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On-line solid-phase extraction with automated cartridge exchange for liquid chromatographic determination of lipophilic antioxidants in plasma

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ABSTRACT

A fully automated liquid chromatographic method is described based on a Prospekt solid-phase extraction unit for the analysis of lipophilic indenoindolic antioxidants in plasma. Plasma samples, mixed with internal standard, were injected onto C_8-cartridges. After washing, the samples were eluted and transferred to a C_8-analytical column, where separation was performed. The eluent was monitored by electrochemical detection. Owing to the nature of the drugs investigated care had to be taken to avoid adsorption losses in vials and capillaries. The method, which was found to give excellent recoveries (100.9%, $n = 8$) and repeatability (R.S.D. ± 1.7%), is time-saving compared to a previously used assay with sample work-up by liquid–liquid extraction.

INTRODUCTION

The fully automated sample handling system Prospekt was first described in 1987 by Nielen *et al.* [1] and so far only a few papers regarding applications using this system have been published [1–3]. We found the Prospekt system attractive as it allows a complete on-line analysis with disposable extraction cartridges at reasonable cost. The cartridge is automatically replaced for every new sample, which means that plasma samples can be injected directly into the chromatographic system without giving problems with blockage by protein precipitate. A similar system OSP-2 with automated cartridge exchange is commercially available from E. Merck [4–6]. Automated techniques are also available using the robotic Zymark system [7] and the ASPEC system from Gilson [8,9].

In systems where the extraction cartridge is an integrated part of the liquid chromatograph, the flow through the cartridge is constant and well defined. This should lend to better reproducibility compared with conventional liquid–solid extraction systems.

In this study we have used the Prospekt system for analysing an indenoindolic antioxidant, H 290/51, in plasma samples in connection with pharmacokinetic studies, and the method has been evaluated against a procedure employing liquid–liquid extraction.

EXPERIMENTAL

Chemicals and reagents

H 290/51, H 290/39 and H 290/58 as hydrochlorides, and H 261/54 (Fig. 1) were synthesized at Medicinal Chemistry, Astra Hässle AB (Mölndal, Sweden). Hydrochloric acid was of analytical grade, Merck (Darmstadt, Germany) and tris(hydroxymethyl)aminomethane (Tris), analytical grade, was of Fluka quality (Buchs, Switzerland). Acetonitrile and methanol were of

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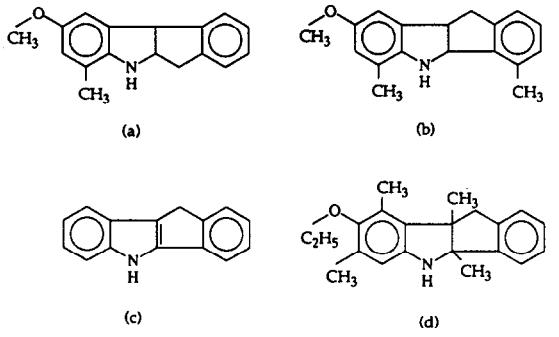

Fig. 1. Structural formulas of (a) H 290/51, (b) H 290/39, (c) H 261/54 and (d) H 290/58.

HPLC grade from Rathburn (Walkerburn, UK) and ethanol (95%) came from Kemetyl (Stockholm, Sweden). Water was purified through an ELGA purification system, ELGA (Wycombe Bucks, UK).

Chromatographic system

A schematic representation of the chromatographic system is shown in Fig. 2. It comprised a Gynkotek Model 480 pump, (Munich, Germany), a CMA/200 refrigerated autosampler (CMA Microdialysis, Stockholm, Sweden), a Spark Holland Prospekt module with a microprocessor, a cartridge transport system, three six-port valves and a solvent delivery unit (SDU) (Emmen, Netherlands), with the capability of delivering up to six solvents. Glass vials (0.3 ml) from Chromacol (London, UK) and plastic vials (0.3 ml) from CMA Microdialysis (Stockholm, Sweden) have been used. Plastic tips, Finntip for pipettes, came from Labsystems Oy (Helsinki, Finland). The solid-phase extraction cartridges (10 × 2 mm I.D.) contained 20 mg of Analytichem C_8, C_{18} or CN packings (30–40 μm) and were distributed by Spark Holland. The analytical columns (150 × 4.6 mm I.D.) Kromasil C_8 (5 μm) and Hypersil CN (5 μm) came from Eka Nobel (Bohus, Sweden) and Shandon (Astmoor, UK), respectively.

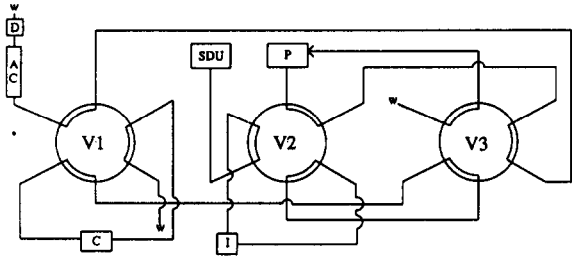

Fig. 2. Schematic representation of the chromatographic system. V1, V2, V3 = valves belonging to the Prospekt unit; SDU = solvent delivery unit; P = pump; I = injector; C = cartridge; AC = analytical column; D = detector; w = waste.

The mobile phase, pH 8.5, contained Tris (50 m*M*), hydrochloric acid (12 m*M*) and 65% of acetonitrile. The three solutions used for conditioning and loading of the cartridge were (1) 100% methanol, (2) 5% of methanol in water and (3) Tris (50 m*M*), hydrochloric acid (12 m*M*), pH 8.5, with 15% of acetonitrile. Prior to use the solutions and the mobile phase were ultrasonicated for degassing. The flow-rate over the analytical column was 1.0 ml/min and the eluent was monitored by a Waters M460 electrochemical detector (Millipore, Milford, MA, USA) at a potential of +0.70 V. Data was collected and processed by a Multichrom chromatographic data system (VG Data Systems, Altrincham, UK).

Sample preparation procedure

The frozen plasma samples were thawed at room temperature, mixed and placed on ice. Aliquots of 100 μl of the samples were pipetted into 1.5-ml glass vials and 100 μl of the internal standard solution of H 290/39 in Tris buffer pH 8.5 with 15% acetonitrile were added. The samples were mixed and centrifuged for 5 min at 3000 *g*. After centrifugation the samples were transferred to glass vials (0.3 ml) and placed in the autosampler. Aliquots of 20–50 μl of the samples were injected.

Plasma reference samples were prepared by mixing 100 μl of a solution containing H 290/51 and H 290/39 in Tris buffer pH 8.5 with 15% of acetonitrile, and 100 μl of drug-free plasma.

Solid-phase extraction procedure

The extraction cartridge connected to valve 1 (see Fig. 2) was activated with 2 ml of solvent 1 (100% methanol) followed by 2 ml of solvent 2

(5% methanol in water) and *ca.* 4 ml of solvent 3 (Tris buffer pH 8.5 with 15% acetonitrile). During the time of activation the autosampler was prepared for injection. Prior to injection the flow-rate over the cartridge was changed from 2 ml/min to 0.5 ml/min. By a signal from the microprocessor the sample was injected into the solid-phase extraction system. The plasma sample was applied to the cartridge and most of the plasma matrix was removed with solvent 3. After 1.5 min of loading valve 1 was switched, and the mobile phase eluted the compounds backwards onto the analytical column for separation and detection. At the same time data collection started. After 5 min of elution, valve 1 switched back and washing of the capillaries started with 3 ml of solvent 2, followed by 4 ml of solvent 1. The procedure is summarized in Table I.

We have used valve 2 to be able to make direct injections onto the analytical column. This was done to check the condition of the analytical column and to determine recoveries of the extraction procedure. By switching valve 2 the sample goes directly from the injector to the analytical column for separation and detection, see Fig. 2.

By switching valve 3 it is possible to utilize the solvent delivery unit for washing of the analytical column after long series of plasma samples to prevent deterioration.

RESULTS AND DISCUSSION

Acid-labile conjugate

H 290/51 can also exist as an acid-labile conjugate in authentic plasma samples. It was therefore necessary to keep the pH above 8 in order not to hydrolyse the conjugate and get falsely increased results. This was examined by adding buffer at pH 2, pH 7 and pH 9 to authentic rabbit plasma samples. The samples were allowed to stand for 20 min before the internal standard in Tris buffer (pH 8.5) was added. The samples were then analysed according to the described method. The results were similar at pH 7 and 9, whereas at pH 2 there was a three-fold increase in concentration. In plasma samples containing less conjugate, such as dog and rat plasma, the increase was not as pronounced as in rabbit plasma. In one strain of rabbits the conjugate existed to a large extent, up to 10 times the concentration of free drug. It was then difficult to stabilize the conjugate and the thawed samples were injected immediately and not allowed to stand in the injector. All

TABLE I

SOLID-PHASE EXTRACTION PROCEDURE

Time (min:s)	Switch valve No.	Solvent	Flow (ml/min)	Comment
00:00		1	2.0	Change of cartridge Activation of cartridge with 100% methanol
01:00		2		Activation of cartridge with 5% methanol in water
02:00		3		Conditioning of cartridge with Tris buffer pH 8.5 containing 15% acetonitrile
03:50			0.5	Adjusting flow-rate through cartridge
04:00				Injection of sample
05:30	1			Start of elution backwards onto the analytical column Start of data collection
10:30	1	2	2.0	End of elution Washing of cartridge and capillaries with 5% methanol in water
12:00		1		Washing with 100% methanol
14:00			0.0	End of washing

aqueous buffer solutions used had a pH of 8.5 to avoid hydrolysing any acid-labile conjugate.

Adsorption

At a pH above 8 adsorption losses in vials have to be taken into consideration. As can be seen in Table II adsorption was significant in plastic vials for both H 290/51 (3.5 μM) and H 290/39 (3.5 μM). The figures show adsorption losses in plastic vials compared with glass vials. The amount adsorbed increased with increasing pH but the presence of plasma decreased the adsorption losses considerably. No adsorption could be seen in glass vials containing a buffer of pH 8.5 with 7.5% of acetonitrile.

Adsorption may also occur in the capillaries between the injector and the cartridge. Adding more than 20% of acetonitrile to solvent 3 to avoid adsorption in the capillaries decreased the recovery of the solid-phase extraction procedure to below 90%, while with 15% acetonitrile, as used in the method, the adsorption was inhibited without lowering the recovery. One way to demonstrate adsorption effects in the capillaries is to switch valve 2 1 min before elution starts. The mobile phase with its high percentage of acetonitrile (65%) will desorb the amount that has been adsorbed in the injector and onto the capillary walls between the injector and valve 2.

We have demonstrated this by using a Tris buffer at pH 8.5 without acetonitrile as solvent 3. The amount adsorbed can be seen in the chromatogram (Fig. 3) as two minor peaks eluting approximately 1 min before the main components. The sample in the chromatogram contained H 290/51 (3.5 μM) and H 290/39 (3.5 μM). The sample was loaded onto a C_8-cartridge with Tris buffer pH 8.5 for 2 min.

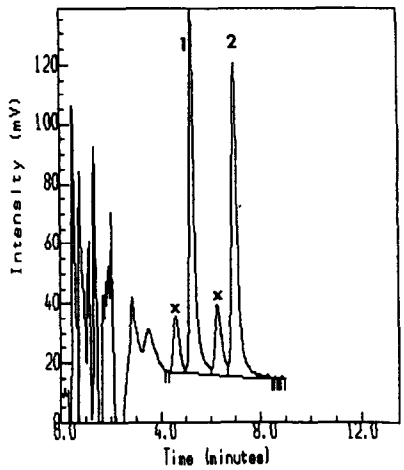

Fig. 3. Adsorption in capillaries when using Tris buffer pH 8.5 without acetonitrile as loading solution (solvent 3). 1 = H 290/51; 2 = H 290/39; × = substance adsorbed in capillaries between injector and valve 2.

In a previously used liquid–liquid extraction method, using 5% butan-2-ol in hexane at alkaline pH, followed by evaporation and reconstitution in an acidic solution, the chromatographic system consisted of a Hypersil CN analytical column and a mobile phase at pH 7 with 40% of acetonitrile. Under these conditions no adsorption occurred in vials or capillaries. When using the Prospekt unit, where plasma is directly injected into the chromatographic system, we had to use a pH above 8 because of the earlier mentioned acid-labile conjugate. To prevent adsorption in the chromatographic system we increased the amount of acetonitrile in the mobile phase by 20%. The analytical column was changed from Hypersil CN to Kromasil C_8 to obtain reasonable retention times.

The original Prospekt unit contained a Marathon autosampler, that we exchanged for a CMA/200 autosampler because of its insufficient washing capability for this particular application. The Marathon washes the needle and the loop, using alternately one out of four vials containing

TABLE II

ADSORPTION LOSSES IN PLASTIC VIALS COMPARED WITH GLASS VIALS

Buffer solutions contained 7.5% of acetonitrile. Plasma samples contained 7.5% of acetonitrile after dilution with buffer solution (1:1).

pH	H 290/51		H 290/39	
	Solution	Plasma	Solution	Plasma
7	13%	8%	41%	12%
9	21%	8%	54%	13%
10	24%	15%	63%	21%
9[a]	39%	16%	80%	23%

[a] No acetonitrile added.

washing solution, and the washing volume was not variable. These lipophilic compounds were adsorbed in the needle and to the rotorseal in the injection valve giving contamination between samples. The CMA/200 sucks washing solution from a separate bottle and there is no risk of contamination of the solution. The washing volume is also variable.

The CMA/200 autosampler cannot fully communicate with the Prospekt microprocessor as the Marathon does, but it is possible to give the CMA/200 a signal to start loading sample and to keep the sample in the loop until it receives an injection signal, by using the Prospekt's auxiliary outputs.

More lipophilic compounds

We have also tested the described method to see if it was possible to handle compounds even more inclined to adsorb in capillaries and vials. H 261/54 which is a dihydroindenoindole (see Fig. 1) gave severe adsorption in capillaries also for plasma samples. Increasing the amount of acetonitrile in solvent 3 to 20% still gave adsorption on capillary walls for H 261/54 along with a decreasing recovery of H 290/51 which was used as internal standard. We changed the internal standard to H 290/58 (see Fig. 1), which is more strongly retarded on C_8 cartridges compared with H 290/51, and could then increase the amount of acetonitrile in solvent 3 to 30% and avoid adsorption in capillaries with a maintained recovery of 99%.

Stability

The stock solutions of H 290/51 (60 μM) and the internal standard H 290/39 (60 μM) dissolved in 95% ethanol were stable in a refrigerator for at least five months. The working standard solutions of H 290/51 (3.5 μM) and H 290/39 (3.5 μM) diluted 17 times in Tris buffer pH 8.5 were stable in a refrigerator for more than 3 days when kept from light. Authentic plasma samples stored at −70°C were stable for up to one year. In the injector with a refrigerated sample tray, authentic plasma samples and standard addition samples were stable for at least 24 h, except for samples from one strain of rabbits, as mentioned earlier.

Solid-phase extraction cartridges

The recovery of H 290/51 on C_8, C_{18} and CN cartridges was investigated as a function of loading time, which is the time between injection of sample onto the cartridge and start of elution. The flow, the amount of acetonitrile in solvent 3 and the elution time were kept constant at 0.5 ml/min, 15% and 5 min, respectively. The recovery was constant for C_8 and C_{18} cartridges when varying the loading time from 0.5 to 2.5 min, but for the CN cartridges it rapidly decreased after 1 min. No change in plate count of the separation column could be seen for any of the cartridges investigated when varying the loading time. The pressure over the analytical column increased at an loading time of less than 1 min.

To ensure that sample work-up conditions do not have to be modified due to inter-batch variation of cartridges under routine analysis, three different batches of C_8 cartridges were tested for recovery and repeatability. There was no difference between the three batches. Since then we have used several batches of C_8 cartridges and not found any differences between them.

Optimization

The importance of the factors which were expected to influence the extraction recovery and the ruggedness of the method was investigated.

A statistical full factorial experimental design [10] was initially performed and the 4 factors studied were (1) amount of acetonitrile in the washing solution (solvent 3 in Table I), (2) flow-rate through the cartridge, (3) loading time, *i.e.* time between injection of sample onto the cartridge and start of elution and (4) elution time, *i.e.* time from start of elution of the cartridge till disconnection from the analytical column.

Each factor was studied at two levels, for factor (1) 10 and 20% of acetonitrile, factor (2) 0.5 and 1.0 ml/min, factor (3) 0.5 and 2.5 min and for factor (4) 1 and 5 min. For 4 factors there are sixteen (2^4) possible combinations of factor levels. The experiments were made in duplicate and moreover one of the recovery experiments was performed at a level in between, *i.e.* (1) 15%, (2) 0.75 ml/min, (3) 1.5

min and (4) 3 min. This was repeated four times to provide an indication of the repeatability of the procedure. A statistical analysis of variance (ANOVA) [10] was performed to evaluate the effects of the experimental variables. The elution time had no statistically significant effect on the recovery, while, as expected, a high amount of acetonitrile in the washing solution in combination with a long loading time and a high flow-rate resulted in low recoveries, 12% for H 290/51 and 53% for H 290/39.

We chose a flow-rate of 0.5 ml/min as it seemed to be advantageous in terms of chromatographic efficiency, calculated as number of theoretical plates. Referring to the tendency of the indenoindolic compounds to adsorb in the capillaries, a high concentration of acetonitrile, 15%, in the washing solution was preferable. The recovery was unaffected by a change in loading time from 0.5 to 2.5 min, giving a very robust method. Even a loading time of 4 min gave as high a recovery as 97%. The front peak was somewhat larger at a loading time of 0.5 min compared with 1.5 min as chosen.

Detection

The response of the electrochemical cell slowly decreased when injecting a long sequence of plasma samples. It was thus important to have an internal standard that behaved similar to H 290/51 and compensated for a minor decrease in response. It was possible to re-establish the response at its former level by polishing the working electrode surface with acetone. This was done every second day.

Quantitation and accuracy

The ratios of the peak height of the analyte to that of the internal standard in the plasma reference samples were measured and used for calculation of plasma concentration. The recoveries were calculated by comparing the peak height ratios of analyte to internal standard of plasma reference samples with those obtained for the same amount injected directly onto the analytical column.

The within-day variability was determined by performing replicate analyses of plasma samples containing 3.2 μM ($n = 10$), 55 nM ($n = 8$) and 20 nM ($n = 8$) of H 290/51 and 3.5 μM ($n = 26$) of H 290/39. The recoveries and coefficients of variation of H 290/51 were $104.1 \pm 0.8\%$, $100.9 \pm 1.7\%$ and $104.0 \pm 3.6\%$, respectively. The extraction yield of the internal standard calculated by direct comparison of peak heights in reference samples to those from direct injection onto the analytical column was $96.0 \pm 4.0\%$ ($n = 10$).

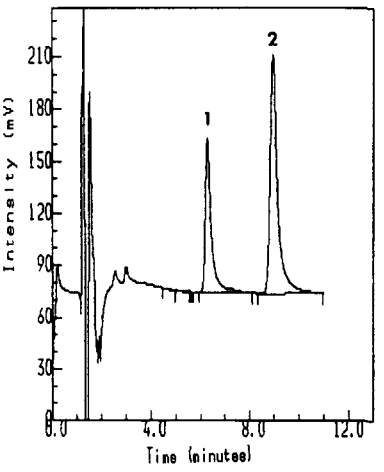

Fig. 4. Chromatogram of dog plasma sample. 1 = H 290/51: 1.75 μM and 2 = H 290/39 (internal standard): 3.5 μM. 20 μl of sample injected.

The within-cartridge variability ($n = 7$) was $101.7 \pm 0.5\%$ for a plasma sample containing 3.2 μM of H 290/51 and thus it was possible to

TABLE III

RESULTS FROM SOLID-PHASE EXTRACTION (SPE) METHOD COMPARED WITH LIQUID–LIQUID EXTRACTION (LLE) METHOD

The values are expressed as nmol/l ± R.S.D.

LLE method	SPE method	±R.S.D.(%)
349	370	4.1
491	526	4.9
900	960	4.6
3680	3860	3.4
4380	4680	4.7
5100	5500	5.3
8700	9000	2.4
9700	9840	1.0
14 100	14 400	1.5

re-use a cartridge at least 7 times without noticing any decrease in capacity. The analytical columns lasted for up to 1.5 months when using a mobile phase at pH 8.5.

The method was linear up to at least 50 μM and the limit of quantitation was estimated at 20 nM when injecting 10 μl of plasma. Fig. 4 shows a chromatogram of an authentic dog plasma sample containing 1.75 μM of H 290/51.

The method was evaluated by comparison analyses of dog plasma samples made with the earlier mentioned liquid–liquid extraction method. Good agreement between the two methods was found, as shown in Table III. The analyses with the liquid–liquid extraction method were performed one year earlier than those with the solid-phase extraction method.

CONCLUSION

Using the Prospekt solid-phase extraction unit for laboratory routine analyses is simple, straight forward and time-saving compared to regular liquid–liquid extraction procedures. The described method for determination of lipophilic indenoindolic compounds yielded high recoveries, good precision and accuracy.

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