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Determination of amiodarone and desethylamiodarone in plasma with a standardised extraction and chromatographic optimisation procedure

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Abstract: A previously described optimisation procedure was tested by developing suitable HPLC systems for the determination of amiodarone and desethylamiodarone in plasma. Chromatography is performed on a cyano-column (CN-column) in the reversed-phase mode. The optimisation strategy consists of carrying out a gradient elution from which an appropriate solvent strength for isocratic elution is determined. Binary and ternary mobile phase compositions with the same solvent strength but with different solvent selectivity are then used to evaluate selectivity. The anti-arrhythmic drugs are extracted from plasma using an ion-pair extraction procedure with sodium-*n*-octyl-sulphate as ion-pairing agent.

Keywords: *Optimisation; solvent strength; solvent selectivity; amiodarone and desethyl-amiodarone; plasma.*

Introduction

Optimisation procedures have been studied for many different analytical procedures. Recently, they have also been applied in high-performance liquid chromatography by several workers [1–13]. Recently an optimisation strategy for the selection of suitable HPLC systems for the separation of pharmaceutical compounds has been reported [13]. The use of a single stationary phase and six mobile phase solvents form the basis of this strategy, that can be applied for chromatography in both the reversed-phase and the normal phase mode. The stationary phase is a CN-column and the six solvents are water, methanol, acetonitrile, tetrahydrofuran, methylene chloride and *n*-hexane. The first four solvents are used in reversed-phase chromatography and the last four in normal phase chromatography. The selection of these solvents is based on Snyder's solvent selectivity classification scheme, which classifies the solvents into eight groups, according to their solvent properties: proton donor, proton acceptor and dipole interactor [14].

The optimisation strategy consists of two steps. In the first instance a suitable solvent strength is determined from a gradient elution experiment so that the pharmaceutical substances elute with k' values in an appropriate range. The second step consists of

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changing the solvent selectivity of the eluting agent to influence resolution, while the solvent strength of the mobile phase is kept the same. This procedure is applied in the reversed-phase mode for the determination of amiodarone, desethylamiodarone and an internal standard in plasma.

Amiodarone is an effective anti-arrhythmic drug and has been used for several years [15–18]. It is characterised by a slow turnover and a long half-life. The steady state is only reached after 1 month and the therapeutic effect may persist for 10 days to 1 month after withdrawal of the drug [16–18]. However, amiodarone has important side-effects (sinus brachycardia, thyroid dysfunction, corneal microdeposits, photosensitivity rash) which are dose-related [16, 19, 20]. Hence it is important to have an analysis method which allows the determination of amiodarone and its most important metabolite, desethylamiodarone, in plasma, so that the optimal therapeutic dose can be determined.

A few HPLC methods are described in the literature using normal phase [21–26] or reversed-phase chromatography [26–27]. In all these papers, the selection of a suitable combination of stationary and mobile phase composition was performed by trial and error experiments, which are very time-consuming and dependent upon the chromatographer's experience and intuition. By the use of an optimisation strategy, a lot of experiments can be avoided and an optimal HPLC system is obtained in a shorter length of time and with a minimum of effort. Most of the HPLC methods described in the literature for these drugs are normal phase separations, while the reversed-phase mode is usually considered to be more attractive by most chromatographers. This paper covers the application of this strategy in a reversed-phase system.

Experimental

Chemicals and reagents

Methanol, acetonitrile, tetrahydrofuran and *n*-hexane were of HPLC grade and were purchased from E. Merck (Darmstadt, FRG). Double distilled water, which was further purified with a Water-I-system (Gelman Sciences, Ann Arbor, MI, USA), was used. Sodium-*n*-octylsulphate, H_3PO_4, $NaH_2PO_4 \cdot H_2O$ and chloroform were obtained from E. Merck. Propylamine was *pro analysi* quality and purchased from Fluka AG (Buchs, Switzerland).

Amiodarone, desethylamiodarone and the internal standard (IS) 2-ethyl-3-(3,5-dibromo-4-dipropylaminopropoxybenzoyl)benzothiophene (L 8040) were kindly supplied by Labaz (Brussels, Belgium).

Apparatus

A Varian 5060 liquid chromatograph, equipped with a Rheodyne injector (sample loop: 50 μl) and a UV-detector with fixed wavelength (254 nm) was used. Chromatograms were recorded with a Varian 9176 recorder and integrated with a Varian Vista CDS 401 chromatographic data system.

Chromatography was carried out on a Lichrosorb CN-column (250 × 4 mm i.d.) with particle size of 5 μm. The flow rate was 1 ml min^{-1}. All experiments were carried out at ambient temperature.

Extraction procedure

One millilitre of plasma sample or standard was pipetted into a glass centrifuge tube equipped with a PTFE-covered screw-cap and 100 μl internal standard solution were

added. Deproteinisation was carried out with 2 ml acetonitrile, added dropwise with continuous vortexing. After centrifugation for 15 min, the supernatant was transferred to a clean centrifuge tube. The centrifuge tube with the protein pellet was gently washed with a minimum volume of acetonitrile, added to the supernatant. Afterwards, the acetonitrile layer was evaporated under a gentle stream of nitrogen at 60°C. Ten millilitres of phosphate buffer, pH 3 (μ = 0.4) containing 0.05 M sodium-*n*-octyl-sulphate and 5 ml chloroform–hexane (3:2, v/v) was added and the tubes were shaken for 30 min. After centrifugation, the aqueous layer was discarded and 4 ml of the organic phase was evaporated at 30°C under a gentle stream of nitrogen. The residue was reconstituted in 200 μl acetonitrile and 50 μl was injected onto the chromatograph.

Stock and standard solutions

Stock solutions of amiodarone, desethylamiodarone and the internal standard were prepared in acetonitrile at a concentration of 500 μg ml^{-1} and stored at 4°C between use. Spiked solutions were prepared on the day of analysis by appropriate dilution. Calibration standards were made by spiking drug-free plasma to give final concentrations in the 0.5–5.0 μg ml^{-1} range. The internal standard was added at a concentration of 5 μg ml^{-1}. Calibration curves were constructed by plotting the peak area ratio of the analytes to the internal standard versus the concentration.

The extraction recoveries from plasma for both substances were determined by interpolation on a calibration curve of unextracted standards in acetonitrile (peak area versus concentration).

Results and Discussion

Optimisation strategy

The separation of the anti-arrhythmic drugs is carried out with a CN-column used in the reversed-phase mode. To the different eluting agents, 0.01% propylamine was added to avoid peak tailing. A gradient elution experiment from 50% methanol in water to 100% methanol in 20 min, was carried out to select a suitable solvent strength for isocratic elution, resulting in k' values smaller than 10. Figure 1 represents the resulting chromatogram of the gradient elution. Taking the dead-time of the gradient system into account, one can calculate the volume percentage of methanol at which each solute elutes. From all these percentages (C_1, C_2, ... , C_n) the geometric mean, multiplied by 3/4 (C), was calculated using the following equation [13]:

$$C = 3/4\ \sqrt[n]{C_1 \cdot C_2 \cdot \ldots \cdot C_n} \qquad (1)$$

In this case, n equals to 3 and C is 69% v/v methanol in water. The factor 3/4 is an experimentally determined factor to lower the solvent strength of the isocratic methanol–water composition [13]. If the geometric mean were not multiplied by this factor, amiodarone would elute in the resulting isocratic eluent composition with a k' value smaller than 1. For the determination of drugs in plasma, in general, one should take into account interference by endogenous substances, especially in the first few minutes of the chromatogram. Therefore, k' values should be as high as possible, consistent with the presence of interferences, but one should also take the total analysis time into account. Figure 2a illustrates the separation obtained with the appropriate mobile phase composition, which corresponds to 69% v/v methanol in water. All peaks

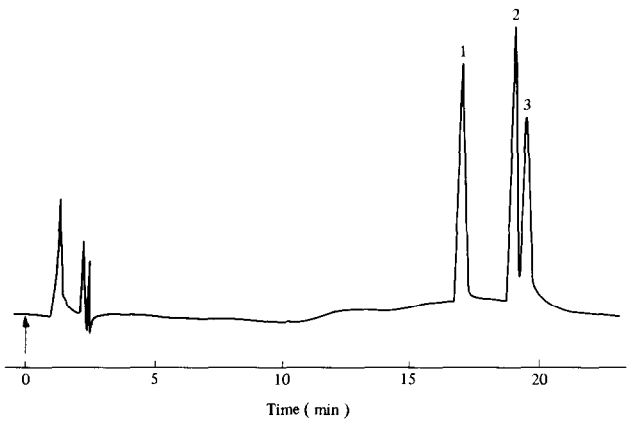

Figure 1
Chromatogram representing the elution of the anti-arrhythmic drugs by gradient elution:methanol–water–propylamine (50:50:0.01) → methanol–propylamine (100:0.01) in 20 min. Flow: 1 ml min^{-1}, a.u.f.s. = 0.08. 1 = amiodarone; 2 = desethylamiodarone; 3 = internal standard.

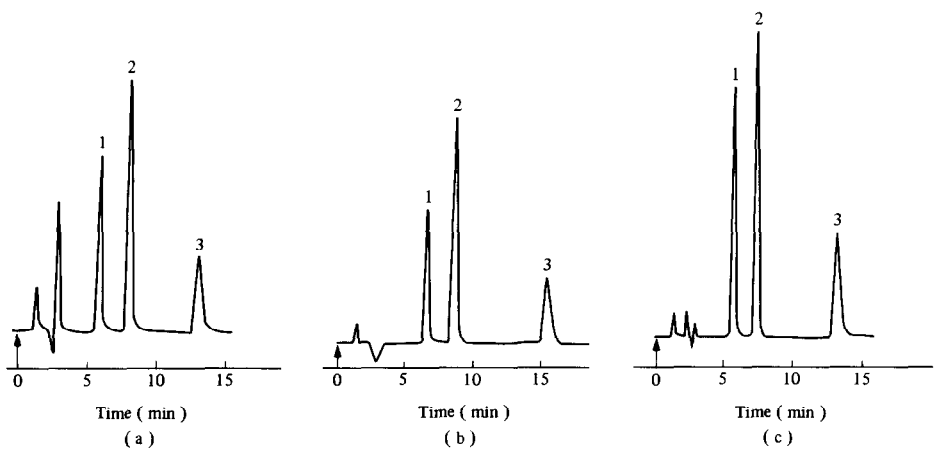

Figure 2
Chromatograms showing the separation in the binary mobile phases with the same solvent strength. (a) methanol–water–propylamine (69:31:0.01); (b) acetonitrile–water–propylamine (56:44:0.01); (c) tetrahydrofuran–water–propylamine (40:60:0.01). Flow: 1 ml min^{-1}, a.u.f.s.: 0.08. 1 = amiodarone; 2 = desethylamiodarone; 3 = internal standard.

have k' values within a suitable range ($k' < 10$). The solvent strength of the methanol–water composition was calculated using the following equation [28]:

$$S_T = \Sigma s_i \psi_i \quad (2)$$

where S_T = the total solvent strength of the mobile phase.
s_i = the solvent strength weighing factor of solvent i.
ψ_i = the volume fraction of solvent i.

The s_i values of the selected solvents are given in [28]. S_T in this case is 1.8. The second step consists of determining binary and ternary mobile phase compositions with the same solvent strength, but with different solvent selectivity. Figure 2 shows the chromatograms obtained using the binary mobile phases with the same solvent strength as the methanol–water composition: 56% v/v acetonitrile in water and 40% v/v tetrahydrofuran in water. The ternary eluting agents are produced by mixing the appropriate binary mobile phases in a 1/1 ratio so that the solvent strength is kept the same. The corresponding chromatograms are shown in Fig. 3.

In all of these HPLC systems, adequate separation is obtained between amiodarone, desethylamiodarone and the internal standard and the elution order remains the same. The k' values, given in Table 1, are within the same range, due to the fact that the eluent components have the same solvent strength. For the determination of the drugs in plasma, one has to select one of these compositions. Since binary solvent mixtures are simpler, ternary mobile phases were not further considered. From the three binary eluents the acetonitrile–water composition is selected, because the baseline of the blank plasma samples returns to zero within 5 min, which was not the case with the methanol–water mobile phase. In this HPLC system various drugs were injected in order to evaluate potential interferences. Their retention times relative to amiodarone and desethylamiodarone are given in Table 2. The selected HPLC system, namely the CN-column and a mobile phase comprising 56% v/v acetonitrile in water, is a suitable chromatographic system for the determination of amiodarone and desethylamiodarone in plasma, since only promethazine has a retention time close to that of desethylamiodarone.

The use of the proposed optimisation strategy facilitates to a large extent the selection of suitable HPLC systems. In the case of amiodarone, a suitable separation was obtained

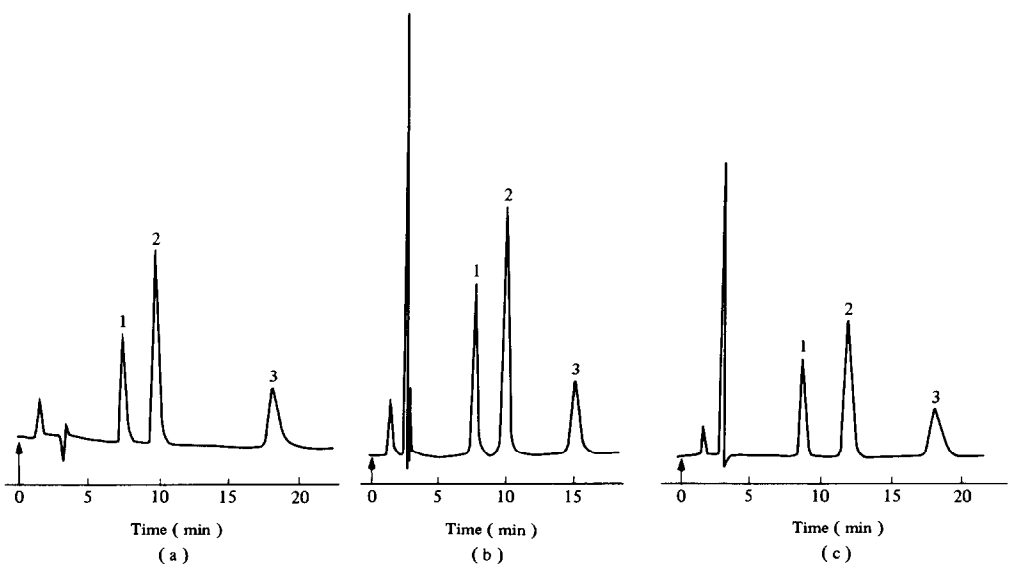

Figure 3
Chromatograms showing the separation in the ternary mobile phases with the same solvent strength. (a) methanol–acetonitrile–water–propylamine (35:28:37:0.01); (b) methanol–tetrahydrofuran–water–propylamine (35:20:45:0.01); (c) acetonitrile–tetrahydrofuran–water–propylamine (28:20:52:0.01). Flow: 1 ml min^{-1}, a.u.f.s.: 0.08. 1 = amiodarone; 2 = desethylamiodarone; 3 = internal standard.

Table 1
k' Values in mobile phase compositions with the same solvent strength but with different solvent selectivity

Mobile phase composition* vol % CH_3OH	CH_3CN	THF	H_2O	Solutes A	DA	IS
69	0	0	31	2.9	4.4	7.8
0	56	0	44	2.9	4.0	8.1
0	0	40	60	2.8	3.9	7.9
35	28	0	37	3.0	4.3	8.4
35	0	20	45	3.5	4.9	8.3
0	28	20	52	3.5	5.2	8.4

*0.01% propylamine was added to all the mobile phase compositions.
A = amiodarone; DA = desethylamiodarone; IS = internal standard.

Table 2
Relative retention of other drugs

Drug	Amiodarone	Desethylamiodarone
Amiodarone	1.00	0.75
Desethylamiodarone	1.34	1.00
Aprindine	7.12	5.32
Imipramine	3.44	2.57
Lidocaine	0.65	0.49
Amitryptiline	0.73	0.54
Nortryptiline	8.74	6.53
Nitrazepam	0.53	0.39
Diazepam	0.53	0.39
Melperone	3.89	2.87
Acebutolol	3.90	2.91
Diacetolol	4.37	3.26
Chlordiazepoxide	0.57	0.42
Cinnarizine	0.59	0.44
Cimetidine	0.61	0.46
Phenobarbital	0.29	0.22
Diphenylhydantoine	0.39	0.29
Thioridazine	3.15	2.31
Mesoridazine	5.13	3.76
Diphenhydramine	2.20	1.64
Metoclopramide	4.24	3.10
Promethazine	1.42	0.98
Procainamide	5.11	3.55
Caffeine	0.65	0.48

by carrying out only three experiments using binary mobile phase compositions. The ternary mobile phases were only used to confirm the optimal composition of the binary solutions and for purposes of demonstration. It is probable that the selection of a suitable HPLC system by trial and error experiments would have required more time due to more extensive work required using various different eluent components.

Extraction procedure and precision

The extraction procedure used was an ion-pair extraction with sodium-*n*-octylsulphate, which has already been applied in the authors' laboratory for the extraction of many basic drugs from plasma [29]. The extraction recovery for each substance, determined at the 2 $\mu g\ ml^{-1}$ level, is given in Table 3. For this application too, it seems

Table 3
Recovery of the anti-arrhythmic drugs from plasma

Drug	Concentration ($\mu g\ ml^{-1}$)	Recovery ($N = 6$)	C.V.
Amiodarone	2	78.5%	3.2%
Desethylamiodarone	2	79.8%	2.8%
Internal standard	2	67.9%	3.3%

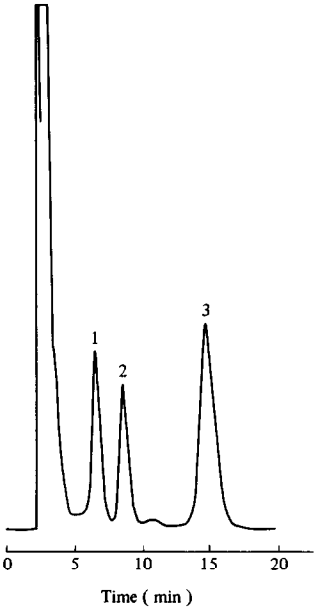

Figure 4
Chromatogram of an extracted plasma sample from a patient receiving 400 mg amiodarone daily. 1 = amiodarone (3.0 $\mu g\ ml^{-1}$); 2 = desethylamiodarone (2.2 $\mu g\ ml^{-1}$); 3 = internal standard. Mobile phase: acetonitrile–water–propylamine (56:44:0.01).

that ion-pair extraction with sodium-*n*-octylsulphate at pH 3 gives acceptable results for the determination of amiodarone and desethylamiodarone in plasma. Figure 4 shows the chromatogram of an extracted plasma sample from a patient receiving 400 mg amiodarone daily. The substances of interest are completely resolved from each other and no interference from endogenous compounds is observed.

Replicate spiked samples ($N = 6$) were analysed to evaluate the within-day precision, determined at two concentration levels (1 and 5 μg ml, respectively). For the determination of amiodarone in plasma, the relative standard deviation (RSD) was 3.5% and 3.2%, respectively, while the RSD values for desethylamiodarone were 6.2% and 4.1%, respectively. The detection limit was estimated to be 0.02 $\mu g\ ml^{-1}$ at a signal-to-noise ratio of 3 for the quantity injected.

Conclusion

The use of the proposed optimisation strategy allows the determination of optimal mobile phase compositions by carrying out the minimum number of experiments. First of all, column choice is omitted since the separation of all kinds of drugs is carried out on a single column type, namely a CN-column [13]. The application of a gradient elution

experiment permits the selection of an appropriate solvent strength for isocratic elution. Then, six isocratic experiments are performed, three with binary solvent mixtures and three with ternary solvent mixtures. All of these mobile phase compositions are of the same solvent strength, but possess different solvent selectivity. For the separation of amiodarone, desethylamiodarone and the internal standard in plasma, a suitable HPLC system was obtained using binary solvent mixtures. A combination of a CN-column with a mobile phase composed of acetonitrile–water–propylamine (56:44:0.01, v/v/v) gave an optimal separation and no interference was observed from plasma compounds or from many other drugs. The drugs were extracted by ion-pair extraction, with which a recovery of approximately 80% was obtained. With the HPLC developed system, complete separation was obtained in the reversed-phase mode, which is more advantageous than the normal phase systems previously described. The whole procedure has the desired sensitivity for therapeutic drug monitoring.

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