Journal of Chromatography, 568 (1991) 145–155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5712

Determination of benzyl alcohol and its metabolite in plasma by reversed-phase high-performance liquid chromatography[a]

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(First received May 2nd, 1990; revised manuscript received March 25th, 1991)

ABSTRACT

A study undertaken following recent reports of deaths in neonatal children associated with the use of benzyl alcohol resulted in the development of a stability-indicating high-performance liquid chromatographic assay of benzyl alcohol in plasma using benzocaine as internal standard. Thawed plasma samples were diluted and subjected to solid-phase extraction using Extrelut and eluted with ethyl acetate. The evaporated eluate was reconstituted with mobile phase and chromatographed on a C_{18} column with water–acetonitrile–glacial acetic acid as mobile phase and detection at 254 nm. Baseline separation was achieved within 12 min for benzyl alcohol, benzaldehyde, benzoic acid, hippuric acid and benzocaine. Peak-height ratios were linear over 80–640 ng of benzyl alcohol injected ($r = 0.998$) and over 10–80 ng of benzoic acid injected ($r = 0.999$). Benzaldehyde and hippuric acid were not quantitated because these compounds were not detectable in actual dog plasma. Validation studies by spiking dog plasma with benzyl alcohol and benzoic acid gave overall percent recoveries (± relative standard deviation, $n = 4$) of 98.3 ± 3.0 and 101.4 ± 7.6%, respectively. The method was applied to the assay of actual plasma samples. Since benzyl alcohol is very susceptible to oxidation to benzaldehyde and benzoic acid, its purity in bulk liquid samples can be determined by this method.

INTRODUCTION

Benzyl alcohol is commonly used as an antibacterial agent in a variety of pharmaceutical formulations for intravenous administration. Recently, there have been reports of deaths in neonatal children associated with the use of benzyl alcohol [1,2]. Deaths in immature animals have also been reported after administration of Ringer's solution containing benzyl alcohol [3,4]. It has been postulated that benzyl alcohol is metabolized to benzoic acid. However, the quantity of benzoic acid exceeds the detoxification abilities of the immature liver. To support

[a] This paper has been partially presented before the Analysis and Pharmaceutical Quality Section at the Third Annual Meeting of the American Association of Pharmaceutical Scientists, Orlando, FL, 1988.

0378-4347/91/$03.50

a further study of the use of benzyl alcohol in our laboratory, a method developmental work on the assay of benzyl alcohol in dog plasma was undertaken.

Benzyl alcohol with benzaldehyde traces have been assayed by high-performance liquid chromatography (HPLC) in formulations containing hydrocortisone [5] and etoposide [6]. DiPietra *et al.* [7] have reported both an HPLC method and a second-order derivative UV spectroscopic method for the determinations of benzyl alcohol and benzaldehyde traces. None of these reports included the determination of benzoic acid. Block and Levine [8] reported a qualitative method for the detection of benzoic acid, benzal chloride and nitrobenzene in benzaldehyde U.S.P. samples by normal-phase HPLC on a μPorasil column. No quantitation was attempted.

Benzyl alcohol, benzaldehyde and benzoic acid along with hippuric acid and benzylamine had been separated by gradient HPLC in a study of the oxidation products of benzylamine in cell suspensions [9]. In this study only benzaldehyde, benzoate and hippurate were determined quantitatively. Several papers reported the quantitative assay of benzoic acid in biological samples. Sioufi and Pommier [10] extracted benzoic acid with diethyl ether and, after derivatization, subjected the derivative to gas chromatography. HPLC had also been applied to the assay of benzoic acid in biological fluids in the presence of benzoyl peroxide [11] or hippuric acid [12].

This paper reports a reversed-phase internal standard HPLC assay method for benzyl alcohol and benzoic acid in dog plasma following solid-phase extraction. In addition, the methods allows for the baseline separation of benzyl alcohol, benzaldehyde, benzoic acid, hippuric acid and benzocaine.

EXPERIMENTAL

Apparatus

The following were used: an Altex Model 330 liquid chromatograph with Model 110A pump, Model 210 sample injection valve (20-μl loop) and a Model 153 fixed-wavelength UV detector (Beckman Instruments, Fullerton, CA, USA). Eluate evaporation were done using a six-port Mini-Vap concentrator–evaporator (Supelco, Bellefonte, PA, USA). Peak heights were measured with a Model 4270 electronic integrator (Varian Instruments, Walnut Creek, CA, USA). Samples were introduced with a 50-μl Type 705-SNR syringe (Hamilton, Reno, NV, USA).

Reagents and materials

The following reagents and materials were used: benzyl alcohol (certified grade), benzoic acid (primary standard), benzaldehyde (certified, chlorine free), benzocaine, ethyl acetate, methanol (Fisher Scientific, Fair Lawn, NJ, USA), hippuric acid (Sigma, St. Louis, MO, USA), and Extrelut QE solid-phase extrac-

tion cartridges (E.M. Science, Cherry Hill, NJ, USA). All other chemicals were analytical grade.

HPLC conditions

A 25 cm × 4.6 mm I.D. Ultrasphere ODS 5-μm column (Beckman Instruments) was used with a 4 cm × 4.6 mm I.D. guard column, packed with Co:Pell ODS, 25–37 μm (Whatman, Clifton, NJ, USA). Mobile phase consisted of acetonitrile–water–glacial acetic acid (120:380:2.5, v/v) and was pumped at a flow-rate of 2 ml/min. Detection was made at 254 nm (0.02–0.08 a.u.f.s.) with the attenuation of the electronic integrator set at 1.0.

Sample stock solutions

Benzyl alcohol. About 40 mg of benzyl alcohol were accurately weighed in a 50-ml volumetric flask and diluted to volume with methanol. Exactly, 5.0 ml of the resulting solution were pipetted into a 50-ml volumetric flask and diluted to volume with methanol.

Benzoic acid. About 25 mg of benzoic acid were accurately weighed in a 250-ml volumetric flask, dissolved in and diluted to volume with methanol. Exactly, 5.0 ml of the resulting solution were pipetted into a 50-ml volumetric flask and diluted with methanol to volume.

Internal standard. About 25 mg of benzocaine were accurately weighed in a 100-ml volumetric flask, dissolved in and diluted with methanol to volume. A volume of 10 ml of the resulting solution was pipetted into a 50-ml volumetric flask and diluted with methanol to volume.

Standard solution

Volumes of 1.0 ml of the above benzyl alcohol, benzoic acid and internal standard solutions were each pipetted into a 10-ml volumetric flask. The mixture was diluted with methanol to volume.

Assay of plasma samples

Thawed dog plasma (0.50 ml) was diluted with 0.5 ml of 0.9% (w/v) sodium chloride solution and 1.0 ml of internal standard solution. The mixture was quantitatively transferred onto a dry Extrelut cartridge and eluted twice with 4-ml portions of ethyl acetate. The eluate was collected in a calibrated 2-ml tube and evaporated to about 0.5 ml by means of a Mini-Vap concentrator using a gentle stream of nitrogen gas. The resulting contents were then made up to volume with acetonitrile.

Chromatographic procedure

Using a 50-μl syringe, about 45 μl of the prepared sample solution or standard solution were introduced to the sample injection valve to completely rinse and fill the 20-μl sample loop and chromatographed under the conditions described

above. Quantitation was based on relating the compound to internal standard peak-height ratio of the sample to that of the standard.

Linearity determination

From the benzyl alcohol and benzoic acid stock solutions described above, seven different aliquots were pipetted into separate 10-ml volumetric flasks, containing 1.0 ml of internal standard solution, to contain 4–32 μg/ml benzyl alcohol and 0.5–4 μg/ml benzoic acid after dilution to volume with methanol. Each standard solution was chromatographed as described above and the compound to internal standard peak-height ratios were calculated and plotted against the amount of compound injected.

RESULTS AND DISCUSSIONS

Preliminary studies with actual dog plasma showed an absence of benzaldehyde and hippuric acid. This is not surprising since the metabolic pathway involves a rapid oxidation of benzyl alcohol to benzoic acid [1,3,4]. However, to ensure that benzaldehyde and hippuric acid, if present, do not interfere with the compounds of interest in the liquid chromatogram, an HPLC run was made of a standard solution containing benzyl alcohol, benzaldehyde, benzoic acid, hippuric acid and benzocaine. This will also ensure that the method can be applied for the purity determination of benzyl alcohol bulk material because benzaldehyde is always present in aged benzyl alcohol bulk samples. Under the described experimental conditions benzyl alcohol, benzaldehyde, benzoic acid, hippuric acid and benzocaine were well separated from one another with resolutions between peaks greater than 2 (Fig. 1). The approximate retention times were 2.2 min for hippuric acid, 3.4 min for benzyl alcohol, 4.8 min for benzoic acid, 6.6 min for benzaldehyde and 9.5 min for benzocaine. The capacity factors, k', were approximately 0.6, 1.4, 2.4, 3.7 and 5.8 for hippuric acid, benzyl alcohol, benzoic acid, benzaldehyde and benzocaine, respectively. With the exception for hippuric acid, these k' values indicated that the separation was well within the optimum range of 1–10 [13]. To ascertain that the benzyl alcohol does not contain benzaldehyde, an aliquot of the bulk material was subjected to capillary gas chromatography on a 15 m × 0.524 mm I.D. DB-17 (1 μm) column with flame ionization detection. Only one sharp peak was obtained at retention time 3.0 min. When the sample was spiked with a trace of benzaldehyde, two additional sharp peaks were observed at 2.2 and 4.0 min. The results indicated that the benzyl alcohol used does not contain benzaldehyde.

The detection wavelength of 254 nm is suitable for monitoring the HPLC eluate as shown by the UV absorption spectra of benzyl alcohol, benzoic acid and benzocaine (Fig. 2).

Initially, the mobile phase consisted of acetonitrile and water only. Although the compounds were separated, the benzoic acid peak showed a shoulder. Since

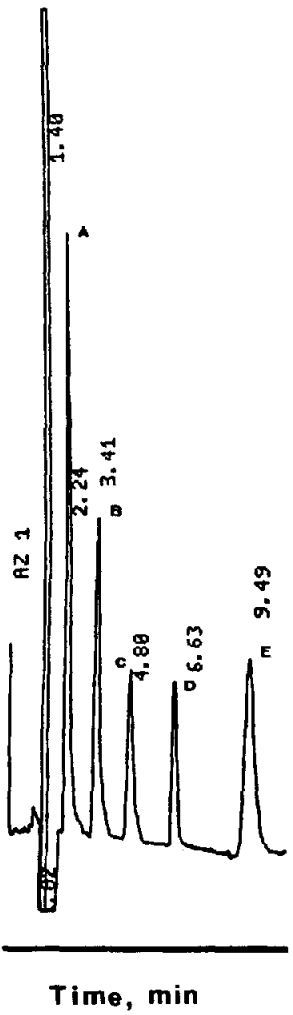

Fig. 1. Liquid chromatogram of a standard solution run under conditions described in text. Peaks: A = hippuric acid (10 ng); B = benzyl alcohol (80 ng); C = benzoic acid (10 ng); D = benzocaine (5 ng); E = benzaldehyde (10 ng injected).

the mobile phase has a pH of about 6 and benzoic acid has a pK_a of 4.19 at 25°C [14], the acid was predominantly dissociated. The conjugate base differs slightly in elution characteristics than the undissociated acid and thus gave rise to the shoulder. Addition of glacial acetic acid decreased the pH of the mobile phase to about 2, whereby dissociation of the acid became suppressed, and under this condition the shoulder disappeared.

The relationship between the compound to internal standard peak-area and peak-height ratios and amount of compound injected were established for benzyl

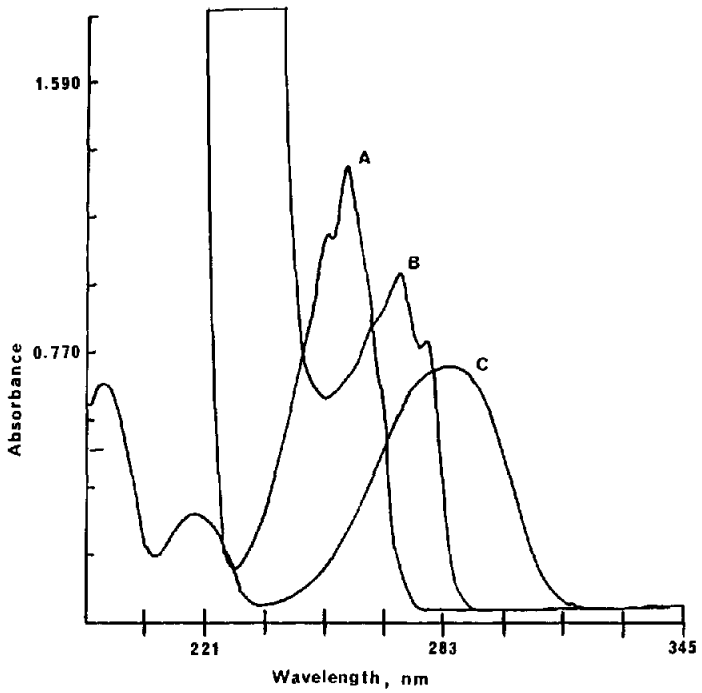

Fig. 2. Ultraviolet absorption spectra of benzyl alcohol (A), benzoic acid (B) and benzocaine (C) in methanol.

alcohol and benzoic acid over a range of 0.08–0.64 μg of benzyl alcohol injected and over 0.01–0.08 μg of benzoic acid injected. The following regression equations were obtained. For benzyl alcohol: $A = 0.53C - 0.01$ ($r = 0.999$) and $H = 1.01C + 0.004$ ($r = 0.999$); and for benzoic acid: $A = 2.10C + 0.004$ ($r = 0.996$) and $H = 3.94C + 0.001$ ($r = 0.998$), whereby A = peak-area ratio, H = peak-height ratio and C = amount (μg) of compound injected. The equations show that the slope values for the peak-height ratio plots are considerably steeper, indicating that the assay by peak height will be more sensitive. Consequently, the peak height was measured in subsequent studies.

The sample clean-up by solid-phase extraction provided relatively clean sample solutions as shown by the liquid chromatogram of blank dog plasma. This chromatogram showed no peaks at the retention times of the compounds of interest (Fig. 3). The clean-up step is simple and less time-consuming than conventional solvent extractions. One analyst can handle twelve samples simultaneously. By adding the internal standard to the samples prior to passing the samples through the solid-phase extraction cartridge, loss of the analyte(s), if any, were compensated. Precautionary measures were taken, however, to prevent the loss of benzyl alcohol during the evaporation of the ethyl acetate eluate as this will not

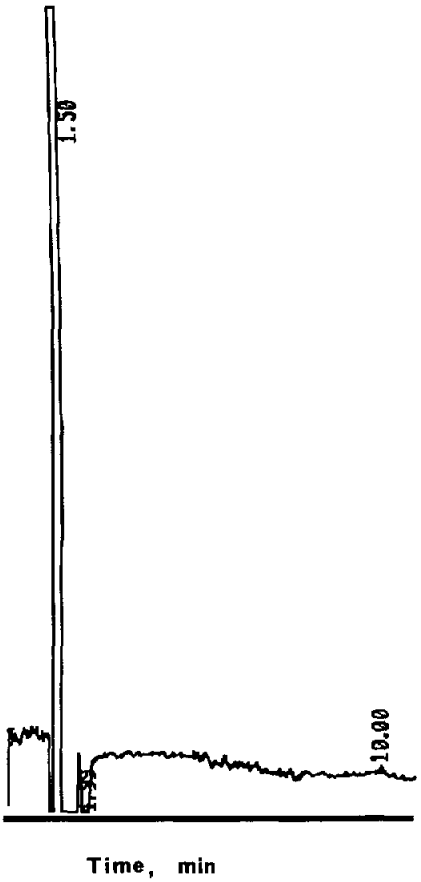

Fig. 3. Liquid chromatogram of a blank dog plasma run under conditions described in text.

TABLE I

RECOVERY DATA FROM SPIKED DOG PLASMA

Amount added (μg/ml of plasma)		Amount found[a] (μg/ml of plasma)		Recovery (%)	
BZOH[b]	BZAC[b]	BZOH	BZAC	BZOH	BZAC
60.2	57.5	60.1	57.4	99.8	99.8
80.1	9.6	80.0	9.1	99.9	94.5
122.4	18.7	114.8	18.5	93.8	98.9
138.7	72.9	138.0	82.0	99.5	112.5
Overall recovery (%)				98.3	101.4
R.S.D. (%)				3.0	7.6

[a] Average of duplicate assays.

[b] BZOH = benzyl alcohol; BZAC = benzoic acid.

be compensated for by the internal standard. These measures included the application of a gentle stream of nitrogen gas and evaporation to a volume of about 0.5 ml and not to dryness. Furthermore, the tip of the Mini-Vap nitrogen gas inlet tube was positioned at least 1 cm above the initial ethyl acetate eluate. The evaporation of the eluate (~8 ml) took about 10 min.

The limit of detection was determined for qualitative purposes. The sensitivity, as determined by the method of Kaiser [15] utilizing twenty blank dog plasmas and measured in terms of peak heights at 0.02 a.u.f.s. and 10 mV integrator input, was 4 ng for benzyl alcohol and 0.1 ng for benzoic acid injected.

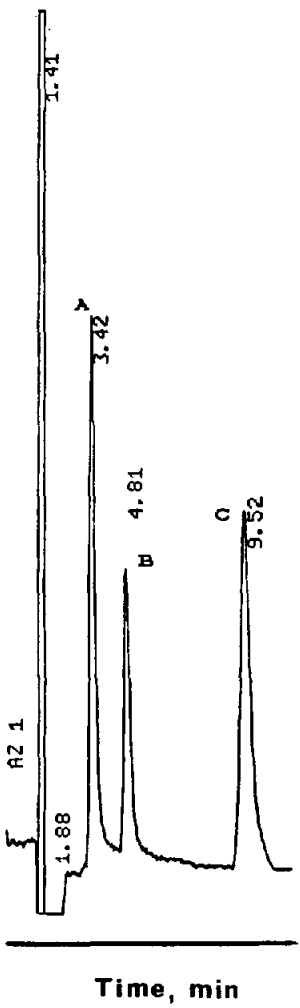

Fig. 4. Liquid chromatogram of spiked dog plasma run under conditions described in text. Peaks: A = benzyl alcohol (122 μg/ml of plasma); B = benzoic acid (18 μg/ml of plasma); C = benzocaine.

Recovery studies were performed by spiking dog plasma with various amounts of benzyl alcohol and benzoic acid. Results are shown in Table I. The overall recovery (± relative standard deviation, R.S.D.) was 98.3 ± 3.0% for benzyl alcohol and 101.4 ± 7.6% for benzoic acid (n = 4). A typical liquid chromatogram of spiked plasma is shown in Fig. 4.

The method was applied to the assay of actual dog plasma in the second phase of this study with dogs. The results will be reported elsewhere. Fig. 5 shows a typical liquid chromatogram of actual dog plasma, sampled 30 min after administration of sodium pentobarbital (65 mg/ml) containing 2% benzyl alcohol. Although no attempt was made to control the amount of benzyl alcohol administered, the amount of benzyl alcohol administered was that contained in about 20 mg/kg pentobarbital required to reach anesthesia in the dog. Preliminary studies

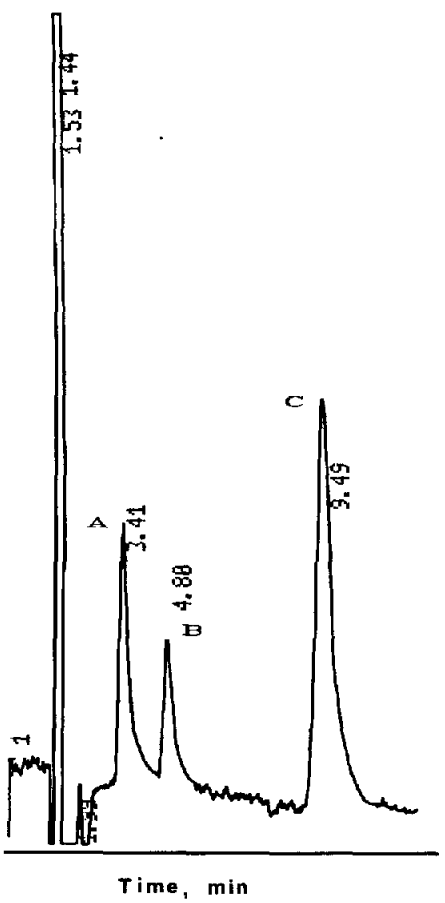

Fig. 5. Liquid chromatogram of dog plasma after administration of benzyl alcohol (A); B = benzoic acid; C = benzocaine. Concentrations and chromatographic conditions as described in text.

indicated that, under the experimental conditions, pentobarbital did not interfere with the compound of interest.

In vitro, benzyl alcohol is also very susceptible to oxidation to benzaldehyde and then to benzoic acid. The latter can be seen as crystals in old benzyl alcohol bulk samples whereas the benzaldehyde imparts the "almond" odor to these samples. The HPLC method described in this paper can be used as a stability-indicating method to determine the purity of benzyl alcohol raw material or bulk samples. Fig. 6 is a typical liquid chromatogram of a six-year old sample of benzyl alcohol taken from a half-full bottle.

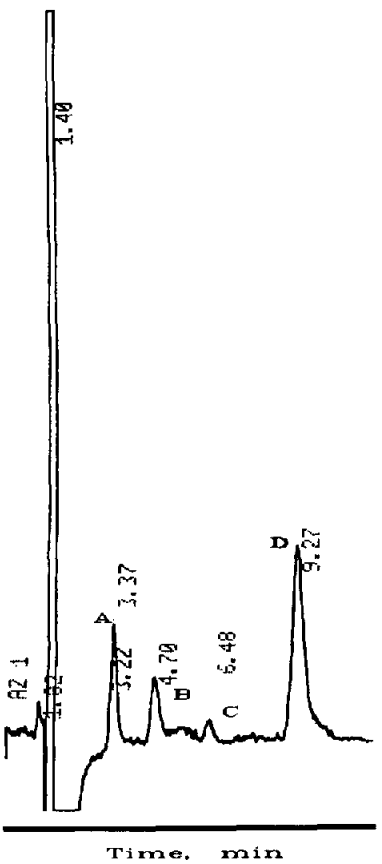

Fig. 6. Liquid chromatogram of a six-month old benzyl alcohol sample from a half-full bottle. Peaks: A = benzyl alcohol; B = benzoic acid; C = benzaldehyde; D = benzocaine.

REFERENCES

1 J. Gershanik, B. Boecler, H. Ensley, S. McCloskey and W. George, *N. Engl. J. Med.*, 307 (1982) 1384.
2 W. J. Brown, N. R. Buist, H. T. C. Gipson, R. K. Huston and N. G. Kennaway, *Lancet*, i (1982) 1250.
3 J. H. Schleifer and T. L. Carson, *J. Am. Vet. Med. Assoc.*, 181 (1982) 853.
4 R. F. Cullison, P. D. Menard and W. B. Buck, *J. Am. Vet. Med. Assoc.*, 182 (1983) 61.
5 A. Rego and B. Nelson, *J. Pharm. Sci.*, 71 (1982) 1219.
6 B. J. Floor, A. E. Klein, N. Muhammad and D. Ross, *J. Pharm. Sci.*, 74 (1985) 197.
7 A. M. DiPietra, V. Carrini and M. A. Raggi, *Int. J. Pharm.*, 35 (1987) 13.
8 J. H. Block and H. L. Levine, *J. Chromatogr.*, 166 (1978) 313.
9 D. P. Jones, *J. Chromatogr.*, 305 (1984) 256.
10 A. Sioufi and F. Pommier, *J. Chromatogr.*, 181 (1980) 161.
11 C. Ehinger and C. Andermann, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 143.
12 K. Kubota, Y. Horai, K. Kushida and T. Ishizaki, *J. Chromatogr.*, 425 (1988) 67.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, p. 50.
14 *Handbook of Chemistry and Physics*, CRC Press, Cleveland, OH, 59th ed., 1979, p. D202.
15 H. Kaiser, *Anal. Chem.*, 42 (1970) 26A.