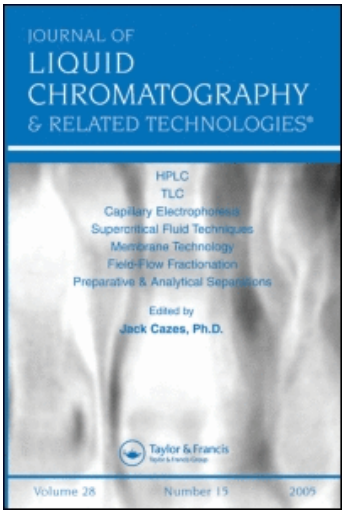

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AROMATIC POLY-AMIDINES: FORMULATORY AND PRECLINICAL APPLICATIONS

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ABSTRACT

A high-performance liquid chromatographic procedure has been developed for the simultaneous determination of aromatic poly-amidines (with 2, 3 or 4 benzamidine residues or different 2'-halogen substitutions). A Supelcosil LC-18-DB column with UV detection at 261 nm and an eluent consisting of phosphate buffer (200 mM, pH 3) - methanol - tetrahydrofuran - triethylamine (75:25:3:0.5, v/v) were employed. The proposed method was found suitable both in cellular biology/pharmacology studies, for the quantification of aromatic poly-amidines in serum and serum containing cell culture medium, as well as in preformulatory studies for the determination of drug release kinetic from delivery systems as in the case of microspheres.

INTRODUCTION

Poly-amidines are a class of compounds of great pharmaceutical interest since, as recently published, they can be proposed in the experimental and clinical

treatment of a large variety of pathologies (1-8). In this respect, pentamidine, an aromatic di-amidine derivative with antiprotozoal activity, has been demonstrated to be effective in the treatment of *Pneumocystic carinii* pneumonia (PCP) in patients with acquired immunodeficiency syndrome (AIDS) (1-3). Moreover other poly-amidinic compounds, with 2, 3 or 4 benzamidine residues in their chemical structure and their 2' halo-derivatives, possess interesting biological activities. These compounds were found (a) to inhibit the activity of serine proteinases involved in thrombosis and hemostasis, such as factor Xa and thrombin (4, 5) and (b) to be potent antitumor agents, both "in vitro" (6) and "in vivo" (7) on a variety of cell lines, including breast, kidney and colon carcinoma cells. In addition, these polyamidines were reported (c) to inhibit the "in vitro" metastatic activity of a highly tumorigenic cell lines (8) and (d) to specifically bind to DNA molecules interfering with DNA-protein recognition (9-11).

On the other hand, despite the large spectrum of biological activities, polyamidines, due to their peculiar chemico-physical properties (i.e. solubility, charge density, affinity for protein and nucleic acid), represent a class of compounds difficult to analyse. For these adverse properties, reliable methods for their determination in formulatory and preclinical studies are still lacking.

The development of an accurate and precise analytical protocol is a prerequisite both for pharmacological and clinical trials (e.g. determination of drug pharmacokinetic, tissue distribution and bioavailability) and for pharmaceutical studies aimed to the design of efficient drug delivery systems such as liposomes and microspheres.

To this purpose, in this paper we describe a high-performance liquid chromatographic (HPLC) procedure based on the use of a ternary solvent system containing a high ionic strength buffer and an organic competing base. The analytical method, here presented, is suitable for the simultaneous determination of different aromatic polyamidines with two (1,3-di-(*p*-amidinophenoxy)-propane, shortly, propamidine or DAPP), three (1-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxy-methyl)-butane, shortly TAPB) and four (1,3-di-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxymethyl)-propane, shortly TAPP) Benzamidine residues as well as for 2'-halo derivatives (TAPP-Cl, TAPP-Br and TAPP-I) of the tetra-benzamidine TAPP (see chemical structures in Fig.1) (12-14). The application of the method to the determination of poly-amidines in human serum and in cell culture media is also described. In addition, this HPLC based analytical method can be efficiently utilized in preformulatory studies such as the

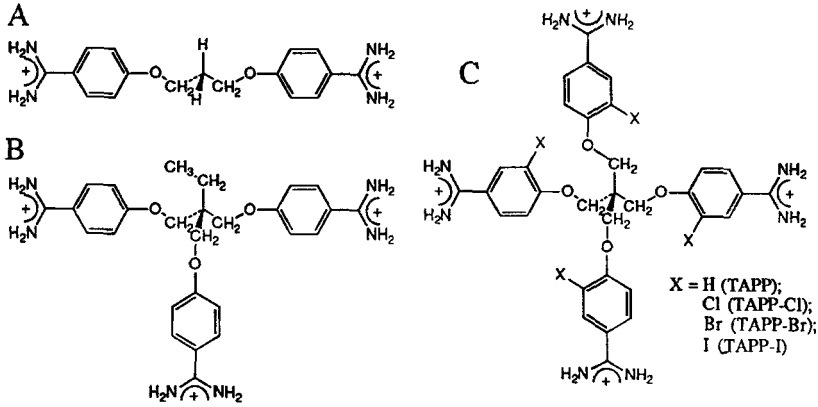

FIGURE 1. Chemical structures of the poly-benzamidines. A: 1,3-di-(p-amidinophenoxy)-propane (DAPP); B: (1-(*p*-amidinophenoxy)-2,2-bis-(*p*-amidinophenoxy-methyl)-butane (TAPB); C: 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP) and its 2'-halo derivatives (TAPP-Cl, TAPP-Br and TAPP-I).

determination of TAPP-Br release kinetic from gelatine-microsphere, especially designed for the controlled delivery of this drug (15).

MATERIALS AND METHODS

Chemicals

Synthesis, melting points, yields, crystallization solvents and analytical data of the aromatic polyamidines (see Fig.1 for chemical structures) have been reported elsewhere (12-14). Methanol, tetrahydrofuran and water were all HPLC-grade as supplied by J.T.Baker (Phillisburg, USA). Cell culture medium was α-medium (GIBCO, Grand Island, New York, USA), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, USA). All other chemicals were of analytical grade (Farmitalia Carlo Erba, Milan, Italy)

Chromatographic Instrumentation

The RP-HPLC analyses were performed utilizing a HPLC system consisting of a Bruker LC21-C chromatographic pump (Bruker, Bremen, Germany), a

Rheodyne 7125 sample injection valve, equipped with a 50-µl loop (Rheodyne, Cotati, USA), and a Chrom-A-Scope rapid scan UV detector (Carlo Erba Strumentazione, Milan, Italy) able to measure and store 10 spectra/min.

Chromatographic Conditions

Samples were chromatographed on a 150 x 4.6 mm stainless steel column packed with 5 µm particles (Supelcosil LC-18-DB, Supelco, Bellefonte, USA) and eluted with phosphate buffer (200 mM, pH 3) - methanol - tetrahydrofuran (75:25:3, v/v) containing 0.5% triethylamine. Chromatography was performed at room temperature, at a flow rate of 1 ml/min.

The polyamidine content of sample solutions was obtained from calibration curves constructed from polyamidine standard solutions. The detection was carried out at 261 nm and the wavelengths scanned between 220 and 340 nm.

Determination of TAPP and TAPP-Br in Culture Medium and Serum Samples

Sample were prepared by adding 10 µl aliquots of tetra-amidine standard solutions in water (10 µg/µl) to 0.5 ml of α-medium or human serum and mixing them thoroughly. These solutions were passed through a pre-conditioned (1 ml of methanol and then 1 ml of water) C_{18}-cartridge (sorbent weight, 200 mg; Baker) which was eluted successively with 1 ml of water, 1 ml of methanol and 3 ml of phosphate buffer (300 mM, pH 3) - methanol - tetrahydrofuran (60:30:5, v/v) containing 3% triethylamine. The last fraction was directly analysed by HPLC. The percentage recovery was determined by comparing the peak areas of TAPP and TAPP-Br extracted from samples with those obtained by direct injections of equivalent amounts of tetra-amidines.

Determination of TAPP-Br Release Kinetic from Microspheres

The determination of the TAPP-Br "in vitro" release kinetic from microspheres was carried out by horizontal shaker method as previously described (15). Typically, 20-25 mg of microspheres were poured into a dialysis tube (molecular weight cut off 10,000-12,000; Medi Cell International, England) placed into 100 ml of 50 mM borate buffer, pH 7.5 and then in a horizontal shaker. Afterwards, samples were withdrawn at regular time intervals from the receiving buffer. The

amount of released drug was determined by reversed-phase HPLC using the analytical method described above.

RESULTS AND DISCUSSION

Until now the aromatic poly-benzamidines DAPP, TAPB and TAPP, have been determined by spectrophotometric techniques (16), which have been proved to be unsatisfactory due to interference problems caused by the intrinsic lack of specificity of the method especially at the low absorption wavelength (260 nm) used for determination of poly-benzamidines.

The present investigation describes an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous identification and quantification of different aromatic polyamidines (see Fig. 1 for chemical structures). The analytical procedure here presented is suitable for the determination of poly-amidines both in cell culture medium or in human plasma as well as for preformulatory pharmaceutical studies.

Chromatographic Separation

Preliminary experiments revealed that the foregoing poly-amidine compounds were not eluted from a cation exchanger column even when a mobile phase buffer concentration up to 1 M was employed. A previously published report indicated that optimal retention for pentamidine, which is structurally similar to the poly-amidines examined in this study, is achieved on a C_{18} column with an eluent consisting of acetonitrile - water and containing tetramethylammonium chloride and phosphoric acid (17).

However, under these conditions, unsatisfactory separation of DAPP, TAPB and TAPP was obtained. In addition, severe peak tailing for the compounds with three and four benzamidine residues was observed. This was probably due to the higher number of positive charges present in their structure as compared to pentamidine. In order to overcome these problems, other mobile phase systems, in combination with a Supelcosil LC-18-DB column, were examined to find conditions were efficient and baseline separations of the above polyamidines could be obtained.

Concerning the choice of the mobile phase pH, we utilized a pH 3 buffer since at acidic pH, interactions between positively charged compounds and silanolic groups are reduced.

In order to diminish residual silanophilic interactions that were observed even at low pH, a high molarity phosphate buffer (200 mM) was used in conjunction with an organic competing base (triethylamine). The influence of a third mobile phase component was also evaluated. When tetrahydrofuran was added to the eluent with the methanol - phosphate buffer ratio kept constant, a decrease in retention for all poly-amidines was observed due to an increase of the eluotropic strength of the mobile phase. Furthermore an enhanced resolution of the above compounds was obtained with this ternary solvent conditions.This finding is in agreement with other reports in the literature showing that mobile phases containing more than two solvents are required for the maximum exploitation of selectivity in RP-HPLC (18).

Figure 2 shows a typical chromatogram of the separation of poly-amidines with 2, 3 or 4 benzamidine residues (DAPP, TAPB and TAPP) obtained with the optimized mobile phase described above. Under the same chromatographic conditions, the 2' halo derivatives of TAPP (TAPP-Cl, TAPP-Br and TAPP-I respectively) are also resolved (see Fig. 3). In addition, Figures 2 and 3 report the spectra of the compounds recorded by the rapid scan UV detector. All spectra obtained in the mobile phase showed an absorption band of different intensity localized at 261 nm and were respectively identical to the spectra of the pure compounds recorded (off line) with a double beam spectrophotometer (data not shown).

Determination of TAPP-Br in Cell Culture Medium and Serum

Since tetra-benzamidine were found the most active poly-benzamidines in both in vitro and in vivo studies (6-9) they were chosen as model compounds to evaluate the applicability of the proposed method to the determination of poly-benzaminides in cell culture media and human serum.

Prior to RP-HPLC assay of TAPP and TAPP-Br, a sample clean-up step was introduced to remove matrix peaks which were found to interfere with the analyte determination. Because of the strong affinity of poly-benzamidines for proteins (in particular, serine proteinase) (4, 5), and their scarce solubility in organic solvents, classical sample pretreatment methods, were found uneffective. For instance, protein precipitation with ethanol or trichloroacetic acid produced unsatisfactory recoveries (less than 20%). For this reason, purification procedures based on solid-phase extraction were examined. Tetra-benzamidines were quantitatively extracted from α-medium or human serum on a octadecyl-bonded (C_{18}) silica cartridge. In

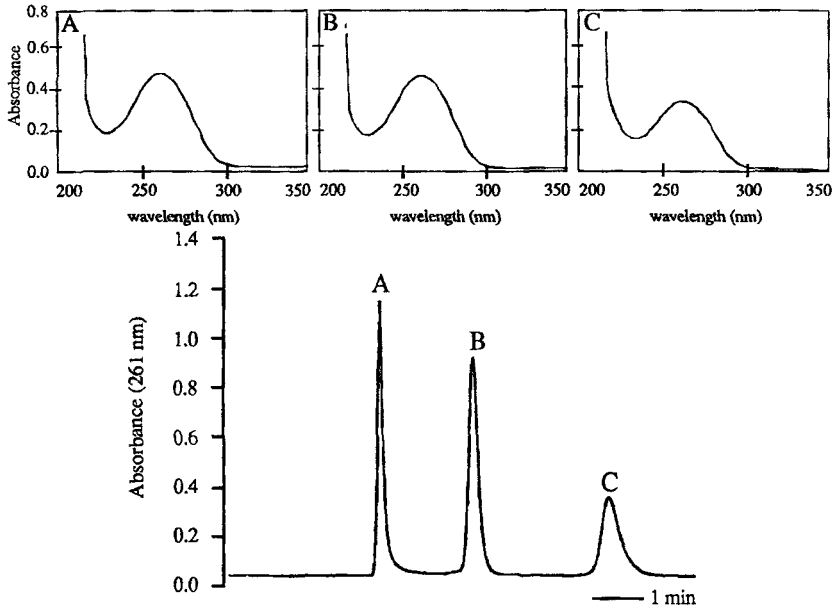

FIGURE 2. Chromatogram and spectra of aromatic poly-amidines. A: 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP); B: 1,3-di-(p-amidinophenoxy)-propane (DAPP); C: 1-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxy-methyl)-butane (TAPB). Operating conditions as described under Materials and Methods.

order to eliminate interfering substances, the C_{18} sorbent was flushed with water and methanol. This latter washing step could be used because of the high affinity of poly-benzamidines for the stationary phase. Complete desorption of the tetra-amidines from the cartridge was then obtained with phosphate buffer (300 mM, pH 3) - methanol - tetrahydrofuran (60:30:5, v/v) containing 3% triethylamine. The recovery of TAPP and TAPP-Br from cell culture medium and serum, are reported in Table 1. Representative HPLC traces are shown in Figure 4.

Determination of TAPP-Br Release from Microspheres

Starting from the consideration that the therapeutic effect of a microencapsulated drug is function of the free drug concentration at the site of action, the analysis of

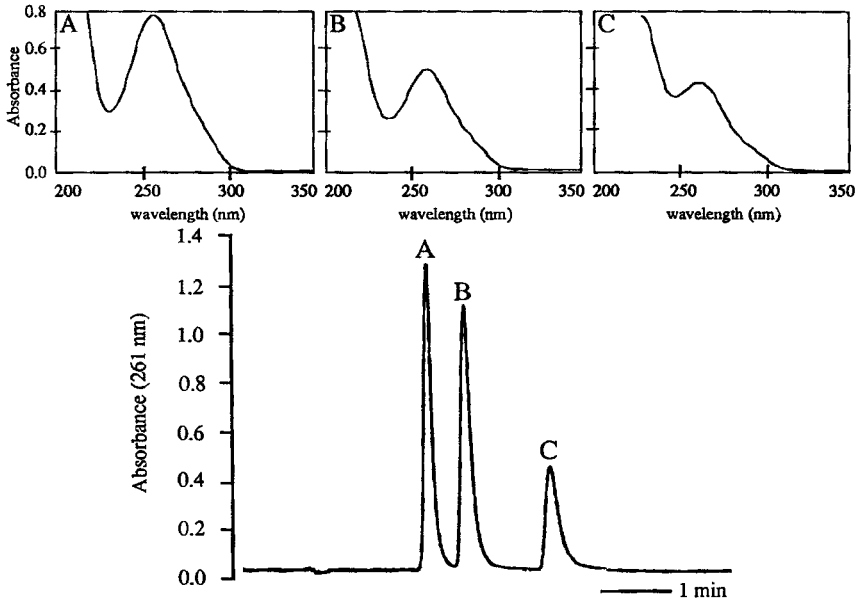

FIGURE 3. Chromatogram and spectra of 2' halo derivatives of the aromatic tetra-amidine 1,3-di-(p-amidinophenoxy)-2,2-bis-(p-amidinophenoxymethyl)-propane (TAPP). A: TAPP-Cl; B: TAPP-Br; TAPP-I. Operating conditions as in Fig. 2.

TABLE 1
Tetra-amidines Recovery from Cell Culture Medium and Human Serum

	α-medium	Human serum
TAPP	91.2±3.2	78.4±7.0
TAPP-Br	88.6±2.1	81.0±5.6

Data represent the average of percentage recovery (n=5) ± SD.

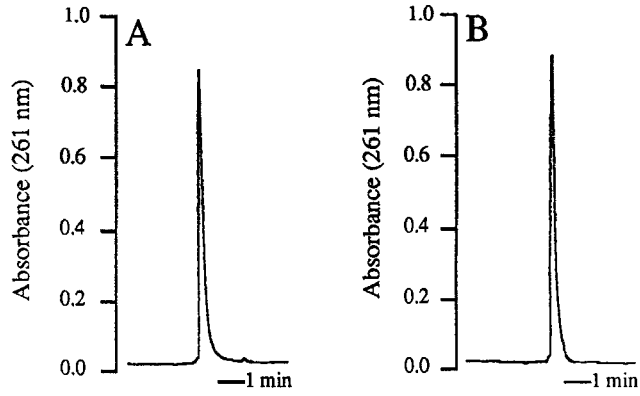

FIGURE 4. Representative HPLC traces of TAPP (A) and TAPP-Br (B) from human serum. Operating conditions as in Fig. 2. Control serum did not show any chromatographic interference in the areas of interest.

both amount and mechanism(s) of drug release from microparticulate systems, represents a fundamental step for the rationale design of nano- and microsphere based delivery systems (19).

In order to obtain reproducible and reliable quantitations of TAPP-Br during release experiments, the above reported HPLC method was employed. Figure 5 shows typical chromatograms of TAPP-Br containing solution from release experiments and the relative release profile obtained.

It is important to underline that the comparison of the TAPP-Br spectra collected by the rapid scan UV detector with that of pure compound suggests that interferences due to TAPP-Br degradation products or microsphere impurities, possibly contained in the release buffer are not present.

In this respect, the use of rapid scan UV detectors to identify and quantitate drugs by both retention time and absorption spectrum is more reliable than the use of HPLC with a conventional UV detector or simply UV determinations. This is especially true in the case of release experiments where drug degradation products and/or components released from microsphere matrix could (a) interfere with the analytical determination or (b) represent bioactive or toxic compounds playing important pharmacological roles after in vivo administration.

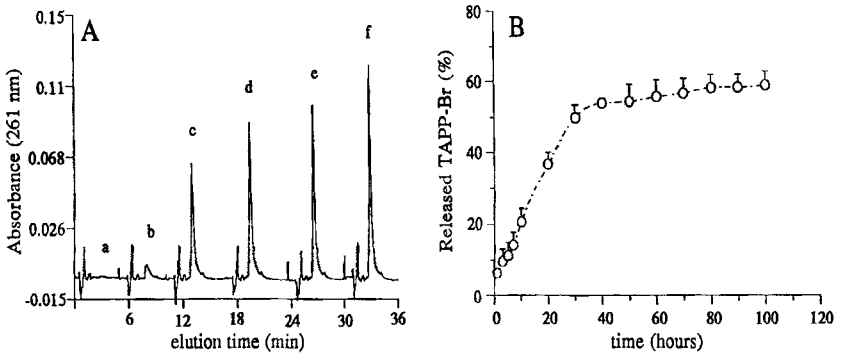

FIGURE 5. A: Typical chromatograms obtained from release experiments. Receiving buffer from gelatin microspheres was analysed for TAPP-Br content after 5 (a) 10 (b), 20 (c), 30 (d), 60 (e) and 100 (f) minutes. B: Resulting TAPP-Br release profile; the reported values represent the average of 6 independent experiments, bars = SD.

Concluding Remarks

In conclusion, a relatively simple and sensitive HPLC method for the simultaneous analysis of different poly-benzamidines has been developed. In addition a sample clean-up procedure is described for the determination of the compounds in plasma, suitable for pharmacokinetic studies. Further work is in progress to validate the method here proposed for the assay of poly-amidines in pharmaceutical formulations, in order to ensure batch to batch homogeneity in quality control tests.

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