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SIMULTANEOUS ANALYSIS OF DAPSONE AND MONOACETYLDAPSONE EMPLOYING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A RAPID METHOD FOR DETERMINATION OF ACETYLATOR PHENOTYPE

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1 A rapid, accurate and convenient technique for determination of acetylator phenotype of patients or subjects has not been available for routine clinical application.

2 An improved method for rapid and convenient determination of acetylator phenotype is described.

3 The plasma concentrations of dapsone (DDS) and monoacetyldapsone (MAD) were measured 3 h after a single oral 100 mg dose of dapsone using a specific and sensitive high performance liquid chromatographic assay.

4 The plasma concentration ratio of monoacetyldapsone to dapsone can accurately assess acetylator phenotype in patients or subjects.

5 The clinical applications for this method are discussed.

Introduction

The incidence of adverse reactions to several therapeutic agents has been shown to be related to the rate of acetylation by hepatic N-acetyltransferase (Devadatta, Gangardharam, Andrews, Fox, Ramakrishnam, Selkon & Velu, 1960; Evans, Davison & Pratt, 1965; Woosley Nies, Drayer Reidenberg & Oates, 1977) and knowledge of acetylator phenotype is often clinically important. The rate of drug acetylation is bimodally distributed, known to be dependent upon the activity of hepatic N-acetyltransferase, and is genetically determined in man (Evans, Marley & McKusick, 1960; Evans, 1969) and rabbit (Frymoyer & Jacox, 1963). The rate of drug acetylation is genetically determined in a simple Mendelian fashion and the frequencies of the genes which control the slow or rapid acetylation of drugs vary greatly in the different races. Slow acetylators are homozygous for an autosomal allele demonstrating a recessive inheritance pattern, whereas the rapid acetylator phenotype is dominant. Therefore, rapid acetylators may be either homozygous or heterozygous for the determining allele. This genetic polymorphism has recently been recognized as a clinically important factor by affecting the plasma concentration of drugs (and therefore their actions) (Devadatta *et al.*, 1960; Evans & White, 1964; Gelber, Peters, Gordon, Glazko & Levy, 1971; Zacest & Koch-Weser, 1972) and the incidence of adverse effects (Devadatta *et al.*, 1960; Evans *et al.*, 1965; Perry, Tan, Carmody & Sakamoto, 1969; Woosley *et al.*, 1977). These relationships have led to the need for a rapid, convenient and safe method for determining acetylator phenotype. Determination of the ratio of monoacetyldapsone (MAD) to dapsone (DDS) in plasma 3 h after a single 100 mg dose of DDS has been found to be a safe and convenient method for determination of phenotype (Gelber *et al.*, 1971; Reidenberg, Drayer, De Marco & Bello, 1973; Reidenberg, Drayer, Levy & Warner, 1975). We describe below an improved analytical method for rapid and specific analysis of MAD and DDS simultaneously in plasma using monopropionyldapsone as an internal standard.

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Methods

Fifty healthy caucasian consenting volunteers received a single 100 mg dose of dapsone (Avlosulfon®) after a 2 h fast. Three hours later a 5 ml sample of heparinized blood was obtained for analysis. All subjects refrained from eating solid foods for 2 h after taking dapsone. Subjects with known allergy to sulfa drugs or glucose-6-phosphate-dehydrogenase deficiency were excluded from participation.

A Waters Associates High Performance Liquid Chromatograph (HPLC) was employed for all

analyses. An M6000-A solvent delivery system, a U6K injector and a μ Bondapak® C_{18} column were coupled to a model 440 detector with a 280 nm filter. A dual channel Linear Instruments Corporation recorder using 10 mv and 100 mv full scale was used to provide a chromatographic pattern.

Dapsone (4,4′ diaminodiphenyl sulfone) (DDS) was obtained from ICN, K and K Laboratories, Inc., Plainview, N.Y., U.S.A. for analytical purposes. Dapsone 100 mg tablets (Avlosulfon®) were obtained from Ayerst Laboratories, Inc., New York, N.Y., U.S.A. Monoacetyldapsone (MAD) for analytical purposes was kindly supplied by Dr Anthony J. Glazko of Parke Davies Laboratories, Inc., Ann Arbor, Mich., U.S.A. Monopropionyldapsone (MPD) was synthesized in our laboratory by reacting dapsone with propionic acid at 60°C for 12 h. MPD was isolated from unreacted dapsone and by-products by the high performance liquid chromatographic method described below. Glass distilled acetonitrile was purchased from Burdick and Jackson Laboratories, Muskegon, Mich., U.S.A. and all other chemicals were ACS reagent grade.

Analytical methods for simultaneous determination of DDS and MAD in plasma

Plasma was obtained from blood collected in Vacutainer® heparinized glass tubes. All plasma samples were stored at −20°C until analyzed. Plasma (0.5 ml) in a 15 ml polypropylene tube was mixed with 100 μl H_2O containing approximately 2 μg of MPD as internal standard. To this were added, 100 μl 1N NaOH, 400 μl water or standards for standard curves and 10 ml diethyl ether. The tube was sealed with a Caplug® and the mixture was shaken for 5 min and centrifuged for 2 min at approximately 800 *g*. Seven ml of the organic phase was transferred to a Teflon-lined screw cap vial with the aid of a 10 ml Mohr pipet. The solvent was evaporated to dryness with a gentle stream of dry nitrogen at 30°C. Three hundred μl of the mobile phase solvent system (water; acetonitrile; acetic acid; 1000:300:25 v/v/v) was added to dissolve the residue and an aliquot of this was injected for HPLC analysis. The mobile phase was delivered through a μ Bondapak C_{18} column at a rate of 2.0 ml/min at room temperature. DDS and MAD were quantified by comparing peak height ratios of DDS to MPD and MAD to MPD in plasma samples to peak height ratios for standards (known concentrations of DDS and MAD added to drug-free plasma).

Results

Employing this method for their simultaneous quantification, plasma concentrations of DDS and MAD from 0.02 to 5.0 μg/ml were measured in less than 12 min after a simple extraction process.

Figure 1a is a chromatogram which demonstrates the ability of the method to readily separate and identify 0.05 μg each of DDS, MAD and 2 μg MPD added to 0.5 ml of plasma. The retention times for DDS, MAD and MPD were 4.5, 5.7 and 8.4 min, respectively. An unidentified compound with a retention time of 11.0 min is found in most routine plasma samples. Various solvent systems were evaluated for extraction efficiency and ability to separate the compounds of interest from impurities. Extraction into diethyl ether allowed accurate quantitation in the above range without interference by other compounds. Figure 1b is a chromatogram of plasma free of dapsone or its metabolites demonstrating the absence of interference from compounds with retention times similar to the compounds of interest. Plasma from patients receiving the following medications did not demonstrate interfering peaks: methyldopa, procainamide, triamterene, hydrochlorothiazide, chlordiazepoxide and diazepam.

Monopropionyldapsone (MPD) was synthesized to serve as an internal standard in the assay and structural confirmation was obtained by mass-spectrometry.

Figures 2a and 2b are standard curves obtained after addition of known amounts of DDS and MAD respectively to plasma free of each substance. The coefficients of variation for six determinations at 0.05, 0.5 and 5 μg/ml or either compound were 6.3, 0.97 and 0.75 for DDS and 4.9, 1.2 and 0.56 for MAD.

Plasma samples from fifty normal volunteers receiving single 100 mg oral doses of dapsone for determination of acetylator phenotype were analyzed by the above method. Figures 3a and 3b are examples of chromatograms for a slow and rapid acetylator, respectively.

The designation of acetylator phenotype was based on the ratio of acetylated metabolite (MAD) to parent compound (DDS). Reidenberg *et al.*, (1973) found that subjects who were slow acetylators of isoniazid had MAD/DDS ratios less than 0.30 and the ratios for rapid acetylators were greater than 0.35. Of the fifty normal subjects listed in Table 1, 25 had a MAD/DDS ratio less than 0.30 and were therefore classified as slow acetylators (0.20 ± 0.048, mean ± s.d.). Twenty-two subjects were found to have a ratio between 0.40 and 1.06 and were identified as rapid acetylators (0.73 ± 0.18, mean ± s.d.). Three subjects were intermediate with ratios of 0.32, 0.33 and 0.34. This distribution is in excellent agreement with the distribution reported for the American population in much larger series (Evans *et al.*, 1960; Evans, 1969). Although investigators have been able to identify a distinct group of individuals who were intermediate acetylators of isoniazid and probable genetic heterozygotes

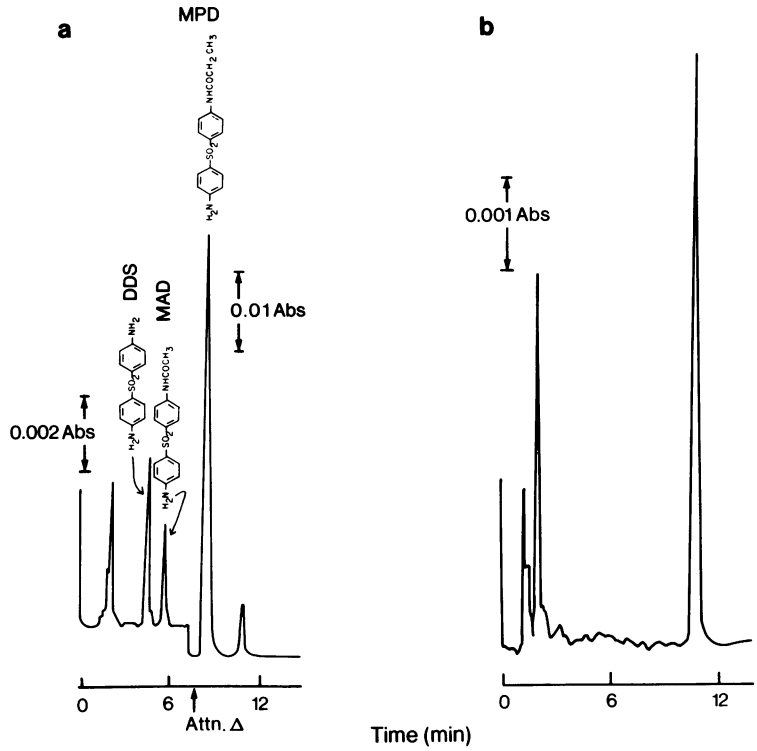

Figure 1 Chromatogram of (a) blank plasma with 0.05 μg each DDS and MAD and 2 μg MPD added. (b) blank plasma. Column: Waters Associates, μBondapak C_{18}, 10 μ particles, 300 mm x 3.9 mm i.d.
Pressure: 2,000 psi
Temperature: Ambient
Solvent: Isocratic, 1000 ml H_2O); 300 ml acetronitrile (Burdick and Jackson Laboratories, Inc.); 25 ml acetic acid (Mallinckrodt Cat. No. 2504)
Flow-rate 2.0 ml/min.
Sample Size: 100 μl
Detector: Waters Associates U.V. 280 nm Model 440
Compounds separated: Dapsone, monoacetyldapsone, monoproprionyldapsone

(Sunahara Urano & Ogawa, 1961; Dufour, Knight & Harris, 1964), no clear distinction of three groups was possible from our data. Three patients were classified as indeterminate based upon the data of Reidenberg *et al.* (1973) which compared the acetylation of isoniazid and dapsone in a group of individuals.

Discussion

Increasing evidence supports the concept that the rational use of several drugs is facilitated by knowing the acetylator phenotype of the patient. The plasma concentration, and therefore drug action, has been found to be influenced by the rate of acetylation for several drugs, such as isoniazid (Devadatta *et al.*, 1960), sulfamethazine (Evans & White, 1964) and hydralazine (Zacest & Koch-Weser, 1972). The incidence of adverse drug reactions is also influenced by acetylator phenotype as best illustrated by the higher incidence of isoniazid-induced peripheral neuritis in slow acetylators (Devadatta *et al.*, 1960) and isoniazid-induced hepatitis in rapid acetylators (Alarćon-Segovia, Fishbein & Alcalá, 1971). It has also been demonstrated that slow acetylators are at increased risk of developing a lupus erythematosus syndrome, or antinuclear antibodies when treated with hydralazine (Perry *et al.*, 1970) isoniazid (Alarćon-Segovia *et al.*, 1971) or procainamide (Woosley *et al.*, 1977). Slow acetylators are also especially susceptible to adverse effects of other drugs such as phenelzine

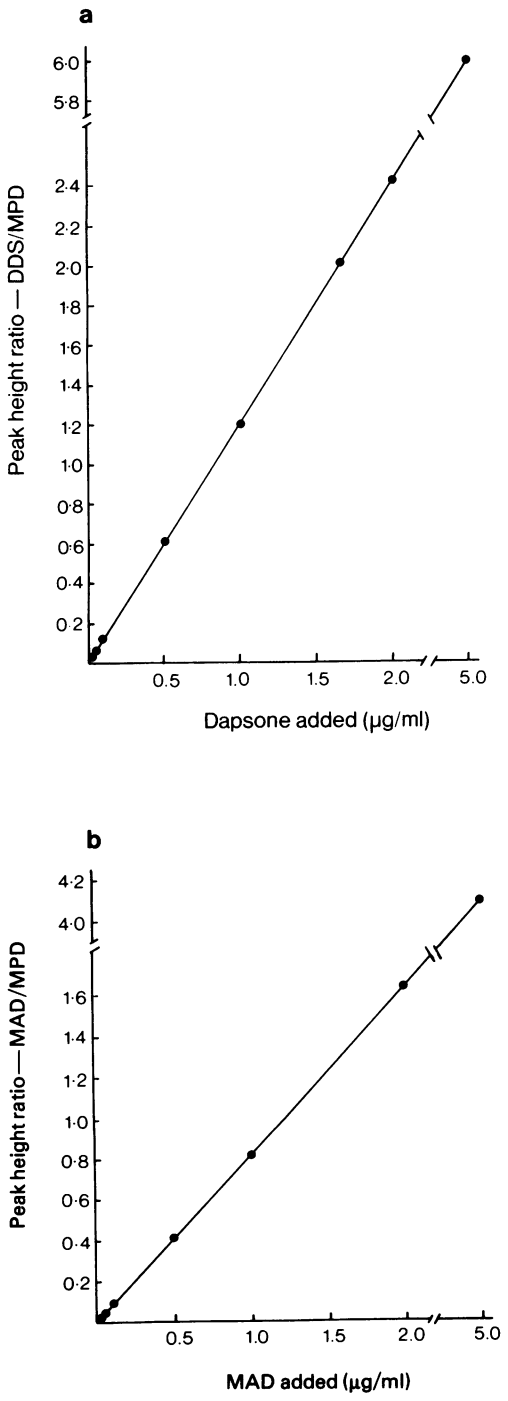

Figure 2 Standard curves for (a) dapsone and (b) monoacetyldapsone from drug free plasma

Table 1 Acetylator phenotype in a normal population determined with dapsone

Slow acetylators			
Subject	*Sex*	*Age (years)*	*MAD/DDS**
D.L.	M	30	.28
R.M.	F	32	.20
D.W.	F	22	.16
R.P.	M	26	.19
R.F.	M	32	.24
A.W.	M	29	.17
J.G.	M	31	.19
J.F.	M	24	.20
J.G.	M	30	.26
R.W.	M	34	.14
M.H.	F	42	.15
J.H.	M	67	.16
F.K.	M	66	.18
I.T.	M	62	.23
W.G.	M	55	.26
J.P.	M	69	.16
V.B.	M	57	.14
J.H.	F	44	.24
C.M.	M	60	.15
W.C.	M	55	.14
W.H.	M	43	.18
E.C.	M	54	.27
P.B.	F	30	.22
E.S.	M	52	.28
I.F.	M	62	.26
Intermediate acetylators			
Subject	*Sex*	*Age*	*MAD/DDS**
S.H.	F	30	.32
R.V.	M	32	.33
G.K.	M	32	.34
Rapid acetylators			
Subject	*Sex*	*Age*	*MAD/DDS**
D.R.	M	31	1.06
B.R.	F	22	.70
G.P.	M	22	.52
R.H.	M	32	.85
J.C.	M	34	.71
R.B.	M	34	.54
B.R.	M	28	.54
L.M.	M	29	.90
T.J.	M	33	.60
S.H.	M	27	.42
O.R.	M	68	.65
L.W.	F	33	.73
R.O.	M	63	.66
R.G.	M	62	.89
R.E.	M	72	.66
C.H.	M	60	1.00
A.B.	F	68	.92
R.S.	M	68	.81
J.L.	M	68	1.02
B.B.	M	60	.46
G.L.	M	30	.81
W.I.	M	56	.62

*Plasma concentration ratio of monoacetyldapsone to dapsone 3 h after a 100 mg tablet administered orally.

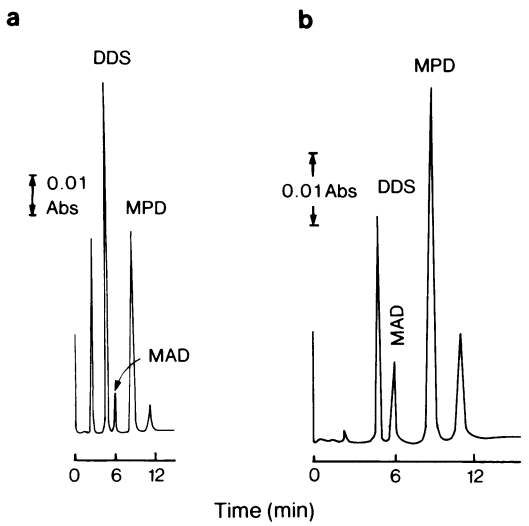

Figure 3 (a) Chromatogram of plasma from a patient who is a 'slow acetylator' of dapsone
DDS–1.75 μg/ml
MAD–0.21 μg/ml
MAD/DDS–0.12
(b) Chromatogram of plasma from a patient who is a 'fast acetylator' of dapsone
DDS–0.55 μg/ml
MAD–0.35 μg/ml
MAD/DDS–0.64

(Evans *et al.*, 1965) and sulfapyridine (Das, Eastwood, McManus & Sircus, 1973) and to some adverse drug-drug interactions. For example, they are at increased risk of developing phenytoin intoxication while receiving this drug in combination with isoniazid (Kutt, Verebely & McDowell, 1966). Because of these relationships the ability to determined acetylator phenotype should allow better estimation of the risk-to-benefit ration in may therapeutic decisions. It has also been proposed as a test to detect susceptibility to the development of arylamine-induced bladder cancer (Lower & Bryan, 1973).

There are several established techniques for determination of acetylator phenotype. One of the first was proposed by Evans *et al.* (1960) and was based upon the concentration of free isoniazid in the blood 6 h after oral administration of a single dose. Another method also used by Evans *et al.* (1969) determined the ratio of free to acetylated sulfamethazine excreted over an 8 h period. Other investigators have used the elimination half-life of isoniazid or sulfamethazine in plasma to determine phenotype (Scott, Wright & Weaver, 1969; Mattila, Tiitinen & Alhava, 1969). These techniques are not generally useful because of the lack of a simple analytical method for isoniazid and the lack of an available pharmaceutical preparation containing only sulfamethazine. Das & Eastwood (1975) have recently reported the ability to identify acetylator phenotype by determining the percent of acetylated metabolites of sulfasalazine (Azulfidine®) in serum or urine. However, this requires multiple dose administration of the drug and quantitation of the metabolites. Elson, Strong & Lee (1974) reported the bimodal distribution of the urinary ratio of N-acetylprocainamide (NAPA) to procainamide (PA) in urine and Reidenberg *et al.* (1975) have been able to determine the phenotype of patients receiving procainamide by the ratio of NAPA to PA in plasma 3–6 h after a dose at steady-state. This requires multiple dose administration of procainamide which is not without side effects in many people. Frislid, Berg, Hansteen & Lunde (1976) found that phenotype could be established by determining the NAPA/PA ratio after a single dose of procainamide but the dose employed was a sustained-release tablet which is not available in all countries. We have recently found that these methods can be facilitated by using an HPLC method which allows simultaneous measurement of PA and NAPA (Carr, Woosley & Oates, 1976).

The use of dapsone for determination of acetylator phenotype was first proposed by Gelber *et al.* (1971). Reidenberg *et al.* (1975) used the ratio of monoacetyldapsone to dapsone three hours after a single 100 mg dose of dapsone to determine acetylator phenotype. Phenotypic classification by this method was found to agree with classification using the elimination half-life of isoniazid or percent excretion of acetylsulfamethazine (Gelber *et al.*, 1971; Reidenberg *et al.*, 1973) or the NAPA/PA ration in plasma (Reidenberg *et al.*, 1975). Side effects after the administration of a single 100 mg dose of dapsone are exceedingly rare if individuals with prior allergy to sulfa drugs are excluded. No subjective symptoms were reported in the 50 normal subjects in this study or in another 35 patients previously given 100 mg dapsone during phenotyping for various clinical indications. Chronic administration of dapsone has been reported to cause several types of adverse reactions, the most common one being haemolytic anemia in individuals with glucose-6-phosphate dehydrogenase deficiency (Degowin, Eppes, Powell & Carson, 1966). However, haemolysis has not been reported until after more than a week of continuous therapy.

Analysis of DDS and MAD in plasma was initially performed by Ellard (1966) using the nonspecific Bratton-Marshall technique. Glazko, Dill, Montalbo & Holmes (1968) measured DDS in plasma using fluorometric detection and a complex extraction technique to separate DDS from metabolites. Peters, Gordon & Colwell (1970) also used fluorometric detection to measure DDS and MAD simultaneously. However, a complex extraction procedure was required and overlapping fluorescent spectra required

a mathematical correction for non-specificity of the technique. Specific analysis of DDS and MAD was reported by Murray, Gordon & Peters (1971) using liquid chromatography and flurometric detection. The utility of the mthod was severely limited by retention times of DDS and MAD from 40–60 min. The complex extraction procedure could benefit from the use of an internal standard which was not employed. A liquid chromatographic method has been utilized by Reidenberg *et al.* (1975) but a complete description and evaluation of the methods was not provided.

We have described a specific and sensitive method for analysis of DDS and MAD in plasma after a single procedure and separation by high pressure liquid chromatography. The use of an internal standard corrects for possible pipet errors and erratic extraction efficiencies and makes the method useful for routine laboratory use. The retention times for DDS, MAD and the internal standard are less than 12 min and permit routine analysis of twenty or more samples daily if necessary. The method quantitatively detects levels as low as 20 ng/ml of either DDS or MAD.

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