger mobile phase: CH_3OH : CH_3CN : H_2O : 0.5 M KH_2PO_4 (480:136:360:24 v/v) for AD and DEAD analysis was pumped at a flow rate of 1.5 mL/min and detection was set at 240 nm.

Sample Preparation for PPF, 5-OHPPF, MEX and DILT

A 0.4 mL of serum was transferred into a 15 mL glass centrifuge tube, then mixed with 200 ng (20 μL) of LU41616 methanol solution (internal standard) and with 50 μL of 10% Na_2CO_3 solution. Next, 4 mL of diisopropyl ether was added and the sample was vigorously shaked for 4 min. After centrifugation and freezing at -20°C the organic layer was quantitatively transferred into a 10 mL conical glass test tube to which 100 μL of 0.01 M HCl

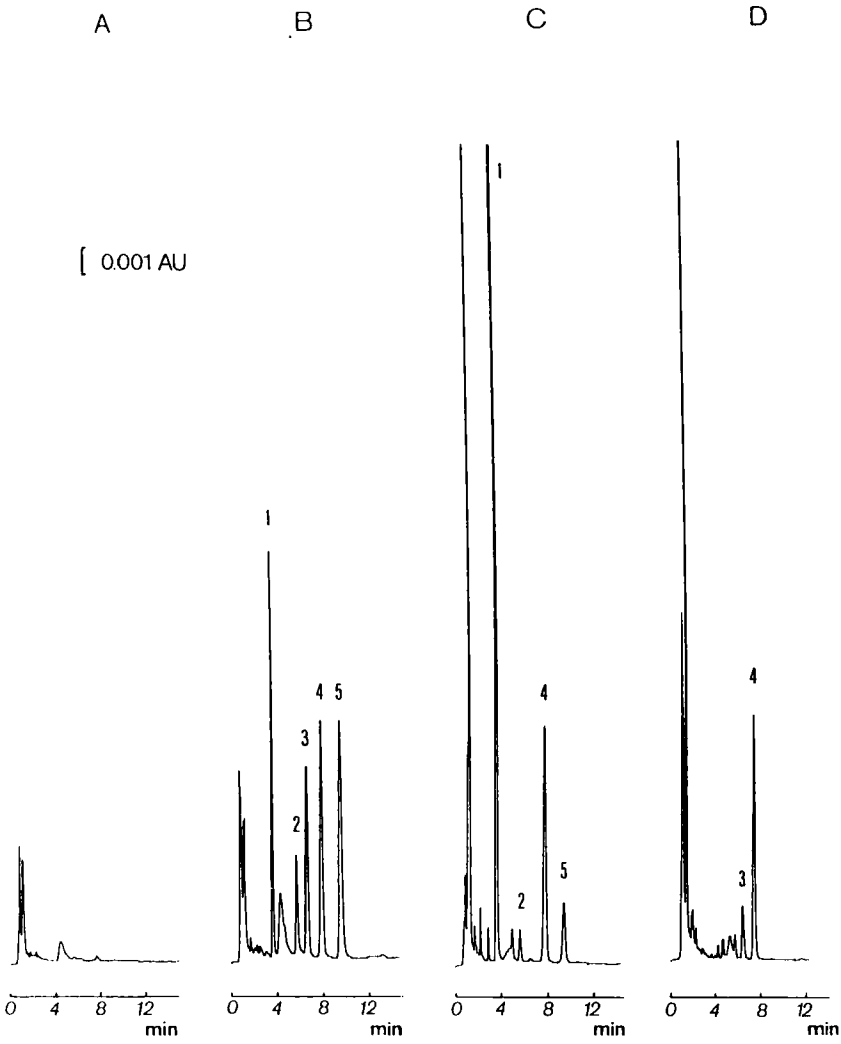

Figure 1. Chromatograms of the serum samples extracted as for PPF, MEX and DILT in Methods: A - drug-free serum; B - drug-free serum spiked with test mixture to obtain the concentrations: PPF - 500 ng/mL, 5-OHPPF - 250 ng/mL, MEX - 500 ng/mL, DILT - 250 ng/mL, LU41616 - 500 ng/mL; C - serum sample from patient treated with PPF and MEX, containing 143 ng/mL of PPF, 85 ng/mL of 5-OHPPF and 2311 ng/mL of MEX; D - serum sample from patient treated with DILT, containing 96 ng/mL of DILT; 1 - MEX, 2 - 5-OHPPF, 3 - DILT, 4 - LU41616 (internal standard), 5 - PPF.

were added. Then the tube was carefully vortex-mixed for 45 s using micro-shaker, centrifuged and the organic phase was removed to waste. The aqueous residual was evaporated to dryness in a water bath at 56°C under a stream of argon. The dried extract was then reconstituted in 100 μL of a mobile phase and a 50 μL aliquot was injected onto the column.

Sample Preparation for AD and DEAD

A 0.2 mL of serum was transferred into a 15 mL glass centrifuge tube, then mixed with 500 ng (20 μL) of L8040 methanol solution (internal standard) and with 100 μL of 0.5 M KH_2PO_4 solution. Next, 4 mL of hexane was added and the sample was shaked for 3 min. After centrifugation and freezing at -20°C the organic layer was quantitatively transferred into a 10 mL conical glass test tube and evaporated to dryness in a water bath at 37°C under the stream of argon. The dried extract was then reconstituted in 100 μL of methanol and a 50 μL aliquot was injected onto the column.

Calibration

Working solutions for calibration and controls were prepared from the stock solutions by an adequate dilution in methanol. Working solutions were added to drug free serum to obtain the concentration levels of: 20, 100, 250, 1000, 4000 ng/mL for PPF; 40, 200, 500, 1000, 4000 ng/mL for MEX and 20, 50, 100, 250, 500 ng/mL for 5-OHPPF and DILT and 50, 200, 500, 1000, 4000 ng/mL for AD and DEAD. The following procedures were as described above for sample preparation.

RESULTS AND DISCUSSION

PPF, 5-OHPPF, MEX, DILT and LU41616 were well separated from the biological background at retention times of: 9.3, 5.6, 3.5, 6.4 and 7.7 min, respectively (Figure 1). In described chromatographic conditions we did not observe any interference with metabolites of analyzed drugs available for tests which were found at retention times: p-hydroxyMEX and hydroxymethylMEX at 2.2 min, their alcohols at 1.6 min, deacetylDILT at retention time of 4.5 min and N-depropylPPF at 4.9 min (Table 1). Because of co-eluting peak of endogenous origin we did not calibrate the assay for determination of

Table 1

Retention Data for Interference Studies

Compound	Relative Retention Time (Internal Standard = 1.00)	Capacity Factor
MEX	0.45	3.4
5-OHPPF	0.73	6.0
DILT	0.83	7.0
LU41616 (internal standard)	1.00	8.6
PPF	1.21	10.6
HydroxymethylMEX-ol + p-hydroxyMEX-ol	0.21	1.0
HydroxymethylMEX + p-hydroxyMEX	0.29	1.8
DeacetylDILT	0.58	4.6
N-depropylPPF	0.64	5.1
Theophylline	0.17	0.6
Phenacetine	0.22	1.1
Aprobarbital	0.22	1.1
Atenolol	0.22	1.1
Nadolol	0.25	1.4
N-acethylprocainamide	0.25	1.4
Procainamide	0.26	1.5
4-Hydroxydebrisoquine	0.26	1.5
Pentobarbital	0.26	1.5
Hexobarbital	0.27	1.6
Sotalol	0.27	1.6
N-desmethylclobazam	0.31	2.0
Acebutolol	0.32	2.1
Mephenytoin	0.34	2.3
Metopolol	0.36	2.5
Clobazam	0.36	2.5
Celiprolol	0.38	2.6
Debrisoquine	0.39	2.8
Diazepam	0.39	2.8
Lidocaine	0.40	2.9
Prazosin	0.43	3.1
Progesteron	0.55	4.3
Quinidine	0.55	4.3

Table 1 (Continued)

Retention Data for Interference Studies

Compound	Relative Retention Time (Internal Standard = 1.00)	Capacity Factor
Propranolol	0.66	5.4
Bupranolol	0.69	5.6
Flecainide	1.13	9.9
Verapamil	1.38	12.3
Gallopamil	1.42	12.6
Desethylamiodarone	4.91	46.3
Amiodarone	7.23	68.6

Analytical conditions as described in Methods for PPF, 5-OHPPF, MEX and DILT analysis.

N-depropylPPF and deacetylDILT which similarly to pharmacologically inactive MEX metabolites are all of minor clinical importance. However, the goal of the presented assay is the fact that in case of PPF parent drug monitoring is linked with the estimation of individual CYP2D6 activity.

Several cardiac agents and other drugs were checked for possible interference with the method. Their solutions were injected onto the column and detected under the described analytical conditions. Retention data for tested compounds are presented in Table 1. None of these drugs interfered with the assay. Additionally, the extraction procedure was established as efficient (75-90%) for quinidine, verapamil, gallopamil, flecainide and a few beta-blockers (acebutolol, propranolol, metoprolol). After only small changes in mobile phase composition the assay may be adapted for the determination of these cardiac drugs. On the other hand, celiprolol was extracted in 40%, atenolol and sotalol were practically unextractable.

AD, DEAD and L8040 were all eluted "far away" after other tested drugs (Table 1). That was the reason for introducing the stronger mobile phase composition. After that modification AD, DEAD and L8040 were also completely resolved and separated from the biological background at retention times of: 6.7, 5.9 and 8.0 min, respectively (Figure 2).

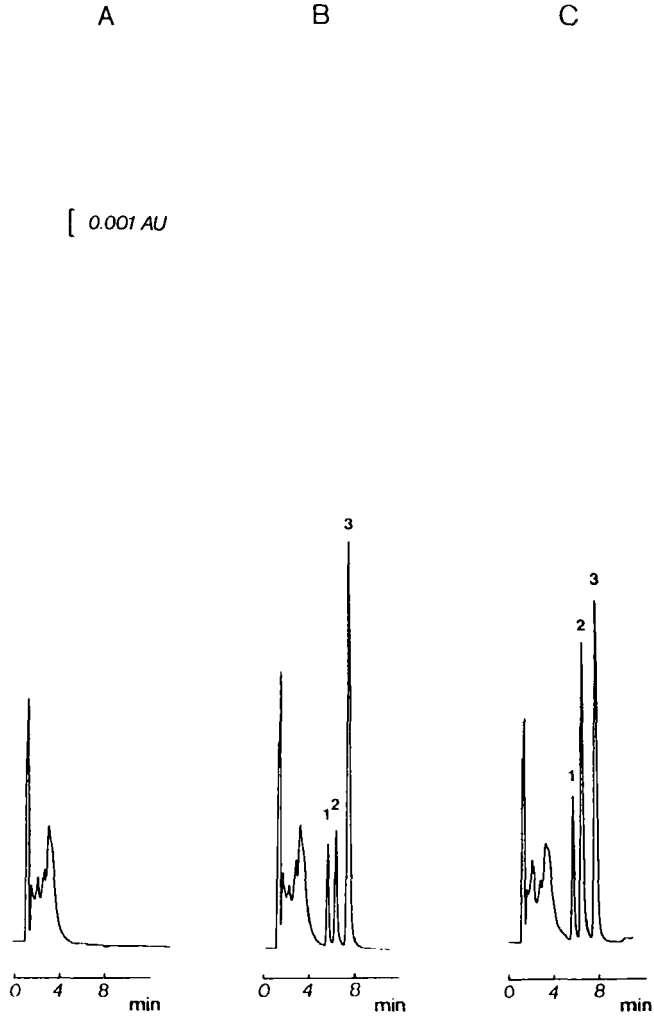

Figure 2. Chromatograms of the serum samples extracted as described for AD in Methods: A - drug-free serum; B - drug-free serum spiked with test mixture to obtain the concentrations: AD - 500 ng/mL, DEAD - 500 ng/mL, L8040 - 2500 ng/mL; C - serum sample from patient treated with AD, containing 1403 ng/mL of AD and 790 ng/mL of DEAD; 1 - DEAD, 2 - AD, 3 - L8040 (internal standard)

The calibration curves were obtained by analyzing four serum samples for each tested concentration. The curves were linear and described by the following equations: y=0.00244x-0.0405 (r=1.0000) for PPF; y=0.00135x-0.0115 (r=0.9993) for 5-OHPPF; y=0.00160x-0.0259 (r=1.0000) for MEX; y=0.00249x-0.0075 (r=1.0000) for DILT; y=0.00056x-0.0183 (r=1.0000) for AD and y=0.00040x-0.0069 (r=0.9999) for DEAD.

The absolute extraction recovery was analyzed by comparing the peak areas for extracted calibration standards with those obtained from direct injection of equivalent quantities of standards. Satisfactory recovery was acquired for all the compounds i.e. 90.2% (89.3-92.6) for PPF, 78.2% (72.5-84.2) for 5-OHPPF, 88.9% (85.9-94.2) for MEX, 80.2% (76.3-83.9) for DILT, 93.3% (88.3-96.9) for AD and 63.5% (62.3-64.9) for DEAD being stable for the concentrations covering the calibration range (data in parentheses). The alkalization of serum sample to pH 10.5 using Na_2CO_3 solution was chosen because of its value for efficient PPF, 5-OHPPF, LU41616 and MEX extraction. The back-extraction step decreased the chromatographic interference from serum. In contrast, our early findings showed that simple one-step extraction with hexane from the acidified serum gives good results for AD and DEAD analysis what is in opposition to the findings of Arranz Pena et al.[25] Some investigators noted the phenomenon of DILT hydrolysis occurring when pH>9 was achieved during analytical procedure.[12-14] In our experiment DILT was extracted in about 80 % with good repeatability and we keep considering such a recovery satisfactory for quantification of this drug. We did not observe any significant influence of the preparation time up to 3 hours from the start of the analytical procedures on extraction recovery. Both internal standards were extracted in a high percent i.e. LU41616 - 90.8 ± 4.4 % (n=20) and L8040 - 96.1 ± 5.6% (n=20).

The precision of the assay was examined using the data from calibration for intraassay and analyzing standard samples in duplicates on 4 different days for interassay precision. Coefficient of variation (CV) for all the tested concentrations was always below 10%. Detailed information is presented in Table 2. The sensitivity of the method (signal-to-noise ratio 3:1) was set at 5 ng/mL for PPF and MEX and at 10 ng/mL for 5-OHPPF, DILT, AD and DEAD.

Verbesselt et al.[28] gave a valuable script for HPLC assays of 12 antiarrhythmics (including PPF, MEX and AD) applying the same apparatus, solid-phase extraction system and general rules but different analytical

Table 2

Precision of the Method (n=4)

	Concentration Added [ng/mL]	Intraassay Factor Determined*	CV [%]	Interassay Factor Determined*	CV [%]
	20	0.046 ± 0.0034	7.51	0.046 ± 0.0045	9.84
	100	0.214 ± 0.0072	3.37	0.212 ± 0.0106	5.01
PPF	250	0.556 ± 0.0187	3.36	0.555 ± 0.0095	1.71
	1000	2.355 ± 0.1062	4.51	2.371 ± 0.0974	4.11
	4000	9.734 ± 0.1667	1.71	9.577 ± 0.1402	1.46
	20	0.027 ± 0.0014	5.61	0.027 ± 0.0017	6.38
	50	0.053 ± 0.0027	5.11	0.056 ± 0.0034	6.10
5-OHPPF	100	0.123 ± 0.0064	5.18	0.122 ± 0.0025	2.05
	250	0.312 ± 0.0146	4.69	0.319 ± 0.0264	8.27
	500	0.672 ± 0.0249	3.70	0.680 ± 0.0153	2.25
	40	0.061 ± 0.0022	3.65	0.062 ± 0.0015	2.43
	200	0.304 ± 0.0068	2.26	0.305 ± 0.0217	7.12
MEX	500	0.758 ± 0.0144	1.91	0.780 ± 0.0310	3.97
	1000	1.546 ± 0.0595	3.85	1.607 ± 0.1150	7.17
	4000	6.371 ± 0.1651	2.59	6.116 ± 0.2170	3.55
	20	0.046 ± 0.0033	7.22	0.043 ± 0.0040	9.51
	50	0.115 ± 0.0043	3.76	0.108 ± 0.0081	7.52
DILT	100	0.239 ± 0.0149	6.26	0.237 ± 0.0049	2.08
	250	0.615 ± 0.0439	7.13	0.603 ± 0.0578	9.59
	500	1.236 ± 0.0681	5.51	1.226 ± 0.0573	4.68
	50	0.020 ± 0.0018	9.19	0.020 ± 0.0007	3.41
	200	0.097 ± 0.0026	2.66	0.100 ± 0.0027	2.66
AD	500	0.254 ± 0.0056	2.19	0.259 ± 0.0047	1.81
	1000	0.527 ± 0.0061	1.16	0.541 ± 0.0121	2.23
	4000	2.213 ± 0.0241	1.09	2.268 ± 0.0427	1.88
	50	0.019 ± 0.0019	10.23	0.019 ± 0.0006	3.22
	200	0.075 ± 0.0065	8.64	0.076 ± 0.0027	3.50
DEAD	500	0.198 ± 0.0173	8.70	0.188 ± 0.0110	5.83

Table 2 (continued)

Concentration Added [ng/mL]	Intraassay Factor Determined*	CV [%]	Interassay Factor Determined*	CV [%]
1000	0.375 ± 0.0028	0.73	0.379 ± 0.0292	7.72
4000	1.591 ± 0.0545	3.42	1.574 ± 0.0467	2.97

*Factor determined - the ratio of peak area of analyzed compound to peak area of internal standard.

conditions for particular drugs. Additionally, of some importance may be large (1 mL) sample volume and for PPF analysis also insufficient (25 ng/mL) limit of quantitation. The analysis of PPF, DILT and AD simultaneously with DEAD on LC-CN column was also described by Mazzi,[27] unfortunately, the sensitivity of that assay was poor (50 ng/mL for PPF, AD, DEAD and 20 ng/mL for DILT).

The presented method provides the possibility of simultaneous measurement of PPF, its 5-hydroxymetabolite, MEX and DILT on LC-CN column. After changing the mobile phase followed by only twenty minutes system washing, detection wavelength change and, obviously, applying different extraction procedure we are successfully able to determine AD with DEAD using the same HPLC system. Described procedure may be the basis for establishing the analysis of verapamil, gallopamil, quinidine, flecainide and beta-blockers.

Concluding, the paper presents simple assay which may be recommended for therapeutic monitoring as well as for pharmacokinetic studies on PPF, AD, MEX and DILT.

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