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Note

High-performance liquid chromatographic assay of benzalkonium in plasma

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Benzalkonium chloride is a widely used general antiseptic. Owing to its high potency towards several types of microorganisms (bacteria, algae and viruses), benzalkonium is present in very low concentrations in almost all the pharmaceutical formulations that contain this antiseptic. Other commercial preparations (e.g. Zephiran) contain much higher levels of benzalkonium, and the possibility of local and systemic toxic effects resulting from accidental ingestion or topical exposure, particularly in children, is a cause of concern. Poisoning and even fatalities have been reported [1–5].

In Canada, algicide solutions for use in swimming pools by the general public may contain as much as 5–10% benzalkonium. Even though these solutions are sold by the millions of liters each year, the effect on health is not documented. This cationic detergent is also an extremely potent spermicidal agent [6], and this property was the basis for the development of new vaginal contraceptives that are widely used in European countries [7]. Recently, we have demonstrated that benzalkonium is extremely potent in killing the AIDS virus in vitro [8] and this compound might prove to be useful in decreasing the risk of heterosexual and homosexual transmission of the virus. All of this raises concern about the possibility of the absorption of the compound into the blood (via oral, vaginal, rectal and percutaneous routes), which could result in toxic systemic effects. In order to detect trace amounts of absorbed benzalkonium and thus evaluate the potential risk, an accurate and sensitive method to measure benzalkonium in biological fluids was required.

The determination of benzalkonium in relatively pure aqueous solution can easily be performed. The most commonly used techniques involve ion-pairing

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with an acidic dye followed by extraction into an organic solvent and measurement of the optical density.

However, several difficulties were encountered during attempts to measure this compound in more complex formulations. Meyer [9] described a high-performance liquid chromatographic (HPLC) method applicable to ophthalmic systems: benzalkonium was first extracted into 1,2-dichloromethane as the methyl orange complex (in order to remove interfering substances), which was then assayed by HPLC. We tested this method, as well as all the other existing techniques [10–19], in an attempt to measure nanogram amounts of benzalkonium in human plasma, and none proved to be adequate. A major problem was the non-recovery of benzalkonium (at less than 100 μg/ml) as the ion-paired molecule in the organic phase whenever plasma was present. A good recovery could be obtained only when relatively large amounts of benzalkonium were added to plasma. Another formidable difficulty was the extreme diversity and large quantities of interfering substances present in plasma.

By using tritiated benzalkonium added to plasma, it became possible to trace the compound during its isolation and purification by column chromatography. This enabled us to develop a rapid and efficient solid-phase procedure for extracting benzalkonium from plasma. The method allows a high recovery and an extremely high purity of the compound, which can then be measured by HPLC.

EXPERIMENTAL

Assay of benzalkonium in plasma

Extraction. Plasma (2 ml) was diluted with 4 ml of distilled water and applied to a 3-ml Baker disposable extraction C_{18} column (J.T. Baker, Phillipsburg, NJ, U.S.A.). Vacuum was applied to a solid-phase extraction vacuum manifold (Supelco, Toronto, Canada), which allowed a dropwise elution from the column. The column was then washed sequentially with three 3-ml portions of water, two 3-ml portions of methanol and two 3-ml portions of ethyl acetate. Benzalkonium was recovered from the column by elution with 4 ml of methanol–ethyl acetate (1:1) containing 0.01% ammonium chloride. The eluate was evaporated to dryness under a stream of nitrogen.

Partition. In order to eliminate the ammonium chloride, that would otherwise interfere in the HPLC assay, the residue from the extraction was partitioned between 1 ml of ethyl acetate and 1 ml of 10% sodium carbonate containing 200 μl of 0.1% bromophenol blue (extraction by the dye-pairing method). The organic phase was dried under nitrogen and the resulting clean extract was suitable for HPLC.

HPLC assay. Plasma extracts as well as standards of benzalkonium were dissolved in 50 μl of mobile phase and injected into the HPLC system. The conditions for the HPLC assay of benzalkonium are essentially those described by Meyer [9], except that the working HPLC conditions are in normal phase. We used a Waters Assoc. HPLC system with a 6000A solvent-delivery system, a U6K injector, a Model 481 detector (214 or 254 nm) and a Maxima 820 module with Maxima Full Control software.

A Spherisorb-CN column (Chromatographic Sciences, Montreal, Canada) was used at ambient temperature. The mobile phase was a mixture of 90% acetonitrile (HPLC grade, BDH, Montreal, Canada) and 10% propionate buffer (0.161 *M*, pH 5.4; 75 ml of 10% sodium carbonate were diluted with 1.5 l of water and mixed with 12 ml of propionic acid, and the solution was diluted to 2 l with water). The mobile phase was filtered on a 0.45-μm filter using a Millipore all-glass filter apparatus (Millipore, Missisauga, Canada). The flow-rate in the column was 2 ml/min.

Analysis of benzalkonium

Benzalkonium chloride (Pharmelac, France) was obtained as a 50% stock solution (w/v) of the compound in water. The purity of this solution was ascertained by combined gas chromatography–mass spectrometry (GC–MS; Kratos instrument MS-25; SE-30 column of 30 m; temperature was 27 °C for 2 min with an increase of 10 °C/min to 250 °C). On injection of the standard benzalkonium chloride, seven peaks were detected (by total ion current) and found to correspond to the thermal degradation pattern of the compound during GC. The ther-

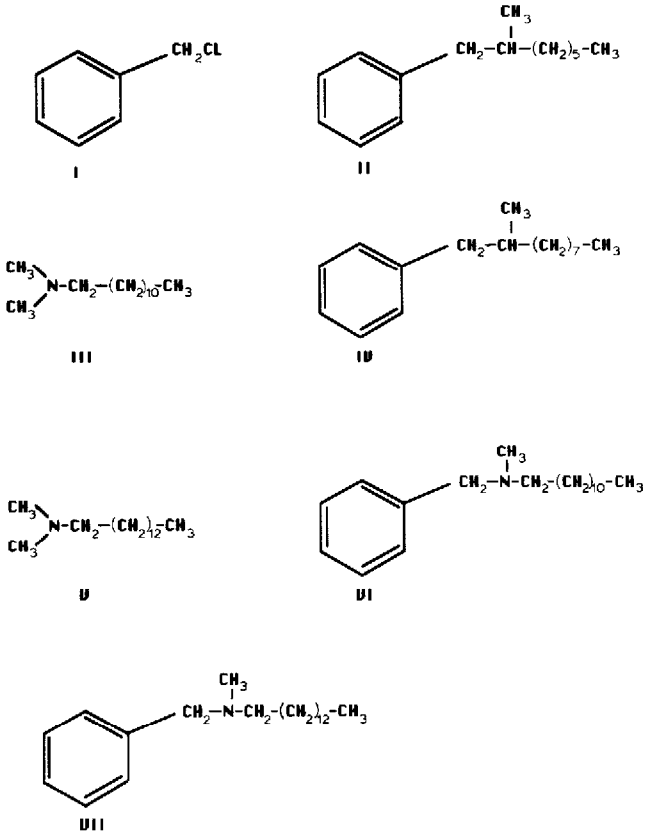

Fig. 1. Proposed structures of the thermal degradation products of benzalkonium chloride, derived from MS data (see Table I); II and IV are probable structures.

TABLE I

GC-MS OF THERMAL DEGRADATION PRODUCTS OF BENZALKONIUM CHLORIDE

Thermal degradation products[a]	Retention time (min)	Molecular ion (*m/e*)	Principal ion (*m/e*)	Other important ions (*m/e*)
I	7.5	126	91	
II	14.5	204	91	119, 105, 83
III	15.0	213	58	
IV	17.2	232	57	119, 105, 91, 85, 71
V	17.7	214	58	
VI	19.3	289	137	91
VII	23.5	317	134	91

[a] The corresponding structures are presented in Fig. 1.

mal degradation products (Fig. 1) were identified by MS (Table I). This analysis by GC–MS confirmed the identity of the parent compound and established the purity of the standard solution that was used: it is composed of a mixture of C_{12}-alkylbenzyldimethylammonium chloride (72%) and C_{14}-alkylbenzyldimethylammonium chloride (28%). A second lot of benzalkonium (from the same supplier) was also used during this study: it contained the C_{12} and C_{14} homologues at 65 and 35%, respectively.

Preparation of tritiated benzalkonium chloride

An aliquot of the standard solution of benzalkonium chloride (100 mg of the compound) containing 72% C_{12} and 28% C_{14} was lyophilized and dried over phosphorus pentoxide. The compound was labelled with tritium by catalytic exchange (New England Nuclear, Boston, MA, U.S.A.) After labelling, an aliquot of the [^{3}H]benzalkonium (100 μg in 50 μl of methanol) was dried under nitrogen, and the residue was applied to a 3-ml disposable extraction column-CN (J.T. Baker). The mini-column was eluted sequentially with 3 ml of ethyl acetate, 3 ml of acetonitrile and 3 ml of 80:20 acetonitrile–propionate buffer (0.161 *M*, pH 5.4). The buffer contained the bulk of the radioactivity when it was eluted from the column. It was evaporated to dryness under nitrogen, and the residue was further purified by HPLC. The tracer was radiochemically pure after this chromatography and its specific activity was calculated to be 0.95 TBq/mmol.

Assay for the recovery of benzalkonium

Known amounts of tritiated (10 000 cpm, equivalent to 1.3 ng) or non-radioactive benzalkonium chloride (dissolved in water) were added directly to 2 ml of plasma. These samples were then processed using the assay method for benzalkonium in plasma.

RESULTS AND DISCUSSION

This method for the assay of benzalkonium in plasma is a three-step procedure: solid-phase extraction, partition as the bromophenol blue complex and quantification by HPLC. The solid-phase extraction step yields a very high recovery of benzalkonium (90% of the [^{3}H]benzalkonium added to plasma). In addition, it allows the separation of the compound from all interfering plasma components. It is only through the use of the tritiated material that this simple and rapid column extraction procedure could be developed. Benzalkonium in diluted plasma is firmly retained on the C_{18} column by strong hydrophobic and electrostatic interactions, and washing with water and organic solvents (both polar and non-polar) then eliminates close to 100% of the other plasma components. The elution of benzalkonium can be achieved only through the use of an organic solvent (methanol–ethyl acetate, 1:1) containing ammonium chloride. With this solvent, both the hydrophobic and the electrostatic interactions of benzalkonium with the column are eliminated. The ammonium chloride is separated from benzalkonium by a partition between ethyl acetate and 10% sodium carbonate containing bromophenol blue.

An alternative means of purifying the extract is to dissolve it in 1 ml of water followed by two extractions with 1 ml of ethyl acetate–2-propanol (10:1). The latter procedure allowed the recovery of more than 90% of the [^{3}H]benzalkonium in the organic phase with no detectable trace of contaminant. For the subsequent HPLC assay of benzalkonium, its partition as the bromophenol blue complex was preferred because the HPLC results were more reproducible.

When human or dog plasma samples were spiked with 250, 500 or 1000 ng/ml benzalkonium chloride, the overall recovery, as measured by HPLC, was 73%. This value is identical with that measured using [^{3}H]benzalkonium as tracer. In view of the high reproducibility of the percentage recovery over this wide range of concentrations (correlation coefficient $r=0.99$, $n=6$), the use of a tritiated internal standard is not obligatory inasmuch as standards are also prepared in plasma. Once a clean extract of benzalkonium from plasma has been obtained, a modification of the HPLC method of Meyer [9] can be applied with excellent results.

Under the HPLC conditions described, the homologues of benzalkonium migrate with a retention time of ca. 6 min; the C_{14} and C_{12} homologues are separated by 0.4 min (Fig. 2A).

Peak heights for both homologues (at 214 and 254 nm) were linearly related to the benzalkonium chloride concentration (in spiked plasma samples) over the 25–1000 ng/ml range (correlation coefficient $r=0.99$, $n=12$ for each curve). In the range 5–25 ng/ml benzalkonium chloride, the C_{14} and C_{12} homologues could still be detected at 214 nm, although their relative peak heights were not constant and this precluded any precise quantitative measurements at these low concentrations. We found a lower detection limit of ca. 5 ng/ml (peak for the C_{14} homologues at 214 nm different from background).

The coefficient of variation was calculated at 250 ng/ml of benzalkonium chlo-

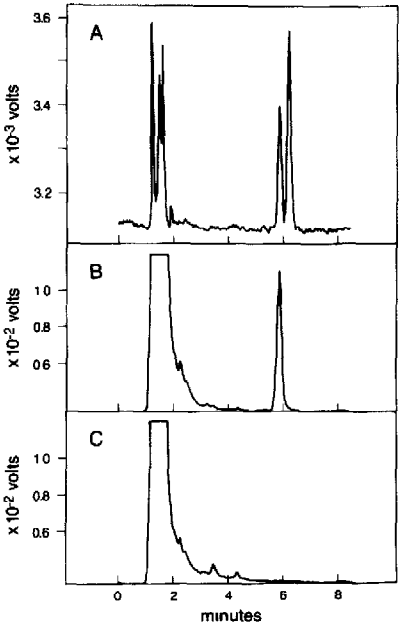

Fig. 2. Chromatograms of (A) pure benzalkonium chloride (250 ng) dissolved in the mobile phase and serum samples from a dog (B) 1 min following the intravenous injection of benzalkonium chloride and (C) before the injection. Detection, UV at 254 nm.

ride. When absorbance was measured at 214 nm, the coefficients of variation were 4.1% ($n=7$) and 6.4% ($n=6$) for the C_{14} and C_{12} homologues, respectively. At 254 nm, the corresponding values were 7.5% ($n=8$) and 6.0% ($n=8$).

The application of this assay for benzalkonium in plasma is demonstrated for a female dog injected intravenously with the compound at 2.5 mg/kg (Fig. 2B and C). Only one peak, corresponding to the retention time of the C_{14} homologue, was detected in the plasma of the animal at 1, 2, 3, 5 and 10 min (Fig. 2B); this peak was absent from the chromatograms of blood samples taken before the injection (Fig. 2C). The material in the peak was recovered and analysed by GC–MS; it was found to correspond to the unchanged C_{14} homologue of benzalkonium. The concentration (as ng/ml C_{14} homologue) in plasma was plotted versus time after the injection (Fig. 3); a half-life of 2.6 min was found for the C_{14} homologue. Similar injections into two other animals confirmed these results; furthermore, the analysis of whole blood from these animals also showed the presence of only the C_{14} homologue. At times beyond 15 min after injection, minor peaks that could possibly represent metabolites of benzalkonium started to appear. The fate of the C_{12} homologue in vivo is under investigation; a rapid uptake by endothelial cells is possible since histologic evidence of vein damage around the site of injection was observed.

Preliminary data also showed that our assay method can be successfully applied to diverse biological samples such as saliva, milk, urine and tissue homogenates. Less complex pharmacological formulations containing trace amounts of benzalkonium can also be easily analysed by this method.

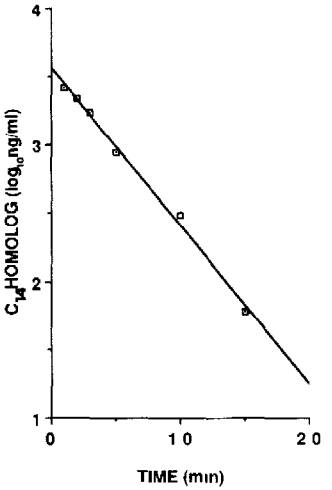

Fig. 3. Disappearance of the C_{14} homologue of benzalkonium from plasma in a dog. The concentration (ng/ml) was measured by HPLC and plotted (expressed as $\log_{10}$) as a function of time after the intravenous injection. Detection, UV at 214 nm.

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