Journal of Chromatography, 187 (1980) 391–398
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,333

RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF AMIODARONE IN BLOOD PLASMA OR SERUM AT THE CONCENTRATIONS ATTAINED DURING THERAPY

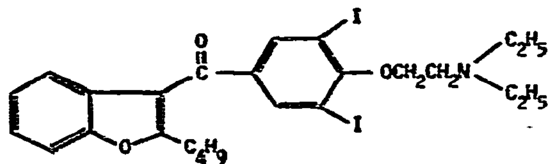
R. J. FLANAGAN, G. C. A. STOREY and D. W. HOLT
Poisons Unit, Guy's Hospital, St. Thomas' Street, London SE1 9RT (Great Britain)
(Received August 20th, 1979)

SUMMARY

A simple high-performance liquid chromatographic method has been developed for the measurement of the antiarrhythmic drug amiodarone in small (200 μl) volumes of plasma or serum. After addition of 2 mole/l phosphate solution, pH 4.5 (20 μl), containing the internal standard, the sample is vortex-mixed with diisopropyl ether (200 μl) for 30 sec. A portion (100 μl) of the resulting extract is analysed on a microparticulate (5 μm) silica column using methanol–diethyl ether (85:15) containing perchloric acid (0.02% v/v) as the mobile phase, and the absorption of the column effluent is monitored at 240 nm. No endogenous sources of interference have been observed, and interference from other drugs is minimal. The procedure is rapid, an analysis in duplicate taking less than 15 min to complete. The limit of sensitivity of the assay is 0.05 mg/l, and the concentrations of amiodarone measured in plasma samples from patients under treatment with this compound ranged from 0.15 to 4.5 mg/l.

INTRODUCTION

Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran; Fig. 1] is an orally effective antiarrhythmic agent. It is thought that this compound may have a relatively long half-life in man since optimal therapeutic effects may only be seen some days after the commencement of therapy[1], and may persist for some time after withdrawal of the drug[2]. However, the pharmacokinetics of amiodarone have received only scant attention using radio-labelled drug[3].



Amiodarone

Fig. 1. Structural formula of amiodarone.

The method described here for the measurement of plasma amiodarone concentrations is based upon the principle of solvent extraction of a relatively small plasma volume, followed by the direct chromatographic analysis of a portion of the resulting extract[4]. In this case, the primary chromatographic system chosen was a microparticulate (5 μm) silica column using methanol–diethyl ether containing 0.02% (v/v) perchloric acid as the mobile phase. However, in the absence of an established reference technique the results of sample analyses were compared to those obtained on a nitrile-bonded microparticulate silica column.

EXPERIMENTAL

Materials and reagents

Amiodarone and the internal standard, fenethazine [10-(2-dimethylaminoethyl)phenothiazine hydrochloride], were obtained from Labaz (Brussels, Belgium) and Rhône-Poulenc (Paris, France), respectively. The internal standard was used as a 12.5 mg/l solution in 2 mole/l aqueous potassium dihydrogen orthophosphate (analytical reagent grade), pH 4.5. Methanol and diethyl ether were both HPLC Grade (Rathburn Chemicals, Walkerburn, Great Britain), and diisopropyl ether (laboratory reagent grade) and perchloric acid (70%) (analytical reagent grade) were obtained from BDH (Poole, Great Britain).

High-performance liquid chromatography (HPLC)

The solvent delivery system was a constant-flow reciprocating pump (Applied Chromatography Systems, Model 750/03) and sample injection was performed using a Rheodyne Model 7120 syringe-loading valve fitted with a 100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the valve to the analytical column, a stainless-steel tube 125 × 5 mm I.D. packed with Spherisorb 5 silica (Hichrom, Woodley, Great Britain), which was used at ambient temperature (normally 22°). The column effluent was monitored at 240 nm (Applied Chromatography Systems, Model 750/11) and integration of peak areas was performed using a Hewlett-Packard 3352 data system. The mobile phase was methanol–diethyl ether (85:15) containing 0.02% (v/v) perchloric acid, and this was helium-degassed before use. The flow-rate was 2.0 ml/min, maintained by a pressure of *ca.* 40 bar.

The chromatography on this system of an extract from a plasma standard containing amiodarone and fenethazine is illustrated in Fig. 2. The retention times of these compounds and some additional drugs on this system are given in Table I.

Sample preparation

Plasma or serum (200 μl) was pipetted into a small (Dreyer) test-tube (Poulten, Selfe and Lee, Wickford, Great Britain). Internal standard solution (20 μl) and diisopropyl ether (200 μl) were added using Hamilton repeating mechanisms fitted with 1.0- and 5.0-ml Hamilton gas-tight luer-fitting glass syringes (Field, Richmond, Great Britain), respectively. Everett stainless-steel needles (No. II serum) were fitted to these syringes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged at 9950 *g* for 2 min in an Eppendorf 5412 centrifuge (Anderman, East Molesey, Great Britain) which was modified to accept Dreyer tubes

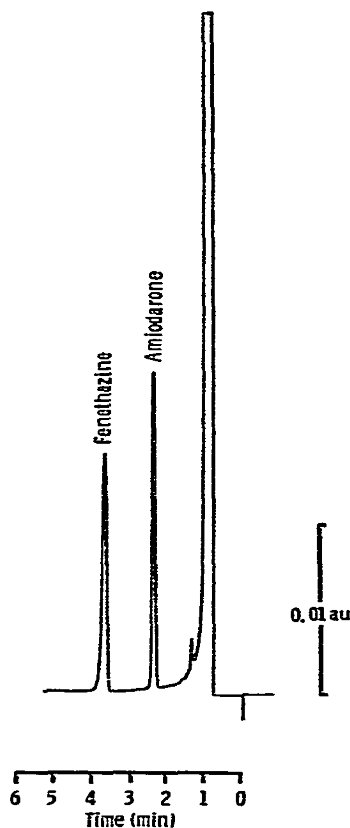


Fig. 2. Chromatogram obtained on analysis of a diisopropyl ether extract of heparinised bovine plasma containing amiodarone (1.0 mg/l) on the 5-μm silica column; 100 μl injection. See text for chromatographic conditions.

by slightly drilling-out the 0.4-ml test-tube centrifuge adapters. Subsequently, *ca.* 110 μl of the extract were taken and used to fill the sample loop of the injection valve. Analyses were performed in duplicate and the mean result taken.

Instrument calibration

Standard solutions containing amiodarone at concentrations of 0.10, 0.20, 0.50, 1.00, 2.00 and 5.00 mg/l were prepared in heparinised bovine plasma by dilution of a 1 g/l solution of this compound in methanol. The plasma and methanolic solutions were stable for at least 1 and 3 months, respectively, if stored at 4° and in the absence of light.

On analysis of these solutions, the ratio of the peak area of drug to the peak area of the internal standard, when plotted against drug concentration, was linear and passed through the origin of the graph. The calibration gradient normally obtained was 1.12 l/mg.

TABLE I

RETENTION TIMES RELATIVE TO FENETHAZINE OF AMIODARONE AND OTHER COMPOUNDS ON (i) THE MICROPARTICULATE SILICA AND (ii) THE NITRILE-BONDED COLUMNS

Compound	*Relative retention times*	
	(i) 5 µm Silica column	*(ii) 5 µm Nitrile column*
Glutethimide	0.20	0.23
Butobarbitone	0.21	0.24
Quinalbarbitone	0.21	0.24
Barbitone	0.22	0.23
Phenytoin	0.22	0.24
Cyclobarbitone	0.23	0.25
Ethotoin	0.23	0.27
Phenylbutazone	0.23	0.25
Amylobarbitone	0.24	0.24
Chlorpropamide	0.24	0.24
Ethosuximide	0.24	0.23
Feprazone	0.24	0.28
Heptabarbitone	0.24	0.23
Methoin	0.24	0.25
Methosuximide	0.24	0.27
Pentobarbitone	0.24	0.24
Pheneturide	0.24	0.23
Phenobarbitone	0.24	0.25
Phenylethylmalondiamide	0.24	0.25
Thiopentone	0.24	0.27
Azapropazone	0.26	0.25
Phenacetin	0.26	0.25
Primidone	0.26	0.23
Caffeine	0.27	0.31
Carbamazepine	0.27	0.29
Lorazepam	0.29	0.33
Oxazepam	0.36	0.55
Temazepam	0.37	0.49
Desalkylflurazepam	0.38	0.72
Nitrazepam	0.44	0.81
Nordiazepam	0.44	0.85
"Amiodarone metabolite"	0.45	0.78
Lignocaine	0.53	0.81
Carbamazepine-10,11-epoxide	0.54	0.82
Chlordiazepoxide	0.55	0.90
Diazepam	0.56	1.00
Propylphenazone	0.60	0.73
Lorcainide	0.64	1.03
Methaqualone	0.62	0.80
Amiodarone	0.68	0.90
Chlormethiazole	0.76	0.75
Chlorpromazine	0.81	0.96
Promethazine	0.83	1.00
Medazepam	0.84	1.00
Phenazone	0.85	0.95
Prochlorperazine	0.85	0.97
Fenethazine	1.00	1.00
Promazine	1.06	1.00
Flurazepam	1.75–1.90*	1.55

* Tailing peak; retention times measured at 1 g/l and 10 mg/l, respectively.

RESULTS AND DISCUSSION

Choice of extraction and chromatographic conditions

Under the extraction conditions chosen (pH 6), the recovery of amiodarone from heparinised bovine plasma was 98.6% ± 3.3 (S.D.) over the range 0.5, 1.0, 2.0 and 5.0 mg/l ($n = 3$ at each concentration). In contrast, the recovery was only 21.0% ± 3.9 (S.D.) at pH 9. Solutions prepared in heparinised human plasma gave analogous results. These findings were obtained by direct comparison of the peak areas obtained on analysis of 100 μl portions of methanolic solutions of amiodarone to those obtained from freshly-prepared sample extracts. However, an internal standard was incorporated in the final analytical procedure in order to minimize possible errors due to partial evaporation of the extract prior to analysis, and also to facilitate the use of injection volumes of less than 100 μl should this prove necessary. For example, duplicate results can be obtained where only 100 μl of sample is available for each extract by using the other reagents in proportion.

Diisopropyl ether was found to be a suitable extraction solvent since it is considerably less volatile than solvents such as diethyl ether and, thus, evaporation of the extract prior to analysis was minimised. On the other hand, diethyl ether was used as the lipophilic constituent of the mobile phase since commercially available diisopropyl ether contains 0.01% (w/v) hydroquinone resulting in a very high absorbtion at 240 nm, a convenient $\lambda_{max.}$ of amiodarone. The presence of hydroquinone in the extracts did not interfere in the analysis since it gave rise in part to the "solvent front" observed (Figs. 2–4).

It was necessary to add perchloric acid in low concentrations (0.02%, v/v) to the mobile phase in order to promote elution of the compounds under study. Under these conditions, the relative retention times of these compounds (Table I) varied by ±0.01, although during routine use the absolute retention times did vary by up to 15%. A higher perchlorate concentration than that indicated gave very rapid elution but with some loss of resolution, whilst lower concentrations gave longer retention times and permitted some peak tailing. Acetic acid, at concentrations up to 1% (v/v), was ineffective in promoting the elution of these compounds; higher concentrations than 1% could not be used since the absorption of the solvent at 240 nm became limiting.

Specificity

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 3, and the chromatogram obtained from a plasma specimen from a patient treated chronically with amiodarone is given in Fig. 4. In both cases, analyses of these and other specimens performed without the addition of fenethazine have not revealed the presence of compounds which could co-elute with this standard.

The compound which eluted with a retention time of 0.45 relative to fenethazine (Fig. 4) has only been observed in extracts from patients treated chronically with amiodarone, and increased in concentration with time in samples obtained from a patient soon after the start of amiodarone therapy. This presumed metabolite of amiodarone appears to have a similar UV absorption spectrum to the parent compound, but is extracted more efficiently at pH 7.4 than under the conditions used in the assay.

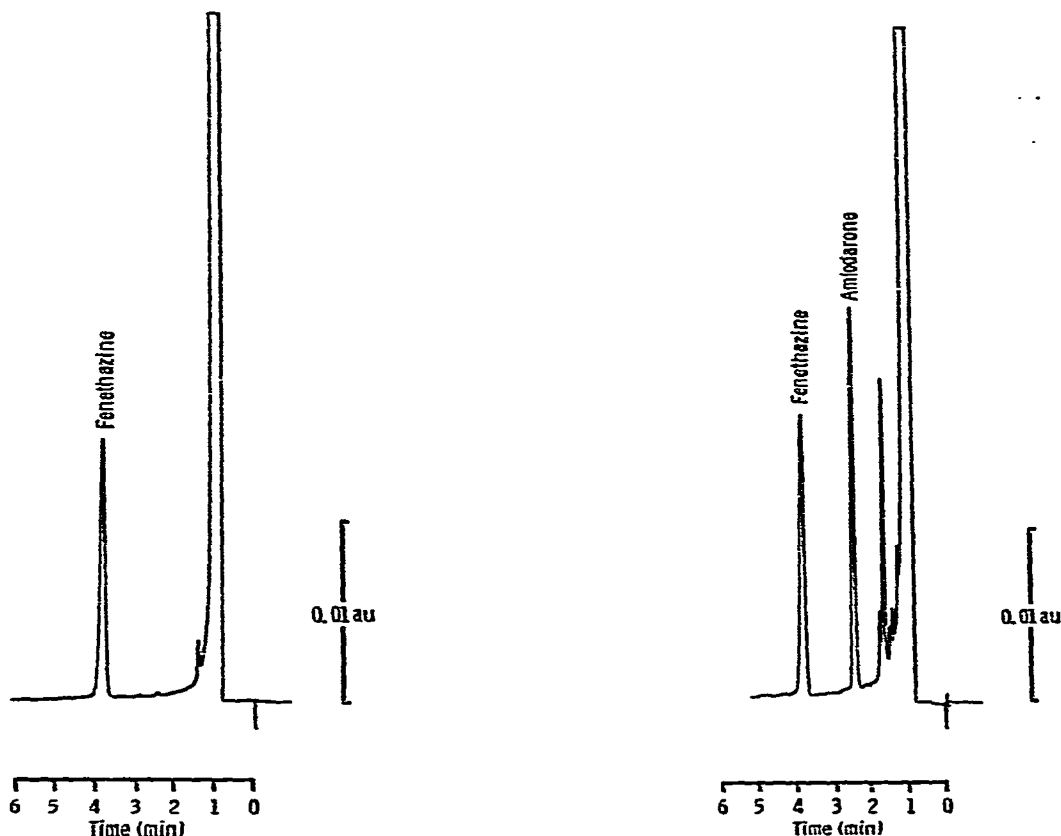


Fig. 3. Chromatogram obtained on analysis of an extract of drug-free human plasma on the 5 μm silica column; 100 μl injection.

Fig. 4. Chromatogram obtained on analysis of an extract of plasma from an amiodarone-treated patient on the 5 μm silica column; 100 μl injection. The amiodarone dose was 200 mg/day, and the plasma amiodarone concentration was found to be 1.1 mg/l.

Neither amiodarone nor this presumed metabolite have been detected in urine specimens from two of the amiodarone-treated patients studied.

Additional cardio-active drugs which might have interfered (disopyramide, mexiletine, procainamide, quinidine and tocainide) were not extracted under the conditions of the assay. The potential interference from a number of other drugs which are extracted under these conditions and have significant absorption at 240 nm has been studied (Table I). The concentrations of lorcainide attained during oral therapy are normally below 0.4 mg/l (ref. 5) and since this compound is poorly extracted at an acidic pH, such interference as may occur is unlikely to prove serious. Of the remaining compounds, only methaqualone and promazine eluted with amiodarone and fenethazine, respectively. Should interference from promazine occur, quantitative analyses can be performed by direct comparison of sample and standard peak areas of amiodarone from freshly-prepared extracts as discussed previously. Interference from methaqualone may be countered by using an alternative chromatographic system, such as that discussed in the following paragraph.

Comparison with results obtained on a second chromatographic system

The results of sample analyses were compared to those obtained on a second chromatographic system in order to provide additional evidence as to the specificity of the assay. The extraction and instrument calibration were identical to those described previously. The column used was a stainless-steel tube (250 × 5 mm I.D., packed with Spherisorb 5 nitrile (Hichrom), and the mobile phase was methanol–propan-2-ol (AR Reagent grade), 75:25, containing 0.02% (v/v) perchloric acid. The other chromatographic conditions were stated previously for the Spherisorb 5 silica column, except that a pressure of 120 bar was required to give a flow-rate of 2.0 ml/min.

The retention times of amiodarone and its presumed metabolite together with those of some other compounds on this nitrile-bonded column are given in Table I. The retention time of fenethazine under these conditions was 6.9 min. No compounds in addition to amiodarone and this presumed metabolite were observed in sample extracts from patients treated with amiodarone. Of the commonly-used drugs, only diazepam interfered in the assay on this column since it was not resolved from the internal standard (Table I). Thus, for the purposes of the comparison specimens which contained diazepam were assayed by direct measurement of amiodarone peak areas from freshly-prepared sample and standard extracts.

Twenty-eight plasma specimens (five containing diazepam) were analysed using both the nitrile and silica columns and the results of this comparison are presented in Fig. 5. The mean results obtained on each system were almost identical [silica: 1.32 mg/l ± 1.03 (S.D.); nitrile: 1.30 mg/l ± 1.02 (S.D.)] and the regression coefficient (using the data from the silica column as the independent variable) was 0.994 with an intercept of −0.011 mg/l.

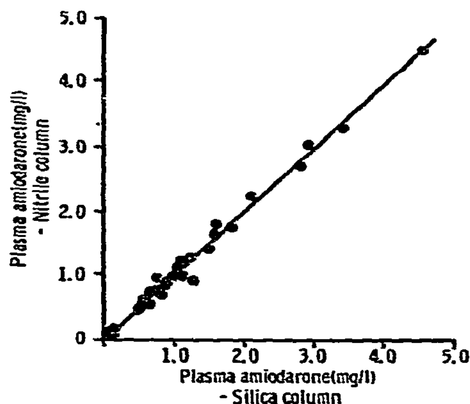


Fig. 5. Results of amiodarone assays performed independently on two different column systems. (The regression line shown is that of x on y.)

Reproducibility

The intra-assay coefficients of variation (C.V.) measured from replicate analyses ($n = 10$) of standard solutions prepared in heparinised bovine plasma containing amiodarone at concentrations of 0.2, 1.0 and 2.0 mg/l were 3.8%, 2.5% and 2.2%, respectively. The inter-assay C.V. at 1.0 mg/l was 6.9% ($n = 10$).

Limit of sensitivity

The limit of accurate measurement of the assay was 0.05 mg/l; the intra-assay C.V. at this concentration was 7.1% ($n = 10$). The plasma amiodarone concentrations measured in specimens obtained from patients receiving from 200 to 600 mg/day of this compound ranged from 0.15 to 4.5 mg/l (Fig. 5); no amiodarone was detected in a blood sample from one patient prescribed 200 mg/day, non-compliance with therapy being the most likely explanation.

CONCLUSIONS

The method described here has been found to be suitable for the measurement of the plasma amiodarone concentrations attained during therapy and may prove useful in single-dose pharmacokinetic studies. Only 400 μl of specimen are required for a duplicate analysis, which can be completed within 15 min, and sources of interference are minimal. In addition, although no amiodarone was detected in two urine specimens from patients treated with this compound, the method may prove useful in the analysis of amiodarone in other biological fluids such as saliva. Finally, the nature of the presumed metabolite of amiodarone observed on sample extract chromatograms is the subject of active investigation.

ACKNOWLEDGEMENTS

We would like to thank Labaz and Rhône-Poulenc for their gifts of pure amiodarone and fenethazine, respectively, Dr. M. Shenasa and Dr. M. Tynan (Guy's Hospital) for supplying blood and urine samples from patients receiving amiodarone and Dr. B. Widdop (Poisons Unit) for his helpful criticism of the manuscript.

REFERENCES

1 Leading Article, *Lancet*, i (1979) 592.
2 M. B. Rosenbaum, P. A. Chiale, M. S. Halpern, G. J. Nau, J. Przybylski, R. J. Levi, J. O. Lazzari and M. V. Elizari, *Amer. J. Cardiol.*, 38 (1976) 934.
3 J. Broekhuysen, R. Laruel and R. Sion, *Arch. Int. Pharmacodyn.*, 177 (1969) 340.
4 R. J. Flanagan and D. J. Berry, *J. Chromatogr.*, 131 (1977) 131.
5 U. Klotz, P. Müller-Seydlitz and P. Heimburg, *Int. J. Clin. Pharmacol. Biopharm.*, 17 (1979) 152.