A sensitive and specific method for the detection of phenylbutazone in biological samples

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In the course of studies on the absorption of phenylbutazone from topical applications, a more specific and sensitive method of detecting the drug was required. The commonly used ultraviolet spectrophotometric method of Burns, Rose & others (1953) lacks specificity since many other acidic drugs including the barbiturates absorb strongly at 265 μm and the limit of detection is about 5 μg ml^{-1} plasma. Phenylbutazone is one of the most commonly used doping agents in animals and the ultraviolet method is particularly unsuitable for use in the analysis of equine samples since these commonly contain substances (possibly coumarins) which absorb strongly in this region (Bogan, 1972). Phenylbutazone is often used to study the kinetics of liver microsomal drug metabolizing enzymes and here also a method which measures phenylbutazone specifically without interference from either its metabolites or its degradation products is desirable (Bellward, Morgan & others, 1972).

In the method described below, phenylbutazone is converted to azobenzene using permanganate oxidation in a method similar to that of Wallace (1968) and the azobenzene measured using gas chromatography.

Methods. To plasma (3 ml) add hydrochloric acid (1 ml N) and hexane (20 ml). Shake the mixture for 5 min. Remove 15 ml of the upper hexane layer and extract with sodium hydroxide (5 ml 5 N). Discard the upper hexane layer and add potassium permanganate (2 ml 2% w/v) in water to the sodium hydroxide layer. Heat for 5 min in a boiling water bath. Cool the mixture rapidly and shake for 5 min with 10 ml hexane. Azobenzene in the final hexane layer is determined by chromatography. The concentration of phenylbutazone in the original sample is calculated from known aqueous standards of phenylbutazone taken through the procedure.

Gas chromatography conditions: Column: 10% DC-200 on Gas-Chrom Q (80–100 mesh); 0·3 m × 4 mm i.d.; temperature: column 180°; detector 200°; injection 150°; carrier gas flow: 60 ml min^{-1} nitrogen; detector: flame ionization. Under these conditions, azobenzene has a retention time of 3·2 min.

Wistar rats weighing between 100 and 120 g were anaesthetized with pentobarbitone. Hair was removed from an area on the side of the animals by shaving. 0·2 g of an ointment containing 5% phenylbutazone in a base containing 20% dimethylsulphoxide (DMSO) was applied to a 25 × 25 mm area on the shaved side. At intervals after application of the ointment, the rats were decapitated and blood collected for phenylbutazone assay. Because of the limited amounts of plasma available from each rat, the assay was carried out using 1·5 ml plasma and the amounts of reagents were reduced accordingly.

This method is specific for phenylbutazone. Under these conditions only phenylbutazone of the commonly used acidic drugs including the phenylbutazone metabolite, oxyphenylbutazone, forms azobenzene (Wallace, 1968). Further, the extraction technique from hexane into alkaline solution before oxidation and the extraction of the oxidation product azobenzene from the reaction mixture without adjustment of pH will eliminate other interferences from 'normal' constituents.

Under the conditions outlined, using samples of plasma with phenylbutazone added to give concentrations of 1, 2, 5, 10, 20 and 100 μg ml^{-1}, the recoveries of azobenzene on duplicate samples were between 65 and 78% of the theoretical yield. These losses were not due to incomplete extraction but to incomplete conversion of phenylbutazone to azobenzene. When the length of heating time was examined it was found that 5 min was optimal and thereafter yields of azobenzene declined. Under the conditions outlined there was a linear relation between phenylbutazone in samples and azobenzene found for phenylbutazone concentrations between 1 and 100 μg ml^{-1}.

Azobenzene gives a sharp symmetrical peak on g.c. and its concentrations could be calculated readily using peak heights. Phenylbutazone itself is difficult to chromatograph giving 'tailed' peaks and the limit of detection of phenylbutazone is higher than that of azobenzene.

The limit of detection of the ultraviolet method of Burns & others (1953) for phenylbutazone is about 5 μg ml^{-1} plasma with blank plasma values of 3–5 μg

Table 1. *Phenylbutazone in rat plasma after topical application.*

Time after application (h)	Phenylbutazone concentration ± s.e.m. (μg ml^{-1} plasma)*
0	0
0·5	3·4 ± 1·0
1·0	5·2 ± 0·7
1·5	6·6 ± 1·5
2·0	10·9 ± 2·6
3·0	12·9 ± 3·2

* Groups of six rats were used at each time.

ml^{-1}. By concentrating the final hexane extract it was possible to readily detect 0·1 $\mu g\ ml^{-1}$ in plasma.

Phenylbutazone was well absorbed after topical application in an ointment containing DMSO (Table 1). In all plasma samples, the drug could be measured readily. The half-life in Wistar rats is 6 h (Burns & others, 1953). Since the concentration of phenylbutazone in plasma from the rats increased during the 3 h of the experiment this would indicate that absorption of the drug occurred throughout the 3 h. From these results the possibility of using ointments containing phenylbutazone for the treatment of localized musculoskeletal lesions deserves further investigation.

October 15, 1976

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Identification of hexadecanamide in cannabis resin

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More than three hundred non-cannabinoid compounds have been identified in various parts of the cannabis plant or in cannabis resin, essential oil or smoke. These compounds include alkaloids, alkanes, amines, amino acids, carbohydrates, carboxylic acids, cyclitols, phenols, sterols and terpenes (Slatkin, Doorenbos & others, 1971; Skelton & Witschi, 1974; Burstein, Varanelli & Slade, 1975; El-Feraly & Turner, 1975; Hendriks, Malingré & others, 1975; Jones & Foote, 1975; Lotter, Abraham & others, 1975; Lee, Novotny & Bartle, 1976; Novotny, Lee & others, 1976a,b; Turner, Hsu & others, 1976). Earlier work has been reviewed by Mechoulam, McCallum & Burstein (1976).

We wish to report the identification of hexadecanamide in a sample of cannabis resin thought to be of Pakistan origin. The parent acid, palmitic acid, has previously been identified in cannabis smoke (Fentiman, Foltz & Kinzer, 1973; Jones & Foote, 1975). One other amide, *N*-(*p*-hydroxy-β-phenylethyl)-*p*-hydroxy-*trans*-cinnamamide, and its parent acid, *p*-hydroxycinnamic acid (*p*-coumaric acid; melilotic acid), have been found in cannabis root and leaves respectively (Bate-Smith, 1962; Slatkin & others, 1971).

Hexadecanamide was present in the resin in a trace quantity. Since it has a low ultraviolet absorbance, it was not observed on high-pressure liquid chromatography (h.p.l.c.) of a resin extract (even a moderately concentrated solution of the pure compound could not be detected). It was first noticed on gas-liquid chromatographic (g.c.) examination of a concentrated fraction prepared from a methanol–chloroform (9:1) extract of the resin (0·5 g resin in 1 ml solvent). The extract was fractionated by h.p.l.c. using methods previously described (Smith, 1975). When the fraction from the cannabidiol-cannabinol region of the chromatogram was examined by g.c. on OV-17 at 240°, a peak with a retention of 0·29 relative to Δ^1-tetrahydrocannabinol was detected. This was further examined by gas chromatography–mass spectrometry (g.c.–m.s.). The electron impact spectrum, obtained under conditions described previously (Smith, 1975), had a base peak of 59 and a second most abundant peak of 72, features characteristic of an amide. The molecular weight was found to be 255 by chemical ionization mass spectrometry using a source temperature of 160°, an electron energy of 100 eV, an emission current of 200 μA, an accelerating voltage of 4 kV and isobutane as the reactant gas. The electron impact and chemical ionization spectra corresponded to literature data for hexadecanamide (*Eight Peak Index of Mass Spectra*, 1974) and the identity of the compound was confirmed by synthesizing a sample of hexadecanamide and examining it by g.c.–m.s. The g.c. retention time and the mass spectral data matched those of the compound found in cannabis resin.

September 14, 1976

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