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Salting-out solvent extraction for pre-concentration of benzalkonium chloride prior to high-performance liquid chromatography

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ABSTRACT

Benzalkonium chloride (BAK) can be readily concentrated prior to chromatographic analysis by the addition of appropriate amounts of sodium chloride and acetonitrile to the sample. This affords a two-phase system the upper of which contains the BAK and which can be directly injected onto the chromatographic column. Using this approach analytical methods with and without an internal standard, have been developed and validated that reproducibly afford an approximately ten-fold pre-concentration of the sample to be analysed thereby improving analytical sensitivity.

INTRODUCTION

Benzalkonium chloride (BAK) is an antibacterial preservative widely used in pharmaceutical products. It consists of a variable mixture of the C_8–C_{18} even-numbered alkyl homologues of alkylbenzyldimethylammonium chlorides with the C_{12} and C_{14} homologues predominating [1,2].

A variety of chromatographic procedures have been reported for BAK [3–7]. Because of the relatively low absorbance of the benzyl chromophore at longer wavelengths [6], the necessity to quantitate total BAK by determining the sum of the areas of up to six peaks and as many of the homologues are present in small proportions in the mixture, it is difficult to analyse at concentrations employed as antibacterial preservatives in pharmaceutical products (0.005–0.01%, w/v).

Workers have addressed this problem by monitoring at short wavelength [5–7], by injection of larger volumes (up to 200 μl) [4,5] and by quantitating only the major peaks $C_{12}/C_{14}/C_{16}$ which together make up approximately 95% of most samples of BAK [3–5].

There have been a number of reports of the use of salting-out procedures of solvents such as acetonitrile and acetone with high concentrations of inert electrolytes [8–13]. These procedures have been used to pre-concentrate metal complexes to improve the sensitivity of metal determinations by atomic absorption spectroscopy [8,9] or high-performance liquid chromatography (HPLC) [10,13] and explosive residues from environmental samples by HPLC [11,121.

Studies in these laboratories involving pre-concentration into acetonitrile using sodium chloride as electrolyte of phenylmercuric nitrate in ophthalmic products [13] suggested that this approach may be appropriate for the pre-concentration of BAK into an acetonitrile-water extract suitable for direct injection onto the HPLC column and this paper reports the results of these studies.

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EXPERIMENTAL

Materials

The two samples of BAK which were employed in this study were 50% (w/v) solutions, Vantoc CL (ICI, Melbourne, Australia) (sample A) and from TCI (Tokyo, Japan) (sample B). Authentic C_{12}, C_{14} and C_{16} homologues were also employed (Aldrich, Milwaukee, WI, USA). The dibenzyldimethylammonium chloride (DBDMAC) used as internal standard was prepared by the reaction of benzylbromide (Ajax Chemicals, Sydney, Australia) and N,N-dimethylbenzylamine (Aldrich) [14]. All other chemicals were analytical reagent grade and solvents were HPLC grade.

Chromatographic equipment and conditions

The liquid chromatograph consisted of a Model 501 pump (Waters Assoc., Milford, MA, USA), Rheodyne Model 7125 loop injector (Cotati, CA, USA), Model 484 variable-wavelength absorbance detector (Waters Assoc.) and Model 3396A integrating recorder (Hewlett-Packard, Palo Alto, CA, USA) together with a column of cyanopropyl bonded silica 10 μm particle size, 10 cm x 8 mm I.D. (Waters Assoc., RCM 8 x 10) with a mobile phase of acetonitrile-tetrahydrofuran-0.1 *M* acetic acid/triethylamine buffer (made by adjusting the pH of 0.1 *M* acetic acid to pH 3.8 with triethylamine) (375 : 20 : 605) at a flow-rate of 1.5 ml min^{-1}. The injection volume was 20 μl and the monitoring wavelength 262 nm.

Spectra of individual peaks were obtained by use of a Model 991 photodiode array absorbance detector (Waters Assoc.).

Spectrophotometric equipment

UV spectra were obtained using an HP 8450 UV-Vis spectrophotometer (Hewlett-Packard).

Sample preparation for routine analysis

Without internal standard. To a 20-ml glass tube with plastic cap and PTFE wad were added sodium chloride (2.7 g), the sample to be analysed (10 ml) and acetonitrile (3 ml) and the solution was shaken to dissolve the sodium chloride. The layers were allowed to separate and the upper phase was immediately submitted to analysis by HPLC.

With internal standard. To a 20-ml glass tube with plastic cap and PTFE wad were added sodium chloride (2 g), sample to be analysed (10 ml), a 2.5 $\cdot 10^{-2}$% (w/v) solution of DBDMAC (1 ml) and acetonitrile (4 ml) and the solution was shaken to dissolve the sodium chloride. The layers were allowed to separate and the upper phase was immediately submitted ot analysis by HPLC.

Validation of analytical methods

Both HPLC methods were applied to BAK solutions containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 $\cdot 10^{-2}$% (w/v) BAK. The relative standard deviations (R.S.D.s) were determined by analysis of 6 replicates at a concentration of 0.5 $\cdot 10^{-2}$% (w/v) total BAK.

Composition of BAK samples and identity of homologue peaks

An 0.1% (w/v) solution of both BAK samples was submitted to HPLC analysis by direct injection. Accurate retention times were determined and the void volume determined by injection of a solution of uracil (0.1%, w/v) and capacity factors were calculated for the individual homologues.

Identity of the peaks was confirmed by obtaining UV spectra at peak maxima using the photodiode-array detector and by comparison of the retention times with authentic samples for the C_{12}, C_{14} and C_{16} homologues.

The homologue composition was determined from relative peak-areas of the homologues in the sample.

Effect of quantity of sodium chloride on salting out

The sample preparation method without internal standard was performed substituting sodium chloride 2.25, 2.50, 2.75, 3.00 and 3.25 g and the amount of BAK extracted determined by HPLC. Relative phase-volumes were determined by performing these experiments, scaled up by a factor of 3, in 50-ml measuring cylinders and measuring the two phase-volumes.

Efficiency of extraction by W spectrophotometry

To a 50-ml glass vial with screw-cap and PTFE wad were added an 0.05% (w/v) solution of BAK (30 ml), water (3 ml), sodium chloride (6 g) and acetonitrile (12 ml). The tube was shaken to dissolve the sodium chloride and the layers allowed to separate. The upper layer (1 ml) was diluted with water (2 ml) and the resulting solution submitted to analysis by UV spectrophotometry at 262 nm using the original solution as the standard. The concentration of BAK in the lower layer was also determined by UV spectrophotometry without dilution.

The efficiency of extraction of the DBDMAC was determined by an identical experiment using a solution of DBDMAC (0.025%, w/v) instead of BAK.

Efficiency of extraction of total BAK and individual homologues by HPLC

Samples of BAK (0.01%, w/v) were submitted to analysis by the sample preparation methods without and with internal standard and the relative peak areas compared with the same BAK samples at a concentration of 0.1% (w/v) by direct injection.

RESULTS AND DISCUSSION

Previously reported HPLC methods for BAK [3,4,6,7] have all used a cyanopropyl-bounded silica column and acetonitrile-buffer systems. The solvent system chosen for this study incorporating a small proportion of tetrahydrofuran afforded improved peak shapes for the higher homologues. The method of Ambrus *et al.* [6], which uses a perchlorate counter-ion in the mobile phase, when used with the work-up procedure outlined here, results in unacceptable peak-broadening of the BAK homologue peaks but the other reported solvent systems have been found to be compatible.

Two samples of BAK solutions were used in this study: sample A which contains all six homologues and sample B which contains only four (C_{10}–C_{16}). Details of the exact composition of each were determined and are given in Table I. Total peak areas for 0.1% (w/v) solutions of both samples of BAK came within 2% of each

TABLE I

STATISTICAL DATA ON PRECISION OF ASSAY WITH AND WITHOUT INTERNAL STANDARD PERFORMED ON $5 \cdot 10^{-3}$% (w/v) BAK

Homologue	Sample A				Sample B			
	Mol fraction of homologue	Concentration of homologue (%, w/v)	R.S.D. (%)		Mol fraction of homologue	Concentration Concentration (%, w/v)	R.S.D. (%)	
			Without internal standard	With internal standard			Without internal standard	With internal standard
C_8	0.0860	$4.30 \cdot 10^{-4}$	±2.00	±1.85	–	–	–	–
C_{10}	0.0943	$4.71. 10^{-4}$	±1.82	±2.10	0.0053	$0.26. 10^{-4}$	±11.40	±14.40
C_{12}	0.5056	$25.28. 10^{-4}$	±1.04	±0.98	0.4619	$23.09. 10^{-4}$	±0.95	±1.00
C_{14}	0.1695	$8.47 \cdot 10^{-4}$	±1.84	±1.40	0.4602	$23.01. 10^{-4}$	±1.53	±1.99
C_{16}	0.0835	$4.22. 10^{-4}$	±2.04	±2.35	0.0762	$3.81. 10^{-4}$	±3.70	±4.47
C_{18}	0.0603	$3.02. 10^{-4}$	±3.40	±3.85	–	–	–	–
Total	1.0008	$50.00. 10^{-4}$	±1.97	±2.10	1.0008	$50.00 \cdot 10^{-4}$	±2.40	±2.37
Calibration lines	With internal standard: peak area ratio = $2.42. 10^2$ (concn. in %, w/v) $- 3.0 \cdot 10^{-3}$ ($r = 0.9996$, $n = 8$)				With internal standard: peak area ratio = $2.39 \cdot 10^2$ (concn. in %, w/v) $+ 1.5 \cdot 10^{-2}$ ($r = 0.9995$, $n = 8$)			
	Without internal standard: peak area = $5.22. 10^8$ (concn. in %, w/v) $+ 2.6. 10^4$ ($r = 0.9995$, $n = 8$)				Without internal standard: peak area = $5.12 \cdot 10^8$ (concn. in %, w/v) $+ 3.5 \cdot 10^4$ (r = 0.9998, $n = 8$)			

other and therefore they were considered interchangeable in the study. The identity of the peaks as being due to individual homologues was confirmed by comparison of retention times for the C_{12}, C_{14} and C_{16} homologues with authentic samples, comparison of the UV spectra using photodiode-array detection of all homologues with that of an authentic sample of the C_{12} homologue and by obtaining a linear plot of the logarithm of the capacity factor for the homologues versus carbon number as would be expected for such a series in the reversed-phase mode (r = 0.9967).

In previous studies of this salting-out phenomena a variety of salts have been investigated [8,9]. Sodium chloride was chosen for this study on the basis of cost and purity.

The phase-volumes produced by salting-out of acetonitrile by sodium chloride is a complex function of volume of acetonitrile and sample and quantity of sodium chloride. By the use of convenient volumes of sample and acetonitrile, 10 ml and 3 ml, respectively, the quantity of sodium chloride to achieve a suitable pre-concentration could be selected (Table II). As the quantity of sodium chloride in the system is reduced the volume of the upper phase becomes smaller leading to greater sampling difficulties but also greater pre-concentration of the BAK.

TABLE II

RELATIONSHIP BETWEEN UPPER-PHASE VOLUME, CONCENTRATION FACTOR AND AMOUNT OF SODIUM CHLORIDE

The concentration factor is defined as the ratio of the concentration of BAK in the original sample to that found in the upper layer.

Mass of sodium chloride (g)	Concentration factor	Phase volumes upper/lower (ml)	Theoretical concentration factor[a]
2.25	18.60	1.5/39.5	20
2.50	14.10	2.0/39.5	15
2.75	11.46	2.5/39.2	12
3.00	10.20	2.7/38.3	11
3.25	9.51	3.0/38.2	10

[a] Determined from the ratio of upper phase volume to volume of sample and assuming complete extraction.

When 2 g of sodium chloride are employed the system becomes homogeneous with no phase-separation and at quantities of sodium chloride greater than 3.25 g the sodium chloride fails to completely dissolve. Therefore the amount of sodium chloride chosen was on the basis of affording an approximately lo-fold concentration factor this being defined as the ratio of the concentration in the sample to that found in the upper-phase. Obviously, quantities of sodium chloride may be chosen such that greater pre-concentration can be achieved should that be considered necessary.

As changes in electrolyte content of the sample may change the volume of the upper phase it was considered desirable to incorporate an internal standard into the analytical method. DMDBAC was found to have appropriate characteristics as it elutes prior to the C_8 homologue and has similar chemical and spectral characteristics to BAK. The addition of the internal standard in water (1 ml) necessitated changes in the acetonitrile (4 ml) and sodium chloride (2.0 g) to maintain relative phase-volumes.

When both samples of BAK were submitted to analysis by both methods linear relationships

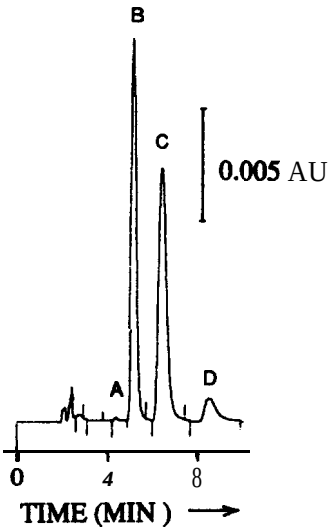

Fig. 1. Chromatogram obtained with BAK sample B (0.01%, w/v) pre-concentrated by salting-out acetonitrile without inclusion of an internal standard. Peaks: A = C,,; B = C_{12}; C = C_{14}; D = C_{16} homologue.

between peak-area and concentration of total BAK over the concentration range **0–0.015%** (w/v) with satisfactory **R.S.D.s** at a concentration of $5 \cdot 10^{-3}$% (w/v) (Figs. 1 and 2) (Table I).

An assessment has also been made of the efficiency of extraction of the BAK homologues and total BAK into the upper acetonitrile layer using the quantities prescribed for the assay incorporating an internal standard (see section ***Sample preparation for routine analysis).*** Measurement of the phase-volumes upper/lower was found to be 3 **ml/43.7** ml, which, based on a 30-ml sample should afford an approximately lo-fold increase in concentration of BAK assuming complete extraction into the upper layer. This efficiency of extraction was determined by UV spectrophotometry following partitioning of an 0.05% (w/v) solution of both samples of BAK and an 0.025% (w/v) solution of DMDBAC used as an internal standard and also by HPLC by comparison of peak-area response of the individual homologues against the peak-area response of an 0.1% (w/v) solution of the BAK samples and the DMDBAC internal standard (Table III). Almost quantitative extraction is achieved for BAK and for its individual homologues. The internal standard (DBDMAC) is less efficiently extracted but is satisfactory for its purpose.

This procedure is capable of effectively performing an approximately ten-fold **preconcentration** in a controlled manner and by adaptation higher concentration factors should be achievable. It provides a quick and convenient procedure for the analysis of BAK at concentrations encountered in pharmaceutical products and

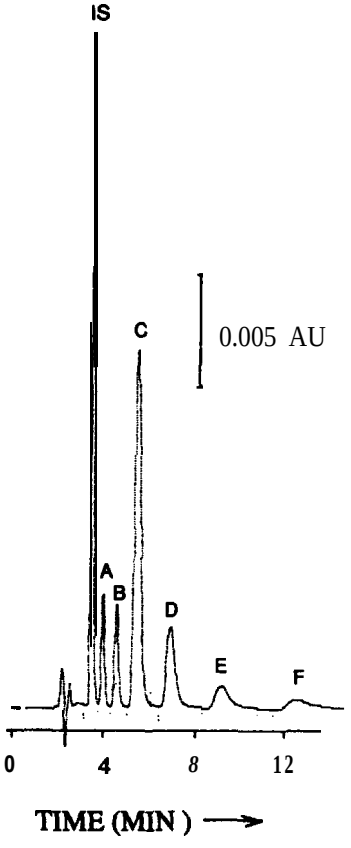

Fig. 2. Chromatogram obtained with BAK sample A (0.01%, w/v) pre-concentrated by salting-out acetonitrile with the inclusion of an internal standard. Peaks: IS = internal standard (dibenzyldimethylammonium chloride); A = C_8; B = C_{10}; C = C_{12}; D = C_{14}; E = C_{16}; F = C_{18} homologue .

TABLE III

PARTITION COEFFICIENTS AND CONCENTRATION FACTORS FOR BAK, HOMOLOGUES AND INTERNAL STANDARD

The concentration factors are defined as the ratio of the concentration of BAK arid homologues in the original samples to that found in the upper layer.

Material		Partition coefficient	Concentration factor
BAK sample A	C_8		9.15
	C_{10}		9.57
	C_{12}		9.55
	C_{14}		9.43
	C_{16}		8.82
	C_{18}		8.74
	Total		9.38
		>100[a]	9.15'
BAK sample B	C_{10}		9.00
	C_{12}		9.33
	C_{14}		9.56
	C_{16}		8.67
	Total		9.43
		>100[a]	9.27"
Internal standard, DBDMAC		14.30"	4.93"

[a] **Determined by ultraviolet spectrophotometry (for the total sample).**

should be readily adaptable to other materials where pre-concentration prior to analysis is desirable.

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