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Determination of dapsone in serum and saliva using reversed-phase high-performance liquid chromatography with ultraviolet or electrochemical detection

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Abstract

A simple, extractionless method for the determination of dapsone in serum and saliva is described. Reversed-phase high-performance liquid chromatography is used with UV detection at 295 nm or electrochemical detection at 0.7 V. Diazoxide in buffer is the internal standard for UV detection and practolol for electrochemical detection. Sample preparation is minimal with protein precipitation of serum samples whilst saliva samples are simply diluted with addition of an internal standard. Low-level serum and saliva samples are front-cut on-line with a 3 cm laboratory-made precolumn in the loop position on a standard Valco injection valve. Isocratic separation is achieved on a 250 mm × 4.6 mm I.D. stainless-steel Spherisorb S5 ODS-1 column. The mobile phase for high levels of dapsone is acetonitrile–elution buffer (12:88, v/v) at 2 ml/min and a column temperature of 40°C for both serum and saliva separations. For the low-level assays using electrochemical detection and solid-phase clean-up, the mobile phase is acetonitrile–methanol–elution buffer (9:4:87, v/v/v). The UV and electrochemical detection limits are 25 ng/ml and 200 pg/ml, respectively, in both serum and saliva. This simple method is applicable to the routine monitoring of dapsone levels in serum from leprotic patients and electrochemical detection gives a simple, reliable method for the monitoring of trough values in subjects on anti-malarial prophylaxis.

1. Introduction

Dapsone (diaminodiphenyl sulphone, **I**) is an important antileprotic drug and is a component of the World Health Organization's (WHO) recommended multi-drug regimen for the treatment of leprosy [1]. For the past three decades it has also been used as a prophylactic against malaria and, in particular, against chloroquine-resistant strains of *Plasmodium falciparum* [2]. A few dermatological disorders with an auto-immunological origin, such as dermatitis herpatiformis [3] and miliary lupus [4], also respond well to **I**, as is also found for arthritic conditions [5].

The WHO recommends the use of 50–100 mg of **I** daily as part of their antileprotic regimen and these dosages generate steady-state serum levels in the region of 1–3.5 μg/ml [1], whilst maximum serum levels of 1.1–2.3 μg/ml are attained with a single 100-mg dose [6]. The low dosing of 100 mg of **I** once a week for malarial control yields trough levels of 2–10 ng/ml on the seventh day [7]. For dermatological problems up to 400 mg per day are administered [8], yielding

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maximum serum concentrations of **I** up to 15 μg/ml. This wide range of serum concentrations applicable to the different uses of **I** means that any general method for its assay must be adaptable to determination over more than three orders of concentration.

Dapsone was originally assayed spectrophotometrically by a 1939 method for the assay of sulphones [9], which was later modified by Ellard [10]. The poor specificity with these methods meant that the major metabolite of **I**, monoacetyldapsone (MAD), was included in the measurement of the parent drug, giving results that could be more than double the true value. Hence pharmacokinetic data on **I** published prior to 1969 are no longer regarded as reliable. Only in 1969 was the more specific fluorimetry introduced for the assay [11], but even then the resulting improvement was small compared with the superior specificity of modern chromatographic methods.

A sensitive gas chromatographic (GC) method using electron-capture detection was noted by Burchfield *et al.* [12] and later described more fully [13]. Since then, various methodologies utilizing high-performance liquid chromatography (HPLC) [14–26] or thin-layer chromatography (TLC) [27] have been published. Ion exchange methods [14–16] do not provide sufficient resolution in the presence of other drugs and, whilst normal-phase separation was used by Jones and Ovenell [19] and Pieters *et al.* [20], the method of Jones and Ovenell was criticized [23,24] for poor reproducibility owing to the use of ammonia solution and diisopropyl ether in the mobile phase. Reversed-phase separation [17,18,21–26] seems to have provided the most satisfactory methods to date. All the published HPLC methods for serum assay require a long extraction procedure except those of Carr *et al.* [18], Zuidema *et al.* [21] and Philip *et al.* [22], which use modifications of Carr *et al.*'s protein precipitation method. Although dapsone is excreted into the saliva, only two of the methods, one using normal-phase separation [20] and the other using TLC [27], investigated the determination of dapsone in saliva. No methods were found where either solid-phase extraction or electrochemical detection were used.

This paper reports a simple, extractionless method for the determination of dapsone in serum and saliva using reversed-phase HPLC and UV or electrochemical detection.

2. Experimental

2.1. Reagents

All solvents used were of spectroscopic grade from Burdick and Jackson (Muskegon, MI, USA) and all water was purified with a Milli-Q system (Millipore, Milford, MA, USA). Standardized dapsone was supplied by Wellcome (Johannesburg, South Africa) and the internal standards, diazoxide and practolol, were supplied by Sherag (Johannesburg, South Africa) and ICI (Johannesburg, South Africa), respectively. All other chemicals were of analytical-reagent grade. The elution and sample buffer was 0.05 *M* ammonium dihydrogenphosphate (pH 4.6).

2.2. Internal standard

Ultraviolet detection

Diazoxide was found during a previous study to exhibit similar polarity and retention behaviour to dapsone and a reasonable molar absorptivity at the detection wavelength of dapsone, and thus was applicable to the use of non-programmable UV detectors. A 100 μg/ml solution of diazoxide in buffer was used as the serum internal standard and 5 μg/ml as the saliva internal standard solution.

Electrochemical detection

Previous experience had shown that practolol showed a similar polarity to dapsone at the pH being used for the separation, and further investigation showed that it was oxidizable under the same conditions as dapsone. Therefore, practolol was used as the internal standard for the electrochemical detection at 100-μg/ml for serum assays, 10 μg/ml for low-level serum assays and high-level saliva assays and 2 μg/ml

for very low serum and saliva levels more than 3 days after dosing.

2.3. Sample collection

Both single-dose and steady-state samples of serum and saliva were collected.

Single dose
Dapsone (100 mg) was ingested with 200 ml of water at zero time by informed staff volunteers. No dietary restrictions were enforced. Blood samples were collected in plain Vacutest tubes just before dosing, then at 30-min intervals for 4 h after dosing, 1-h intervals for the next 5 h and then once daily until 4 days after dosing. Blood sampling was performed via a cannula with heparin lock in the antecubital vein for the continuous samples on the day of dosing. Fifteen minutes after collection, each clotted blood sample was centrifuged and the serum transferred into a clean borosilicate vial. Immediately prior to blood collection, salivary excretion was stimulated by twice rinsing the mouth with water and then chewing on a plug of Parafilm. Saliva was then collected in a sterile borosilicate tube. All samples were stored at −18°C until assayed, within 1 month of collection.

Steady state
Serum samples were obtained 4 h after the daily dosing from leprosy patients who had been dosed daily with 100 mg of dapsone and also 150 mg of rifampicin and 100 mg of clofazimine according to WHO recommendations for at least 1 month. Blood was collected in a plain Vacutest tube from the antecubital vein and treated as described for the single-dose samples.

2.4. Sample preparation

With ultraviolet detection

Serum. A 100-μl volume of internal standard solution (100 μg/ml diazoxide) and 100 μl of elution buffer were added to 200 μl of serum in a borosilicate centrifuge tube, then 50 μl of 20% perchloric acid were added to precipitate the protein. The mixture was shaken on the vortex mixer, centrifuged at 2000 *g* for 5 min and the clear supernatant injected on to the column via a 250-μl loop.

Saliva. The defrosted saliva samples were centrifuged at 3000 *g* for 4 min to remove cellular debris and undissolved materials. A 100-μl volume of the saliva supernatant was added to 150 μl of water and 100 μl of internal standard (5 μg/ml diazoxide) in a test-tube and shaken on a vortex mixer, then 250-μl of this saliva solution were injected without further treatment.

With electrochemical detection

Serum. Practolol at 10 or 100 μg/ml, depending on the assay range, was used as the internal standard in place of the diazoxide used for UV detection.

Saliva. Practolol at 10 μg/ml was used as the internal standard for expected levels >50 ng/ml and the samples were injected without further treatment as for UV detection. For the low levels found in saliva 5 or 6 days after dosing, 2 μg/ml of practolol was used as the internal standard and a 250-μl volume of the prepared saliva was loaded in the forward-flush mode into a 250-μl loop connected to a 3-cm laboratory-made precolumn in the loop position on a standard Valco six-port autoinjector valve (Fig. 1). The precolumn was packed with 30–40 μm C_{18} packing. After loading the sample into the loop, it was manually flushed on to the precolumn with 700 μl of buffer using a gas-tight syringe and the valve was immediately turned to forward-flush the remaining front-cut sample on to the analytical column. After 2 ml of mobile phase had flushed through the precolumn on to the analytical column (1-min flow), the valve was turned again to the loading position and manually backwashed with 2 ml of 50% methanol in buffer using a flushing syringe. Just prior to loading the next sample, the precolumn system was forward flushed with 1 ml of elution buffer. This clean-up procedure was also used for very low (<20 ng/ml) serum samples and allowed the removal of the highly polar matrix components of both

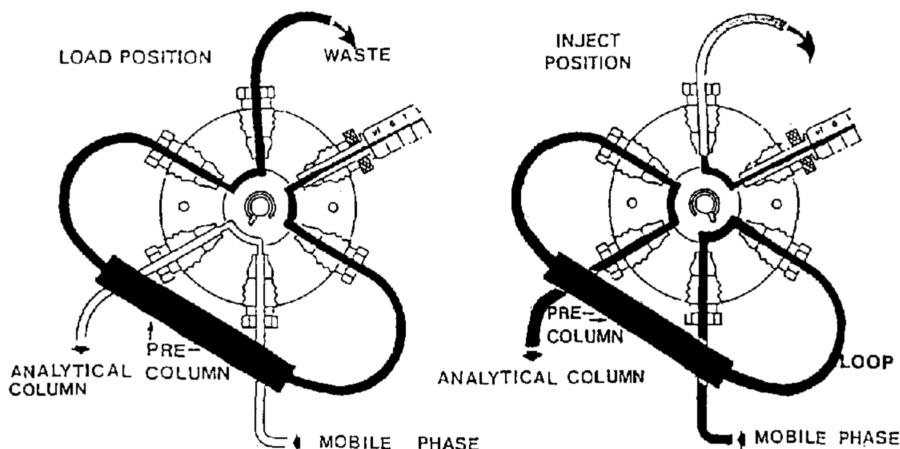

Fig. 1. Valco injector valve showing arrangement of the 250-μl loop and precolumn for forward flush on-line front-cutting of the low level samples.

serum and saliva without any breakthrough loss of analyte.

2.5. Preparation of standards

Serum

Standard (100 μl) in elution buffer (range 0.01–5 μg/ml) and 100 μl of internal standard solution (100 μg/ml diazoxide or practolol, depending on the detector used) were added to 200 μl of drug-free serum in a borosilicate centrifuge tube and shaken. Then 50 μl of perchloric acid (20%) were added and the mixture was shaken and centrifuged and the supernatant injected as for the serum samples.

Saliva, high levels

A 50-μl volume of standard solution (range 50 ng/ml–1 μg/ml) together with 50 μl of internal standard solution (5 μg/ml diazoxide or 10 μg/ml practolol depending on the detector used) were added to 100 μl of centrifuged defrosted drug-free saliva in a borosilicate tube and shaken manually. A further 100 μl of water were then added and the mixture shaken on a vortex mixer. A 250 μl volume of the solution was injected as for the serum samples.

Saliva, low levels

The concentration range of the standards was 5–100 ng/ml and 2 μg/ml of practolol was used as the internal standard. The sample was prepared as for the high-level standards, but was front-cut on the precolumn as described for the low-level saliva samples.

2.6. Chromatography

Initial investigations of UV detection and elution conditions were performed on a Hewlett-Packard Model 1050 quaternary chromatograph with diode-array detection (Hi Performance Systems, Johannesburg, South Africa), with monitoring simultaneously on a Perkin-Elmer Model 56 strip-chart recorder (Vacutec, Johannesburg, South Africa), and a Spectra-Physics SP 4200 integrator. All subsequent HPLC was performed on a Spectra-Physics SP 8100 liquid chromatograph (Anatech Instruments, Randburg, South Africa), with a pneumatically controlled six-port Valco loop injector valve fitted with a 250-μl loop or an additional precolumn at the loop position. A 250 mm × 4.6 mm I.D. stainless-steel Spherisorb S5 ODS-1 column (singly end-capped 5-μm C_{18} on a spherical silica packing) (Phase Separations, Clwydd, UK), was used for the separation of the dapsone from serum and saliva matrix components. The column was preceded by a laboratory-made 10 mm × 4.6 mm I.D. guard column filled with Spherisorb S5 ODS-1 packing. The mobile phase for the isocratic elution of high levels of dapsone was acetoni-

trile–elution buffer (12:88) at 2 ml/min and a column temperature of 40°C for both serum and saliva separations. For the low-level assays in serum and saliva (<20 ng/ml), using electrochemical detection, the mobile phase was changed to acetonitrile–methanol–elution buffer (9:4:87). The peak purity for dapsone was tested by measuring absorption spectra at 25%, 50% (full peak) and 75% of the peak width and by the ratio of the peak widths at 10% and 50% of the peak height for sample and standard peaks.

Ultraviolet detection

The UV–Vis absorption spectrum for dapsone showed a λ_{max} at 295 nm when scanned in the range 220–600 nm, so this was used as the detection wavelength. A Spectra-Physics Spectra-System UV 2000 scanning UV–Vis detector (Anatech Instruments) with an 8-μl flow cell was used for subsequent investigations and routine assays.

Electrochemical detection

An ESA 5100A Coulochem dual-electrode detector (Anatech Instruments) was used. The voltammogram of dapsone showed oxidation occurring from 0.3 to 0.9 V. The best response was obtained in a window from 0.5 to 0.7 V, so these values were used for the first and second electrodes respectively.

Quantification

Serum and saliva dapsone levels were calculated from the daily UV and electrochemical calibration graphs for serum and saliva on the basis of the peak-height ratio of dapsone to internal standard.

3. Results

Using the above methods, baseline separation of **I** was achieved, free of interference from matrix components. Using UV detection, no interfering components were found in either the predosing serum (Fig. 2) or the saliva (Fig. 3) from fifteen volunteers. The retention time for dapsone was 6.8 min ($k' = 3.57$) and that for diazoxide was 8.5 min. For the low-level assays the retention times were 7.0 and 11.1 min for dapsone and practolol, respectively. M, an unidentified metabolite of **I**, probably monoacetyldapsone, eluted at 11.8 min and the total assay time was 12 min.

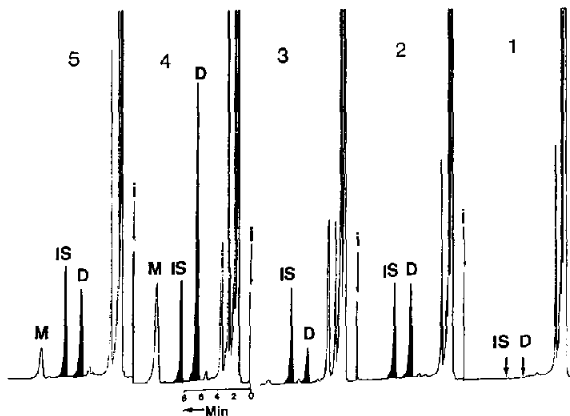

Fig. 2. Chromatograms of serum using UV detection. (1) Undosed serum; (2) 1 μg/ml dapsone standard with 100 μg/ml diazoxide as internal standard; (3) steady-state low-level sample 4 h after daily 100-mg oral dose; (4) steady-state normal-level sample 4 h after daily 100-mg oral dose; (5) serum sample 4 h after single 100-mg oral dose. D = dapsone; IS = diazoxide (100 μg/ml); M = metabolite of dapsone; i = injection.

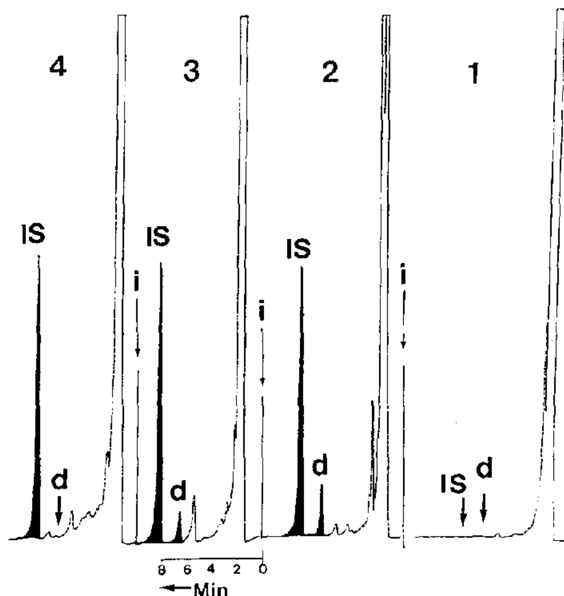

Fig. 3. Chromatograms of high levels of dapsone in saliva using UV detection. (1) Undosed saliva; (2) 250 ng/ml dapsone standard with 5 μg/ml diazoxide as internal standard; (3) saliva 8 h after a single 100-mg oral dose; (4) very low dapsone 3 days after a single 100-mg dose. d = Dapsone; IS = diazoxide (5 μg/ml); i = injection.

Using UV detection, the standard response curves were linear. The detectability (peak = three times the system noise) was 25 ng/ml for **I** in both serum and saliva. The intra-sample relative standard deviations (%) (R.S.D.s) were <1.0% at 5 μg/ml and 1.3% at 0.5 μg/ml in serum and 2.1% and 5.2% at 500 and 50 ng/ml, respectively, in saliva for eight assays of each. The inter-sample R.S.D.s in serum were 1.3% and 1.5% at 5 and 0.5 μg/ml, respectively, for eight samples. In saliva the inter-sample R.S.D.s were 2.8% and 5.9% at 500 and 50 ng/ml, respectively. These results are given in Table 1. As no fixed-volume extraction of the analytes is made, the accuracy and analyte recovery for the method cannot be determined by simple comparison of identically spiked aqueous and matrix results. However, by assuming that 100 μl of serum produces 60 μl of supernatant when precipitated with perchloric acid and by spiking aqueous samples accordingly, the intra-sample recovery or accuracy of the method was found to be 89 ± 1.1% for six determinations, whereas the inter-sample result was 91 ± 1.9%.

Using electrochemical detection at +0.7 V, the standard response curves were linear and the detectability was 200 pg/ml in serum and saliva. The intra-sample R.S.D. at 50 ng/ml in serum was 3.8% and the inter-sample R.S.D. was 6.1% at the same level. At 50 ng/ml the intra-sample R.S.D. was 2.6% and the inter-sample R.S.D. was 3.1% in saliva, whereas at 5 ng/ml they were 4.7% and 7.5%, respectively.

The pharmacokinetic profiles of dapsone in both serum and saliva revealed that although the saliva profile in an individual was a lower reproduction of the serum profile, the factor by which it was lower differed greatly between individuals.

4. Discussion

Trial determinations with some of the HPLC methods found in the literature for the assay of **I** in serum showed they were not capable of assaying the very low levels of **I** found 6 days after a single 100-mg dose of dapsone. Moreover, many of the sample clean-up methods were very time consuming. Investigation on a singly end-capped C_{18} column showed that baseline separation of dapsone from serum components was possible when using a binary mobile phase of acetonitrile–elution buffer (12:88, v/v). The best peak shape occurred at pH 4.6 and a column temperature of 40°C. The UV spectrum of dapsone showed two major absorption peaks; the larger broad peak occurred below 220 nm whereas the other more discrete peak was at 295 nm. Investigation at 210 nm gave numerous matrix peaks for serum, which proved difficult to separate. However, with 295 nm as the detection wavelength, a clean baseline for dapsone in both serum and saliva was found. Dapsone could be measured down to 25 ng/ml in both serum and saliva using UV detection.

As dapsone is part of a once-weekly antimalarial regimen, pharmacokinetic studies of this regimen require a method that can measure dapsone down to 5 ng/ml. Investigation of the electrochemical behaviour of dapsone showed it

Table 1
Validation results for determination of dapsone in serum and saliva

Body fluid	Relative standard deviation (%)									
	UV detection						Electrochemical detection			
	Intra-sample			Inter-sample			Intra-sample		Inter-sample	
	5 μg/ml	500 ng/ml	50 ng/ml	5 μg/ml	500 ng/ml	50 ng/ml	50 ng/ml	5 ng/ml	50 ng/ml	5 ng/ml
Serum	<0.1	1.3		1.3	1.5		3.8		6.1	
Saliva		2.1	5.2		2.8	5.9	2.6	4.7	3.1	7.5

could be oxidized above +0.3 V. The best signal-to-background response was achieved between 0.5 and 0.7 V and these voltages were used for the leading and measuring electrodes, respectively.

At the low levels detectable with an electrochemical detector and using only protein precipitation and centrifugation for sample preparation, the background in serum and saliva proved too high for dapsone to be resolved [Fig. 4(1)]. Previous experience [28] had shown that on-line clean-up of serum and saliva samples could decrease this problem, so an on-line clean-up method was developed. At low levels, interfering matrix components appeared in undosed serum and saliva and the mobile phase had to be adjusted to a ternary solution of acetonitrile–methanol–elution buffer (9:4:87, v/v/v) to achieve separation.

During the testing of this methodology, sera taken for steady-state dapsone levels in non-responsive leprosy patients revealed that more than 25% of the levels measured were below therapeutic level and well below the normal levels found after this dosage, as can be seen in Fig. 2(3) and (4). This could be due to poor compliance, poor absorption or rapid metabolism. This important finding is now being further investigated and to date has revealed that, after dosing with either solid tablets or tablets dissolved in 200 ml of water, a smaller peak increment in serum levels occurs with these patients than in patients exhibiting normal serum levels.

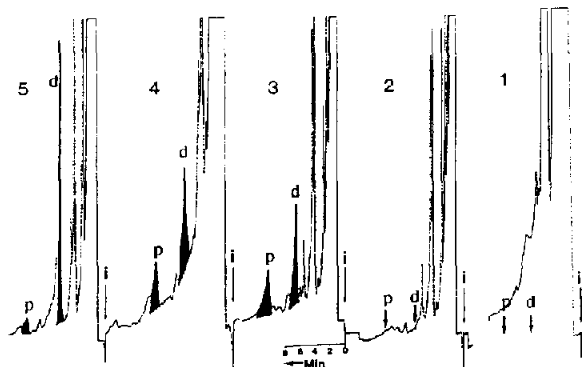

Fig. 4. Chromatograms of saliva using electrochemical detection. (1) Undosed saliva without front cut; (2) undosed saliva after front cut; (3) 5 ng/ml standard in serum with 2 μg/ml practolol; (4) 6 days after a single 100-mg oral dose of dapsone; (5) 3.5 h after a single 100-mg oral dose of dapsone. d = Dapsone; p = practolol (2 μg/ml); i = injection.

5. Conclusion

The simple HPLC method reported here is very applicable to the routine monitoring of dapsone levels in serum from leprotic patients. The use of electrochemical detection results in a simple, reliable method for the monitoring of trough values in persons on anti-malarial prophylaxis.

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