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Short Communication

Analysis of sulthiame in serum by narrow-bore high-performance liquid chromatography

Comparison of direct sample injection with pre-column switching and extrelut extraction

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ABSTRACT

Two high-performance liquid chromatographic methods for the analysis of sulthiame in serum are described. In the first method direct injection of serum samples onto a 4 × 4 mm I.D. (C_{18}, 25 μm) precolumn in a column-switching device was used. After a purge step, the adsorbed analytes were eluted onto a 250 × 3 mm I.D. (C_{18}, 5 μm) narrow-bore column for chromatographic separation. In the second method the sample pretreatment was an Extrelut extraction with dichloromethane–propanol-2 (95:5). After evaporation of the solvents, the residue was dissolved in methanol. The chromatographic separation was carried out on the same analytical column as used in the column-switching method. Both sample pretreatment methods were compared with respect to their suitability of routine analysis of sera from patients also receiving other antiepileptic drugs.

INTRODUCTION

Sulthiame (tetrahydro-2-*p*-sulphamoyl-phenyl-2H-1,2-thiazine-1,1-dioxide) is a product of the condensation of a sulphonic acid and an amino group, with the elimination of water [1]. Sulthiame (SULT, trade name "Ospolot"] is used as an antiepileptic drug (AED) in the treatment of psychomotor and grand mal seizures [2]. Although it is seldom prescribed, we have received more than 200 serum samples containing this AED in the last year. It seems that Ospolot is again being prescribed in our epilepsy centre most often.

In addition to some gas chromatographic methods, only a few high-performance liquid chromatographic (HPLC) methods for the determination of SULT in body fluids were published in the late 1970s [2–5].

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The determination procedure of Berry *et al.* [2] seems to be the best developed. They found linearity in the concentration range 1.25–10 mg/l and were able to carry out a chromatographic separation of SULT and several other AEDs. Ethyltolylbarbituric acid (ETB) was used as an internal standard (I.S.). The same I.S. was used for our routine and research analyses of the common AEDs and their clinically relevant metabolites [6–8].

At first we developed a pre-column switching method for direct sample injection based on our system for the determination of drugs in serum and ultrafiltrates [9,10]. We mixed the serum samples with an appropriate buffer in order to lower protein binding and sample viscosity. The buffer included ETB as an I.S. in a concentration which was suitable for detection at a wavelength of 245 nm (UV maximum of SULT). We found linearity in the concentration range of SULT 1–40 μg/ml and a good reproducibility of the results.

But in some samples with additional AEDs we observed sample background peaks (supposedly endogenous compounds) which could interfere with the separation of SULT. We therefore developed an alternative method using serum extraction with Extrelut columns (diatomaceous earth) with which sample extracts free of the interference mentioned above were available in all cases.

MATERIALS AND METHODS

Apparatus

An HP 1090 LC with a workstation and column-switching software was obtained from Hewlett-Packard (Waldbronn, Germany). The precolumns used for the direct serum injection were LiChroCART guard columns (4 × 4 mm I.D.) filled with LiChrospher-100 RP-18 (25 μm) obtained from Merck (Darmstadt, Germany). The analytical column was a "narrow-bore" column (250 × 3 m I.D.) filled with Nucleosil-100 C_{18} (5 μm) supplied by MZ-Analysentechnik (Mainz, Germany).

Chemicals and reagents

Acetonitrile ChromAR was obtained from Promochem (Wesel, Germany), and all other reagents (including the Extrelut-1 columns) were obtained from Merck.

Sulthiame was obtained from Bayer (Leverkusen, Germany) and ETB from Aldrich (Steinheim, Germany).

Internal standard buffer. A 22-g aliquot of sodium dihydrogenphosphate monohydrate and 1 g of sodium azide were dissolved in 1000 ml of HPLC water to give an acidic buffer of pH 5. ETB (25 mg) was dissolved in 3 ml of methanol and mixed with the buffer.

Extraction solvent. Dichloromethane (950 ml) and propanol-2 (50 ml) were mixed.

Mobile phase buffer. A 1000-ml volume of 0.01% phosphoric acid was adjusted to pH 4.5 with a 10% triethylamine solution in water, mixed with acetonitrile (90:10 or 40:60, v/v), and filtered for storage at room temperature. With a minimum content of 10% acetonitrile the buffer mixtures are stable for a long time.

Calibration and control samples

SULT (10.0 mg) was dissolved in 5 ml of methanol and diluted with water

(containing 0.1% sodium azide to prevent the growth of micro-organisms at room temperature) to 100 ml in a measuring flask. From this stock solution (having a concentration of 100 μg/ml SULT) 40 ml, 20 ml, 10 ml, 5 ml and 1 ml were pipetted into 100-ml measuring flasks and filled up with the 0.1% sodium azide solution.

As control samples a 5% solution of bovine albumin in water with a concentration of 4 μg/m SULT and pooled patient samples were used. Portions of 500 μl were transferred to 2-ml vials and frozen at −18°C.

Sample preparation

A 500-μl portion (calibration samples, patient sera and thawed controls) was mixed with 1000 μl of the I.S. buffer.

Direct sample injection with pre-column switching

A volume of 100 μl of the above mixture was injected by the autosampler of the HPLC apparatus into the precolumn (see above), placed outside of the column heating chamber, and flushed with 0.01% phosphoric acid.

After washing for 2 min the valve was switched and the compounds retained were eluted onto the analytical column. The valve configuration was as given in ref. 14.

Extrelut extraction

A 1000-μl portion of the mixture (sample and I.S. buffer) was pipetted onto an Extrelut-1 column. After 10 min 5 ml of the extraction solvent (see above) were added. The solvents were evaporated under vacuum at 30°C and the analytes dissolved in 100 μl methanol. A 10-μl sample of the extract was injected into the same analytical column as used above.

Chromatographic parameters

The chromatographic separations were carried out as gradient elutions with a mobile phase consisting of acetonitrile and an alkylamine–phosphoric acid mixture [11].

The gradient–time profile is shown in Table I (A = direct sample injection with pre-column switching, B = Extrelut extracts). The stop time was set at 14.00 min (A) or 12.00 min (B) and the post time at 3.00 min. The flow-rate was 0.6 ml/min, the oven temperature 45°C and the detection wavelength 245 nm.

RESULTS AND DISCUSSION

First of all it was determined whether the common AEDs prescribed in our epilepsy centre and their metabolites (MBs) interfere with the chromatographic separation of SULT. As Table II shows, SULT is well separated from AEDs and their MBs, which could be found in the patient samples.

The next step was the investigation of the linearity of the calibration curve. Although SULT concentrations higher than 20 μg per millilitre of serum were not found in any of the patient samples, the calibration curve was measured with 40 μg/ml as the highest level of detection. The linearity was found to be sufficient in the range 1.0–40.0 μg/ml SULT with the slope of the regression line 0.997 and the coefficient of correlation of 0.998.

TABLE I

GRADIENT PROFILES OF THE 1090 LC FOR BOTH METHODS

A = Column-switching; B = Extrelut extracts; SDS = Solvent delivery system.

Time (min) SDS	Orthophosphoric acid (0.01%) I	Buffer–acetonitrile (%)	
		90:10 II	40:60 III
A			
0.00	100%	0	0
2.00	100%	0	0
2.01	0%	60	40
4.00	0%	60	40
9.00	0%	20	80
9.01	0%	60	40
B			
0.00	[a]	60	40
2.00	[a]	60	40
7.00	[a]	20	80
7.01	[a]	60	70

[a] Solvent delivery system A is shut off.

At the outset of our determinations of SULT in patient sera we used only the method of direct sample injection with pre-column switching. A lot of time for sample pretreatment can be saved using this method. Hence it can be recommended for pharmacological studies with Ospolot using volunteers who do not suffer from epilepsy, if it is checked that the blood of the volunteers contains no interfering compounds.

Direct sample injection is not recommended for the analysis of patient samples.

TABLE II

RETENTION TIMES RELATIVE TO ETB OF SULT, COMMON AEDs AND MBs

A = Taken from the column-switching method; B = taken from the Extrelut results.

Substance	Relative retention time	
	A	*B*
2-Ethyl-2-phenylmalone diamide (PEMA)	0.59	0.33
Ethosuximide (ET)	0.64	0.36
Primidone (PRI)	0.66	0.39
CBZ-10,11-diol (DIOL)	0.65	0.45
Sulthiame (SULT)	0.70	0.52
Phenobarbital (PB)	0.85	0.74
CBZ-10,11-epoxide (EPO)	0.87	0.79
N-Desmethyl methsuximide (DM)	0.89	0.78
5-Ethyl-5-(*p*-tolyl) barbituric acid (ETB)	1.00	1.00
Phenytoin (PT)	1.05	1.11
Carbamazepine (CBZ)	1.07	1.21

Interfering sample background was found in the first half of the chromatograms of some of the blood samples of patients in our epilepsy centre. Sometimes the baseline was not clean in the retention time window of SULT. Similar observations were made earlier, when we developed a direct injection method for the analysis of the common AEDs in serum [9].

The reason might be that the C_{18} pre-column material is used for solid phase extractions when a large number of serum samples are injected. The same effect was not observed when solid phase extractions with Bond-Elut cartridges were used. For the off-line extractions the cartridges were used only once, whereas the reversed-phase (RP) material in the precolumn was loaded with many serum injections in sequence.

Some of the proteins injected with the serum samples are irreversibly adsorbed on the RP materials of the precolumn [12]. The surface of the packing in the precolumn is "protein-coated" and hence has a higher adsorption capacity than the freshly packed RP material. Unknown endogenous compounds in the patient samples (of sera and to an even higher degree of saliva samples [13]) are adsorbed in the column-switching methods. They are not observed, however, when using C_{18} cartridges off-line. These compounds can interfere with the analytes of interest in the first part of the chromatograms. These interferences can affect all substances eluted before phenobarbital in the separation of the AEDs.

The patients in the epilepsy centre Bethel are normally not on monotherapy, but take two, three or more AEDs and additional co-drugs. For this reason direct sample injection with pre-column switching was, in spite of its time-saving property, not the best choice for our routine determinations of SULT in patient samples.

From our experience of the different off-line extraction procedures available, the sample extraction using Extrelut clean-up columns affords the cleanest drug extracts from biological samples. Thus we developed an extraction using Kieselguhr as an alternative to direct sample injection for the determination of SULT.

The chromatographic parameters for the separation of the analytes in the solvent extracts remained basically the same as for the column-switching method (see Materials and methods section). Nevertheless, the chromatograms look slightly different (compare Figs. 1 and 2), because in one case the compounds were flushed

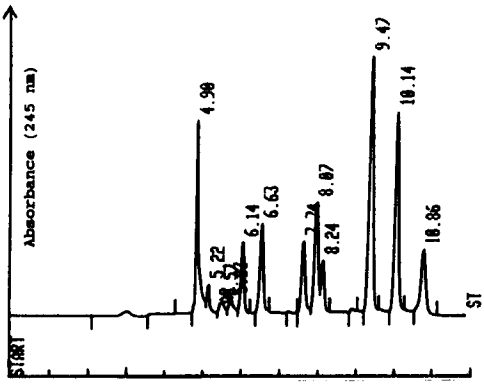

Fig. 1. Chromatogram from the direct injection of a serum containing Ospolot and other AEDs. Numbers at peaks indicate retention times in min; SULT (4.7 μg/ml), 6.63 min; ETB = I.S., 9.47 min. For the relative retention times of other AEDs, see Table IIA.

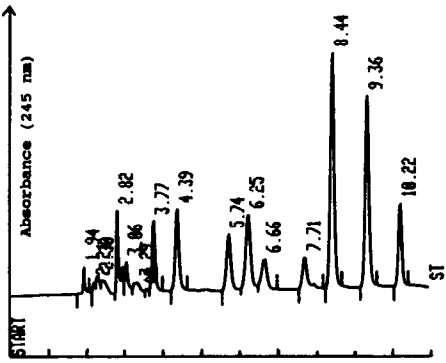

Fig. 2. Chromatogram from an Extrelut extract of the same serum as in Fig. 1. Numbers at peaks indicate retention times in min. SULT (4.8 μg/ml), 4.39 min; ETB = I.S., 8.44 min. For the relative RTs of other AEDs, see Table IIB.

through the precolumn in the straight-flush mode and in the other they were injected as methanolic solutions.

Recovery and reproducibility using Extrelut extraction

Since no control serum with SULT is commercially available, we made up control samples containing 4.0 μg/ml SULT with a bovine albumin matrix (5% in water). Portions of 500 μl were frozen at −18°C and thawed out before analysis of each of the sample series. In addition we checked the day-to-day precision of the analyses of a sample which was pooled from patient sera.

TABLE III

DAY-TO-DAY PRECISION OF A CONTROL AND A POOL SAMPLE ANALYSED USING THE EXTRELUT EXTRACTION METHOD

CS = Controle samples; PS = pool samples.

	n	Mean	S.D.	C.V. (%)
CS	58	3.95	0.164	4.15
PS	40	2.85	0.141	4.95

The method described is suitable for routine analyses having coefficients of variation which are lower than 5% for day-to-day determinations of SULT in both control and pooled samples. The mean value of 3.95 μg/ml which was found in the self-made control sample (see Table III) shows a 98.8% recovery of the spiked concentration of 4.0 μg/ml SULT.

CONCLUSION

Both of the methods described for the HPLC determination of sulthiame in

serum samples, direct injection with pre-column switching and solvent extraction using Extrelut columns, are in principle suitable for routine work.

In the case of samples from healthy volunteers taking SULT, the column-switching method gives the convenient option of automated sample pretreatment.

But, if the blood samples are taken from epileptic patients, especially from those on polytherapy with AEDs and other drugs, the Extrelut extraction, which yields cleaner extracts, is recommended for routine analyses.

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