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Evaluation and comparison of colorimetric, radiometric and high performance liquid chromatographic assays for aminopyrine-*N*-demethylation by rat liver microsomes

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Aminopyrine (DMAP) is metabolized by two successive *N*-demethylations to monomethyl-4-aminoantipyrine (MMAP) and 4-aminoantipyrine (AAP). Separation and quantification of DMAP and its metabolites in microsomal incubation mixtures by h.p.l.c. showed that other reactions also occur. In the radiometric method where [Me_2-^{14}C] DMAP is used as substrate, [^{14}C]formaldehyde is formed during the *N*-demethylation. However since commercial [^{14}C]DMAP, is not completely double labelled, and both MMAP and AAP are formed, it is impossible to calculate the formaldehyde formation accurately from the specific activity of [^{14}C]DMAP. Moreover, it was shown that DMAP, AAP and particularly MMAP may all develop considerable colour intensity with the Nash reagent, which is used to determine formaldehyde. Despite this difficulty, DMAP may still be used as a model substrate in vitro, with the Nash assay being used to determine formaldehyde if low substrate concentrations and a short incubation time are used. Thus the interference of DMAP or its metabolites with the Nash assay is negligible. As the same K_m and V_{max} values were obtained from both the radiometric and the relatively precise colorimetric assay it is suggested that there is little wrong with either method, at least under our experimental conditions. The h.p.l.c. method however, underestimates formaldehyde formation, probably because metabolites other than MMAP and AAP are formed. The latter method however may be used to analyse the aminopyrine metabolism in more detail.

Aminopyrine (DMAP) is used as a substrate to assess hepatic monooxygenase activity. Its metabolism by liver microsomal monooxygenase involves a two-step oxidative *N*-demethylation (Gram et al 1968; La Du et al 1955; Pechtold 1964) initially to monomethyl-4-aminoantipyrine (MMAP) and formaldehyde and subsequently to 4-aminoantipyrine (AAP) and formaldehyde. Formaldehyde or AAP production is usually taken as a measure of the reaction velocity (e.g. Mazel 1972; Pederson & Aust 1970). That the reaction proceeds via two separate steps is often disregarded. An additional complicating factor is that alternative metabolic pathways for both of the reaction products and for DMAP may play a role (Mazel 1972).

We have critically evaluated the analytical methods commonly used to determine formaldehyde or AAP. The microsomal DMAP-*N*-demethylation reaction has also been examined by measurement of the formation of the reaction products using a high-performance liquid chromatographic (h.p.l.c.) method, the results of which have been compared with those obtained with other common assay methods for DMAP-*N*-demethylation.

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MATERIALS AND METHODS

Chemicals

Aminopyrine was purchased from Brocacef, Rotterdam, The Netherlands; 4-Aminoantipyrine from E. Merck, Amsterdam, The Netherlands; [^{14}C]dimethylaminopyrine (specific activity 9·6 mCi mmol^{-1}, radiochemical purity 97%) and [^{14}C]-formaldehyde (specific activity 4·4 mCi mmol,$^{-1}$ radiochemical purity 99%) from the Radiochemical Centre, Amersham, Great-Britain; $NADP^+$, glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase were obtained from Boehringer/Mannheim GmbH, Mannheim, Federal Republic Germany; bovine serum albumin from Poviet Producten NV, Amsterdam, The Netherlands; dioxane scintillation fluid from Packard-Becker BV, Groningen, The Netherlands; monomethyl-amino-antipyrine was a gift from Hoechst Pharma, Amsterdam, The Netherlands; 4-formylamino-

antipyrine (FAA) was a gift from Prof. S. Iguchi, Fukuoka, Japan. All other chemicals and solvents were of analytical grade purity.

Preparation of microsomes

Liver microscomes from at least six rats, prepared as described by Bast & Noordhoek (1980b), were pooled and stored at −70 °C after quick-freezing in liquid nitrogen.

Enzyme assays

Microsomes (2 mg protein ml^{-1} incubate) were incubated at 37 °C, with shaking, air being freely admitted. The incubation mixture contained mM $NADP^+$ 0·5, $MgCl_2$ 4·2, glucose-6-phosphate 4·2, 0·3 I.U. ml^{-1} incubate, glucose-6-phosphate dehydrogenase and substrate. Semicarbazide was omitted because formaldehyde remains stable in microsomal incubates (Savenije-Chapel & Noordhoek 1980).

Colorimetric assays

The incubation was carried out in a total volume of 3 ml and stopped after 5 min (unless otherwise stated) by addition of 0·5 ml $ZnSO_4$ (40% w/v) and 1 ml $Ba(OH)_2$ (saturated at 37 °C). After mixing and centrifugation, 2 ml of the supernatant was mixed with 1 ml Nash reagent at double strength (Nash 1953) and incubated at 60 °C for 30 min. The amount of formaldehyde formed was estimated by determining the absorbance at 415 nm relative to that at 500 nm and subtracting the amount of Nash-positive material formed in the appropriate blank, which contained all additions except the substrate and the metabolites.

Radiometric assay

The assay was carried out in a total volume of 0·5 ml, as described by Poland & Nebert (1973) with some minor modifications. The reaction was terminated after 5 min incubation by adding 0·5 ml semicarbazide (20 mM), 0·5 ml 0·2 M NaOH and 4 ml cold $CHCl_3$. After mixing and centrifugation, 1·2 ml and subsequently 0·8 ml of the aqueous layer were again extracted with 4 ml $CHCl_3$. A 0·5 ml sample of the aqueous phase of the third extraction was then added to 10 ml dioxane scintillation fluid and counted in a Packard TriCarb scintillation counter model 3255.

H.p.l.c. assay

The incubations were described under colorimetric assay. They stopped with 0·1 M HCl—bringing the mixture to pH 6·0 (Goromaru et al 1976)—and 15 ml $CHCl_3$. The mixture was centrifuged, and 12 ml of the $CHCl_3$ layer was evaporated under reduced pressure at 35 °C. The residue was redissolved in 1 ml methanol. A sample (25 μl) was chromatographed on a Lichrosorp 5 RP 8 (150 × 4·6 mm) column (Chrompack, The Netherlands) using a Hewlett-Packard 1084 B liquid chromatograph equipped with a fixed wavelength u.v. (254 nm) detector. The elution system consisted of methanol (35% v/v) and 0·05 M phosphate buffer pH 6·5 (65% v/v). The flow rate was 1 ml min^{-1} and the oven temperature was set at 40 °C.

Protein assay

Microsomal protein was assayed according to the method of Lowry et al (1951), with crystalline bovine serum albumin as a standard.

RESULTS

H.p.l.c. assay

The method described allowed the separation of DMAP, MMAP, AAP and 4-formylaminoantipyrine (FAA) (Fig. 1B). When DMAP was added to the incubate, MMAP and AAP were clearly detectable. In addition, two unknown components (I,II) were eluted (Fig. 1A). Incubation of MMAP with liver microsomes yielded AAP. Some unknown components were also present in the extract, apparently only in small quantities (III, IV, V) (Fig. 1B).

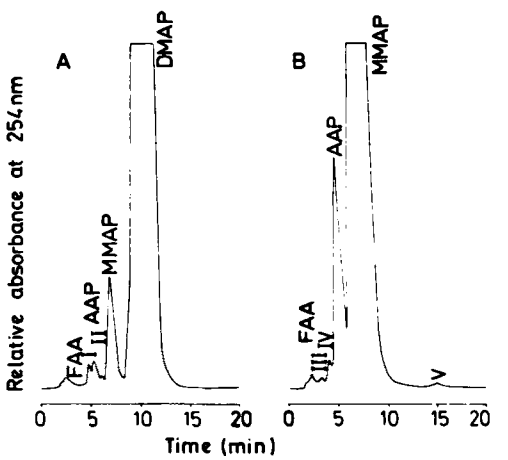

FIG. 1. A Chromatogram obtained by separation of DMAP and its metabolites with h.p.l.c. (as described under Materials and Methods) after incubation of rat liver microsomes with 3·33 mM DMAP for 5 min. B As A, with 3·33 mM MMAP as substrate.

FAA, also a known metabolite of DMAP (Noda et al 1976), sometimes appeared after incubation of both DMAP and MMAP, but only in small quantities. The theoretical position of FAA is indicated in the chromatograms (Fig. 1AB). All the uncharacterized peaks (I–V) are probably metabolites because they could not be detected at zero time. Moreover, their appearance and their concentrations were dependent upon the substrate used, the concentration of this substrate and the incubation time used.

From the progress curves (Fig. 2AB) for the metabolism of 0·2 mM DMAP and 0·2 mM MMAP it is clear that much of the substrate which disappears is

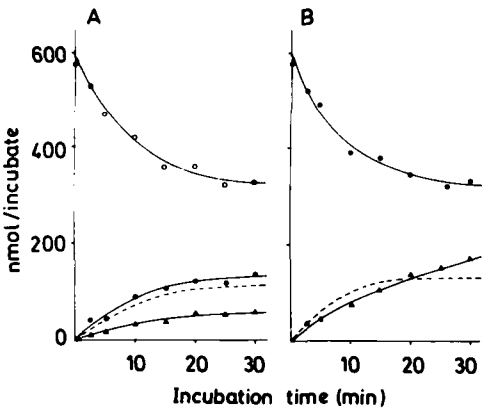

FIG. 2. A: Progress curves for the metabolism of 0·2 mM DMAP (○) incubated with rat liver microsomes (2 mg protein ml^{-1}) and the formation of MMAP (●) and AAP (▲), measured by h.p.l.c. Subtraction of MMAP and AAP formed from the disappearing DMAP yielded the unknown curve (– – –). The data points are the means of two experiments. B: As A, with 0·2 mM MMAP as substrate.

unaccounted for, if only MMAP and AAP formation is taken into account. Incubation with 0·2 mM AAP as substrate revealed no disappearance of AAP, which is in agreement with the results of Gram et al (1968).

The effect of the DMAP concentration on the formation of MMAP and AAP was also measured using the h.p.l.c. assay (Fig. 3). From these data the theoretical formaldehyde production has been calculated and the kinetic constants are shown in Table 1.

Colorimetric assay. We found that DMAP, MMAP and AAP may develop a yellow colour in the presence of the Nash reagent (Fig. 4). The $\Delta A_{415-500nm}$ at different concentrations of DMAP or its metabolites is given as formaldehyde equivalents. The curvature of the plot using MMAP is unusual. The shapes of the absorption spectra with the Nash

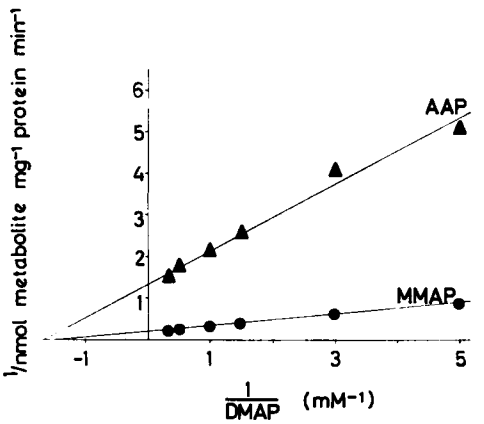

FIG. 3. Double reciprocal plot of the DMAP concentration (0·2–3·33 mM) vs the formation of MMAP and AAP, using DMAP as substrate, during incubation with rat liver microsomes, as described under Materials and Methods. Data points represent the means of three experiments.

reagent of the highest concentrations of MMAP or DMAP (Fig. 5) showed a close similarity to the spectrum of the formaldehyde reaction product (Nash 1953), although the $\Delta A_{415-500\ nm}$ for DMAP was relatively small. A completely different spectrum was obtained for AAP (Fig. 5).

With t.l.c. on silica gel plates and the Nash reagent, it was possible to show a yellow staining at the position occupied by MMAP. This indicated that the staining was probably not due to an impurity of the MMAP. AAP already began to colour under u.v. light without spraying with the Nash reagent. As a consequence of these findings the formaldehyde determination has to be corrected for interference by

Table 1. V_{max} and K_m (± s.e.m.) values for formaldehyde formation[a]

Assay	V_{max} nmol formaldehyde mg^{-1} protein min^{-1})	K_m (mM)	n[e]
Colorimetric	7·29 ± 0·95	0·491 ± 0·088	3
Radiometric[b]	8·62 ± 1·25	0·380 ± 0·076	5
H.p.l.c.[c]	7·21 ± 0·89	1·085 ± 0·013[d]	3

[a] The values were obtained using various assays to determine DMAP (0·2–3·33 mM) demethylation, as described in the methods section. [b] Double labelling of [^{14}C]DMAP was assumed. [c] Formaldehyde was calculated from MMAP and AAP formation. [d] Significantly different from the K_m (radiometric assay), $P < 0·0025$ and the K_m (colorimetric assay), $P < 0·025$, using Student's *t*-test. [e] Number of experiments.

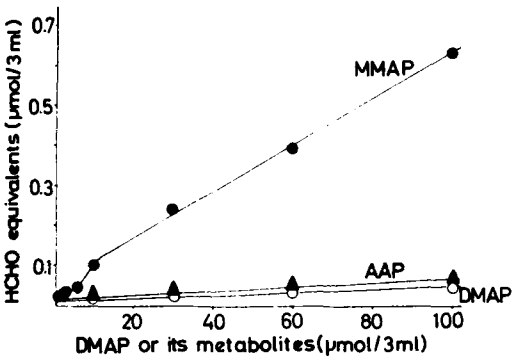

FIG. 4. Development of colour with DMAP, MMAP and AAP. Different concentrations of DMAP, MMAP and AAP are plotted against the formaldehyde concentration, which gave the same absorbance (415–500 nm) in the Nash assay as did the DMAP, MMAP and AAP at each concentration tested.

DMAP, MMAP and AAP *in the colorimetric* Nash assay. However combining the data of Fig. 3 and Fig. 4 shows that under our experimental conditions there is no need for correction of the colorimetric formaldehyde determination. The apparent V_{max} and K_m values, using the uncorrected Nash assay, as described in the methods section, are shown in Table 1.

Radiometric assay

Batch 2 of [^{14}C]DMAP was manufactured using AAP and [^{14}C]methyl chloride with an isotopic abundance of 85·4%, resulting in a product with 85·4% of the activity in the form of doubly labelled molecules and 14·6% of the activity as singly labelled molecules. It was subsequently diluted with inactive DMAP bringing the specific activity down to 9·6 mCi mmol^{-1}. This material contained 6·58% of doubly labelled and 2·25% of singly labelled molecules, i.e. not all of [^{14}C]DMAP is doubly labelled as is generally assumed (Poland & Nebert 1973). The data in Table 1 were however obtained by assuming that all molecules [^{14}C]DMAP are doubly labelled (see discussion). No labelled compounds, except for formaldehyde, remained in the aqueous phase after $CHCl_3$ extraction, as indicated by t.l.c. analysis (data not shown).

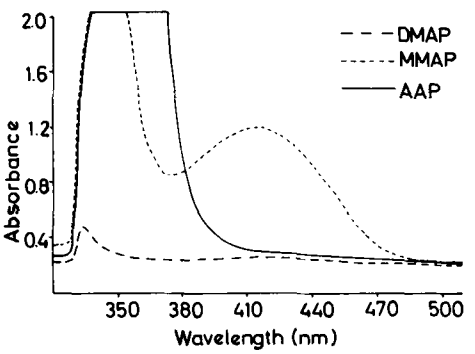

FIG. 5. Absorbancy spectra after reaction of 33·3 mM DMAP, MMAP or AAP with the Nash reagent.

DISCUSSION

The data from this study emphasize the observation that formaldehyde formation during DMAP-*N*-demethylation originates from two *N*-demethylation reactions (Gram et al 1968). This implies that it is fundamentally wrong to determine the velocity of the hepatic microsomal DMAP-*N*-demethylation reaction by measuring formaldehyde or AAP. Consequently only apparent V_{max} and K_m values are obtained.

Furthermore, from this study it is clear that difficulties may be encountered with the *colorimetric formaldehyde determination* according to Nash (1953) who considered this Hantzsch-reaction to be both sensitive and specific. However, amines and some other chemicals (Nash 1953) as well as several naturally occuring compounds (Cinti & Thal 1977) interfere with the Hantzsch-reaction. We have shown that DMAP, AAP and especially MMAP may develop a colour, with an absorbance at 415 nm relative to 500 nm, in the presence of the Nash-reagent. Moreover, the shape of the absorbance spectra of MMAP and DMAP with the Nash-reagent were similar to that for formaldehyde (Fig. 5). This was very striking because the chromogen, 3,5-diacetyl-1,4-dihydrolutidine, obtained from reaction of formaldehyde with the Nash-reagent is specific.

We cannot explain the curvature of the MMAP-Nash concentration plot, because the molecules responsible for the colour are not known. However, when DMAP is incubated in the concentration range, 0·2–3·33 mM, for only 5 min, only minor disturbances of the Nash assay by DMAP or its main metabolites will occur. This was shown by concentration measurements of the metabolites using h.p.l.c. (Fig. 3). The colorimetric method, therefore, gives reliable results under the conditions described and can be used to examine the suitability of other methods.

In the *colorimetric AAP determination*, according to Brodie & Axelrod (1950) or according to Brun (1951), MMAP also develops a considerable colour (De Waide 1971). Because MMAP may accumulate in the reaction mixture to a considerable extent, as

shown in the present work, these methods will not always be reliable. The same problems will occur in these colorimetric assays when MMAP is used as a substrate.

In the *radiometric assay* for [^{14}C]DMAP *N*-demethylation, [^{14}C]formaldehyde is determined (Poland & Nebert 1973) and the specific activity of the substrate is used to calculate the amount of [^{14}C]formaldehyde formed. Poland & Nebert assumed that [^{14}C]DMAP has been labelled at both *N*-methyl groups in the 4-position. However the batch we used was only partially double labelled (see results). Because of this incomplete double labelling the specific activity of [^{14}C]MMAP, formed during the *N*-demethylation reaction differs from the specific activity of [^{14}C]DMAP in an unpredictable way. Measurement of the specific activity of [^{14}C]MMAP during the in vitro incubation of [^{14}C]DMAP with rat liver microsomes is impossible, since [^{14}C]-MMAP is metabolized to unknown compounds (Fig. 2B)—in addition to AAP.

Nevertheless, the same results were obtained from both the colorimetric- and the radiometric formaldehyde assay (Table 1). Because the colorimetric assay is accurate these identical results suggest that there is little wrong with either method, at least under our experimental conditions. Moreover, these results validate the use of the assumption that [^{14}C]DMAP be completely double labelled.

With the aid of the h.p.l.c. method we detected some unknown metabolites, formed during DMAP or MMAP metabolism (Fig. 1A,B). Also, by plotting progress curves for the metabolism of both DMAP and MMAP (Fig. 2A,B), we found that these compounds are metabolized by pathways other than those leading to MMAP or AAP alone. Some of these unknown metabolites may possibly be formed by oxidation of DMAP at the carbon atom in the 3 position (Niwa et al 1975). The FAA metabolite (Noda et al 1976) could sometimes be detected, but then only in very small quantities.

Comparison of the h.p.l.c. method with the other methods reveals that the V_{max} calculated from the formation of MMAP and AAP is similar to that obtained using the other methods, but the apparent K_m is higher (Table 1). Because MMAP is also metabolized by other routes this is not surprising. The h.p.l.c. method used is, therefore, not suitable for determination of aminopyrine demethylation by rat liver microsomes, because it underestimates the formaldehyde formation. However it may be used to analyse DMAP metabolism in more detail.

In addition to biochemical difficulties (such as semicarbazide concentration (Matsubara et al 1977), mutual interference of MMAP and DMAP with the metabolism one of the other (Bast & Noordhoek 1980a), or the two-step *N*-demethylation reaction), the analytical problems outlined should be considered when interpreting the wide variation in the literature for the activity of DMAP demethylating enzyme systems (Matsubara et al 1977). Furthermore, in studies which suggest that more than one cytochrome P-450 species is involved in the metabolism of DMAP or MMAP (e.g. Pederson & Aust 1970), these biochemical and analytical problems are generally not recognized.

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