Derivatization With Acetic Anhydride: Applications to the Analysis of Biogenic Amines and Psychiatric Drugs by Gas Chromatography and Mass Spectrometry

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Acetylation with acetic anhydride, under both aqueous and anhydrous conditions, has been utilized to derivatize various biogenic amines and psychotropic drugs for subsequent analysis by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS). Under basic aqueous conditions, acetic anhydride derivatizes phenols and amines but not alcohols; under anhydrous conditions, all three functions are acetylated. Primary amines, once derivatized with acetic anhydride, can be further derivatized with other reagents; these diderivatives have proven useful for subsequent analysis by GC or GC-MS. Examples of applications of derivatization with acetic anhydride to analysis of biogenic amines, antidepressants, antipsychotics, and some of their metabolites are presented.

Keywords: Acetic anhydride, Gas chromatography, Mass Spectrometry, Biogenic amines, Antidepressants, Antipsychotics

Introduction

Many of the gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS) techniques that have been applied to neurochemical studies in biological psychiatry have involved derivatization of the neurochemicals or drugs of interest. Derivatization may serve a number of purposes: to facilitate extraction of the compounds of interest from biological media; to improve detection sensitivity; to alter chromatographic properties such as polarity and peak shape; and/or to alter retention times on chromatographic columns to facilitate the separation of the compounds of interest from other substances in the extract. Many derivatizing reagents are available, but this review will concentrate on acetic anhydride, a simple molecule that has had relatively wide applications in biological psychiatry. Several aspects of acetic anhydride are very relevant to such analyses and should be emphasized at this point. Amines and phenols (but not alcohols) can be derivatized with acetic anhydride under basic aqueous conditions (Chattaway, 1931; Welsh, 1955; Goldstein et al., 1959; Hagopian et al., 1961; Brooks and Horning, 1964; Sharman, 1969), and the resultant acetylated derivatives are more lipophilic than the parent amines or phenols, thus facilitating extraction of the neurochemicals or drugs of interest into organic solvents. Under anhydrous conditions, amines, phenols, and alcohols can be derivatized with acetic anhydride. It is also important that primary amines, once derivatized with acetic anhydride, can often be further derivatized with another acylating agent; such compounds have proven to have high sensitivity in GC and GC-MS analytical systems. Several examples demonstrating the wide application of acetylation with acetic anhydride to analysis of biogenic amines and antidepressant and antipsychotic drugs and their metabolites are now provided in this review.
Analysis of Endogenous Amines in Tissues and Body Fluids

2-Phenylethylamine (PEA)

This arylalkylamine has been implicated in the etiology and/or pharmacotherapy of a number of neurological and psychiatric disorders (Paterson et al., 1990; Baker et al., 1993). It is present in very low concentrations in brains of drug-free laboratory animals and humans, a factor that has created considerable difficulties in quantitative studies of this amine. However, extractive acetylation with acetic anhydride followed by derivatization with a second reagent under anhydrous conditions has proven to be very useful in overcoming this problem. Martin and Baker (1977) derivatized PEA in basified aqueous brain extracts with acetic anhydride, extracted the resultant N-acetylPEA into an organic solvent, then derivatized further with pentafluoropropionic anhydride (PFPA) under anhydrous conditions. The final derivative, which was shown to be N-acetyl-N-pentafluoropropionylPEA, proved to be very sensitive to analysis by GC with electron-capture detection (GC-ECD). This analytical procedure revealed levels of PEA in brain similar to those previously demonstrated with mass spectrometric and radioenzymatic procedures. Hampson et al. (1984a) utilized aqueous acetylation followed by derivatization with pentafluorobenzoyl chloride (PFBC) under anhydrous conditions to measure PEA in brain and urine samples; final analysis was by GC-ECD. The combination of acetylation with acetic anhydride and subsequent reaction with PFBC has also been used to measure PEA in brain tissue and plasma employing GC-MS with negative chemical ionization (NCI) (Durden, 1991; Durden et al., 1991b). The structures of N-acetyl-N-pentafluoropropionylPEA and N-acetyl-N-pentafluorobenzoylPEA are shown in Figure 1.

5-Hydroxytryptamine (5-HT) and Tryptamine (T)

These two indolealkylamines are both formed metabolically from the amino acid tryptophan, and both have been implicated in the etiology of depression and the actions of antidepressant drugs (Grahame-Smith, 1992; Mousseau, 1993). In a study of levels of T in rat brain, Warsh et al. (1977) employed extractive acetylation of basified brain extracts by adding a solution of acetic anhydride in an organic solvent and shaking the two phases; after taking the solution containing N-acetylT to dryness, the residue was reacted with pentafluoropropionic anhydride (PFPA) in the presence of ethyl acetate. The resultant spirocyclic derivatives (Blau et al., 1977) have excellent properties for GC-ECD analysis (see Figure 2 for structures), and a similar procedure has also been utilized by Martin et al. (1988) and Durden and Boulton (1988) to analyze T in rat brain regions, using GC-MS-NCI for analysis.

Phenolic Phenylethylamines

Because of their amphoteric nature, phenolic amines are often difficult to extract efficiently from aqueous solutions, but they can be extracted in high yield by first acetylating them with acetic anhydride in basified aqueous medium and extracting the acetylated products into organic solvents. Under these conditions, primary and secondary amino groups are converted to amides and phenolic hydroxy groups are converted to O-acetates (Welsh, 1955; Goldstein et al., 1959; Hagopian et al., 1961; Laverty and Sharman, 1965; Sharman, 1969; LeGatt et al., 1981a,b; Wood and Rao, 1990). The resultant lipophilic neutral products are readily extracted by organic solvents.

Figure 1. Structures of N-acetyl-N-pentafluoropropionyl-2-phenylethylamine (I) and N-acetyl-N-pentafluorobenzoyl-2-phenylethylamine (II).
A procedure developed in our laboratories (Coutts et al., 1981; LeGatt et al., 1981a,b) allows for simultaneous assay of PEA, T and the phenolic amines m- and p-tyramine (m- and p-TA), 3-methoxytyramine (3-MT), normetanephrine (NME) and 5-HT in brain tissue and urine. Its application to brain tissue is summarized in the flow diagram in Figure 3. In this procedure, the organic extract of acetylated amines is treated with a small quantity of 10 N NH₄OH to hydrolyze the acetylated phenolic groups of m- and p-TA, 3-MT, and NME (LeGatt et al., 1981b; Coutts et al., 1980, 1981). The NH₄OH is then neutralized with HCl, the samples are vortexed again, and the ethyl acetate phase is retained. The selective hydrolysis step can be omitted and the N-acetylated, phenolic O-acetylated derivatives reacted directly with the perfluoroacylating reagent to produce N-acetyl, N-TFA, and O-acetyl derivatives (in the case of NME, the alcohol group is also derivatized with TFAA). However, it was found in our GC procedure that an interfering substance co-chromatographed with p-TA under these conditions. In addition, the hydrolysis step frees the phenolic group for subsequent reaction with a perfluoroacylating or trimethylsilylating reagent under anhydrous conditions derivatizes the alcohol hydroxyl groups and results in increased sensitivity to GC-ECD compared with the derivative formed when hydrolysis is omitted (see Figure 4 for comparison of the two derivatives). Detailed mass spectral analysis of the structures of the derivatives of the phenolic amines are presented in the paper by Coutts et al. (1984). PEA forms the same derivative when carried through either procedure, but we have found that by incorporating the hydrolysis step, a cleaner blank for PEA is provided. Unfortunately, T and 5-HT cannot be analyzed concomitantly using the hydrolysis technique since they appear to deteriorate to some extent under these conditions; hence a separate aliquot must be taken for analysis of T and 5-HT prior to hydrolysis.

Durden et al. (1991a) used aqueous acetylation with acetic anhydride followed by reaction with PFBC under anhydrous conditions to prepare derivatives of m- and p-TA for subsequent analysis by GC-MS-NCl; the technique was applied to analysis of these amines in rat brain regions.

Acetylation of phenolic substituents with acetic anhydride under basic aqueous conditions followed by derivatization of the alcohol groups under anhydrous conditions has also been employed for analysis of (3-methoxy-4-hydroxyphenyl)ethylene glycol (MHPG) and (3,4-dihydroxyphenyl)ethylene glycol (DHPG), metabolites of noradrenaline, in urine and tissue samples (Sharman, 1969; Kahane et al., 1976; Takahashi et al., 1977; Warsh et al., 1981; Li et al., 1985; Baker et al., 1986; Wood and Rao, 1990). Acetylation of the phenolic hydroxyl groups of MHPG and DHPG produces derivatives that are readily soluble in an organic solvent. Subsequent reaction with perfluoroacylating or trimethylsilylating reagents under anhydrous conditions derivatizes the alcohol hydroxyl groups and results in the formation of molecules suitable for analysis by GC or GC-MS (see Figure 5).

**Analysis of Antidepressant and Antipsychotic Drugs and Some of Their Metabolites in Tissues and Body Fluids**

**Antidepressants**

Gas chromatography with nitrogen-phosphorus detection (GC-NPD) has proven to be a popular technique for analysis of tricyclic and other antidepressants and their metabolites extracted from plasma or serum samples (Cooper, 1988 for review). The procedure commonly utilized involves basification of the samples, extraction of the drugs into an organic solvent, back-extraction into acid, basification of the acid phase and extraction with an organic solvent, and injection of a portion of the extract onto the GC. However, some workers have found it useful to include an acetylation step with acetic anhydride in the assay to provide improved separation of the parent drugs and their metabolites (Jorgensen, 1975) and avoid adsorption of primary and secondary amines onto GC columns (Gupta et al., 1977). The acetylation assay procedure permits simultaneous analysis of tertiary amine antidepressants (e.g., imipramine), which are not derivatized, and their corresponding secondary amine metabolites (e.g., desipramine) [Gupta et al., 1983]. Furthermore, secondary amines, such as maprotiline (Gupta et al., 1977; Drebit et al., 1988) and fluoxetine (Goodnough...
can be analyzed simultaneously with their N-desmethylated metabolites. The procedure has also been applied to the analysis of the secondary amine viloxazine (Fazio et al., 1984). Reaction with acetic anhydride followed by GC-MS analysis has also been employed for simultaneous analysis of doxepin and N-desmethyldoxepin (Frigerio et al., 1977) and for separation of the tricyclic antidepressant trimipramine from a number of its desmethylated and hydroxylated metabolites (Maurer, 1989). Most assays (e.g., Weder and Bickel, 1968; Belvedere et al., 1975; Jorgenson, 1975; Gupta et al., 1976, 1977; Sioufi and Richard, 1980; Maurer, 1989) employ reaction with acetic anhydride under anhydrous conditions after previous extraction of the drugs, but we have found that the acetylation step can be conducted under aqueous conditions early in the procedure (Drebit et al., 1988; Goodnough and Baker, 1994; Goodnough et al., 1993). In an assay for maprotiline and N-desmethyl-maprotiline (Drebit et al., 1988), we basified the plasma and urine samples using NaHCO₃ and acetylated directly. In subsequent studies with other antidepressants, we have found that initial basification of the samples with Na₂CO₃ followed by extraction with an organic solvent such as ethyl acetate may produce cleaner samples; the organic extract can be taken to dryness and the residue taken up in water and basified for acetylation as described by Drebit et al. (1988). This modified procedure with an initial extraction is useful if only the parent drug and its demethylated metabolite are of interest, but the recovery of phenolic hydroxylated metabolites will be reduced.

Although the procedure of acetylation under aqueous conditions has not yet been employed extensively for the analysis of hydroxylated metabolites of the tricyclic antidepressants, we have found that direct aqueous acetylation under basic conditions (produced by the addition of solid NaHCO₃) of plasma and urine samples from patients treated with imipramine provides for simultaneous analysis of imipramine (underivatized), desipramine (N-acetylated), 2-hydroxyimipramine (O-acetylated), and 2-hydroxydesipramine (N,O-diacetylated).
Figure 4. Derivatives formed from p-tyramine by reacting with acetic anhydride (AA) under aqueous conditions followed by trifluoroacetic anhydride (TFAA) under anhydrous conditions. In one scheme (a), hydrolysis of the acetylated phenol is included, while in the other (b) it is omitted (modified from Baker et al., 1981).

(R Drebit et al., unpublished). As described later in this review, this procedure has also been utilized in analysis of these drugs and metabolites in in vitro experiments with CYP2D6 isozyme expressed in a human cell line (Coutts et al., 1993; Su et al., 1993). The structures of the derivatives are shown in Figure 6.

Tranylcypromine (TCP) is a monoamine oxidase (MAO)-inhibiting antidepressant that is similar structurally to amphetamine. This drug is a primary amine, and aqueous acetylation with acetic anhydride followed by reaction with PFPA (Calverley et al., 1981) or PFBC (Hampson et al., 1984b) under anhydrous conditions have been employed for its analysis in extracts of brain tissues; GC-ECD was used for analysis, but the methods should also be applicable to GC-MS analysis.

Antipsychotics

Acetylation of organic extracts of body fluids with acetic anhydride under anhydrous conditions to separate N-desmethylated and hydroxylated metabolites of phenothiazines from the parent drugs prior to analysis using GC has been employed by several researchers (e.g., Driscoll et al., 1964; Johnson et al., 1965; Rose et al., 1964). Hansen and Larsen (1974) utilized reaction with acetic anhydride under anhydrous conditions to derivate perphenazine (which contains an alcohol hydroxy group) after extraction from human whole blood and prior to GC analysis. Javaid and coworkers have reported that reaction with acetic anhydride under anhydrous conditions is a useful workup procedure for subsequent GC-NPD analysis of a number of alcohol-containing antipsychotics (Javaid et al., 1980a,b, 1981; Chang et al., 1985). Reaction with acetic anhydride under anhydrous conditions after drug extraction from plasma has also been used for derivatization of pimozide; analysis was subsequently conducted using GC-MS in the chemical ionization mode (Reed, 1988). Presumably N-desmethylated and phenolic hydroxy metabolites of antipsychotic drugs could be readily acetylated under aqueous conditions, although this procedure has not been reported, to our knowledge, for extraction and analysis of these metabolites.

Use of Acetic Anhydride to Investigate Detailed Metabolism of Psychotropic Drugs

As mentioned previously in this review, acetylation under aqueous conditions provides for simultaneous
analysis of imipramine, desipramine, 2-hydroxyimipramine, and 2-hydroxydesipramine. This procedure, combined with GC-NPD and GC-MS has been utilized to study metabolism of imipramine in vitro by CYP2D6 expressed in human cell lines (Coutts et al., 1993; Su et al., 1993). The fact that acetic anhydride derivatizes phenolic hydroxyl groups under both aqueous and anhydrous conditions but derivatizes alcohol hydroxyl groups only under anhydrous conditions has value in structural elucidation. By making use of this knowledge and comparing mass spectral fragmentation patterns of antidepressants and potential metabolites before and after derivatization with acetic anhydride, under both aqueous and anhydrous conditions, identification of the location of hydroxyl groups in the metabolites of drugs such as the tricyclic antidepressants can be greatly facilitated. This strategy has been employed to examine metabolites of such drugs as imipramine, trimipramine, and iprindole in body fluid samples from rats and humans (Coutts et al., 1990, 1991a,b; Hussain et al., 1991), and investigate CYP2D6-catalyzed formation of metabolites of amphetamines and antidepressants in vitro (RT Coutts et al., unpublished data).

Several antidepressants and antipsychotics are extensively metabolized, and recent reviews have stressed the importance of considering metabolites in the overall therapeutic actions and side effects of psychotropic drugs (Midha et al., 1987; Potter and Manji, 1990; Young, 1991; Caccia and Garattini, 1992). The acetylation strategy should prove to be a very useful one for future studies on metabolism of such drugs.

In summary, the use of acetic anhydride, under both aqueous and anhydrous situations, as a derivatizing reagent, has had important implications for neurochemistry, pharmacology, and biological psychiatry. Acetylation under aqueous conditions has proved to be a rapid and effective means of improving the extractability of amine- and phenol-containing neurochemicals and drugs from biological fluids and aqueous supernatants of tissue homogenates; in doing so, it also offers an additional rapid clean-up stage in assay procedures. In some instances (e.g., with some GC-NPD and GC-MS assays), the acetylated derivatives can be used directly for analysis. However, after acetylation, primary amines can be derivatized further with perfluoroacylating reagents, providing derivatives that often have excellent properties for subsequent analysis by GC-ECD or GC-MS. In addition, esters formed by acetylation of phenols can subsequently be hydrolyzed selectively to free the phenolic function for reaction with a perfluoroacylating reagent in a later stage of an assay procedure. Reaction with acetic anhydride (under aqueous and anhydrous conditions) is also proving to be a useful technique for investigating detailed drug metabolism, and it is anticipated that this application will be important in future research.

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