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Distribution of azapropazone and its principal 8-hydroxy-metabolite in plasma, urine and gastrointestinal mucosa determined by HPLC

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Methods are described for the HPLC determination of azapropazone and its 8-hydroxyl metabolite in plasma, urine and gastrointestinal mucosae after purification of samples on reverse-phase mini columns. Plasma levels of the drug in gouty patients receiving 900–2400 mg daily were relatively constant after 5 days, though overall the values were higher than after a single dose. Gastric absorption of azapropazone in rats is relatively rapid suggesting that the low gastric irritancy is probably due to its weak inhibitory effects on prostaglandin and mucus synthesis rather than slow gastric absorption.

Azaproprazone is one of the few non-steroidal anti-inflammatory (NSAI) drugs having a relatively low incidence of gastrointestinal side-effects (Rainsford 1982a, b). This may be related to its slow absorption into the gastric mucosa, weak inhibitory effects upon the synthesis of prostaglandins (Brune et al 1981) and gastric mucus (Rainsford 1978), and its capacity to stabilize lysosomal membranes (Lewis et al 1971).

Upon reviewing analytical methods for the assay of azapropazone and its principal 8-hydroxy-metabolite (8-HA)* in various tissues, it appeared that all had disadvantages. Thus, the uv and colourimetric procedures (Schatz et al 1970; Farrier 1974; Jones 1976) applied to plasma fail to discriminate between azapropazone and 8-HA. While the combined TLC-densitometric procedure of Geissler et al (1977) although discriminating between these two drugs, lacks the sensitivity of HPLC methods. Moreover, three highly absorbant unknown components are evident in the TLC-densitometric trace including one which interferes with the absorbance of 8-HA (Geissler et al 1977). Kline et al (1983) described an HPLC method but there was a strongly absorbant peak at the solvent front indicating the presence of large quantities of impurities in these non-purified methanolic extracts of plasma which could seriously affect column performance and indeed this was found in pilot studies by the present author. Furthermore, those authors (i) failed to establish if the known degradation products (notably Mi82—see later) which readily occur upon storage of aqueous solutions of azapropazone, and which could interfere with the assay of this drug and 8-HA, (ii) published traces showing peak widths which were rather broad and (iii) did not report assays of clinical samples, so it was difficult to assess their procedure.

Thus methods, reported here, were devised to purify the plasma and tissue samples before HPLC, and to assay more satisfactorily by HPLC all the known and presumed metabolites of azapropazone in plasma/tissue samples at their wavelength maxima free from possible interference from degradation products. They have been applied to the determination of azapropazone and 8-HA in human plasma and urine of normal individuals following a single oral dose of 600 mg of the drug, plasma from patients with gout receiving repeated doses of azapropazone, and plasma and gastrointestinal mucosae of rats given oral azapropazone, so enabling an assessment of the role of drug absorption and distribution in the therapeutic and side-effects of this drug.

Methods

HPLC assay. HPLC was performed using a 25 cm long × 4·5 mm i.d. Lichrosorb RP-18 pre-packed reverse phase column (Jones Chromatography Ltd, Llanbradach, Glamorgan, UK) coupled to either Gilson Model 302 (Anachem Ltd, Luton, UK) or ARL constant flow HPLC (Applied Chromatography Systems, Luton, UK) pumps set at a flow rate of 1 ml min^{-1}. The solvent system was 80% HPLC grade methanol (Blackford Wells Ltd, Coalville, Leics, UK) in triple-glass distilled H_2O adjusted to pH 3·0 with *o*-phosphoric acid (Merck), filtered through 0·2 μm PTFE filters (Whatman Ltd, Maidstone, UK) and degassed before use. Samples were injected in a 20 μl sample loop (Rheodyne In., Cotati, CA, USA). For assay of azapropazone and 8-HA, the eluate was monitored by uv detection (Waters Lambda Max Model 480; Waters Assoc., Milford, Mass., USA) at their maximal absorption (in column solvent) (250 nm).

Possible degradation products or manufacturing impurities were also monitored at this wavelength and/or their respective absorption maxima (in column solvent, see below) to discriminate them from azapropazone and 8-HA. All the known degradation products exhibit broad absorption in the 250 nm region where azapropazone and 8-HA absorb. To enable identification of these impurities/degradation products all the known products from the photo-decomposition in aqueous media, or the degradation in strong acids or alkaline (>pH 8) mixtures were prepared as described

* This metabolite has been known previously as 6-hydroxy-azapropazone (Mixich 1968) but re-assignment of the numbering of the parent drug according to IUPAC rules (where the 2-carbonyl groups are assigned numbers 1 and 3 respectively and the dimethylamino group is located at the 5-position on the benzotriazine nucleus), leads to the hydroxyl being located at the 8-position.

(Mixich 1968). Additionally, pure synthetic samples of the impurities/decomposition products, Mi82 (3-dimethylamino-7-methyl-1,2,4-benzotriazine, λmax = 253 nm), Mi307 (2-(α-carboxy-valeryl)-3-dimethylamino-7-methyl-1,2-dihydro-1,2,4-benzotriazine, λmax = 227 nm), Mi306 (3-dimethylamino-7-methyl-2-valeryl-1,2-dihydro-1,2,4-benzotriazine, λmax = 226 nm) and Mi283 (9-methyl-2-propyl-1-oxo-1H,2H-pyrazolo[1,2-α]-1,2,4-benzotriazine λmax = 225 nm) were generously provided by Dr M. Hewlins, University College of South Wales, Cardiff, UK. Azapropazone and 8-hydroxyazapropazone were provided by Mr F. S. Walker, A. H. Robins Co. Ltd, Horsham, UK. 8-Hydroxyazapropazone was purified before used by TLC described below.

These drugs/standards were freshly dissolved in either methanol or chloroform–methanol (80 : 20) in containers protected from visible light by a covering of aluminium foil and when necessary stored briefly (<24 h) at −20 °C following N_2 gas flushing. These procedures were necessary since exposure to fluorescent or direct sunlight at room temperature (20 °C) for longer than 2–3 h causes appreciable degradation of azapropazone to Mi82.

Thin layer chromatography (TLC) on Merck silica gel 60 F_{254} (0·2 mm) was used to identify the drugs/impurities in aluminium foil-wrapped TLC tanks maintained at 4 °C, with solvent systems: (1) chloroform–methanol (80 : 20 by vol.) (R_F values being: azapropazone = 0·62, 8-hydroxyazapropazone = 0·36, Mi82 = 0·89, Mi307 = 0·47, Mi306 = 0·75, Mi283 = 0·18), and (2) light-petroleum (40–60 °C)–propionic acid–ethanol (10 : 1 : 5 by vol) (R_F values being azapropazone = 0·20, 8-hydroxyazapropazone = 0·12 and Mi82 = 0·86).

A sample of [1,3-^{14}C]azapropazone was provided by Mr F. S. Walker (A. H. Robins Co. Ltd, Horsham, UK) and purified by TLC on chloroform–methanol (80 : 20) immediately before use. The purity of this preparation was confirmed by HPLC (as above) with the radioactivity being continuously monitored by an Isoflo Radioactivity Monitor (Nuclear Enterprises Ltd, Edinburgh, UK).

Plasma and urine levels in man. Plasma and urine levels of azapropazone and 8-HA in three healthy volunteers (who had not received any medication for 7 days previously) were determined after they had ingested 600 mg azapropazone in 50 ml H_2O after breakfast. Blood samples were taken from the antecubital vein at 1, 2, 3, 4, 8 and 23 h following the drug, placed into heparinized vials centrifuged and plasma fractions assayed immediately to minimize degradation. Urine samples were stored at 4 °C in the dark for up to 48 h after the drug dose. This did not affect the stability of the drug or its metabolites. The experiments were subsequently repeated three times on two of the volunteers.

Plasma samples from 21 patients taking repeated oral doses of 900–2400 mg of the drug for 2–5 days for the treatment of gout were obtained in the early morning. They were immediately frozen and subsequently transported packed in dry ice. The samples were generously provided by Dr Clare Higgens, Charing Cross Hospital, London, from patients under her care. Details of these patients including plasma urate levels in response to treatment can be found elsewhere (Higgens & Scott 1984). Samples (67) were taken before initiating treatment with azapropazone, to determine if other drugs received interfered with assay of azapropazone and 8-HA. All but two of these patients were not receiving any other acidic NSAI drug, some were receiving antibiotics, azathioprine, steroids or cimetidine.

Sample work-up. Purification of the samples was by chromatography on Waters Sep Pak C-18 cartridges (Waters Associates, Milford, Mass., USA) which were prewashed with 20 ml HPLC grade methanol followed by 1 mM HCl. Aliquots (0·2 ml) of acidified (pH 3 with 0·2 M citric acid) plasma or urine samples, to which was added 100 μg indomethacin as an internal standard, were placed on the column and subsequently were eluted with 10 ml H_2O acidified to pH 3 with *o*-phosphoric acid or 1 M HCl (to remove polar materials, proteins etc) followed by 4 ml methanol to obtain azapropazone and its metabolites. The recoveries of azapropazone and 8-HA averaged 92% (n = 22) and 86% (n = 9) respectively.

Distribution in rat gastrointestinal mucosa and plasma. The distribution of azapropazone in the gastrointestinal tract of 31 male Sprague-Dawley rats was studied following a 24 h fast and subsequent oral administration of 100 mg kg^{-1} with or without added [1,3-^{14}C]-azapropazone (10 μCi kg^{-1}). Sections of the fundic and upper 12 cm of intestinal tract were removed 10, 60 and 360 min after drug administration and the mucosa scraped off with a glass microscopic slide. The mucosal scrapings were homogenized in 0·2 M citrate, pH 3·0 (1 g/10 ml) and divided for scintillation counting in 40% Lumax (Lumac B.V. Schaesberg, Holland) in toluene, or extraction twice with 5 volumes of chloroform–methanol (80 : 20 by vol). The organic phase was evaporated to dryness over N_2 gas at room temperature and redissolved in either 0·5 ml ethyl acetate for TLC or methanol for HPLC following purification on Sep-Pak C-18 cartridges (as above).

Results and discussion

Azapropazone and its 8-hydroxy metabolite (8-HA) were clearly separable by HPLC on the Lichrosorb RP-18 column both from each other and the principle degradation products and/or commercial impurities (Fig. 1). The drug and 8-HA also separated well from indomethacin, so that by having somewhat similar physicochemical properties (pK_a, log P) with azapropazone, indomethacin proved a satisfactory internal

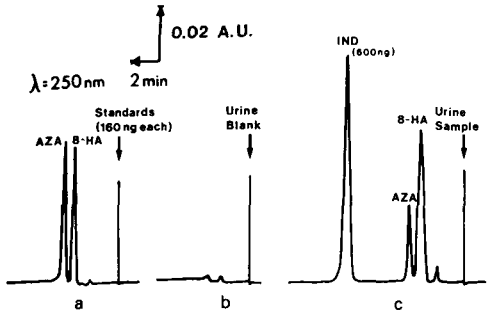

FIG. 1. HPLC recordings of (a) analytical standards of azapropazone (AZA) and 8-HA (8-hydroxyazapropazone) (left), (b) urine blank (middle) and (c) a urine sample containing azapropazone and 8-hydroxyazapropazone and indomethacin as an internal standard (right). The samples were analysed on a Lichrosorb RP-18 column eluted with 80% methanol in H_2O adjusted to pH 3·0 with *o*-phosphoric acid. Typical regression curves calculated for the lowest limit absorbance unit full scale range (AUFS = 0·02) were for: Azapropazone: concn = $(1{\cdot}266 \times 10^3) \times$ absorbance (r = 0·998, n = 12); 8-HA, concn = $(2{\cdot}78 \times 10^3) \times$ absorbance (r = 0·991, n = 9). Limits for detection for both drugs were 0·7–1·5 ng.

standard. The desmethyl metabolite of indomethacin had a retention time (T_R) of 4·7 min so that azapropazone and 8-HA could be assayed in the presence of indomethacin and that metabolite.

Of the reverse- and straight-phase HPLC columns tried, only Lichrosorb RP-18 proved satisfactory for separating azapropazone from 8-HA. Kline et al (1983) showed that 8-HA could be separated from azapropazone on a reverse-phase column (μBondapak) eluted with much lower concentrations of methanol (45%) than used herein. However, in the present studies it was found that those concentrations of methanol led to longer retention times with extensive broadening of the peaks leading to poor resolution of azapropazone and 8-HA even with Sep Pak-C18-purified plasma samples.

In Fig. 2 it can be seen that peak plasma values of 40 μg ml^{-1} azapropazone in man were evident 3 h after ingestion of a single 600 mg dose and were similar to those obtained with a 600 mg dose by Leach (1976) and Jones (1976) as measured by spectrophotometry, and by Geissler et al (1977) with a TLC-densitometric method. While there was no 8-HA in plasma after the single dose of drug, it was present in appreciable quantities in the urine free and as a presumptive glucuronide or sulphate conjugate (detected following incubation with β-glucuronidase/sulphatase preparation (Table 1)). 8-HA was present also in some plasma samples of those gouty patients receiving repeated doses of the drug for up to 5 days (data not shown), the amounts being 5–10% relative to azapropazone, where this was >100 μg ml^{-1}. The elimination half-time of azapropazone from plasma after a 600 mg dose is approximately 8 h, in agreement with other reports (Jones 1976; Leach 1976; Geissler et al 1977). Azapropazone, its presumptive 8-HA and

Table 1. Urinary excretion of azapropazone 8-HA and 8-HA-glucuronide in normal humans. Values are mg total of mean ± s.e.m., n = 4–5 or *2. N.D. = not detected. The 8-HA glucuronide concentrations were determined by HPLC of the 8-HA content before and after hydrolysis of 2 ml urine samples with 1000 units of β-glucuronidase/sulphatase (Sigma) for 30 or 60 min at 37 °C, with appropriate reference blanks (minus enzyme) as controls.

	Time	
Drug/metabolite	0–24 h	24–48 h
Azapropazone	229 ± 59·9	47·8 ± 18·3
8-HA	159 ± 55·8	182 ± 47
8-HA glucuronide	57·9 ± 7·9*	N.D.

8-HA-glucuronide/sulphate metabolites were principally excreted in urine over 48 h (Table 1).

Previously, 8-HA has been found in human urine to the extent of 16% of total azapropazone by combined gas-chromatography–mass spectrometry (Jones 1976). Table 1 shows that a much higher proportion of 8-HA was excreted, being approximately 55% of the total azapropazone + metabolites. Kline et al (1983) failed to observe any conjugates of 8-HA in a 12 h human urine sample after 300 mg azapropazone had been ingested, the drug in its conjugated and non-conjugated forms being determined by difference after overnight digestion of the urine with *Helix pomatia* β-glucuronidase (Sigma type G1512, with low sulphatase activity). Such prolonged incubation would have led to the loss of any 8-HA liberated because of its inherent instability in aqueous solution; indeed, these authors observed a reduction in 8-HA. Moreover, little conjugate is evident such a short time after drug intake (data not shown). The shorter incubation (1 h) protected from light, with

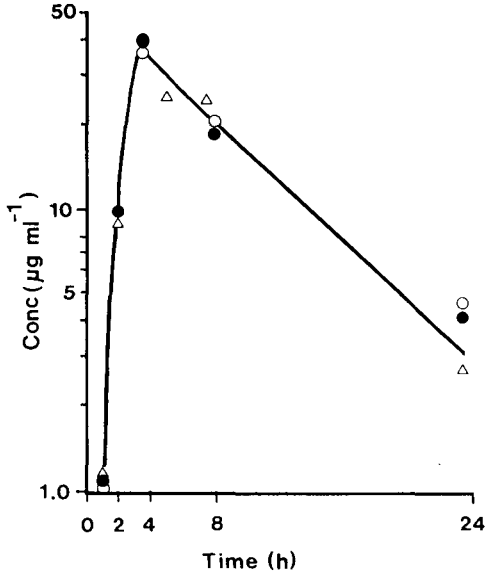

FIG. 2. Plasma concentration of azapropazone in 3 subjects after ingestion of 600 mg azapropazone suspension.

β-glucuronidase/sulphatase, compared with a control without enzyme to account for the small loss in 8-HA occurring over 1 h obviates any difficulties in detecting 8-HA conjugates. Although Mi307 has been found in human urine (Jones 1976), it was not found in these studies.

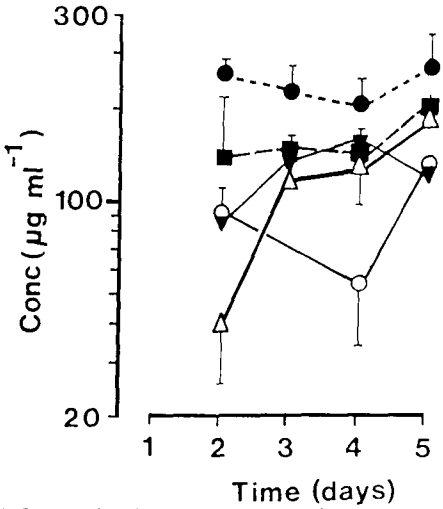

FIG. 3. Plasma levels of azapropazone (mean ± s.e.m.) in gouty patients 2–5 days after repeated daily ingestion of 600–2400 mg azapropazone. (● 2·4, ■ 1·8, △ 0·9, ▼ 1·2, ○ 0·6 g day^{-1}.) Plasma samples from the control period before ingestion of the drug were assayed by HPLC to determine if any of the drugs being taken concurrently interfered with the assay of azapropazone. Only one patient's plasma samples showed evidence of interfering components. This patient was receiving azathioprine, prednisolone, and dihydrocodeine and it appeared that dihydrocodeine was causing the interference.

The results of studies of the gastrointestinal distribution in rats after 100 mg kg^{-1} azapropazone (Fig. 4) show that the drug values peak at 291 ± 88 μg g^{-1} wet weight in 1 h in the gastric mucosa and at 100 ± 1·0 μg g^{-1} wet weight at 2 h in the intestinal mucosa (means ± s.e.m., n = 3). Recoveries of the drug from gastrointestinal tissue averaged 77–81% (i.e. determined after addition of drug in-vitro or by comparison of HPLC data with that from radio-labelled drugs). The results of the assays by HPLC (corrected for losses) were within the statistical range of those achieved from measurement of the radioactively labelled drug.

These results of absorption of azapropazone in both rat and man indicate a relatively rapid rate of absorption of this drug. That by the gastrointestinal tract of rats may be slightly less in comparison with aspirin, but not more ulcerogenic drugs (Rainsford et al 1981; Rainsford 1984). The slight reduction in absorption therefore may not be a major factor accounting for the low ulcerogenicity of azapropazone compared with other NSAI drugs, rather it appears that intrinsic biochemical properties (low PG synthesis and mucus inhibition, lysosomal stability) must be responsible.

It is apparent that, in man, formation of 8-HA is possibly time- and dose-dependent since none of this metabolite is detectable in plasma after single doses of azapropazone, yet it is evident on repeated ingestion of high doses. While higher plasma values of azapropazone are achieved in gouty patients by repeated ingestion than are achieved after a single dose in healthy volunteers (Figs 2, 3), the plasma levels of the drug appear to level, especially after high doses, over 5 days (Fig. 3).

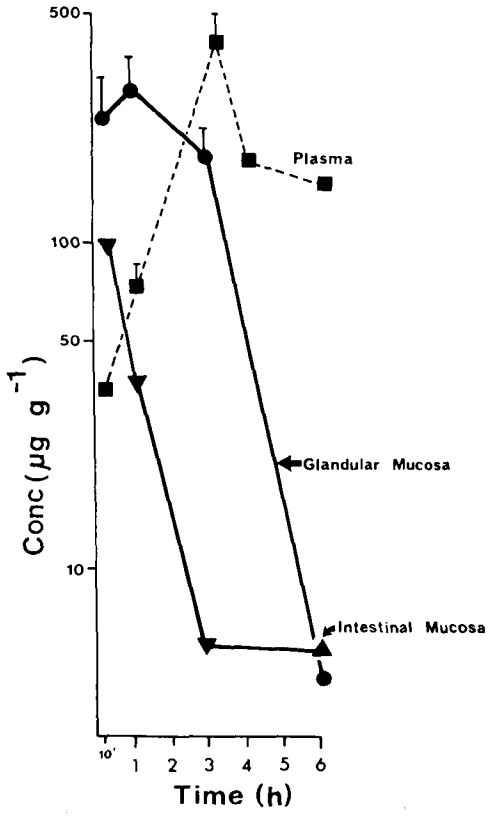

FIG. 4. Azapropazone concentrations in fundic and upper (12 cm) small intestinal mucosa, and plasma, following oral administration of 100 mg kg^{-1} azapropazone to rats. ●—● = mean ± s.e.m.

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Modulation of substance P-induced bronchoconstriction by lipoxygenase metabolites

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In anaesthetized, mechanically ventilated guinea-pigs, substance P induces a bronchoconstrictor response comprising increases in airway resistance and decreases in dynamic compliance. Eicosatetraynoic acid (ETYA, 20 mg kg^{-1} i.v.) or BW755c (20 mg kg^{-1} i.v.) potentiated the substance P-induced bronchoconstriction. Neither indomethacin (1 or 5 mg kg^{-1} i.v.) nor aspirin (20 mg kg^{-1} i.v.) significantly altered the potency of substance P on bronchomotor responses. These observations are consistent with the existence of a bronchodilator lipoxygenase metabolite(s).

Immunohistochemical identification of substance P-containing nerves in the guinea-pig respiratory tract (Nilsson et al 1977) has initiated interest in the possible role(s) of this neuropeptide in the airways. In the guinea-pig, substance P induces bronchoconstriction (Andersson & Persson 1977). Further immunohistochemical studies indicate that the substance P-containing nerves in the guinea-pig lung originate from vagal primary sensory neurons in the nodose ganglion (Terenghi et al 1983). Lundberg et al have reported that pulmonary substance P-containing sensory fibres may be stimulated antidromically to elicit bronchoconstriction and increased pulmonary vascular permeability (Lundberg & Saria 1982a, b; Lundberg et al 1983a; Saria et al 1983). In addition, exposure of the airways to irritants, such as ether or cigarette smoke, also elicits an increase in pulmonary vascular permeability (Lundberg & Saria 1983; Lundberg et al 1983b) which is prevented by a regimen of capsaicin pretreatment reported to deplete vagal substance P (Gamse et al 1981). It has recently been reported that the substance P-induced tone in the guinea-pig isolated trachea may be enhanced by metabolites of arachidonic acid (Regoli et al 1984). It is not known whether a similar effect occurs in-vivo. Since substance P may be an important regulator of bronchomotor tone, we have investigated its actions in anaesthetized guinea-pigs treated with the cyclo-oxygenase inhibitors indomethacin or aspirin, and inhibitors of both cyclo-oxygenase and lipoxygenase enzymes, eicosatetraynoic acid (ETYA) or BW755c.

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Methods

Guinea-pigs of either sex (400–650 g) were anaesthetized by an injection of a mixture of 25% w/v urethane and 0·3% w/v sodium pentobarbitone (4–6 ml kg^{-1} i.p.). The respiratory pump (Palmer) delivered 0·7 ml of air/100 g weight per stroke at a rate of 60 strokes min^{-1}. Airways resistance (R_L) and dynamic compliance (C_{dyn}) were measured according to Diamond (1972) as modified from the method of Amdur & Mead (1958). Transpulmonary pressure (TPP) was obtained by measurement of the difference in pressure between the trachea and the interior of the whole body plethysmograph using a Pye differential pressure transducer. Inspiratory and expiratory flow rates (Q) were measured across a pneumotachograph. The difference in pressure across the pneumotachograph was detected by a Statham differential pressure transducer. The preamplified (Grass Polygraph model 79D) signals representing Q and TPP were fed into a modified EAI 180 analogue hybrid computer onto which was patched a program for breath to breath on-line computation of R_L and C_{dyn} as modified from that of Mindlin (1969). The computed values of R_L and C_{dyn} were displayed and recorded on a two channel Rikadenki recorder. The carotid artery was cannulated and mean arterial blood pressure was monitored on a Grass Polygraph (Model 79D). All drugs were injected in a volume less than 0·5 ml kg^{-1} via a cannula placed in the left jugular vein