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Note

Use of impregnated reagents in the pentafluorobenzylation of indoleamine metabolites

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Analytical derivatization to electrophoretic products[1–3] is obligatory for mass spectrometric (MS) determination of serotonergic cascade metabolites that exist in picogram per sample amounts[4–8]. Elucidation of the physiological role of two of these compounds, N-acetyl serotonin and melatonin[4,5], are based on such analyses[6–8]. In order to simplify and possibly automate sample preparation procedures for these two analytes we investigated the paradigm of an impregnated reagent.

Impregnated reagents have been used extensively in synthesis because of the technical simplicity and the mild reaction conditions[9,10]. In addition, analytical derivatization reactions of carbonyl compounds in air have also been affected with impregnated reagents[11,12]. More recently, we demonstrated the simultaneous extraction and derivatization of organic acids from aqueous solution using impregnated reagents[13] and have now tested this reagent on an organic base (melatonin) and an amphoteric compound (N-acetyl serotonin) which require analysis at picogram sensitivity[6–8,14]. The reagent consisted of pentafluorobenzyl bromide (PFBBr) impregnated on solid beads of XAD-2 resin, a macroreticular polystyrene–divinylbenzene cross-linked co-polymer. The resin served a three-fold purpose: (a) an adsorbant of N-acetyl serotonin and melatonin from aqueous medium; (b) a catalyst for the pentafluorobenzylation of these analytes; and (c) a "chromatographic phase" to separate the pentafluorobenzyl (PFB) derivatives of N-acetyl serotonin and melatonin from excess PFBBr and lipophilic PFB derivatives such as those of palmitic and stearic acid.

Materials

N-Acetyl serotonin and melatonin were purchased from Sigma. The macroreticular resin XAD-2 was purchased from Rohm and Haas and was cleaned by methods described elsewhere[13]. PFBBr and trifluoroacetic acid anhydride (TFAA) were purchased from Supelco (Canada). Gas chromatographic (GC) columns and phases were obtained from Chromatographic Specialties, Brockville, Canada.

Gas chromatographic analysis

GC was performed on a Hewlett-Packard 5710 gas chromatograph equipped with a frequency pulsed electron-capture detector. The analyses were carried out on a 3.4 m × 2 mm I.D. glass column packed with 1.5% OV-17 on Chromsorb W,

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80–100 mesh. The gas was a methane–argon (15:85) mixture and was maintained at a flow-rate of 15 ml/min. The output of the detector was monitored on a Hewlett-Packard 3380A recording integrator.

Gas chromatographic–mass spectrometric analysis

Electron impact mass spectra were obtained on a Finnegan GC–MS–Data System using the INCOS data handling package. The ion source was maintained at 300°C and at an ionizing voltage of 70 eV. The packed column, of identical dimensions described above, was connected to the ion source by a Ryhage jet separator. Prior to use the column and separator were silylated.

Synthetic preparation of pentafluorobenzyl derivatives of analytes

The PFB derivatives of N-acetyl serotonin and melatonin for use as standards were synthesized on the preparative scale using previously reported methods[13], but modified by the substitution of acetone for dimethyl formamide. The PFB derivative for N-acetyl serotonin (NAS-PFB) was purified by crystallization, whereas that for melatonin (M-PFB) was purified by preparative-scale thin-layer chromatography (2 mm silica gel PF_{254}, developed twice in acetone–diethyl ether, 10:90). Yields were 30% and 15%, respectively.

Derivatization of analytes on XAD-2

Derivatization procedure. A 200-mg amount of cleaned XAD-2 resin in a 100 × 16 mm silanized screw-capped glass tube was treated with 4 ml of freon 11 (b.p. 27°C). PFBBr (10 μl) and ethanol (200 μl) were added to the mixture. The freon was removed by shaking at 60 rpm in a waterbath at 30°C for 60 min thus depositing the PFBBr and ethanol uniformly on the beads.

A 4-ml aliquot containing a known amount of N-acetyl serotonin or melatonin in 4 ml 0.1 *N* sodium hydroxide solution was added to 100 mg impregnated resin and the mixture was shaken for 90 min at 40°C. The beads were isolated by filtration through a plug of glass wool and washed with 20 ml of hexane. This removed only excess PFBBr and highly lipophilic PFB derivatives (*e.g.*, PFB ester of palmitic and stearic acids). The hexane washes were retained for future recovery of PFBBr. The PFB derivatives of N-acetyl serotonin and melatonin were eluted with 20 ml of ethanol–diethyl ether (10:90) which was evaporated and the residue reconstituted in 25 μl of TFAA and kept at room temperature for 20 min. The TFAA was evaporated and the residue reconstitueed in 500 μl of toluene containing 1 μg of the PFB ester of ntadecanoic acid (C_{15}-PFB) which served as an external standard for analysis of N-acetyl serotonin.

Calculation of yield. An amount of synthetically prepared NAS-PFB equimolar to 1 μg of underivatized NAS was trifluoroacetylated as described above. To this was added 1 μg of C_{15}-PFB in 500 μl of toluene. The peak height ratio of the GC–ECD peak of NAS-PFB/trifluoroacetyl (TFA) to that C_{15}-PFB was determined. This peak heights ratio was also determined for the experimental samples. Comparison between the ratio obtained from the standard solution and experimental samples directly gave the yield of the NAS-PFB. However the overall yield could not be calculated since there is no easy synthetic access to product B (see Fig. 1) of the NAS derivatization on XAD-2.

RESULTS AND DISCUSSION

Derivatization of N-acetyl serotonin with the impregnated reagent, resulted in two products reflecting polyfunctional nature of that molecule. Prior to GC analysis the mixed PFB/TFA derivatives were prepared to improve the GC properties of the PFB derivatives. These mixed PFB/TFA derivatives are well separated by GC (Fig. 1). The formation of two derivatives is not an inherent disqualifying factor since GC–MS methods[15,16] have been reported where analytical derivatizations give more than one product. Moreover, the yield is constant from 0.5 to 25 μg of N-acetyl serotonin (r = 0.98 for a calibration curve in that range) and multiple determination at the 1-μg level using an external standard gave a relative standard deviation of

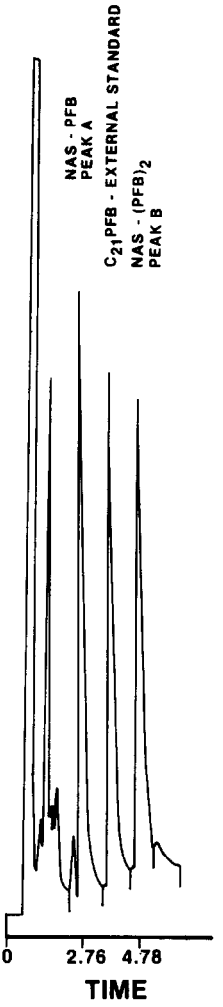

Fig. 1. GC–ECD trace of NAS-PFB derivatives prepared by impregnated XAD-2 extraction and derivatization of NAS from buffer. Time in minutes.

12%. Interestingly, while both products were also formed when N-acetyl serotonin was derivatized with PFBBr in acetone, product A (*cf.*, Fig. 1) compromised approximately 95% of the product reaction. This suggests that the resin exerts a major effect on controlling the rate of different reaction pathways.

The structure of product A (*cf.* Fig. 1) can be readily assigned by comparison to synthetic mono-NAS-PFB which by ^{13}C NMR was shown to be the O-PFB derivative. Product A has the same retention and mass spectrum as the mixed PFB/TFA derivative of the synthetically prepared NAS-PFB. The mass spectrum (Fig. 2A) shows an intense peak at *m/e* 476 which is the molecular ion of the PFB/TFA *spiro* derivative analogous to that observed for the pentafluoropropionyl *spiro* derivative of melatonin[6–8]. As is common for PFB derivatives the base peak is 181 which is the PFB moiety.

The structure of product B is more difficult to assign as it is not accessible by simple synthetic means. The mass spectrum (Fig. 2B) shows an intense peak at 475 and the PFB related peak at 181. This high-molecular-weight fragment is 1 a.m.u. lower than that of the PFB/TFA *spiro* structure. Tentatively, this product is assigned a bis-PFB structure with one PFB group at the phenolic oxygen and one at the *ortho* carbon. This would still permit formation of the *spiro* derivative but upon fragmentation would lose one PFB thus generating the fragment at *m/e* 475.

The mass spectrum of the only mixed PFB/TFA derivative of melatonin is consistent with the formation of the mono-PFB product. The molecular ion at *m/e* 508 and at the PFB ion at 181 indicated that the amide N had been trifluoroacetylated

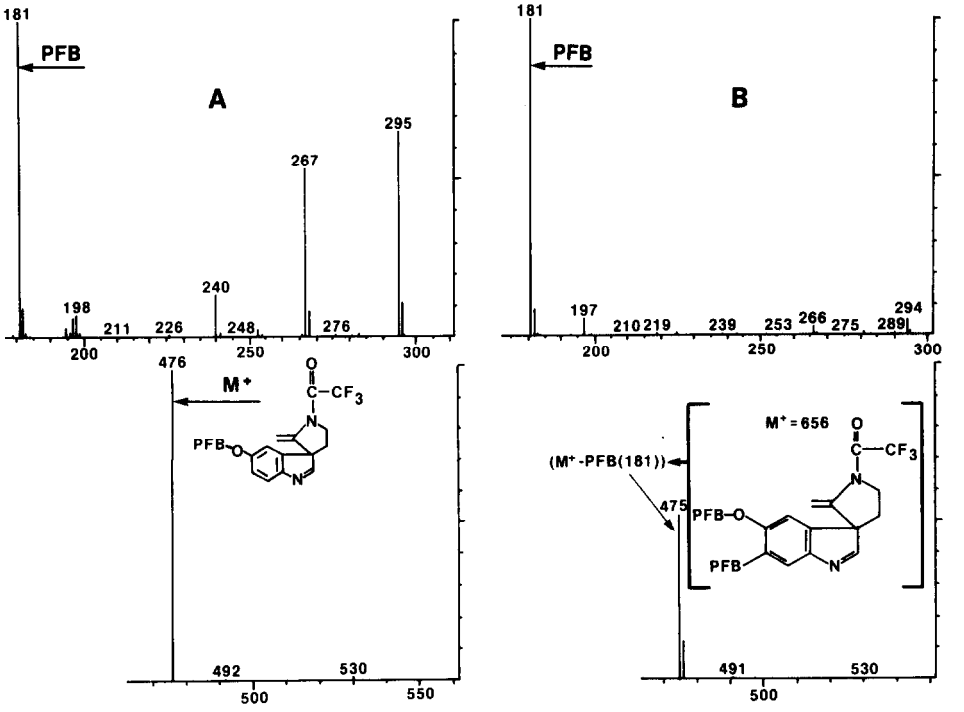

Fig. 2. Mass spectra of (A) product A and (B) product B.

TABLE I

EFFECT OF pH ON YIELD AND RELATIVE REACTION OF PRODUCTS A AND B

pH	*Yield of product A (%)*	*Peak height ratio of product A: product B*
7	0	0
9	3	0
11	20	0.1
0.1 *N* sodium hydroxide solution	38	0.6

but the *spiro* derivative did not form. A similar molecular ion was not observed in product B of the NAS derivatization reaction. This further supports the argument that derivatization for that product was not at the indole nitrogen but rather at the carbon adjacent to the phenolic carbon. The anionic nature of carbons *ortho* to a phenolate anion has been described[17].

In order to simplify the reaction mixture the factors controlling the ratio of product A and B were investigated. The pH of the liquid phase appeared to be a major determining factor both of yield and of the ratio of reaction products (Table I). Possibly this results from the differing pK_a values of the protons which must be ionized to give the different derivatives. However the markedly reduced yield at low pH made these conditions unacceptable.

The elution profile of the PFB derivatives indicates an additional potentially useful property of the impregnated reagent. Elution with hexane removed excess PFBBr (which could then be recovered) as well as lipophilic PFB derivatives but none of the indole PFB products. The ethanol–diethyl ether eluate contained the PFB derivatives of both analytes. This selectivity is unlikely to be chromatographic since the derivatives are equally distributed over the entire surface. This selectivity is probably due to a high affinity between the derivatives and the resin.

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