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Improved method for the simultaneous determination of proguanil and its metabolites by high-performance liquid chromatography and solid-phase extraction of 100-μl capillary blood samples dried on sampling paper

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Abstract

An improved method is presented for the determination of proguanil, cycloguanil and 4-chlorophenylbiguanide in 100-μl capillary blood samples applied to sampling paper. This method also utilises a solid-phase extraction technique and high-performance liquid chromatography. Different kinds of sampling paper, such as ion-exchange and cellulose sampling paper were tested. The best elution recovery (70–80%) was obtained after treatment of cellulose sampling paper with a quaternary ammonium compound. The limit of determination was 50 nmol/l for cycloguanil and 4-chlorophenylbiguanide and 125 nmol/l for proguanil using 100 μl capillary blood. The stability of the analytes and elution performance from sampling paper was validated at different temperature and storage time. Venous blood and capillary blood concentrations of proguanil and metabolites were found to be similar.

Keywords: Proguanil; Cycloguanil; 4-Chlorophenylbiguanide

1. Introduction

The antimalarial drug proguanil (PG) has recently been used in combination with chloroquine for prophylaxis, due to the continued development and spread of resistance of parasites to chloroquine used alone. PG is a prodrug that is activated by the hepatic cytochrome P-450 isoenzyme to its active triazine metabolite cycloguanil (CG) [1,2]. Another metabolite, 4-chlorophenylbiguanide (4-CPB), exhibited weak antimalarial activity in vitro [2]. Structural formulas for PG, CG and CPB are shown in Fig. 1. Differences in metabolism of drugs can lead to therapeutic failure, due to poor metabolism to the active metabolite. PG is activated by the phase I enzyme CYP2C19 in the liver to CG and has been found to show genetic polymorphism as either poor metabolizer or extensive metabolizer [3,4]. The poor metabolizer phenotype is observed in 3–6% of

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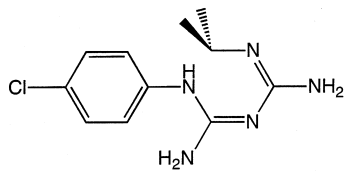
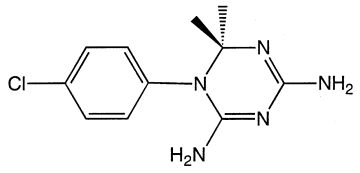
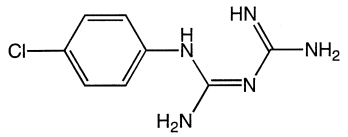


Proguanil (PG)



Cycloguanil (CG)



4-Chlorophenylbiguanide (4-CPB)

Fig. 1. Structural formulas.

Caucasians [5] and Africans [6] and 13–23% of Oriental populations [7]. Recently extremely high frequencies of CYP2C19 mutations have been reported in two malaria endemic islands in Vanuatu [8]. Few data are reported on the relationships between phenotype, PG and metabolite concentration and antimalarial response.

For field studies of antimalarials, it is essential to have a simple, rapid and reliable sampling technique for the determination of drug levels in blood. Dried blood from a finger prick on sampling paper provides a suitable delivery system, as the sampling procedure is less invasive than for venipuncture. Furthermore, the method permits transport when the ambient temperature precludes the use of whole blood. However, in this sampling method, the analytes to be measured must be stable to drying and be efficiently eluted from the sampling paper. Methods have been published for paper sampling and assay of antimalarial in review [9] and later for quinine [10] and mefloquine [11,12]. A paper sampling method for determination of PG and metabolites has been presented by Kolawale et al. [13].

The elution and extraction of the analytes from paper sampling is an important step preceding high-performance liquid chromatography (HPLC) quantification. In recent years, a large variety of solid-phase methods have been developed and evaluated. Solid-phase extraction (SPE) has been used for the isolation of samples collected to sampling paper for PG and its metabolites [14]. Our first test with Whatman ET 31 chromatographic sampling paper, which is commonly used as collection sampling paper for antimalarial drugs [10–12] in the field, gave a very low recovery for PG (about 30–40%) if the sampling paper was kept at +37°C, and about 50% at −20°C. The decrease in recovery was not a stability problem, but rather a question of a very hard binding of PG to the cellulose matrix of the sampling paper. No publications have been presented on testing of different brands or ''compositions'' of the sampling paper e.g., cellulose matrix to achieve optimal elution of the drugs and metabolites.

The aim of the present work was to extend the utility of our improved validated HPLC method for the determination of PG and its metabolites [15], with the development and validation of an acceptable routine clinical assay method for the collection and analysis of capillary whole blood, for use in the field monitoring of PG and its metabolites.

2. Experimental

2.1. Chromatography

The HPLC system consisted of a SP-8810 HPLC pump (Spectra-Physics, San Jose, CA, USA) and a WISP 710B autoinjector (Waters, Milford, MA, USA) with a Spectroflow 757 variable-wavelength UV detector set at 240 nm (Kratos, Ramsey, NJ, USA). The analytical column was a Zorbax SB-CN column (5 μm, 25 cm×4.6 mm I.D.), (Rockland Technologies, Newport, DC, USA). The peaks were evaluated with a Shimadzu C-R5A integrator (Shimadzu, Kyoto, Japan). The mobile phase was acetonitrile–0.1 mol/l phosphate buffer, pH 2.6 (21.5:78.5, v/v) and was degassed by ultrasonic agitation. The flow-rate of the mobile phase through the system was set to 1.0 ml/min.

2.2. Chemicals and reagents

HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). PG, CG, 4-CPB and the internal standard (I.S.) chlorcycloguanil were obtained from Zeneca (Cheshire, UK). Dodecyldimetyletylammonium bromide (DOD) was obtained from Sigma (St. Louis, MO, USA) and Nonidet P40 ethylphenol-poly(ethyleneglycolether) (NP-40) from Merck. All other reagents and chemicals used were of HPLC or analytical grade from Merck.

2.3. Calibration

Stock solutions of PG, CG and 4-CPB were prepared by dissolving appropriate amounts in to distilled water to give 500 μmol/l of each and stored at −20°C. Whole blood calibration samples containing the analytes were prepared by serial dilution over the concentration range of 50–6000 nmol/l and 100-μl volumes of each calibration sample applied to sampling paper, dried and stored at −20°C until calibration. The calibration samples were extracted together with the patient samples in the assay procedure. A linear calibration graph was obtained by plotting analytes–I.S. peak-areas ratios versus concentration.

2.4. Elution of PG, CG and 4-CPB from blood spots dried on sampling paper

The dried blood spots on the various sampling paper were cut into small pieces and transferred to polypropylene tubes, whereby 5.0 ml of 0.9 mol/l aqueous ammonia and 50 μl 5 mmol/l I.S. in water were added. The tubes were vortex-mixed, shaken on a mechanical shaker for 30 min and place in an ultrasonic bath at room temperature for 30 min. The tubes were then left standing for 60 min before the ammonia phase were added to the SPE column.

2.5. Solid-phase extraction

We used an IST VacMaster 20 sample preparation system, (International Sorbent Technology, Hengoed, UK) using endcapped (EC) columns CN, containing 100 mg cyanopropyl bonded silica gel. The SPE columns were preconditioned with 2×1.0 ml methanol followed by 2×1.0 ml water, and vacuum was applied after the addition of each solvent. After centrifugation of the tubes at 3000 *g* for 5 min the ammonia eluate was then applied to preconditioned SPE columns and moved slowly into the columns with vacuum. The columns were then washed with 2×1.0 ml water and 2×1.0 ml methanol. The PG, CG, 4-CPB and I.S. analytes were eluted from the SPE columns with 1×1.0 ml of methanol–HCl (99.9:0.1, v/v), applying the vacuum to dryness at this step only. The eluates were then evaporated to dryness under a stream of air and the residues reconstituted into 200 μl of HCl (1 mmol/l). A 150-μl volume of each eluate was injected onto the HPLC system.

2.6. Recycling of SPE columns

We used the CN (EC) columns for SPE extraction four times by reconditioning the columns with subsequent washings of 2×1 ml of (1) methanol–HCl (99.5:0.5, v/v), (2) acetonitrile, (3) 0.1 mol/l phosphate buffer, pH 2.6, (4) 0.1 mol/l phosphate buffer, pH 10 and (5) distilled water. No variation in recovery or precision was observed in relation to new columns. Recycling for four times was the limit of our evaluation and not necessarily the limit of their utility.

2.7. Source of blood samples for comparison of sampling methods

The paper sampling method was validated by comparing concentrations of PG, CG and 4-CPB in venous and capillary blood samples collected simultaneously.

Samples were taken from two healthy volunteers 2–48 h after oral intake of 200 mg proguanil (Paludrine). Whole blood samples were drawn by venipuncture into heparinized Vacutainer tubes. A 100-μl capillary blood sample was taken by finger prick using 100 μl heparinized precision capillaries; Blodcaps, Kebo-Lab (Stockholm, Sweden) and applied to a 5×5 cm piece of ET 31 Chr DOD-treated sampling paper. Whole blood samples were diluted

1:2 with water and all samples were stored at −25°C until analysis.

2.8. Elution performance of PG and metabolites from the sampling papers

The elution performance for PG, CG and 4-CPB from the various test sampling papers in Table 1 was evaluated by analysing samples of 100 μl whole blood, spiked with the analytes at 1000 nmol/l and applied to a 5×5 cm piece of sampling papers. The elution of the analytes from sampling paper stored at 37°C for five days, 25°C for four days and −20°C for 12 days was determined and compared with the peak heights from direct injection of the analytes stored in 1 mmol/l HCl at −20°C. All the specimens were assayed in duplicate.

2.9. Deactivation of ET 31 sampling paper

The ET 31 sampling paper was treated with 5 or 10 mmol/l DOD and 5 or 10 mmol/l of NP-40 by wetting paper pieces of 50×5 cm during 60 s and then left to dry at room temperature for about 24 h.

2.10. Elution performance of DOD and NP-40-treated ET 31 sampling paper

The elution performance of PG, CG and 4-CPB from DOD and NP-40-treated ET 31 sampling paper was evaluated by analysis of 100 μl whole blood spiked with the analytes at 1000 nmol/l and applied to treated ET 31 sampling paper, stored at 37°C and −20°C for 12 days. The eluted analytes from the treated sampling paper were determined and the results compared with those from the direct injection of the analytes stored in 1 mmol/l HCl at −20°C. Peak height responses of the analytes were used in the calculations. All the specimens were assayed in duplicate.

2.11. Intra- and inter-assay precision of DOD-treated ET 31 sampling paper

Whole blood was spiked with different concentrations of PG, CG and 4-CPB at four concentration levels and 100 μl was applied to DOD-treated ET 31 sampling paper. Three samples for each concentration were analyzed on three separate days. Concentrations were determined using a calibration graph prepared on the day of analysis. Intra- and inter-assay precision was calculated from the results.

2.12. Accuracy of capillary sampling onto DOD-treated ET 31 sampling paper

Accuracy was tested by analysis of simultaneous collected capillary blood applied to treated ET 31 sampling paper and venous whole blood from two volunteers. The whole blood was analyzed by a validated HPLC method [15]. The sampling paper was dried and kept at −20°C until assay according to the procedure.

Table 1
Selection of sampling paper tested for sampling of proguanil and metabolites

Company	Name	Abbreviation	Paper matrix
			Cationic paper
Whatman[a]	C/CM 30	C/CM 30	Cellulose with carboxymethyl groups
	C/CM 50	C/CM 50	Cellulose with carboxymethyl groups
	C/P 30	C/P 30	Cellulose with orthophosphate groups
	P 81	P 81	Cellulose with orthophosphate groups
			Anionic paper
	C/DE 30	C/DE 30	Cellulose with diethylaminoethyl groups
	DE 81	DE 81	Cellulose with diethylaminoethyl groups
			Cellulose
	ET 31 Chr	ET 31	Cellulose

[a] Whatman International, Maidstone, UK.

2.13. Quality control

Quality control (QC) samples were prepared from whole blood spiked with 4-CPB and CG at 60 nmol/l and PG at 500 nmol/l, 100 μl of these QC samples were added to DOD-treated ET 31 sampling paper. The QC samples were analyzed 3–4 times in every assay run over a period of two months to monitor assay performance with regard to accuracy and precision, alongside the assay of clinical blood paper samples with the presented method.

3. Results and discussion

3.1. System performance

Fig. 2 shows chromatograms from DOD-treated ET 31 sampling paper, from blank whole blood and capillary blood from one poor and one extensive metabolizer. The method was free from chromatographic interference from endogenous compounds and the commonly use antimalarial drugs.

3.2. Elution performance of PG and metabolites from the sampling papers

For elution of PG from the various Whatman sampling papers ET 31, C/P 30, C/DE 30 and DE 81 a low recovery (<60%) was observed and elution recovery from for C/CM 30, C/CM 50 and P 81 was >60% at the tested storage temperatures as seen in Table 2. The elution recovery seems to decrease at higher temperatures. We have recently improved the SPE extraction technique for PG, CG and 4-CPB in plasma [15] and we achieve a recovery of about 82% for PG. We believe that the low recovery for PG at pure cellulose sampling paper is due, to very strong

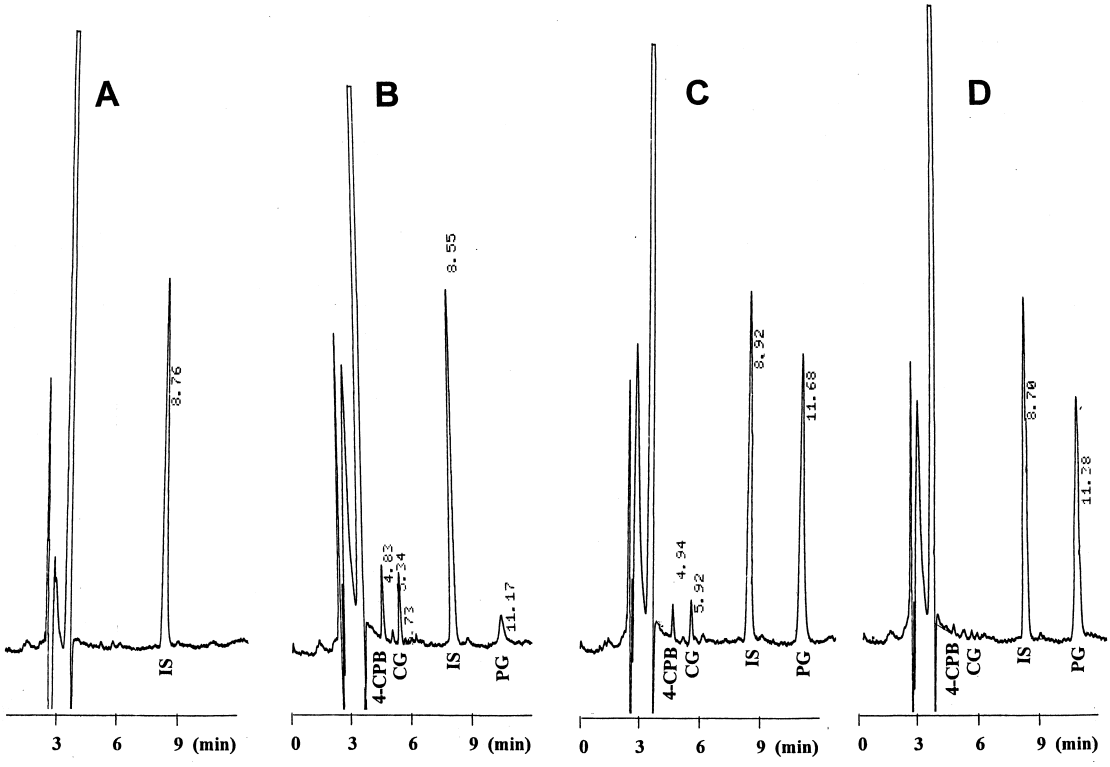


Fig. 2. HPLC chromatograms of separation of PG, CG, 4-CPG and I.S. after elution of capillary blood from ET 31 treated sampling paper and SPE extraktion. (A) Blank blood. (B) Calibration; blood spiked with 300 nmol/l of PG, CG and 4-CPG. (C) Extensive metabolizer: capillary blood obtained from a volunteer after an oral dose of 200 mg proguanil. The sample was taken 6 h after intake of the dose. (D) Poor metabolizer: the same dose and sampling conditions as C. Chromatographic conditions as described in Section 2.1.

Table 2
Influence of elution performance recovery of PG, CG and 4-CPB stored at different sampling papers, temperature and time

	Mean from two replicates (%)								
	+37°C, 5 days			+23°C, 4 days			−20°C, 12 days		
Paper	4-CPB	CG	PG	4-CPB	CG	PG	4-CPB	CG	PG
Cationic									
C/CM 30	79	57	69	86	62	81	79	81	68
C/CM 50	75	53	60	83	60	76	79	79	67
C/P 30	66	48	58	82	59	77	68	69	62
P 81	73	53	67	80	59	81	78	78	73
Anionic									
C/DE 30	70	53	33	82	63	46	82	88	45
DE 81	63	45	29	88	61	44	88	87	46
Cellulose									
ET 31	75	81	32	78	88	42	76	81	46

hydrogen bonding between the amino group in PG and the –OH and –COOH groups of the cellulose matrix. The cationic sampling papers seems to have a higher recovery possible due to an ion-exchange reaction between the PG and the ionic groups in the sampling paper. The recoveries using the anionic sampling papers (C/DE 30 and DE81) were very low, which support this hypothesis. In blood the pH is between 7.30 and 7.50 and the analyte (PG), which is a very strong base, becomes positively charged at the amine group at this pH. The metabolites CG and 4-CPG exhibit higher elution recoveries with all the tested sampling papers.

3.3. Deactivation of ET 31 sampling paper

A drawback with the ion-exchange sampling papers as sampling papers is that these papers are very thin, resulting in relatively long time for applied blood to dry. The pure cellulose sampling papers (ET 31) are thicker and the blood dries faster. To take advantage of the more optimal hydrodynamic properties of the ET 31 cellulose sampling paper, our strategy was to decrease the hydrogen bond activity of the –OH and –COOH groups at the cellulose matrix by treating the ET 31 sampling paper with the non-ionic surfactant Nonidet P-40 (NP-40) or the quaternary ammonium compound (DOD) before application of the blood spiked with PG, CG and CPB. Table 3 shows that the recovery of PG increased if the sampling paper was treated with NP-40 or DOD. The –OH groups in pure cellulose paper give a very high electron density on the surface. The recovery increases with increasing concentration of NP-40 or DOD, as well as with decreasing temperature. The elution recovery for 4-CPB and CG did not increase in the same manner as PG. The conclusion to these improvements in elution recovery for PG is that the surfactant (NP-40) and the quaternary hydrophilic compound DOD deactivate the charged cellulose matrix. The deactivation seems to be more efficient for DOD than for NP-40 since the quaternary ammonium compound has both

Table 3
Comparison of treatment and no treatment of ET 31 sampling paper on elution performance at two different temperatures with spiked analytes at a concentration of 1000 nmol/l

	Recovery (%), (mean of two replicates)					
	+37°C, 12 days			−20°C, 12 days		
Treatment	4-CPB	CG	PG	4-CPB	CG	PG
No treatment	77	84	35	77	84	48
Treatment						
5 m*M* NP-40	78	85	61	83	89	69
10 m*M* NP-40	77	87	66	80	85	69
Treatment						
5 m*M* DOD	77	83	74	80	95	81
10 m*M* DOD	81	96	83	84	87	84

Table 4
Intra- and inter-assay precision of PG, CG and 4-CPB from ET 31 DOD-treated sampling paper

Analyte	Intra-assay			Inter-assay		
	Determined concentration mean, (nmol/l)	No. of replicates	C.V. (%)	Determined concentration mean, (nmol/l)	No. of replicates	C.V. (%)
PG	126	9	19	126	3	22
	202	9	11	202	3	11
	432	9	10	432	3	12
	2100	9	4.4	2100	3	7.2
CG	37	9	17	37	3	18
	83	9	7.1	83	3	7.2
	192	9	5.3	192	3	6.0
	396	9	8.7	396	3	9.4
4-CPB	52	9	5.8	52	3	8.7
	77	9	6.9	77	3	7.4
	183	9	7.2	183	3	7.5
	457	9	13	457	3	15

hydrophobic and hydrophilic moieties, which improve the elution recovery of PG and metabolites.

3.4. Intra- and inter-assay precision of DOD-treated ET 31 sampling paper and limit of determination

The assay precision is summarised in Table 4. The limit of determination is estimated to be 50 nmol/l for CG and 4-CPB and about 125 nmol/l for PG, with an inter-assay precision of 15–20%. We have prioritised the limit of determination for the metabolites, since the level of proguanil in patients sample are 4000–6000 nmol/l. In poor metabolizers the concentration of CG and 4-CPB are less than 50 nmol/l [16]. The precision as coefficient of variation (C.V.) and accuracy of the heparinized 100 μl capillary tubes was <0.5% with a relative volume error of 2.1% ($n=10$). These results are comparable to use of a 100-μl precision pipette used for pipetting whole blood.

3.5. Accuracy of DOD-treated ET 31 sampling paper

No difference was found between capillary blood sampling to ET 31 DOD-treated paper and venous blood sampling. Fig. 3 shows a comparison of results from capillary sampling and venous sampling whole blood from one volunteer who received 200 mg proguanil. The curves have a similar profile for the other volunteer.

3.6. Quality control

The precision and accuracy results from the QC samples analyzed daily in routine assay over a period of two months are presented in Table 5. The QC

Table 5
Quality control in routine assay of PG, CG and 4-CPB from ET 31 DOD-treated sampling paper

Analyte added concentration (nmol/l)	Determined concentration mean, (nmol/l)	Intra-assay		Inter-assay	
		No. of replicates	C.V. (%)	No. of replicates	C.V. (%)
PG; 500	489	21	5.8	7	10
CG; 60	59	24	20	7	21
4-CPB; 60	58	22	16	7	17

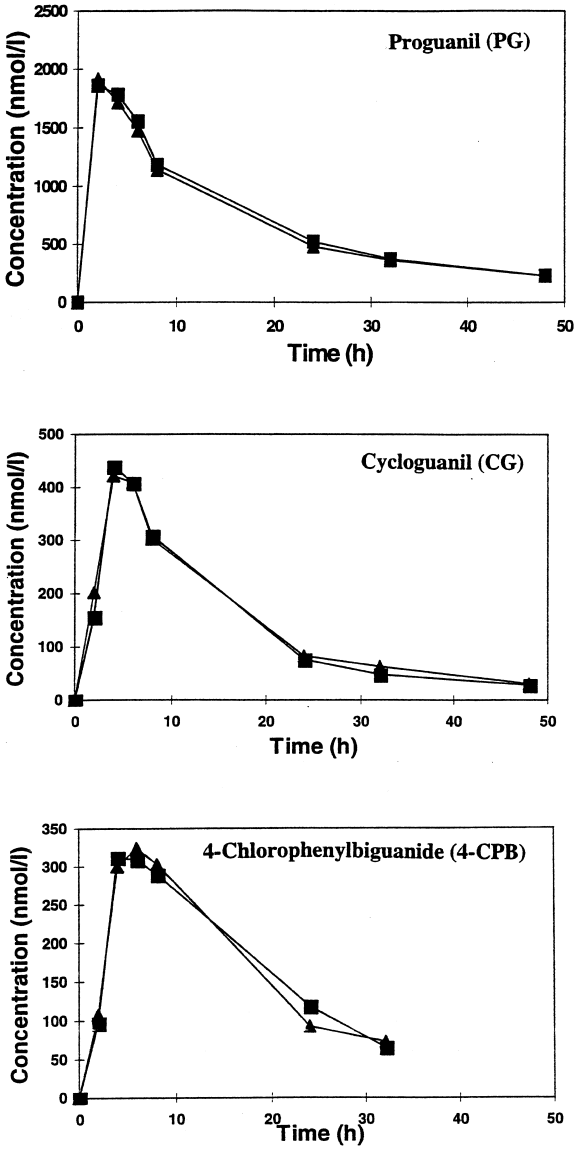


Fig. 3. PG, CG and 4-CPB concentration–time profiles in whole blood (■) and capillary blood (▲) applied to ET 31 DOD treated sampling paper from a healthy human after a single oral dose of 200 mg of Paludrine.

results give an estimation of the precision and the accuracy during the determination of the analytes sampled on to treated ET 31 sampling paper in our routine laboratory.

3.7. Stability of DOD-treated ET 31 sampling paper

No decrease in concentration was found during storage between +37 to −20°C for a storage period of 30 days. The clinically accepted decrease of PG and metabolites during transport of samples will vary somewhat with the application. For most clinically purposes a bias of 10% maximum is acceptable, including the assay precision.

4. Conclusions

The use of SPE with cyanopropyl columns and HPLC with a stable bond cyanopropyl column has improved the determination of PG and metabolites in capillary blood samples onto sampling paper. The DOD-treated ET 31 sampling paper gives high recovery and high stability when the sampling paper is kept at up to +37°C, e.g., tropical temperatures, for several weeks. Our conclusion from the testing of different sampling papers for capillary blood sampling is that the treatment of ET 31 paper with DOD seems to give the highest elution recovery, in comparison to the other sampling papers. Another advantage with the ET 31 sampling paper is that this sampling paper is more suitable for application of capillary blood since this sampling paper is thicker than the ion-exchange sampling papers. A 100-μl capillary blood sample gives a spot of about 20–30 mm diameter, with a rapid absorption of blood into ET 31 sampling paper. Both sides are dry within 30 s. The results from the determination of PG and metabolites in capillary blood sampled onto DOD-treated ET 31 sampling paper agreed quite well with results from the determination of PG and metabolites in venous blood.

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