Further validation of the Mulder and van Doorn kinetic procedure for the measurement of microsomal UDP-glucuronosyltransferase activities

UDP-glucuronosyltransferase (UDPGT; EC 2.4.1.17) is a membrane-bound family of isoenzymes involved in the detoxication of xenobiotics and endogenous compounds. It catalyses the transfer of D-glucuronic acid on to a wide range of acceptors. These acceptors can be of different structure, e.g. monoterpenoid alcohols, phenols, hydroxycoumarins, hydroxysteroids. The sugar moiety can be transferred on different functions such as $-\text{CO}_2\text{H}$, $-\text{OH}$, $-\text{NH}_2$ or $-\text{SH}$ (see Dutton, 1980; Boutin, 1987 for complete reviews).

The study of the microsomal UDPGT activities requires the use of methods of measurement which are not specific for the type of acceptor but rather which can be used with numerous substrates of different nature. Four different types of methods are available for such studies: a t.l.c. method using ^{14}C -labelled UDP-glucuronic acid (Bansal & Gessner, 1980), an isotachophoretic method (Holloway *et al.*, 1981), a g.l.c. method described for eugenol but adaptable for numerous other aglycones (Boutin *et al.*, 1983) and a h.p.l.c. method also using ^{14}C -labelled UDP-glucuronic acid (Hamar-Hansen *et al.*, 1985, 1986; Coughtrie *et al.*, 1987). A fifth method has been described by Mulder & van Doorn (1975) on the model of the creatine kinase assay (Forster *et al.*, 1974). This method is described in Scheme 1. In summary, UDP, the by-product of the glucuronidation reaction, reacts with phosphoenolpyruvate via pyruvate kinase leading to the production of pyruvate. This pyruvate is in turn measured in the presence of NADH and lactate dehydrogenase which lead to the consumption of NADH, monitored in a spectrophotometer at 340 nm. NADH, pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate are added to detergent-activated microsomes with UDP-glucuronic acid and the aglycone. Three major criticisms have been raised against the use of this method (Hanninen *et al.*, 1977; Finch *et al.*, 1979), as follows.

(i) The recycling of UTP (product of the reaction 2) mediated by a microsomal nucleotidase would lead to an

Scheme 1. Schematic representation of the Mulder & van Doorn (1975) method of measurement of UDP-glucuronosyltransferase activities

From Colin-Neiger *et al.* (1982). 1, UDP-glucuronosyltransferase (EC 2.4.1.17); 2, pyruvate kinase (EC 2.7.1.40); 3, lactate dehydrogenase (EC 1.1.1.40).

increased concentration of UDP, giving an artifactually high rate of glucuronidation.

(ii) A microsomal-associated 'NADH oxidase' consumes extremely rapidly the NADH added to the assay, leading to an apparent fast reaction.

(iii) There is no evidence that a given amount of UDP added to an UDP-glucuronic acid- and UDPGT-free medium would lead to a comparable amount of NAD^+ and/or that there would exist a linear relationship between the UDP concentration added and the amount of NAD^+ measured.

Chronologically, the first criticism was answered by Colin-Neiger *et al.* (1982), who assessed the method by showing that the nucleotidase associated with the microsomes was inhibited by Triton X-100, routinely used under the conditions described by these authors. Other detergents tested in this assessment, particularly Tween 80, Brij 20 and Lubrol WX, were inefficient in producing any appreciable inhibition of this pathway.

Secondly, a detailed study of the treatment of rat liver microsomes by detergent (Boutin, 1986) led to the observation that the 'NADH oxidase' was inactivated by, and only by, Triton X-100, as previously suggested by Colin-Neiger *et al.* (1982) whereas, as above, the three other detergents failed to produce such an inhibition of this activity.

Thirdly, the last criticisms are answered by the present data. The measurement of the amount of NADH oxidized in a Mulder and van Doorn medium in presence of known quantities of UDP (0.005, 0.01, 0.05, 0.08 and 0.1 mM) has been conducted with and without rat liver Triton X-100-activated microsomes (10–20 μg of protein per assay). This amount of protein does correspond to the amount used in the automated assay as described in our recent papers (Boutin *et al.*, 1984, 1985, 1987). These

Fig. 1. Consumption of NADH in a Mulder & van Doorn (1975) medium supplemented with known quantities of UDP, in an enzyme-free system or in the presence of Triton X-100-activated microsomal suspension

●, Enzyme-free system; ○, Triton X-100-activated microsomes.

experiments have been conducted as described by Colin-Neiger *et al.* (1982). Briefly, the Mulder and van Doorn medium contained, in 75 mM-Tris/HCl buffer, pH 7.4: 5 mM-MgCl₂, 0.2 mM-phosphoenolpyruvate, 0.2 mM-NADH and 25 µg of pyruvate kinase (200 units/mg; Sigma) and 10 µg of lactate dehydrogenase from beef heart (250 units/mg; Boehringer)/ml. Various amounts of UDP (Sigma) were added to start the reaction which was monitored at 340 nm in a Shimadzu spectrophotometer at 37 °C. The reaction came to completion within 10 min. The decrease in absorbance at 340 nm was a linear function of the added amount of UDP. The total amount of NAD⁺ after 10 min was plotted versus the concentration in UDP at zero time (Fig. 1). Data presented are expressed as the mean for three determinations; standard deviations were systematically under 5%. The linear regression coefficient is 0.99 without microsomes, and 0.98 in the presence of Triton X-100-activated microsomes. In this latter case, the slight shift of the plot towards the NADH axis indicates that NADH is concomitantly oxidized unspecifically, which is in agreement with the incomplete inhibition by Triton of the microsomal NADH oxidation, as previously reported (Boutin, 1986).

It has been argued that the present method could not be used for measurement of UDPGT activities towards aglycones with a low turnover, such as endogenous substrates (e.g. steroids or bilirubin). Measurements done on hamster hepatic microsomes, using testosterone as aglycone, run in parallel with the specific method of Rao *et al.* (1976), clearly showed similar results with the two assays (Jayyosi *et al.*, 1988). Furthermore, the Mulder and van Doorn assay was also assessed, in our laboratory against the t.l.c. method of Bansal & Gessner (1980) clearly showing identical results for the substrates tested. We found the reliable minimum of activities measurable with the present assay to be around 1 nmol/min per mg of protein.

This last study indicates the validity of the method, since most of the data obtained by this method were comparable with the data published by other groups using different, specific assays. Furthermore, Coughtrie *et al.* (1987) assessed their h.p.l.c. method by showing similar results obtained with the present assay (referred to as 'conventional assay' in their paper) compared with their own, for the substrates phenolphthalein and eugenol with specific activities of 5 and 42 nmol/min per mg of protein, respectively. We nevertheless believe that the use of the method of Mulder and van Doorn can lead to relative data and must preferentially be used in comparison with other information obtained with the same methodology, particularly in cases of studies conducted with numerous, alternative substrates for studies of the

active site topology in experiments *in situ* (Thomassin *et al.*, 1987; J. A. Boutin, unpublished work).

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