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Quantification of benzocaine and its metabolites in channel catfish tissues and fluids by HPLC

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Abstract

Methods for extraction and gradient HPLC quantification were developed for benzocaine (BZ) and three of its metabolites to be used in conjunction with a reverse isotope technique. The metabolites were *p*-aminobenzoic acid (PABA), acetyl-*p*-aminobenzoic acid (AcPABA) and acetylbenzocaine (AcBZ). The matrixes studied were white muscle, red muscle, skin, liver, trunk kidney, head kidney, plasma and the bile of channel catfish. Analytes were validated for each of the compounds at 25 and 100 nmol per sample in the various tissues and fluids. The intraday variability (R.S.D.) was less than 13% in all tissues and fluids except for BZ in the liver. Recoveries varied from matrix to matrix for each analyte. The highest recoveries were obtained from plasma which ranged from 82.8–99.8% depending on the concentration. The average recovery of the compounds from tissues was between 50 and 78%, except for liver where the recovery of PABA and BZ was below 30%. Detection was by UV absorbance at 286 nm and the linear range was 2.5–15 nmol 100 ml^{-1} for all analytes. The method was selective; no interference peaks coeluted with the analytes. © 1997 Elsevier Science B.V.

Keywords: Benzocaine; *p*-Aminobenzoic acid; HPLC; Channel catfish; Residue

1. Introduction

Benzocaine (BZ) is used as a topical anesthetic [1] and it has potential for use as a general anesthetic in fin fish aquaculture [2–4]. BZ is extensively metabolized in fish to form acetylbenzocaine (AcBZ), acetyl-*p*-aminobenzoic acid (AcPABA) and *p*-aminobenzoic acid (PABA). BZ has been analyzed in a number of pharmaceutical preparations by reverse phase HPLC [5–9]. BZ and PABA were separated and quantified by reversed phase HPLC in a study of the influence of pH, temperature and phosphate ion on their stability in aqueous solution [10]. An HPLC method for determination of benzocaine in rainbow trout plasma was described [11]. These studies reported benzocaine recoveries of 90% and higher from relatively simple liquid matrices. An in vitro percutaneous absorption and metabolism study involved extraction of BZ from a solid matrix [12]. [^{14}C]Benzocaine and metabolites (AcBZ, PABA

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and AcPABA) were separated from receptor fluid and skin homogenate of hairless guinea pigs. Extracts were applied to TLC plates, analytes were identified by use of unlabeled standards, and for quantification the radioactivity was measured using a plate scanner. A study of [^{14}C]benzocaine uptake in rainbow trout [4] described a method for analysis of exposure water and rainbow trout urine. The parent compound and metabolites were separated by reverse phase HPLC and the radioactivity was quantified in column effluent by liquid scintillation counting. Radioactive compounds were identified by comparison of their retention times with those of authentic PABA, AcPABA, BZ and AcBZ. In these two latter studies the unlabeled compounds were used only to locate the metabolites as they eluted; quantification was based on radioactivity. Previously reported methods were not suitable for analysis of BZ and its metabolites in fish tissues, due to the complexity of these matrices and because most of the methods did not consider metabolites. We developed and validated the method described here for the analysis of BZ, AcBZ, PABA and AcPABA in channel catfish tissues and fluids.

2. Experimental

2.1. Animals

Channel catfish were purchased from The Ohio State University Piketon Research and Extension Center and maintained in a 1000 l flow through raceway at 25°C. The fish used for the validation study was a 396 g female.

2.2. Materials and reagents

BZ, (labeled purity 99%) and PABA (labeled purity 99%) were from Sigma, St. Louis, MO. AcPABA (labeled purity 97%) was from Pfaltz and Bauer, Waterbury, CT. AcBZ (purity 98%) was prepared by reaction of ethyl *p*-aminobenzoate with acetic anhydride [13]. Its structure was confirmed using FT-NMR (Brucker AC250); the following chemical shifts (δ, ppm) were observed downfield from an internal tetramethylsilane standard: ^{1}H-NMR (acetone d_6) δ 1.32 (t, 3H, -CH_3), 2.11 (s, 3H, CH_3CO-), 2.88 (s, 1H, -NH-), 4.30 (q, 2H, -CH_2-), 7.75, 7.95 (2d, 4H, H-aromat.). Elemental analysis calculated for $C_{11}H_{13}O_3N$: C, 63.76; H, 6.33; O, 23.17; N, 6.76. Found: C, 63.71; H, 6.21; O, 23.84; N, 6.58.

Reagents used for sample preparation were analytical grade. The mobile phase included HPLC grade methanol and glacial acetic acid, from Fisher Scientific, Pittsburgh, PA, and demineralized, double distilled water. Cyanopropyl-bonded silica solid-phase extraction (SPE) tubes (3 ml) were supplied by Supelco, Bellafonte, PA.

A methanolic stock solution (5 μmol ml^{-1}) of each analyte was stable for 1 year when stored at 4°C. A spiking solution that contained 1 μmol ml^{-1} of each analyte was prepared monthly by mixing equal volumes of each stock solution and methanol.

2.3. Equipment and instrumentation

For sample preparation the following equipment was used: Omni mixer homogenizer with the 7 mm generator (model 17105, Omni International, Waterbury, CT); centrifugal vacuum concentrator (model SVC 200H, Savant Instruments, Farmingdale, NY); horizontal shaker at 280 excursion min^{-1} (model 6010, Eberbach, Ann Arbor, MI); nitrogen stream evaporator (model 111 from Organomation Associates, Berlin, MA) with a 40°C water bath.

The Beckman HPLC system consisted of two model 110B pumps, a model 406 digital–analog interface, a model 166 UV detector at 286 nm, a Pharmacia model FRAC-100 fraction collector, an IBM model 55 SX PC computer, and a System Gold instrument controller card for PC and System Gold software. The 3 μm, 15 cm × 4.6 mm C-18 analytical column, model LC-18-DB and the 2 cm guard column, model Supelguard LC-18-DB were from Supelco. The mobile phase gradient was:

0–3 Min: mobile phase A; 3–8 min: gradient to 100% mobile phase B; 8–23 Min: mobile phase B; 23–24 min: linear gradient to 100% mobile phase A.

Mobile phase A: 5 v/v% methanol, 94 v/v% water, 1 v/v% acetic acid.
Mobile phase B: 55 v/v% methanol, 44 v/v% water, 1 v/v% acetic acid.

2.4. Sample preparation

Plasma (0.25 ml) and bile (0.15 ml) samples were spiked with 25 or 100 μl of the spiking solution; blank samples were not spiked. Proteins were precipitated with three volumes methanol, separated by centrifugation and methanol was removed from the supernatant by vacuum centrifugal evaporation. The residue was filtered through a 0.22 μm nylon filter, and 100 μl was injected into the HPLC column. Samples of white and red muscle (1.1 and 0.50 g), liver (0.64 g), trunk and head kidneys (0.25 and 0.17 g) and skin (0.32 g) were homogenized in 5 volumes of 100 mM acetic acid, except for skin and red muscle, where 25 and 100 mM sodium acetate at pH 4.5 were used, respectively. The spiking solution (25 or 100 μl) was added during homogenization or samples were not spiked. Methanol at 5 times the tissue volume was added to the homogenate and the slurry was shaken for 15 min on the horizontal shaker. Samples were centrifuged at $3000 \times g$ for 15 min at 15°C. The supernatants were transferred to polypropylene tubes and methanol was removed by vacuum centrifugation. The residue was acidified with 10% acetic acid to a final 1% acetic acid concentration for all samples but skin where the final acetic acid concentration was 2.5%. Acidified samples were loaded onto the SPE tubes and eluted with methanol which was then removed using the nitrogen evaporator. Dry residues were reconstituted in sample solvent (15 methanol, 1 acetic acid, 84% water) to the following volumes: bile and plasma to 1 ml, residues of all tissues to 500 μl. Samples were filtered through a 0.22 μm nylon microcentrifuge filter and 100 μl was injected into the HPLC column.

To validate the method for BZ and its metabolites in all matrices, samples were spiked with either 100 nmol (medium concentration) or 25 nmol of BZ and each metabolite. When recovery was 50% and the reconstitution volume was 500 μl, 2.5 nmol of each compound was injected per 100 μl. The instrument LOQ was 1 nmol per injection.

2.5. Calibration curve

Five point calibration curves were made from 2.5 nmol 100 ml^{-1} to 15 nmol 100 ml^{-1} for all four analytes using dilutions of the spiking solution. Peak areas of benzocaine and each of the metabolites were linearly related to the quantities of the added compounds. Linear regression analysis of the standard curves indicated no significant deviation from linearity ($r^2 = 0.998–0.999$). Representative linear regression values for each of the compounds are indicated in Table 1.

3. Results and discussion

Fig. 1 shows the chromatograms of blank samples and of samples spiked with a mixture of BZ, AcBZ, PABA and AcPABA. The method was selective; no interference peaks were found in the chromatograms of the blank samples, except for bile. It showed a peak with the same retention time as AcPABA, but the area was less than 3% of the peak area that was observed after addition of 100 nmol AcPABA to blank bile. Decomposition of BZ was observed in vitro in bile, but not the other matrices. As a result no BZ peak was found for the spiked bile samples and the recovery of the metabolites was above 100%, Table 1.

The recovery of each analyte was used as the measure of accuracy [14]. Recoveries from the 25 and 100 nmol enriched samples appeared to be similar, except for AcPABA in red muscle, where recovery at the 25 nmol level was 41.9 versus 66.6% at the 100 nmol level, Table 1. Recovery varied from matrix to matrix; the highest recovery was from plasma: 89.3–99.8% for the 100 nmol level and 82.8–94.0% for the 25 nmol level. Recoveries from other matrices were generally above 50% with the exceptions being PABA from white muscle (35.4%), and PABA and BZ from liver (22.6 and 28.3% respectively).

Table 1
Recovery of BZ and its metabolites from tissues and fluids of channel catfish

Tissue or fluid	Amount added (nmol per sample)	Replicates		PABA	AcPABA	BZ	AcBZ
			m	15.50	13.30	16.94	16.77
			b	−5.09	3.93	2.58	0.092
White Muscle	100	5	Recovery	35.4	51.6	51.3	59.5
			R.S.D.	5.78	5.43	11.6	13.3
	25	1	Recovery	35.0	48.6	46.2	59.5
Skin	100	5	Recovery	50.3	67.4	58.7	75.2
			R.S.D.	11.6	5.26	9.66	3.78
	25	1	Recovery	43.7	64.6	41.1	64.1
Red Muscle	100	3	Recovery	55.9	66.6	57.7	68.0
			R.S.D.	4.59	3.37	4.59	7.35
	25	1	Recovery	46.8	41.9	45.5	56.9
Liver	100	5	Recovery	22.6	81.8	28.3	47.1
			R.S.D.	10.8	11.21	18.2	11.2
	25	1	Recovery	16.7	68.7	19.9	64.1
Trunk Kidney	100	4	Recovery	72.2	98.5	65.9	64.5
			R.S.D.	3.02	2.12	4.03	1.16
	25	1	Recovery	71.3	68.8	55.0	73.7
Head Kidney	100	2	Recovery	59.2	78.1	52.6	54.4
			R.S.D.	6.35	10.7	9.51	9.75
Plasma	100	4	Recovery	89.3	99.8	91.7	93.3
			R.S.D.	6.86	9.73	8.73	9.30
	25	1	Recovery	94.0	90.0	82.8	88.4
Bile	100	2	Recovery	122	178	0	124
			R.S.D.	1.81	0.714	0	8.24

Representative linear regression values (from linear regression of absorbance vs. concentration (nmol 0.1 ml^{-1}); m, slope; b, intercept) for each compound are also shown. Recovery and R.S.D. are expressed as percent.

The measure of precision was the relative S.D. (R.S.D.) of multiple determinations [14]. At the 100 nmol level R.S.D. was below 13.3% in all tissues and fluids except BZ in the liver, Table 1. R.S.D. values below 10% were observed in plasma, bile, red muscle and head kidney.

Compared with previously reported methods [5–11], the matrices used in the present study were more complex and diverse. Their biochemical and associated physicochemical complexity provided numerous possibilities for binding of the analytes which probably accounts for their incomplete and variable recoveries. The method described here permits the recovery of benzocaine and its three metabolites from these different matrices using the same extraction and chromatographic separation method. Considering that the LOQ is 1 nmol and the average recovery is 50% for each of the compounds, 1–2 ppm of each compound in the original sample can be detected by UV. In conjunction with isotope dilution the sensitivity of detection is increased depending on the specific activity of the labeled compound. A surrogate internal standard such as *o*- or *m*-aminobenzoic acid may prove useful in the absence of isotopically labeled BZ.

4. Conclusion

A reproducible method was established for the extraction and HPLC quantification of BZ and its

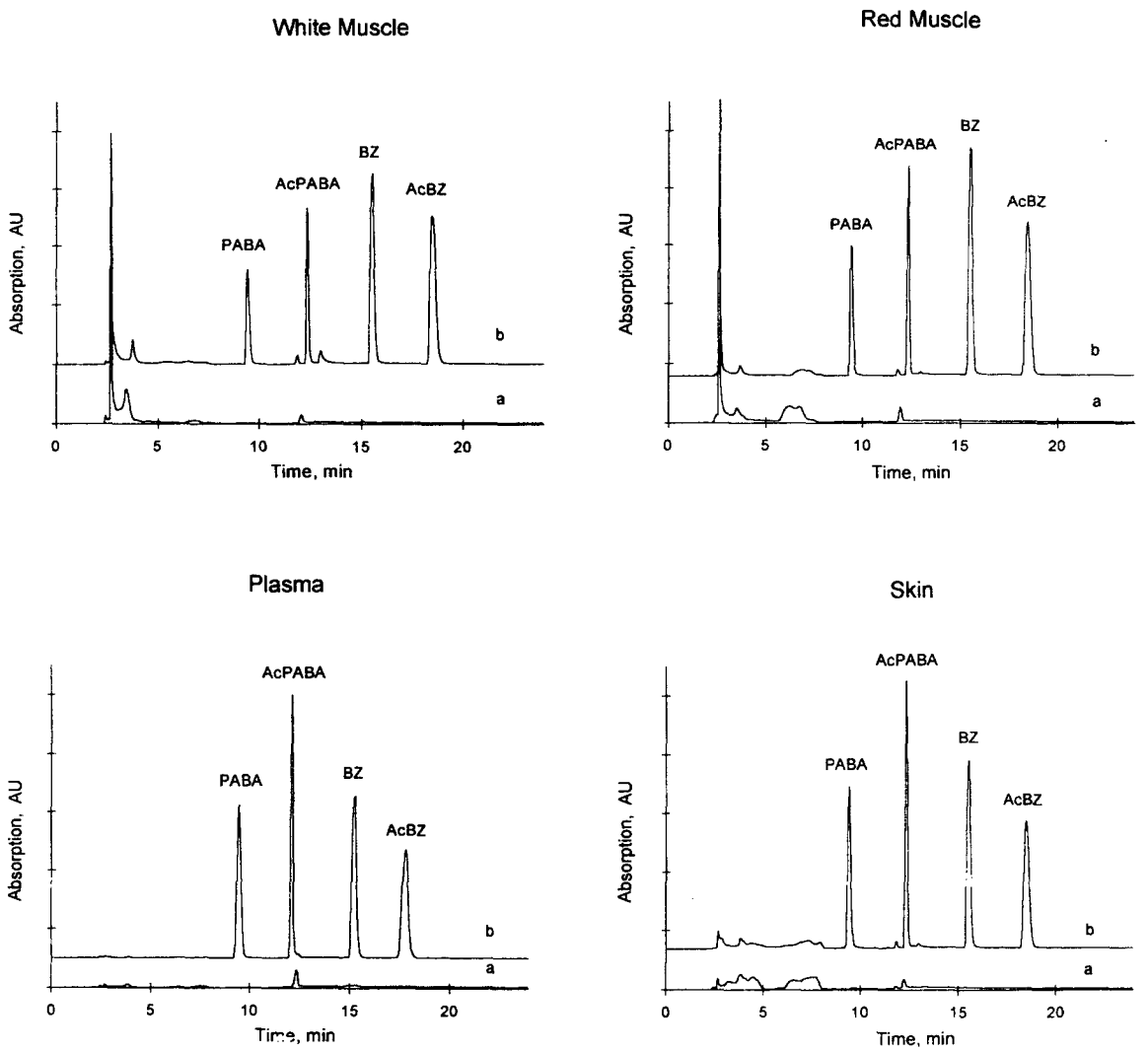

Fig. 1. Chromatograms of various tissues and fluids from channel catfish: (a) not spiked and (b) spiked with 100 nmol each of PABA, AcPABA, BZ, and AcBZ. Extracts were reconstituted in 500 μl sample solvent prior to analysis. HPLC conditions are described in Section 2.

metabolites (AcBZ, PABA and AcPABA) from six tissues (white muscle, red muscle, skin, liver, trunk kidney and head kidney) and two fluids (plasma and bile) of channel catfish. The method was used in a metabolite profile and residue deple tion study in combination with reverse isotope dilution for the analysis of a large number of samples [15].

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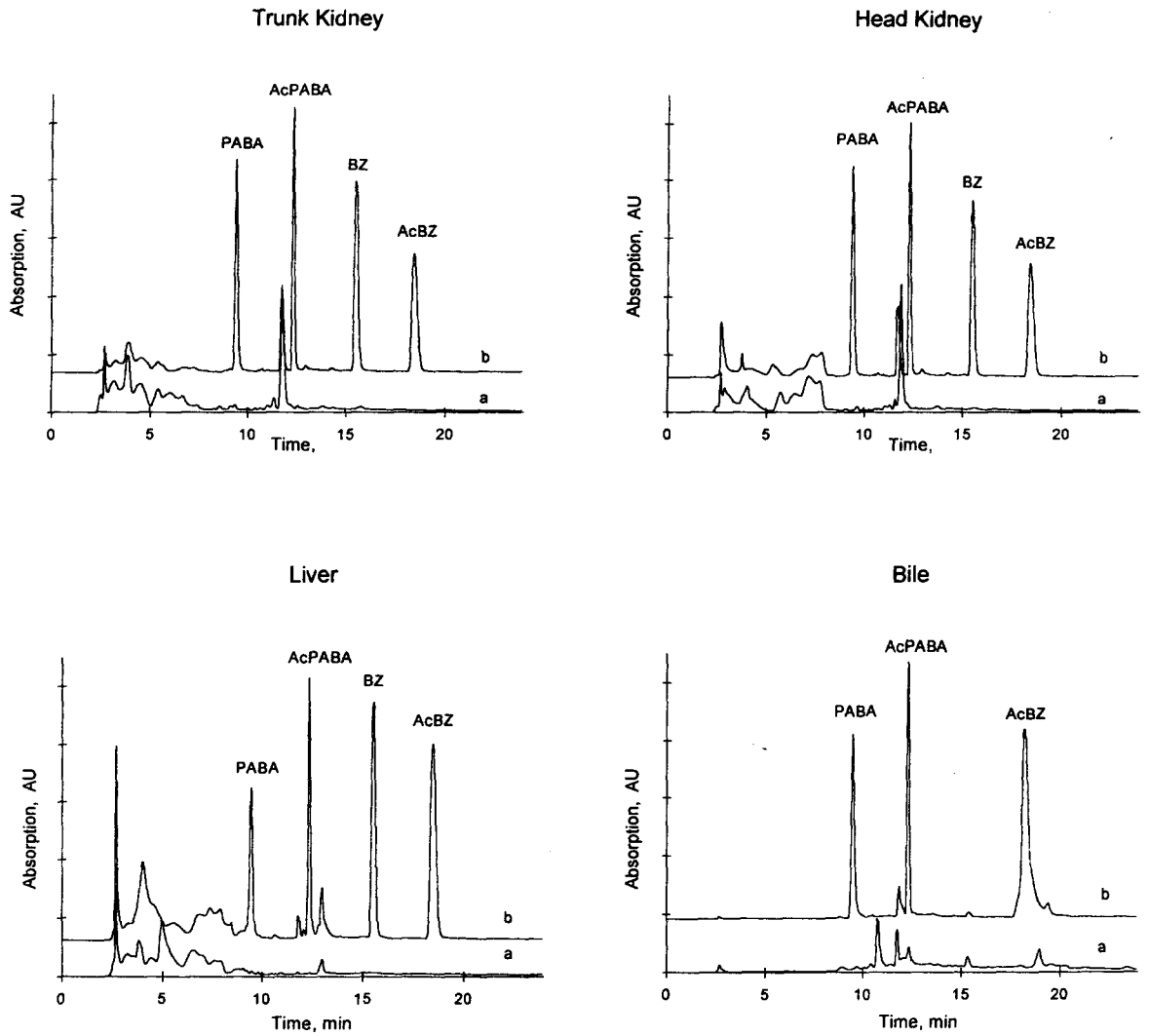

Fig. 1 (continued).

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