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Liquid chromatography with amperometric detection of some sulphonamides and their N_4-acetyl-metabolites in serum and urine*

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Abstract: Sulphonamides separated on a C18 LC column were detected at lower levels using amperometric detection at a glassy carbon electrode in comparison with UV detection at 258 nm. Whereas sulphonamides are detectable at a potential of +1.00 V, their N_4-acetyl-metabolites required a potential of +1.25 V to be detected after their separation by LC. An interference commonly present in serum and urine, which co-eluted with one of the analytes, was detected at 1.25 V. This was overcome with an appropriate sample preparation in which 150 μl of serum or 75 μl of urine were first diluted to 1.5 ml with phosphate buffer (pH 3.0; 0.2 M). A 1.0 ml volume of this solution was then passed through an Extrelut® 1 column. The analytes were eluted with dichloromethane, which was evaporated under vacuum, and redissolving the analytes in an appropriate volume of mobile phase, i.e. methanol–phosphate buffer (pH 6.7; 0.067 M) (25:75, v/v). For sulphamethoxazole (SMX) and sulphamethoxypyridazine (SMP) and their N_4-acetyl-metabolites the calibration curves were linear between 1.5×10^{-7} and 8×10^{-6} M. The recovery ranged between 92.6 and 97.6% in serum and between 80.5 and 99.4% in urine. Detection limits were 10 times lower with amperometric detection than with UV detection. The method has been applied to the quantitation of SMX and SMP and their N_4-acetyl-metabolites in serum and urine after their oral administration.

Keywords: *LC–amperometric detection; sulphamethoxazole; sulphamethoxypyridazine; N_4-acetyl-metabolites; serum and urine.*

Introduction

Sulphonamides were the first chemotherapeutic agents used for the systematic control of bacterial diseases. They still find important applications for this purpose in humans and livestock animals. Most sulphonamides are metabolized mainly by N_4-acetylation [1]. Since 1978, various authors have published procedures to determine different sulphonamides together with their N_4-acetyl-metabolites in body fluids and tissues, mainly by reversed-phase HPLC with UV detection [2–6]. In two other papers, their determination by normal phase HPLC and UV detection [7, 8] was proposed. The separation and determination by GLC [9] or GC–MS [10] is more complicated and time-consuming, because this generally involves a prior derivatization reaction.

Momberg *et al.* have shown that sulphonamides separated on a C_{18} HPLC column can be determined at lower levels using amperometric detection at a glassy carbon electrode in comparison with UV detection at 258 nm [11]. Amperometric detection at a potential of 1.00 V vs Ag/AgCl has been applied to the determination of sulphamethoxazole, sulphathiazole and sulphamethoxypyridazine in serum and urine [12] and sulphadiazine, sulphamerazine and sulphapyridine in plasma [13]. Under these conditions the sulphonamides are detectable, but not their N_4-acetyl-metabolites.

In this paper, it is shown that sulphamethoxazole (SMX), sulphamethoxypyridazine (SMP) and their N_4-acetyl-metabolites (Ac-SMX and Ac-SMP) (Fig. 1) can be determined in serum and urine by isocratic reversed-phase HPLC separation with amperometric de-

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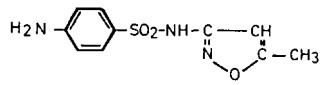
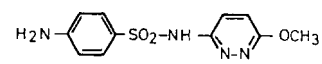


Sulphamethoxazole (SMX)



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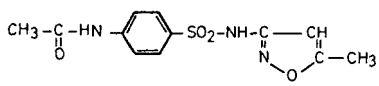
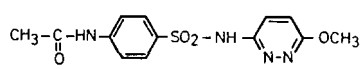


N-4-Acetyl-Sulphamethoxazole (Ac-SMX)



N-4-Acetyl-Sulphamethoxypyridazine (Ac-SMP)

Figure 1
Structures of sulphonamides and their N_4-acetyl-metabolites.

tection, by increasing the applied potential to 1.25 V vs Ag/AgCl and improving the sample preparation procedure.

Experimental

Instrumentation

HPLC: Perkin–Elmer series 2/1 isocratic HPLC with Rheodyne 7125 Injection Valve with 20 µl Loop. UV detector: Perkin–Elmer LC-85 at 258 nm. Amperometric detector: wall-jet type, Metrohm 656/641, with glassy carbon working and auxilliary electrodes and Ag/AgCl reference electrode; applied potential between 1.00 and 1.30 V; cell temperature: 25.0 ± 0.2°C. Both detectors are connected in series. Integrator: Shimadzu Chromatopac CR 1 B. HPLC Column: RP 18 Superspher®, 125 × 4 mm, 4 µm (Merck, Darmstadt, Germany). Mobile phase: methanol–phosphate buffer (pH 6.7; 0.067 M) (25:75, v/v); degassing after filtering during at least 15 min in a Millipore Vacuum Filtration System; flow rate: 1.0 ml min^{-1}.

Synthesis of N_4-acetyl-derivatives of sulphonamides

This was based on the procedure described by Vree *et al.* [14]; the purity of products was checked by IR and NMR spectroscopy and by HPLC with UV-detection.

Sample preparation

A 150 µl volume of serum or 75 µl of urine were diluted with phosphate buffer (pH 3.0, 0.2 M) to a final volume of 1.50 ml; 1.00 ml of this solution was then transferred to an Extrelut® 1 column (Merck). The sulphonamides and their metabolites were eluted with 5 ml of dichloromethane, which was then evaporated under vacuum. The residue was redissolved in 5.00 ml of mobile phase.

Evaluation and application of the method

For each analyte were determined the linear range, the limit of detection (according to Knoll [15]) and the recovery. Serum and urine samples were taken before the oral administration of the respective sulphonamide (blank) and 10 h after their ingestion. RSDs were calculated for five replicates, including extraction procedure.

Results and Discussion

Optimization of detector response

As shown in Fig. 2, SMP and SMX are detectable at 1.00 V, due to the oxidation process reported previously [11], whereas their N_4-acetyl-metabolites require a higher applied potential to give an appropriate signal. This second process has not been studied in detail, but since both the parent compounds and their metabolites give rise to similar behaviour, it can be concluded that the oxidation is occurring probably at the SO_2-NH-R moiety.

The optimum potential for the detection of both metabolites, together with their precursor drug molecules, is 1.25 V. These results explain those found by Hansen [16], who reported a very unfavourable detection limit for amperometric detection for an analogous compound, N_4-acetyl-sulphapyridine (Ac-SP), because the applied potential in that case ranged only between 0.60 V and 1.00 V vs Ag/AgCl, so that for Ac-SP spectrofluorimetric detection was preferred.

Optimization of mobile phase

For the separation of SMP and SMX from

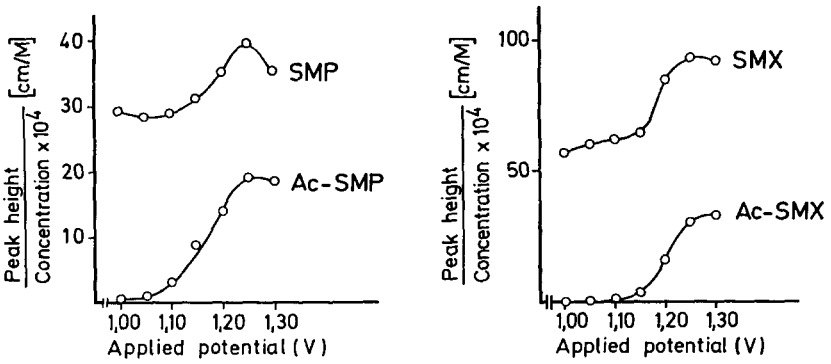


Figure 2
Amperometric detector response for sulphonamides and their N_4-acetyl-metabolites as a function of applied potential.

Table 1
Chromatographic data

Parameter	SMX	Ac-SMX	SMP	Ac-SMP
Retention time (min)	2.04	3.07	3.94	6.03
Capacity factor	1.50	3.00	4.00	6.75
Resolution	4.8		5.5	

their respective N_4-acetyl-metabolites, as well as from endogenous compounds present in serum and urine, the most favourable mobile phase was methanol–phosphate buffer (pH 6.7; 0.067 M) (25:75, v/v). The chromatographic parameters under these conditions are summarized in Table 1.

Development of an extraction procedure

An increase of the applied potential from 1.00 to 1.25 V, necessary to make Ac-SMX and Ac-SMP detectable, decreased the selectivity of the detector, because at higher potentials endogenous compounds present in serum and urine are now also oxidized at the glassy carbon electrode. In fact, with most extraction procedures described previously for UV-detection of sulphonamides and their N_4-acetyl-metabolites from body fluids [2–8], interfering compounds co-extracted from serum and urine blanks were detectable at 1.25 V. An extraction procedure used previously by the present authors for the determination of SMX and SMP alone, and not simultaneously with their N_4-acetyl-metabolites, was affected by the same limitation [12].

This limitation was overcome by extracting the analytes at pH 3.00 with dichloromethane through an Extrelut® 1 column, as proposed in the present work. In Table 2, the results obtained with this procedure after optimizing the pH of the aqueous phase and the composition of the organic extractant are summarized. RSDs for the whole procedure, including extraction and chromatography were between 4.4% for SMX and 7.1% for Ac-SMP.

Table 2
Recovery of analytes added to serum and urine blanks

Analyte	Recovery (%) Serum	Urine
SMX	92.6	99.4
Ac-SMX	96.1	80.5
SMP	97.6	96.6
Ac-SMP	93.8	93.2

Evaluation and application of the method

The calibration curves obtained by LC with amperometric detection were linear ($r > 0.998$) for SMX, Ac-SMX, SMP and Ac-SMP in the assayed concentration range (1.5×10^{-7}–8×10^{-6} M). For amperometric detection, the limits of detection, calculated according to Knoll [15], were between 1×10^{-8} M for SMX and 3×10^{-8} M for Ac-SMP, whereas for UV-detection they were between 1×10^{-7} M for SMX and 3×10^{-7} M for Ac-SMP. Considering the dilution involved in the proposed sample preparation procedure, the detectable concentrations are respectively 50

Table 3
Concentration of sulphonamides and their N_4-acetylmetabolites in serum and urine*

	Serum [M]		Urine [M]	
	Detection mode			
	Amperometric	UV	Amperometric	UV
SMX	1.25×10^{-4}	1.21×10^{-4}	1.93×10^{-4}	1.87×10^{-4}
Ac-SMX	4.70×10^{-5}	4.74×10^{-5}	1.59×10^{-3}	1.59×10^{-3}
SMP	1.59×10^{-4}	1.62×10^{-4}	1.97×10^{-4}	2.03×10^{-4}
Ac-SMP	1.24×10^{-5}	1.25×10^{-5}	2.65×10^{-4}	2.60×10^{-4}

*Samples taken 10 h after oral administration.

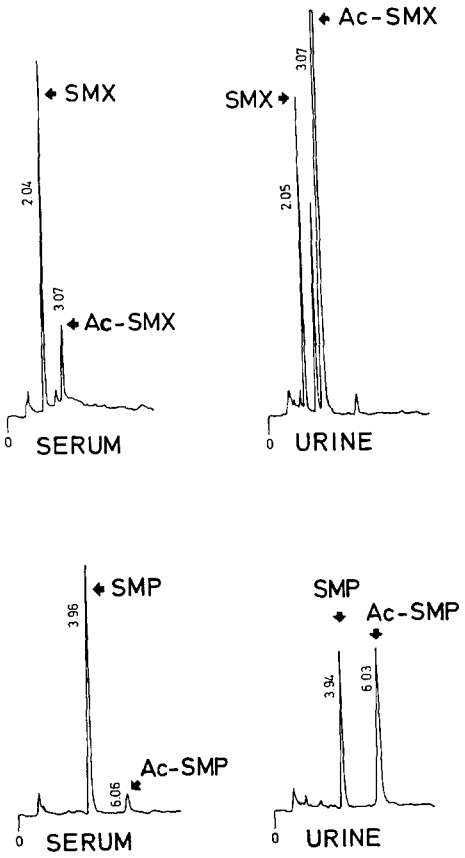


Figure 3
Chromatograms of sulphonamides and their N_4-acetylmetabolites in serum and urine 10 h after oral administration. Amperometric detection was used.

times higher for serum and 100 times higher for urine.

In Fig. 3, chromatograms obtained using amperometric detection for serum and urine samples taken from volunteers 10 h after oral administration of the respective sulphonamides are shown. In Table 3, the concentrations found in both matrices by amperometric and by UV-detection are summarized.

The concentrations obtained by amperometric detection are consistent with those determined by UV-detection. At the levels reported in Table 3, both detection modes can be applied, but amperometric detection can be especially useful for the determination of lower concentrations of sulphonamides and their metabolites when the available sample volume is too small (e.g. infants) or when trace levels of sulphonamides have to be determined in livestock animal meat.

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